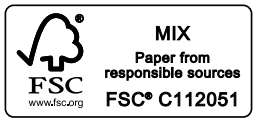
The Dual Face of Endothelial Control of Vascular Tone

ZHICHAO ZHOU



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The Dual Face of Endothelial Control of Vascular Tone

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Ik wil alleen maar weten

Ik wil alleen
maar weten
wie
ik ben.

Een
andere reden
om te schrijven
heb
ik niet.

Maar
wie ik ben
gaat niemand
wat aan.

(Jan Arends, 1925-1974)

For my parents

Contents

Chapter 1

General introduction and outline of the thesis

General Introduction

The circulation and heart

Cells need oxygen (O_2) and nutrients to survive. In single cells or small organisms, the supply of metabolic needs can be achieved by the simple processes of diffusion and convection of solutes from the external to the internal milieu. With increasing size and complexity of multicellular organisms, not all cells are in direct contact with either the air or sources of nutrients, and simple diffusion is not adequate. This is because diffusion is too slow and the concentration of nutrients in the environment is too low to meet the cells' metabolic requirements. Therefore, the requirement for a circulatory system is an evolutionary consequence to deliver O_2 and nutrients close to all cells of multicellular organisms such as the mammals. The circulatory system of mammals integrates three basic functional parts: a pump (the heart) builds up the pressure to drive a mixture of cells and fluid (the blood) to flow through a set of containers (the vessels) [1, 2].

The heart pumps blood around the body continuously and beats on average 2.5 billion times during the lifetime of a human being. With a size of one's fist, and a weight of approximately 300 grams, the human heart ejects about 5 liters of blood out through the whole body every minute, which can increase up to 25 liters during exercise [1].

In order to govern the movement of blood through the body, the heart consists of two pumps, namely the right and the left pump. Both pumps are subsequently divided into two functional compartments: the collecting compartments (atria) and the ejecting compartments (ventricles). Deoxygenated blood from the body is collected in the right atrium, which pumps the blood into the right ventricle. From the right ventricle the deoxygenated blood is pumped into the lung where it gets oxygenated. Then the oxygenated blood goes through the pulmonary vein and enters the left atrium. From the left atrium it is pumped into the left ventricle, which subsequently pumps the blood into the aorta, and finally reaches various organs (Fig. 1).

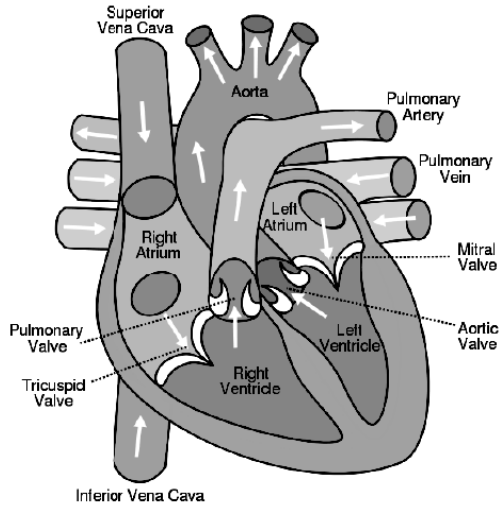

***Fig. 1**. Schematic representation of cardiac anatomy and the direction of blood flow. Adapted from Wikipedia.*

The English physician William Harvey (1578-1657) was the first to describe the cardiovascular system as a closed circuit. It has since been understood that the cardiovascular

system consists of two serial circuits, the pulmonary and the systemic circulations, which both originate from and terminate in the heart, implying that flow through the systemic circulation must equal that in the pulmonary circulation. In contrast, pressure and vascular resistance (calculated as the pressure drop through a circulatory system divided by the blood flow) of the pulmonary and systemic circulations are very different [3]. As the heart pumps approximately once every second continuously throughout our lives, it consumes a large amount of energy and therefore needs a commensurate amount of O_2 and nutrients. To supply the heart itself with O_2 and nutrients, a very important special circulation exists: the high pressure heart (coronary) circulation [1]. The fact that 5% of total blood flow goes through the coronary vasculature (the heart weighs about 300 gram i.e. 0.5% of total body weight) and that 60-80% of O_2 is extracted from blood during a single pass through this circulation, compared with 30-40% in the body as a whole [4, 5], underlines the importance of the coronary circulation.

The different characteristics of pulmonary, systemic and coronary circulations result in marked differences in vessel anatomy as well as vascular function, as will be outlined in more detail.

Pulmonary circulation

Pulmonary circulation refers to the movement of blood from the right ventricle, to the lungs, and back to the left atrium. The main function of the pulmonary circulation is to oxygenate blood and to remove carbon dioxide (CO_2) from the blood. O_2 is supplied by the airways, where the bronchus branches into smaller tubes (bronchioles), ultimately ending in the terminal respiratory units of the lungs: the alveolus (Fig. 2). The two main branches of the pulmonary artery, the only artery in the body that carries deoxygenated blood, follow the two mainstem bronchi into the lungs and bifurcate along with the bronchus to the alveolus. In the capillaries, carbon dioxide diffuses out of the blood cells into the alveoli, and oxygen diffuses from the alveoli into the blood. The blood, now containing high levels of O_2 and low levels of CO_2, is then collected by the pulmonary veins, the only veins in the body that carry oxygen-rich blood, and transported to the heart, where it re-enters at the left atrium. Since the barrier between the air and the blood in the alveolus is very thin, pulmonary capillary pressure must be very low to avoid the consequences of Starling forces; otherwise the alveolus would fill with plasma thereby affecting the efficiency of gas exchange in the lungs [1].

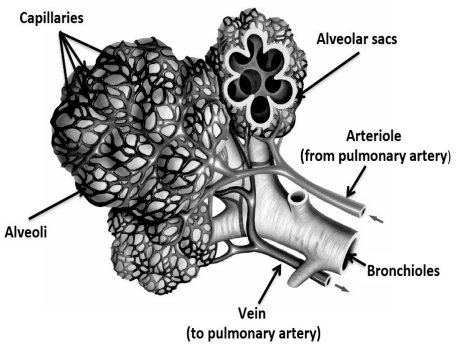

Fig. 2. *The alveolus and its capillary network.*

Indeed, blood is pumped into the lungs under a low mean pressure of ~15 mmHg at rest. In order to flow through the lungs, the blood requires a pressure drop of only 7 mmHg between the pulmonary artery and the end of the capillaries [3], reflecting the low-resistance of the pulmonary circulation system. During exercise, mean pulmonary artery pressure significantly increases up to ~40 mmHg [6], which is a result of (i) increase in left atrial pressure that is transmitted back through the lungs, (ii) the exercise-induced increase in cardiac output. To avoid a detrimental elevation of pulmonary artery pressure, pulmonary vascular resistance can decrease by up to ~50% during exercise [7].

Systemic circulation

In contrast to the pulmonary circulation, the systemic circulation is a high pressure and a high resistance system. Blood is pumped into the aorta under a mean arterial pressure of approximately 90 mmHg, and returns into the right atrium at pressure of ~5 mmHg. This large pressure drop at the same flow implies that systemic vascular resistance is about ten times higher than that of the pulmonary circulation.

Amato Lusitano (Curationum Medicinalium Centuriæ Septem 1st. edition in 1551) firstly described the physiological theory that the systemic circulation is a system that carries oxygenated blood away from the heart to the body, and returns deoxygenated blood back to the heart. Oxygenated blood leaves through the left ventricle to the aorta, that gives branches to all organs and tissues. These big arteries subsequently branch into smaller arteries, arterioles, and finally capillaries. On average, oxygen from the blood diffuses into the cell, while waste and carbon dioxide diffuse out of the cell into the blood. Approximately 30-40% of O_2 is extracted from the blood at rest to accommodate the O_2 demand of various organs in the body [4].

The larger arteries exert minimal resistance to blood flow and function primarily as conduits. However, as arteries approach the organ they perfuse and divide into many small arteries both just outside and within the organ. These small arteries and the arterioles of the microcirculation constitute the resistance vessels, containing up to 80% of total resistance. By adjusting their resistance locally through physical factors, chemical environment and hormones in the blood as well as autonomic system, all organs and muscles can be adequately supplied with oxygen. To ensure that not all organs and muscles increase their perfusion simultaneously, the distribution of systemic blood flow needs to be well coordinated [4, 8].

Coronary circulation

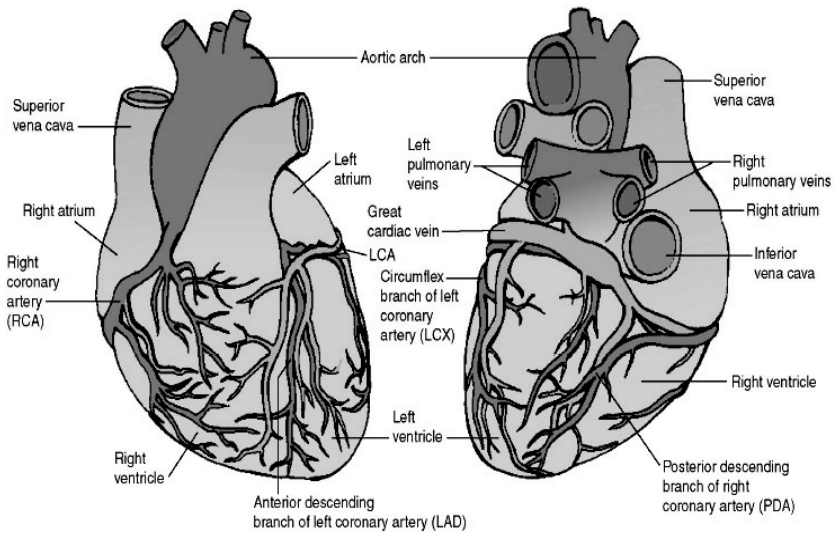

Fig. 3. *Coronary vasculature. Left, anterior view; right, posterior view.*

The coronary vasculature supplies the heart (myocardium) with oxygen and nutrients. The entire blood supply to the myocardium flows through the right and left coronary arteries. The

right coronary artery originates from the right aortic sinus, while the left coronary artery originates from the left aortic sinus. The right coronary artery generally supplies the right ventricle and atrium, and the left coronary artery supplies the left ventricle and atrium. The left coronary artery divides near its origin into two principal branches: the left circumflex artery sends branches to the left atria and ventricle and supplies lateral and posterior wall, while the left anterior descending artery descends to the apex of the heart and predominantly supplies the anterior wall (Fig. 3). Similar to vascular beds of other organs in the body, as the coronary vessels branch further, they become smaller to end in capillaries, where the O_2 and nutrients exchange [9]. Since this network is part of the systemic circulation, the coronary circulation is a high pressure and high resistance system. By regulating coronary resistance, the constantly changing metabolic demands of the myocardium can be fulfilled. The mechanisms (For instance, metabolic vasodilation, myogenic vasodilation and flow-induced vasodilation) which contribute to control of coronary blood flow must serve two purposes: First, blood flow must be suited to the metabolic demands of the myocardium. That is, blood flow must be able to change in parallel with changes in metabolic demand. Secondly, capillary pressure must be regulated and maintained relatively constant so that efficient delivery of oxygen and nutrients is ensured without producing damage to exchange vessels. The majority of coronary vascular resistance which serves to control both local blood flow and capillary perfusion pressure is found in coronary arterioles less than 150 μm in diameter [10]. However, even within the section of coronary resistance vessels, heterogeneity of function by different mechanisms exists. Small coronary arterioles are more sensitive to metabolites such as adenosine, intermediate-sized arterioles are sensitive to myogenic vasodilation, and large-sized arterioles respond more to flow-induced vasodilation [10]. A lack of balance between these mechanisms is often apparent in disease, which is characterized by inadequate control of coronary blood flow [11, 12].

The heart contracts approximately 60 times a minute at rest, which requires a constant high delivery of O_2. The utilization of heart for O_2 from the blood that flow through the coronary circulation is very high, about 80% [13] which contrasts sharply with other organs in the body where the O_2 extraction is relatively low [4]. During exercise, coronary blood flow increases as much as five-fold to meet the increase in oxygen demand, which is determined by contractility, heart rate, muscle shortening, and wall tension. Augmented blood flow during exercise is the primary contributor to O_2 delivery, since the change in O_2 extraction is negligible [5, 14].

Regulation of vascular tone

All circulations (pulmonary, systemic and coronary) have in common that their resistance can be adjusted by the regulation of vascular diameters, and the changes in vascular diameter are the sum of both passive (structural, mechanical) and active (smooth muscle tone) influences [15].

Passive influences

The distension, flow recruitment of the vessels as well as vessels imbedded in muscle by compressive force are all regarded as passive regulation in all the vascular beds. However, different vascular beds may have one or more passive influences pronounced. In the pulmonary circulation, there are two passive mechanisms at work being recruitment and distension of the small vessels [1, 6]. Under normal conditions, when pulmonary arterial pressure is low, some pulmonary vessels are open and conducting blood, some are open but not conducting, and some are closed. As pressure increases, vessels that previously had been open but not conducting

blood may now begin to conduct, and those that were closed may now open. This vessel recruitment will decrease pulmonary vascular resistance and additional distension of these vessels will cause a further reduction in pulmonary resistance [1].

In the coronary circulation, passive influence of vascular diameter is based on extravascular compressive forces occurring during systolic contraction, when the heart contracts and pumps oxygenated blood out through the aorta. At this time, the coronary vasculature is compressed and squeezed. The heart predominantly receives oxygenated blood during diastolic relaxation, when the coronary blood flow is not impeded by the myocardium [5]. Although, passive influence of vascular diameter is crucial and is part of total resistance, this thesis focuses mainly on the active regulation of vascular tone and diameter.

Active regulation

Vascular tone refers to the state of contraction of the vascular smooth muscle cells. The vascular smooth muscle cells are lined circumferentially in the vessel wall just outside the endothelial cells. Changes in vascular tone directly affect vessel diameter, thereby altering vascular resistance. Regulation of vascular tone is the result of an interplay between a myriad of relaxing (vasodilator) and contracting (vasoconstrictor) factors that influence smooth muscle cell relaxation or contraction. Smooth muscle cells are highly specialized cells that express various contractile proteins, ion channels, and signaling molecules. The process of smooth muscle contraction is principally regulated by pharmacomechanic activation: activation by ligands of cell surface receptors, and electromechanic activation: stretch, intraluminal pressure of the contractile proteins myosin and actin [16, 17]. Contraction or relaxation of the smooth muscle cells is directly influenced by the concentration of free calcium (Ca^{2+}) in the cytosol. An increase in cytosolic Ca^{2+} results in formation of the Ca^{2+}-calmodulin complex, which activates myosin light chain kinase (MLCK). MLCK then phosphorylates the myosin light chain, allowing cycling of cross bridges between actin and myosin and resulting in smooth muscle cell contraction [16]. The myosin light chain is dephosphorylated by myosin light chain phosphatase (MLCP), resulting in smooth muscle cell relaxation. Inactivation of MLCP occurs through phosphorylation by Rho-kinase [18]. Ca^{2+} and MLCP act synergistically to regulate vascular tone (Fig. 4).

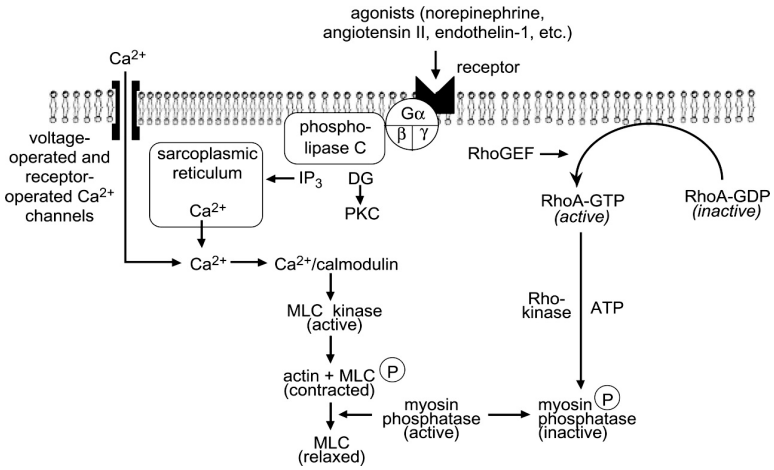

Fig. 4. *Regulation of smooth muscle contraction and relaxation. Adapted from [19].*

As described in the paragraph above, the regulation of vascular tone is the result of a complex balance between a multitude of vasodilator and vasoconstrictor influences. These influences are initially exerted by the endothelium, neurohormonal, and metabolic signals from the myocardium, which result in the (in)activation of intracellular signaling cascade. In addition, many other vasoactive factors such as reactive oxygen species, phosphodiesterases, substance P, and vasoactive intestinal peptide have been shown to influence vascular tone.

Endothelial control of vascular tone

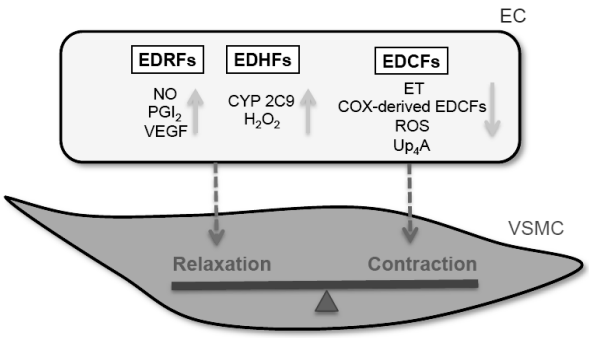

Fig. 5. *The endothelium exerts its effect on vascular tone through the balanced release of EDRFs (endothelium-derived relaxing factor), EDHFs (endothelium-derived hyperpolarization factor) and EDCFs (endothelium-derived contracting factor). EC: endothelial cells; VSMC: vascular smooth muscle cells; NO: nitric oxide; PGI_2: prostacyclin; VEGF: vascular endothelial growth factor; CYP 2C9: cytochrome P450 2C9; H_2O_2: hydrogen peroxide; ET: endothelin; COX: cyclooxygenase; ROS: reactive oxygen species; Up_4A: uridine adenosine tetraphosphate.*

The vascular endothelium releases a variety of vasoactive substances, vasodilators including nitric oxide (NO), prostacyclin, endothelium-derived hyperpolarisation factor (EDHF), and vascular endothelial growth factor (VEGF); as well as vasoconstricotors including endothelin, reactive oxygen species (ROS) and a recently identified novel endothelium-derived contracting factor: uridine adenosine tetraphosphate (Up_4A), all of which contribute to vascular tone regulation (Fig. 5). Importantly, the endothelial lining is not a homogeneous compartment as it is characterized by significant structural and functional heterogeneity [20]. For instance, the endothelium in the pulmonary bed differs in ultrastructure and function from the systemic vascular endothelium. Hence, the influences of endothelium-derived vasoactive factors on the vascular tone in different vascular systems may vary [21, 22].

Nitric oxide. NO was first discovered and described by Furchgott as an EDRF [23, 24]. In the endothelium, this vasoactive agent is synthesized from L-arginine by endothelial NO synthase (eNOS) which uses tetrahydrobiopterin (BH_4) as a cofactor [25, 26]. NO production is dependent on eNOS activity, which can be activated by mechanical forces (i.e. an increase in shear stress exerted by the blood flow on the endothelium) as well as by a host of chemical factors such as bradykinin (BK), acetylcholine, substance P, extracellular nucleotides acting on their respective receptors on endothelium [27, 28]. NO diffuses to the underlying smooth muscle cells, where it activates soluble guanylyl cyclase, resulting in production of cGMP, which leads to a potent vasodilation. However, under conditions of endothelial dysfunction, oxidation or reduction of the eNOS cofactor BH_4 causes eNOS to uncouple from a dimeric to a monomeric form that no longer produces NO but forms superoxide ($O_2^{\cdot}$) instead [29]. $O_2^{\cdot}$ is capable of increasing vascular

tone and results in additional eNOS uncoupling. Furthermore, $O_{2}^{\cdot}$ reacts with NO to form the powerful oxidant peroxynitrite [30]. Consequently, eNOS-mediated $O_{2}^{\cdot}$ production not only results in vasoconstriction but also reduces the bioavailability of NO (Fig. 6). In healthy conditions, we [31] and others demonstrated that NO contributes to both coronary [32, 33] and pulmonary vasodilation [34, 35] in dogs, swine and humans. In the diseased condition, it has been shown that eNOS was uncoupled resulting in production of ROS, which contributes to porcine coronary vasoconstriction after myocardial infarction (MI) [36], while in the condition of pulmonary hypertension, NO bioavailability was decreased and ROS production was increased which enhances pulmonary vasoconstriction thereby increasing the pulmonary arterial pressure [37].

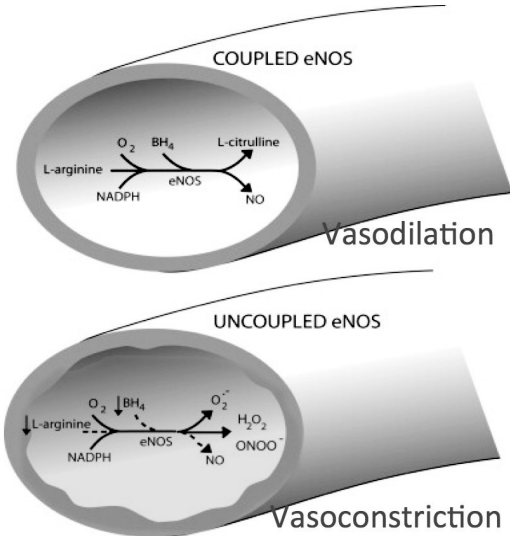

Fig. 6. *NO is produced from L-arginine by eNOS together with cofactor BH_4 resulting in vasodilation; whereas reduction of BH_4 results in eNOS uncoupling leading to vasoconstriction.*

Prostanoids. Prostanoids are metabolites of arachidonic acid produced by the cyclooxygenase (COX) pathway. The vascular effects of prostanoids depend on the balance between vasodilator and vasoconstrictor prostaglandins as well as thromboxane A_2 (TXA_2). After arachidonic acid is converted to the intermediate endoperoxide (PGH_2) by cyclooxygenase, PGH_2 is subsequently converted into prostaglandin I_2 (PGI_2), D_2, E_2 (PGE_2), $F_{2\alpha}$ ($PGF_{2\alpha}$) and A_2 (PGA_2) by prostacyclin synthase, PGD synthase, PGE synthase, PGF synthase and thromboxane synthase, respectively (Fig. 7). PGI_2 is the principal arachidonic acid metabolite released by the endothelium and exerts both antiplatelet and vasodilator effects through interaction with the IP receptor to activate cAMP-dependent pathways [38]. Conversely, PGE_2, $PGF_{2\alpha}$ and TXA_2 cause vasoconstriction through the interaction with their respective receptors (EP, FP, and TP). Under normal conditions, those vasoconstrictor metabolites are unstable, whereas under disease conditions they become stable and predominate over the vasodilator metabolites [39, 40]. In the healthy vasculature, inhibition of COX results in coronary vasoconstriction implying that endogenous COX contributes to coronary vasodilation [31, 41]. In contrast, endogenous prostanoids are not mandatory for the regulation of pulmonary vascular resistance tone [31, 42]. In the diseased vasculature, inhibition of COX improves coronary function after acute myocardial ischemia [43] and attenuates hypoxia-induced pulmonary vasoconstriction [44] indicating COX-derived vasoconstrictor predominated in diseased conditions.

Cytochrome P450 2C9. A significant endothelium-dependent vasodilation persists after inhibition of nitric oxide synthase and cyclooxygenase, suggesting the existence of third vasodilator pathway. The mechanism of this pathway is activated by hyperpolarization of vascular smooth muscle via opening of K^+ channels, thereby reducing the open probability of voltage-dependent Ca^{2+} channels so that intracellular Ca^{2+} is lowered and vasorelaxation is produced [45]. Several factors appear to participate in endothelium-derived hyperpolarization, including cytochrome P450 2C9 (CYP 2C9) metabolites [46], hydrogen peroxide [47], S-nitrosothiols

[48] and K^+ [49] are called endothelium-derived hyperpolarized factors (EDHFs). CYP enzymes are probably best known for their critical contribution to detoxification processes in the liver and kidney. A much less appreciated role of these enzymes has been observed in the vascular system [50]. CYP 2C9 is abundantly expressed in the vasculature [51, 52], producing epoxyeicosatrienoic acids from endothelial cells. Vasodilation induced by CYP 2C9 metabolites has been reported in various vascular beds, particularly after blockade of NO and PGI_2 [53-55], suggesting an interaction between NO/PGI_2 and CYP 2C9 metabolites, and in some diseased condition such as diabetes, the vasodilator influence of CYP 2C9 metabolites can backup and compensate for the loss of NO/PGI_2 [56]. In contrast, CYP 2C9 can also produce vasoconstrictor metabolites and has been suggested as a source of reactive oxygen species [55, 57, 58]. For instance, ROS were generated in isolated porcine coronary arteries in response to BK. ROS were detected by immune/fluorescent staining in situ, and generation of ROS disappeared after incubating with CYP 2C9 specific inhibitor sulfaphenazole [58]. In the coronary vasculature, the role of CYP 2C9 metabolites has been only addressed in isolated arteries in response to BK that CYP 2C9 exerts a vasodilator influence [46, 59]. However, in the systemic vasculature, the forearm blood flow was enhanced after inhibition of CYP 2C9 metabolites in patients with coronary artery disease [60], while in the pulmonary vasculature, studies regarding the role of CYP 2C9 metabolites in vivo and in vitro showed that CYP 2C9 metabolites constrict pulmonary artery in mice [57] and rabbits [61], respectively. However, little is known about the endogenous role of CYP 2C9 in those vascular beds.

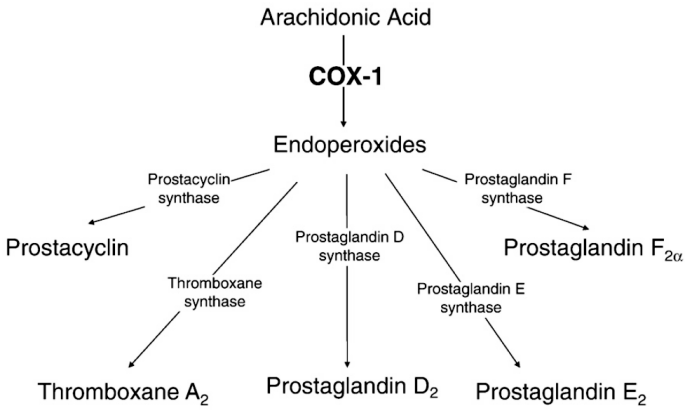

Fig. 7. *Schematic overview of the metabolism of arachidonic acid into various prostanoid metabolites. Adapted from [62].*

Vascular endothelial growth factor. VEGF represents a family of homodimeric glycoproteins. In mammals, five different VEGF ligands have been identified [63]. Through activation of their receptor VEGFR (VEFGR1, 2 and 3) in an overlapping pattern, VEGF targets the tyrosine kinases to stimulate vessel formation (angiogenesis and vasculogenesis) in a variety of physiological and pathophysiological biological processes, including embryogenesis, wound healing, tumor growth, and myocardial ischemia [64, 65]. In addition, VEGF regulates multiple endothelial cell functions such as inflammation [66], vascular tone [67], and is essential for endothelial homeostasis. VEGF production is induced in cells that are not receiving enough oxygen when circulation is inadequate. Therefore, hypoxia is the main stimulus for

increased VEGF expression [67]. However, when VEGF is overexpressed, it can contribute to diseases such as cancer. Since tumor cells are fast growing tissues, cancer overexpresses VEGF resulting in angiogenesis to maintain the blood supply and the tumor growth. With this concept, inhibition of angiogenesis can have beneficial outcomes in certain cancers and a variety of haematological malignancies [68]. For instance, sunitinib, a tyrosine kinase inhibitor, has been applied in clinic and clinical trials to treat patients with renal cell carcinoma, breast cancer, colon cancer and hepatocelluler carcinoma [69, 70]. On the other hand, anti-VEGF therapy has drawn the concern on the rises in mean arterial blood pressure in patients by inducing systemic vasoconstriction [71]. Moreover, anti-VEGF may also result in endothelial damage in healthy conditions, since VEGF plays a critical role in vascular protection by producing both NO and PGI_2 [72]. In additional to high blood pressure induced by sunitinib, little is known about the adverse effect of sunitinib on coronary and pulmonary circulations of cancer patients under the treatment of anti-VEGF therapy.

Endothelin. de Mey and Vanhoutte firstly reported the release of vasoconstrictor factors from endothelial cells in 1982 [73]. Over the next years, the release of a peptidergic vasoconstrictor substance was further investigated and reported [74-76]. Based on pioneering work of Vanhoutte, Yanagisawa and his colleagues named and sequenced this substance as "endothelin" [77]. There are three different forms of endothelins (ET), namely, ET-1, ET-2 and ET-3 [78, 79]. ET-1 is the major isoform expressed and secreted in endothelial cells and is one of the most potent vasoconstrictors known to date, with receptor sensitivities in the nanomolar range [78]. ET is produced by the cleavage of its precursor Big ET by endothelin converting enzyme [80, 81]. ET exerts its vasoactive influence by activation of endothelin receptors. The ET receptors are located on the endothelium and on vascular smooth muscle. Binding of ET to ET_B receptors on the endothelium leads to production of NO and PGI_2, which induce vasodilation. In contrast, binding of ET to the ET_A and ET_B receptors on vascular smooth muscle leads to vasoconstriction [80-82]. Administration of exogenous ET causes ET_B-mediated vasodilation at low doses but ET_A-mediated vasoconstriction at high doses, indicating that the ET_B receptor on the endothelium is more sensitive to ET than the receptors on vascular smooth muscle [80-82]. ET_A receptors are the predominant ET vasoconstrictor receptors in arteries throughout the body. Vasoconstrictor ET_B receptors are present in the veins and pulmonary vessels in larger numbers than in arteries, although ET_A receptors still predominate over ET_B receptors in these vessels [83]. The net effect of ET on vascular tone depends on the relative density and sensitivity of ET_A receptors on smooth muscle cells and of ET_B receptors on smooth muscle and endothelial cells, rather than the plasma level of ET. Because ET secretion is predominantly abluminal [84] and the clearance of ET can be mediated by the endothelial ET_B receptor, a potent vasoconstriction can still be induced with a high density and high sensitivity of ET receptors, despite of a low circulation ET level [85, 86]. In healthy coronary vasculature, we found that ET exerts a potent vasoconstrictor influence via ET_A receptor at rest, while the vasoconstrictor effect of ET wanes during exercise, whereas ET exerts a tonic vasodilation via ET_B receptors. In contrast, ET exerts an ET_B-mediated vasoconstriction during exercise in healthy pulmonary vasculature [87]. In diseased conditions, the ET_A-mediated coronary vasoconstriction is reduced due to a decreased receptor sensitivity in swine after myocardial infarction [88]; whereas increased pulmonary vasoconstriction in swine with secondary pulmonary hypertension after myocardial infarction is an emergent tonic ET_A-mediated as well as the exercised-induced ET_B-mediated that is already present in healthy swine [89].

Uridine adenosine tetraphosphate. Up_4A has been recently identified by Jankowski group as a novel potent endothelium-derived contracting factor [90]. Up_4A is the first dinucleotide found in living organisms that contains both purine and pyrimidine moieties (Fig. 8). This factor is isolated from the supernatant of human endothelium and synthesized by VEGFR2 pathway [90, 91]. Up_4A can be released from endothelium by stimulation such as ATP, UTP, acetylcholine, ET and mechanical stresses, suggesting that Up_4A contributes to vascular autoregulation [90]. Like other nucleotides, Up_4A exerts its vasoactive influence by binding to purinergic receptors (Fig. 9) [90, 92-94]. Specifically, Up_4A induces vasoconstriction in rat renal arteries through $P2X_1$ receptors [90], in rat aorta through P1 and P2X receptors [95], and in rat pulmonary large arteries through P2Y receptors [96]. In addition to vasoconstriction induced by Up_4A, there is also evidence that Up_4A can produce vasodilation in isolated aortic rings of rats [95] and induce hypotension in conscious rats [97]. However, which specific purinergic receptor in response to Up_4A to induce vasodilation is currently unknown. As a nucleotide, Up_4A has been proposed to be degraded by ectonucleotidase to ATP and/or UTP [90], the latter of which can be further degraded to ADP/UDP, AMP/UMP and adenosine/uridine [98-101].

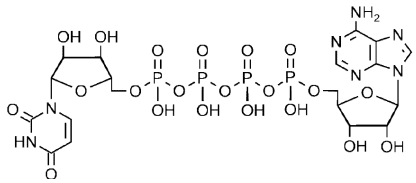

Fig. 8. *The structure of Up_4A with a purine moiety and a pyrimidine moiety connected by four phosphates.*

Plasma concentrations of Up_4A detected in healthy subjects are in the vasoactive range. Since Up_4A level is significantly elevated in hypertensive subjects, a role for Up_4A in the development of hypertension and other cardiovascular diseases has been proposed [102]. Indeed, Up_4A induces vascular calcification [103] and proliferation [104]. Vasoconstrictor responses to Up_4A in basilar arteries, in renal arteries, and in femoral arteries from DOCA-salt hypertensive rats are enhanced, as compared to those from normotensive rats [105]. Moreover, Up_4A-induced vasoconstriction in renal artery is enhanced via thromboxane in diabetic rats [106], suggesting a role for Up_4A in the diabetes-mediated vascular complications. However, the role of Up_4A in other vascular beds (coronary vasculature) and different segments (pulmonary small artery) in health and disease is currently unknown. The vasoactive influence of Up_4A in the coronary vasculature in health and disease will be discussed in detail in Chapter 7 through Chapter 10.

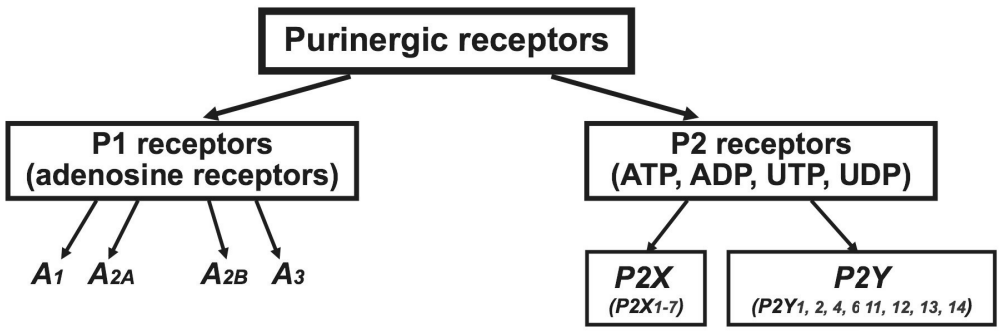

Fig. 9. *The classification of P2 receptor subtypes. Adenosine is the ligand for P1 receptors, whereas ATP is the ligand for most P2X receptors, ADP is the ligand for $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors, UTP is the ligand mainly for $P2Y_2$ and $P2Y_4$ receptors, and UDP is the ligand for $P2Y_6$ and $P2Y_{14}$ receptors.*

Neurohumoral control of vascular tone

Sympathetic control. The vasculature expresses both alpha (α) and beta (β) adrenergic receptors. These receptors primarily bind norepinephrine that is released from sympathetic nerve fibers, as well as norepinephrine and epinephrine that circulate in the blood. Activation of α-adrenergic receptors produces vasoconstriction, while the activation of β-adrenergic receptors mediates the relaxation of smooth muscle cells, resulting in vasodilation [107]. Normally, α-adrenergic receptors predominate in smooth muscle of resistance vessels, thereby sympathetic stimulation generally results in vasoconstriction. Specifically, vascular smooth muscle has two primary types of α-adrenergic receptors: α_1 and α_2. The α_1-adrenergic receptors are located on the vascular smooth muscle, and α_2-adrenergic receptors are located on the sympathetic nerve terminals as well as on vascular smooth muscle. Smooth muscle α_1 and α_2-adrenergic receptors are linked to a G-protein, which activates smooth muscle contraction. The downstream signaling events in α_1-adrenergic stimulation are to increase in Ca^{2+} levels by at least two mechanisms: (1) coupling to specific G proteins on the cell membrane (α_1/Gq; α_2/Gi); (2) blockade of potassium ion channels [108]. Prejunctional α_2-adrenergic receptors located on the sympathetic nerve terminals serve as a negative feedback mechanism for norepinephrine release [109, 110]. There are two β adrenergic receptor subtypes of β_1 and β_2, with β_2-adrenergic receptors being predominant subtype [111]. β2-adrenergic receptors located on the smooth muscle cells are normally activated by norepinephrine released by sympathetic adrenergic nerves or by circulating epinephrine. These receptors are coupled to a G_S-protein, which stimulates the formation of cAMP, thereby resulting in vasodilation [112]. In the coronary vasculature, both α_1 and α_2 receptors can mediate vasoconstriction at rest, and α_1 adrenergic mechanisms act to blunt the increase in coronary blood flow that occur during exercise in some species such as the dog, and possibly humans, but appears to be absent in swine [5]. On the other hand, both β_1 and β_2 adrenergic receptors do not contribute to coronary vasomotor tone at rest but become predominant during exercise, indicating that both β_1 and β_2 adrenergic receptors contribute to the β-feedforward coronary resistance vessel dilation during exercise [5, 113]. In the pulmonary vasculature, despite marked activation of the sympathetic nervous system during exercise, its net influence on pulmonary vascular tone is similar at rest and during exercise in sheep and swine [6], suggesting that the sympathetic nervous system exerts a net vasoconstrictor influence on the pulmonary vasculature at rest and that exercise results in a balanced increase in α- and β-adrenergic influences on pulmonary vascular tone.

Parasympathetic control. The vasculature is also innervated by the parasympathetic division of the autonomic nervous system [28, 114]. Parasympathetic activation exerts weak species-dependent effects on the coronary and pulmonary vessels [5], the extent of innervation as well as the size of vessels that are innervated is also species-dependent [27, 115]. Acetylcholine released from the parasympathetic nerve endings can act either directly on the vasculature (endothelium and vascular smooth muscle) and/or can inhibit norepinephrine released from the sympathetic nerve terminals [109, 110, 116]. Acetylcholine exerts its vasoactive effect by interacting with muscarinic (M) receptors. There are 5 subtypes of M receptors, namely M1-M5 [117]. Binding of acetylcholine to M receptors located on vascular smooth muscle cells leads to vasoconstriction, whereas binding of acetylcholine to those located on the endothelium leads to vasodilation by activating production of NO and prostanoids [118]. The net effect of acetylcholine on vascular tone regulation depends on the relative number of receptors on the endothelium and vascular smooth muscle [119].

In the coronary circulation, in animals in which acetylcholine causes vasodilation, parasympathetic activity causes a weak dilator influence at rest when vagal tone is high, but vagal tone is withdrawn during exercise. In species in which acetylcholine causes coronary vasoconstriction, as in swine where the acetylcholine-induced NO-mediated vasodilation is outweighed by a direct vasoconstrictor effect on smooth muscle cells, withdrawal of vagal tone may augment the increase in coronary flow during exercise [5]. In the pulmonary circulation, selective stimulation of the parasympathetic nervous system results in pulmonary vasodilation, and the activity of the parasympathetic nervous system is reduced during exercise, suggesting that exercise-induced pulmonary vasodilation in swine is blunted by the loss of M receptor-mediated vasodilation [6].

Angiotension. Angiotensin II (ANG-II) is a vasoactive octapeptide of the renin-angiotensin system (RAS). It is formed by two enzymatic conversions from its precursor angiotensinogen that circulates in the plasma. Angiotension I is cleaved from angiotensinogen by rennin or rennin-like enzymes. ANG-I is then converted into ANG-II by the angiotensin converting enzyme (ACE) [120]. Besides the well-known kidney-based RAS, many other tissues including the vascular wall contain their own local RAS [121]. Thus, all components of the RAS have been shown to be present in the vascular wall [121]. ANG II exerts important cardiovascular effects, including positive inotropism and vasoconstriction, as well as causing norepinephrine and aldosterone release, all of which are mediated by the AT1 receptors. In contrast, ANG-II binding to AT2 receptors leads to vasodilation [122]. More recently, ACE2 (a homologue of ACE) was identified. ACE2 cleaves a single amino-acid from both ANG-I and ANG-II, thereby generating angiotension-(1-9) and angiotension-(1-7), respectively. Angiotension-(1-7) preferentially binds to the AT2 receptors, thereby evoking vasodilation [123, 124]. In the coronary vasculature, blockade of AT1 receptor results in coronary vasodilation, implying an endogenous vasoconstrictor system of ANG-II [125]. Importantly, a cross-talk between ANG-II and ET emerges and acts synergistically on coronary resistance control after MI [126], despite a lack of interaction between ANG-II and ET in the healthy vasculature [126, 127]. In the pulmonary vasculature, blockade of AT1 receptor had no effect on vasomotor tone in male, whereas it enhanced pulmonary vascular tone in female, suggesting the contribution of ANG-II in the pulmonary circulation depends on gender [128]. Moreover, ANG-II inhibits vasoconstrictor influence of ET in female, and the interaction between ANG-II and ET occurs locally in the vasculature [128].

Metabolic messengers

Adenosine. Adenosine is an endogenous purine nucleotide that modulates many physiological processes such as vascular tone regulation. Adenosine predominantly dilates arterioles less than 100 μm in diameter. Vessels of this size correspond to the site at which vascular metabolic regulation [129, 130] and autoregulation occur [131, 132]. There are two ways by which adenosine is produced: adenosine is mainly generated via extracellular pathways from ATP and ADP via the combined action of ecto-nucleoside triphosphate diphosphohydrolase (ecto-NTPDase-1 [CD39]) or possibly other NTDPases- and ecto-5′-nucleotidase (CD73). Secondly, adenosine can be generated by *S*-adenosylhomocysteine hydrolase [133]. The effect of adenosine on the vascular tone regulation is both species-dependent and vascular bed-dependent [134-137]. These vascular effects of adenosine occur through adenosine receptors (also known as purinergic P1 receptors): A_1, A_{2A}, A_{2B} and A_3 (Fig. 9) [92, 94]. Thus, in the pulmonary vasculature, adenosine induces vasoconstriction through A_1 and A_2 receptors in cats and sheep [138, 139], but induces vasodilation through A_{2A}

receptors in lamb [140]. In renal vasculature, adenosine induces vasoconstriction through A_1 receptors in rats [141] and in rabbits [142], whereas adenosine induces vasodilation through A_{2B} receptors in rats [143] and through A_{2A} and A_{2B} in mice [144]. In the coronary vasculature, adenosine produces vasoconstriction through A_1 receptors in rabbits [145], but produces a very potent vasodilation through A_{2A} in mice [137] and pigs [146]. Adenosine, with half life less than 10 seconds, is broken down by adenosine deaminase, a purine salvage pathway enzyme that degrades adenosine to inosine and adenosine kinase [147].

Second messenger: cyclic nucleotides

The cyclic nucleotide second messengers cAMP and cGMP cause smooth muscle relaxation by lowering intracellular Ca^{2+} and by activating myosin phosphatase, thereby decreasing the sensitivity of the contractile apparatus to Ca^{2+} [27, 148]. The amplitude and duration of their signaling depends on their rate of production and their rate of degradation [149]. cAMP, a potent vasodilator, is produced by adenylyl cyclase [27, 148]. Adenylyl cyclase is activated by G-protein coupled receptors including the β-adrenergic receptors and the IP and DP receptors [27]. cAMP exerts the majority of its actions through activation of protein kinase A (PKA). cGMP that also exerts a potent vasodilator influence is produced by guanylyl cyclase (GC) [27, 148]. There are two types of GC, being particulate GC and soluble GC [149]. Particulate GC is activated by ligand binding to the natriuretic peptide receptor-A and receptor-B. Soluble GC is activated by NO and carbon monoxide. cGMP exerts the majority of its actions through activation of protein kinase G (PKG) [149]. These cyclic nucleotide second messengers are short-lived, and can be hydrolyzed by phosphdiesterases (PDE) [149]. At least 11 different gene families of PDE are currently known to exist in mammalian tissues. The tissue distribution of the PDE-isoforms as well as their specificity for cAMP and cGMP varies [150]. Therefore, inhibition of PDE in vascular smooth muscle provides a powerful tool to reduce vascular resistance by prolonging the half-life of cAMP and/or cGMP. The PDE isoforms that are predominantly present in vascular smooth muscle cells are PDE1, 3, 4, 5, 7 and 9 [151, 152]. Among them, PDE1, 4 and 7 are cAMP-specific, while PDE3 has a 4-10 times higher affinity for cAMP than for cGMP. PDE5 and 9 are cGMP-specific [150]. However, PDE9 is abundantly expressed throughout the body, whereas PDE5 expression is 10 times more abundant in the pulmonary vasculature as compared to the heart [153, 154]. Inhibition of PDE5 has been clinically validated as an effective treatment for pulmonary arterial hypertension. Indeed, in the pulmonary vasculature, PDE5 inhibition resulted in pulmonary vasodilation in both health [155] and in patients with pulmonary hypertension [156]. In the coronary vasculature, PDE5 limits coronary blood flow to hypoperfused myocardium during exercise [157] and contributes little to regulation of coronary hemodynamics in congestive heart failure [158]. However, little is known about the endogenous role of PDE5 in the healthy coronary vasculature. Consequently, the vasoconstrictor influence of PDE5 both in the coronary and pulmonary circulation as well as its interaction with ET system will be discussed in detail in Chapter 4, Chapter 5 and Chapter 6, respectively.

Reactive oxygen species

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen [159]. ROS form as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis [160]. However, during times of environmental stress (e.g., UV or heat exposure), ROS levels can increase dramatically. This may result in significant damage to

cell structures. Cumulatively, this is known as oxidative stress. The free radical superoxide ($O_2^{\cdot}$) is produced by normal aerobic mitochondrial respiration and is converted to H_2O_2 by superoxide dismutase (SOD) [161]. H_2O_2 is further degraded by catalase to O_2 and H_2O [159]. In the presence of free iron, the hydroxyl radical ($OH^{\cdot}$) can be formed [162, 163]. $OH^{\cdot}$ is the most reactive ROS and will react with surrounding tissues before diffusion takes place, whereas both $O_2^{\cdot}$ and H_2O_2 can diffuse away and function as signaling molecules in physiological and pathological cardiovascular processes, including hypertension, atherosclerosis, myocardial infarction and diabetes [164, 165]. In addition to mitochondria, there are some enzymatic sources for ROS generation including nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, uncoupled NO synthase, CYP 450, xanthine oxidase and lipoxygenases [166, 167]. Through their actions on K^+ channels in the vascular smooth muscle cells, both $O_2^{\cdot}$ and H_2O_2 exert an effect on vascular tone regulation. In general, $O_2^{\cdot}$ is regarded as a vasoconstrictor, while H_2O_2 acts as a vasodilator [168, 169]. In the pulmonary vasculature, $O_2^{\cdot}$ induces contraction of pulmonary artery, triggering Rho-kinase-mediated Ca^{2+} sensitization and is involved in hypoxia-induced pulmomary vasoconstriction and development of pulmonary hypertension [37, 170], whereas more controversial is the effect of H_2O_2 on the effect of pulmonary vascular tone that constricts both isolated perfused rat lung and rabbit lung, and also isolated rat and rabbit pulmonary arteries. H_2O_2 is also responsible for the elevation of intracellular calcium concentration in pulmonary artery smooth muscle cells during hypoxia, and multiple similarities have been drawn between H_2O_2-induced vasoconstriction and hypoxic pulmonary vasoconstriction. In contrast, it has been shown that H_2O_2 produces concentration-dependent relaxation of precontracted isolated bovine arteries as well as in isolated perfused rabbit lungs by a mechanism independent of the endothelium or prostaglandin mediators but related to soluble guanylate cyclase and cGMP [170]. In the coronary vasculature, $O_2^{\cdot}$ contributes to vasoconstriction, and the generation of $O_2^{\cdot}$ in the coronary vasculature is likely from either uncoupled eNOS [36] or CYP 2C9 [58]. H_2O_2 again results in a dual vasoactive effect that exogenously and endogenously produced H_2O_2 may elicit vasodilation by different mechanisms in human coronary arterioles [171] and a tachypacing-induced metabolic coronary vasodilation in rabbits in vivo [172], whereas H_2O_2 impairs endothelium-dependent NO-mediated dilation of porcine coronary microvessels by reducing L-arginine availability through upregulation of arginase [173].

End-effectors: K-channels

K-channels are classically recognized as key-regulators of vascular tone [174], which determines the resting membrane potential and serves as targets of endogenous and therapeutic vasodilators [175]. Opening of these channels results in an outward flux of potassium that increases the membrane potential of the sarcolemma. This hyperpolarization closes voltage-dependent calcium channels, leading to a decreased influx of calcium, thereby causing vasodilation [175, 176]. Four large families of K-channels have been identified: the inward rectifier K-channels, the two pore channels, the voltage sensitive K-channels, and the Ca^{2+}-sensitive K-channels [177], the latter two of which are the most prevalent classes [178]. These isoforms that determine the resting membrane potential vary among cells, segments and vascular beds [179]. In the coronary vasculature, BK_{Ca}, K_{ATP} and Kv channels are the major contributors to the vascular tone regulation. K_{ATP} and Kv channels are important in determing basal microvascular tone, whereas BK_{Ca} channels do not appear to contribute to resting coronary flow. BK_{Ca} and Kv channels participate in endothelium-dependent coronary vasodilation, while K_{ATP} appear to play no role [175]. In the pulmonary vasculature, activation of endothelial BK channels causes endothelium-

dependent vasodilation in micro- and macrovasculature in the lung [180], whereas K_{ATP} channels predominantly regulate conduit pulmonary arteries [181].

Cardiovascular diseases and coronary microvascular tone

With a simple monolayer, the endothelium is optimally placed and is able to respond to physical and chemical signals by production of a wide range of factors that regulate vascular tone, cellular adhesion, thromboresistance, smooth muscle cell proliferation, and vessel wall inflammation. The importance of the endothelium was first recognized by its effect on vascular tone. This is achieved by production and release of several vasodilators and vasoconstrictors, as well as by response to and modification of circulating vasoactive mediators such as bradykinin and thrombin. The vasomotion mediated by an interplay of those vasoactive factors plays a direct role in the balance of tissue oxygen supply and metabolic demand by regulation of vessel tone and diameter, and is also involved in the remodeling of vascular structure and long-term organ perfusion. Together, these factors work in concert to maintain vascular homeostasis [182]. On the other hand, impaired modulation of vascular growth, disregulation of vascular remodeling, altered anti-inflammatory response result from endothelial dysfunction. Among those an impairment of endothelium-dependent vasodilation caused by loss of NO-bioavailability has proposed to be the major feature of endothelial dysfunction. For example, impaired endothelium-dependent vasodilation in the coronary circulation of humans has profound prognostic implications in that it predicts adverse cardiovascular event and long-term outcome [183].

Cardiovascular disease is one of the main causes of deaths worldwide [184], though over the last two decades, cardiovascular mortality rates have declined in many high-income countries. At the same time, cardiovascular deaths and disease have increased at a fast rate in low- and middle-income countries [184]. In the Netherlands, cardiovascular diseases are responsible for 30% of all deaths and are the main cause of death in women [185]. In China, the mortality of heart disease in rural regions has approximatey doubled since 1988, with stroke as the largest cardiovascular killer at about 40% of total mortality [186]. On the other hand, improved treatment of cardiovascular diseases reduced acute mortality, but effectively increased the number of patients suffering from heart failure, which occurs when the adaptation mechanisms of the heart fail to compensate for stress factors, such as myocardial infarction (MI), pressure overload, and diabetes [187].

Heart failure is the final phase of cardiac dysfunction, which can be classically distinguished by systolic and diastolic heart failure. Systolic heart failure hearts undergo progressive expansion of the ventricle. This eccentric hypertrophy induced by volume-overload or MI is characterized by myocyte lengthening with sarcomeres added in series, resulting in a dilated heart [188]. Conversely, diastolic heart failure hearts undergo hypertrophy induced by pressure-overload (hypertension or aortic stenosis) with a marked increase in fibrosis, which leads to concentric remodeling without ventricular expansion [189] (Fig. 10).

Myocardial infarction. The most important cause of heart failure is MI. MI is caused by occlusion of a coronary artery resulting in ischemia of the downstream myocardial tissue. The reduction of coronary blood flow results in tissue hypoxia [190], an accumulation of metabolic products [191] and failure in substrate delivery [192] both in the ischemic area and in remote, non-infarcted areas. In the first hours after MI, myocyte necrosis, inflammation and edema are restricted to the ischemic area. This process is followed by scar formation in which fibroblast

proliferation and collagen deposition occur to replace the damaged tissue. Until the strengthening of the scarred region has completed, the infarcted region becomes thin and elongate without additional cell death, the so-called infarct expansion [193]. With the dilation of the ventricle, eccentric hypertrophy occurs in the remote non-infarcted area. Long-term ischemia and decompensated hypertrophy will lead to heart failure [187].

As tissue hypoxia occurs during the first phase of ischemia, coronary vasodilation is observed. Hypoxia is a well-documented vasodilator of coronary arteries and triggers the activities of several signaling pathways due to its effects on endothelial cells, vascular smooth muscle cells and cardiomyocytes [194]. On the one hand, the signals released from endothelial cells including NO and PGI_2, as well as the increased production of adenosine [9] account for the hypoxia-induced coronary vasodilation; on the other hand, changes in O_2 levels affect the production of superoxide and superoxide anion-derived hydrogen, and also a cGMP-mediated relaxation response [195]. However, as the coronary blood flow is reduced due to a constant ischemia, a paradoxical increase in microvascular resistance occurs [9]. This is evidenced by hypotension-induced increased coronary resistance blunted by ROS scavenge; however, vasodilation occurred when either synthesis of the EDRF NO or activity of the EDCF ET was inhibited, suggesting that both the NO and the ET systems are required for this vasoconstrictive response [196, 197]. Given the important role of NO-cGMP and ET systems in MI, the coronary vasoconstrictor role of PDE5, a hydrolyzed enzyme for cGMP, in health and MI will be discussed in Chapter 4, and the interaction between PDE5 and ET system will be discussed in Chapter 5; as a novel EDCF, the vasoactive role of Up_4A in the coronary vasculature in MI will be discussed in Chapter 8.

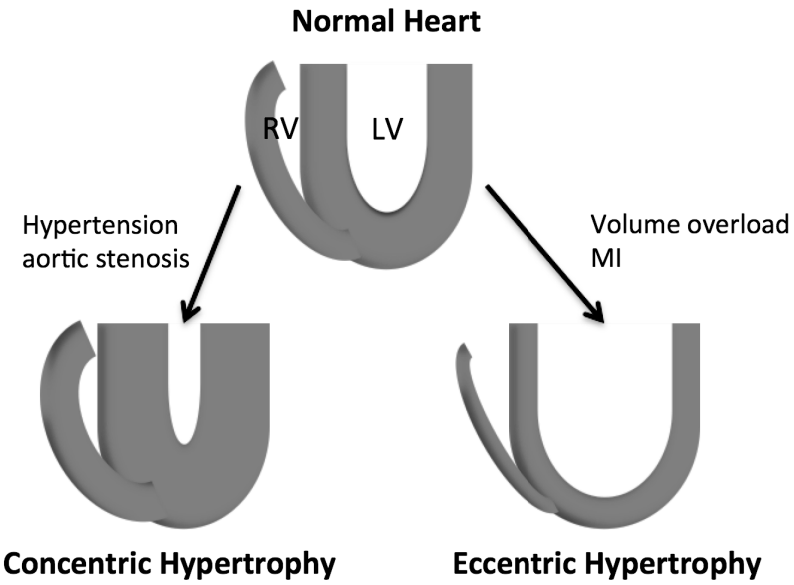

Fig. 10. *Different etiology (i. e. pressure-overload vs. volume-overload) results in different cardiac hypertrophy. Inadaptation to these two types of hypertrophy eventually leads to heart failure.*

Pressure overload. One of another major causes of heart failure is pressure overload, which is commonly caused by hypertension and aortic stenosis. Pressure overload-induced cardiac remodeling is characterized by myocyte thickening, normal or slightly decreased left ventricular

volume and parallel addition of sarcomeres [198, 199]. With pressure overload, the heart undergoes an increase in cardiomyocyte size, enhanced protein synthesis, and a higher organization of the sarcomere to sustain cardiac output. However, prolonged pressure overload, which becomes maladaptive and fails to maintain cardiac output, will evolve into pathological remodeling and thereby leads to heart failure [200, 201].

Long-term pressure-overload induced cardiac hypertrophy keeps the O_2 demand of the heart increasing, which results in a decrease in coronary flow reserve. Coronary flow reserve is a parameter that indicates the capacity of the coronary circulation to meet the O_2 demands of increased workloads of the heart; it depends on the resting and maximal flows. In other words, the arteries and arterioles fail to increase their cross-sectional areas to compensate for increased cardiac mass [9]. Thus, there are two ways that coronary vasodilator reserve can be compromised: 1) if coronary maximal flow is limited, e.g., by a failure of the resistance vessels to expand during hypertrophy and 2) if maximal flow is preserved but resting flow is increased to meet metabolic demands, e.g., when basal heart rate is increased. One of the major contributors to impaired coronary reserve is proposed to be locally generated substances, e.g., locally produced increased vasoconstrictors angiotensin II, endothelin, prostanoids, and some cytokines from the inflammatory cells and endothelial cells [202], as well as decreased vasodilator NO [203]. These substances not only increase coronary vascular tone but, in the long term, also cause vascular smooth muscle cells hypertrophy and perivascular collagen deposition. As one of those locally produced substances, the role of Up_4A in the coronary vasculature in the condition of pressure-overload will be discussed in detail in Chapter 9.

Diabetes mellitus. Diabetes is a group of metabolic diseases in which a person has high blood sugar, either because the pancreas does not produce enough insulin or because cells do not respond the insulin that is produced [204]. This high blood sugar produces the classical symptoms of frequent urination, increased thirst and increased hunger. There are three main types of diabetes: Type 1, type 2 and gestational diabetes, with different characteristics of the failure of insulin release from pancreas, the failure of utilization of insulin properly, and a high blood glucose level in pregnant women without previous diagnostics of diabetes, respectively. One of the main complications of diabetes is the presence of endothelial dysfunction, which is characterized by releasing more vasoconstrictors and less vasodilators from the endothelium of vessels. Due to the unique position at the border between blood and vascular tissue, endothelial cells are the first vascular cells to sense humoral changes. In diabetes, impaired insulin signaling through either phosphoinositide 3 kinase-Akt or RAS-mitogen activated protein pathway in the endothelial cells, with reduction of insulin-induced eNOS phosphorylation or insulin-mediated ET production causes attenuation of the insulin-induced capillary recruitment and insulin delivery, which in turn reduces glucose uptake by the skeletal muscle [205]. Therefore, endothelial dysfunction is an early sign of diabetic vascular disease. Since hyperglycemia has been identified as a well-recognized risk factors for vascular complications, hyperglycemia changes the balance of release of the endothelium-derived contracting and relaxing factors including NO, PGI_2, TXA_2 and ET [40, 206], which can further increase the risk of cardiovascular diseases including atherosclerosis, ischemic heart diseases and heart failure [207].

The heart of the diabetic individual faces a number of unique challenges, with the most notable feature of increased cardiac output [208]. Therefore, oxygen consumption and the need for perfusion increase. Parallel to these increases, the deleterious effects of the diabetes eventually lead to altered control of coronary vasomotor tone [209, 210]. One of the common mechanisms underlying increased coronary vascular resistance in diabetes is the production of ROS [56]. As

outlined in the earlier paragraph, ROS have been shown to react directly with NO. This consequently and directly reduces NO bioavailability but also alters eNOS functioning and leads to eNOS uncoupling. In this case, the important role of EDHF manifests where NO production is reduced. Thus, a reduced NO-mediated coronary vasodilation can be observed [211, 212], and the diminished NO-dependent dilation is compensated by EDHF [213]. In addition, an exaggerated secretion of constrictors is also involved. In the presence of ROS, peroxynitrite inhibits the prostacylcin synthase reducing PGI_2 release. This induces a shift in the arachidonic acid metabolism to generate vasoconstrictor prostanoids such as TXA_2 to promote coronary vasoconstriction [214, 215]. As a novel EDCF, the vasoactive role of Up_4A on coronary adaptation to diabetes, as well as the involvement of EDRF (NO and PGI_2), and EDHF (CYP 2C9 metabolites) in response to Up_4A will be discussed in Chapter 10.

Chemotherapy-induced hypertension

Chemotherapy uses chemical substances as part of a standardized regimen that act electively on cells in mitosis aiming to destroy cancer cells. The majority of chemotherapeutic drugs can be divided into alkylating agents, antimetabolites, anthracyclines, plant alkaloids, topoisomerase inhibitors, and other antitumor agents [216]. All of these drugs affect cell division or DNA synthesis. This means that chemotherapy also harms cells that divide rapidly under normal circumstances: cells in the bone marrow, digestive tract, and hair follicles, which results in the most common side-effects of myelosuppression, mucositis, and alopecia. During the last years novel-anticancer treatments targeting specific molecules or genes involved in cancer development are being developed to improve outcome and reduce side-effects. In particular several tyrosine-kinase inhibitiors (TKIs, gefitinib, erlotinib, sorafenib and sunitinib) have been approved for the treatment of certain tumors [217]. For instance, sunitinib, an orally available small-molecule multikinase inhibitor, is thought to act as an anti-tumor agent by inhibiting angiogenesis and potentially inhibiting VEGFR1/2, PDGFR and c-kit in addition to other kinases in biochemical and cell-based assays. In phase I studies, oral administration of doses up to 50 mg/day were reasonably tolerated and resulted in plasma concentrations in the range of targeted levels needed for sustained kinase inhibition. Biomarker and functional imaging studies showed a reduction in tumor metabolism. Sunitinib also showed clinical activity in patients with renal cell cancer, imatinib-resistant gastrointestinal stromal tumors, as well as in patients with breast, colon and lung cancer [69, 70, 218].

Unfortunately, inhibition of angiogenesis with tyrosine-kinase inhibitors is frequently accompanied by adverse effects including: the development of hypertension and cardiac and renal toxicity [218]. Hypertension has been observed in up to 60% of patients treated with sunitinib. In addition, impaired cardiac function, as reflected by a decrease in left ventricular ejection fraction of 10% to 15%, has been reported in up to 28% of patients receiving sunitinib [219]. Angina pectoris and increased levels of biomarkers reflecting ischemic myocardial damage may also occur during sunitinib treatment [219, 220]. In contrast to diseased conditions, VEGF plays a critical role in physiological vascular protection by inducing cell survival, synthesis of NO and PGI_2, and together with other vasoactive factors maintain vascular homeostasis [72]. Therefore, anti-VEGF in health can also lead to endothelial dysfunction resulting in vasoconstriction. Thus far, clinical and experimental studies on the cardiovascular effects of angiogenesis inhibition only focused on the systemic vasculature. Whether adverse effects of angiogenesis inhibition with sunitinib also occur in the pulmonary and/or coronary circulation is unknown.

Aim and outline of the thesis

By releasing multiple vasoactive factors, the endothelium plays a primary role in the regulation of vascular tone. However, the same vasoactive factor released from the endothelium may result in different vascular influence, due to the heterogeneity of the endothelium as well as different vascular conditions. Knowledge about the regulation of vascular tone by a number of individual EDRFs and EDCFs and other vasoactive substances, as well as the different vascular reactions by those factors is necessary to treat diseases. The general aim of this thesis is 1) to investigate the complex regulation of pulmonary, systemic and coronary circulations, in health and disease (MI, pressure-overload-induced cardiac hypertrophy and diabetes) of an intact animal model of swine as well as in isolated small arteries from pulmonary and coronary vascular beds; and 2) to compare the different vasoactive influence, as well as to elucidate underlying mechanisms behind the phenomenon.

VEGF plays a critical role in physiological angiogenesis and acts as a vasodilator by producing NO and PGI_2 [67, 72]. Similarly, tumor growth is accompanied by increased pathological angiogenesis to supply efficient nutrient. With this concept, anti-VEGF therapy has been widely used in clinic for various cancer treatments. Although it is, to some extent, beneficial to cancer patients, the side effect of development of systemic hypertension in a long-term treatment by inducing vasoconstriction, has caused much concern [221]. Given the role of VEGF in vascular protection by producing NO and PGI_2, anti-VEGF in healthy condition can also result in endothelial dysfunction, thereby inducing vasoconstriction [72]. However, whether the anti-VEGF therapy also has adverse effects on the pulmonary and coronary circulations is largely unknown. Consequently, in **Chapter 2** we investigated the effects of sunitinib, a tyrosine kinase inhibitor, on the pulmonary, systemic and coronary circulations, as well as the mechanisms underlying the development of hypertension.

The vasodilation persists after blockade of two major vasodilator pathways: NO and PGI_2, suggesting a third vasodilator pathway that is independent on endothelium exists. This third pathway is recognized as EDHF [45]. An increasing number of studies demonstrate that EDHF plays an important role in the regulation of vascular tone, particularly its compensatory function for the loss of NO in many diseased conditions [45, 52, 56]. However, most of the studies regarding the role of EDHF are performed in vitro; little is known about its endogenous function in vivo. Therefore, we studied the endogenous contribution of CYP 2C9 metabolites, one of the putative source of EDHFs [59], to the coronary circulation (**Chapter 3**) in vivo, and elucidated the role of CYP 2C9 metabolites in a more mechanistic manner in vitro.

NO-mediated vasodilation is through the production of downstream second messenger cGMP. The cGMP is short-lived, because it is hydrolyzed by PDE [149]. The Activity of those enzymes determines the amount of cGMP thereby regulating vascular tone. In **Chapter 4** we studied the effect of PDE5 inhibition on the coronary circulation in healthy swine as well as in swine with myocardial infarction. Since either PDE5 inhibitor such as sildenafil or ET blocker such as bosentan has been used as a therapeutic tool in treating patients with heart failure and pulmonary hypertension [222], little is known about whether the combination of those two treatments may have additional therapeutic effects, as well as the controversial concern about the combined treatment on induction of systemic hypotension. Therefore, we investigated the effects of combined treatment of PDE5 inhibition and ET blockade in the coronary (**Chapter 5**) and pulmonary circulations (**Chapter 6**), as well as the mechanism of interaction between the PDE5 and ET systems.

In addition to ET, Up_4A has been recently identified as a potent endothelium-derived contracting factor [90]. This factor is endogenously released from endothelium upon stimuli such as shear stress, acetylcholine and ET [90], and exerts a vasoconstrictor influence in various vascular beds including pulmonary, aortic, renal and mesenteric vasculatures [223]. Since the effect of Up_4A on the coronary vasculature had not been addressed previously, we investigated the effect of Up_4A on the coronary vascular tone by using isolated coronary small artery from healthy swine (**Chapter 7**). The plasma concentration of Up_4A dramatically increases in hypertensive subjects as compared to healthy subjects, suggesting a role for Up_4A in the development of cardiovascular diseases [102]. Consequently, in the last part of the thesis we focused on the effects of Up_4A on the coronary vascular adaptation in diseased conditions including myocardial infarction (**Chapter 8**), pressure overload-induced hypertrophy (**Chapter 9**) and diabetes (**Chapter 10**).

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Chapter 2

Sunitinib induced systemic vasoconstriction in swine is endothelin-mediated and does not involve NO or oxidative stress

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Abstract

Angiogenesis inhibition with agents targeting tyrosine kinases of vascular endothelial growth factor receptors (VEGFR) is an established anticancer treatment, but is, unfortunately, frequently accompanied by systemic hypertension and cardiac toxicity. Whether VEGFR-antagonism also has adverse effects on the pulmonary and coronary circulations is presently unknown. In chronically instrumented awake swine, the effects of the VEGFR-antagonist sunitinib on the systemic, pulmonary and coronary circulation were studied. One week after sunitinib (50 mg p.o. daily) mean aortic blood pressure (MABP) had increased from 83±5 at baseline to 97±6 mmHg (P<0.05) due to a 57±20% increase in systemic vascular resistance as cardiac output decreased. In contrast, sunitinib had no discernible effects on pulmonary and coronary hemodynamics or cardiac function. We subsequently, investigated the mechanisms underlying the sunitinib-induced systemic hypertension. Intravenous administration of NO-synthase inhibitor N^{ω}-nitro-L-arginine increased MABP by 24±1 mmHg under baseline conditions, while it increased MABP even further following sunitinib administration (32±3 mmHg, P<0.05). Reactive oxygen species scavenging with a cocktail of antioxidants lowered MABP by 13±2 mmHg before, but only by 5±2 mmHg (P<0.05) after sunitinib administration. However, intravenous administration of the dual ET_A/ET_B receptor blocker tezosentan, which did not lower MABP at baseline, completely reversed MABP to pre-sunitinib values. These findings indicate that sunitinib produces vasoconstriction selectively in the systemic vascular bed, without affecting pulmonary or coronary circulations. The sunitinib-mediated systemic hypertension is principally due to an increased vasoconstrictor influence of endothelin, with no apparent contributions of a loss of NO-bioavailability or increased oxidative stress.

Introduction

Angiogenesis inhibition, by targeting the tyrosine kinases of the vascular endothelial growth factor (VEGF) receptors (VEGFRs), has become an established treatment of several tumor types. This therapy is associated with adverse effects including the development of hypertension and cardiac and renal toxicity [1]. Hypertension has been reported in up to 60% of patients treated with sunitinib, an orally active multitarget receptor tyrosine kinase inhibitor (RTKI), targeting amongst others the VEGFR-1 and -2, that is used as first-line treatment of metastatic renal cell carcinoma or imatinib-resistant gastrointestinal stromal tumors [1]. In addition, impaired cardiac function, as reflected by a decrease in left ventricular ejection fraction (LVEF) of 10-15%, has been observed in up to 28% of patients treated with sunitinib [2]. Angina pectoris and increased levels of biomarkers reflecting ischemic myocardial damage may also occur during sunitinib treatment [2, 3]. Thus far, clinical and experimental studies on the cardiovascular side effects of angiogenesis inhibition only focused on the systemic vasculature. Whether adverse effects of angiogenesis inhibition with sunitinib also occur in the pulmonary and/or coronary circulation is unknown. It should be mentioned that pulmonary arterial hypertension has recently been reported during treatment with dasatinib, a RTKI used for the treatment of chronic myeloid leukemia [4].

VEGF, through activation of VEGFR-2, stimulates endothelial NO synthase (eNOS) resulting in enhanced NO production and vasodilation [5, 6]. It has therefore been suggested that NO bioavailability is reduced during inhibition of the VEGF-pathway, resulting in vasoconstriction and the development of hypertension. Indeed, in mice administration of an anti-VEGFR-2 antibody, attacking the same receptor as RTKIs, caused a rapid increase in mean aortic blood pressure (MABP) and a marked reduction in the expression of endothelial and neuronal NOS in the kidney [7]. Furthermore, prior administration of the eNOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) in mice abolished the difference in MABP between vehicle and anti-VEGFR-2 treated groups, suggesting that decreased NO-bioavailability in response to anti-angiogenic agents is one of the mechanisms causing hypertension [7]. However, clinical studies, using flow-mediated dilatation (FMD) as an index of NO-bioavailability, do not unequivocally support the hypothesis that a decrease in NO-bioavailability underlies the increase in MABP, as decreases in both endothelium-dependent and endothelium-independent vasodilatation have been reported in patients treated with RTKIs [8, 9]. In previous clinical and experimental studies we and others have shown that activation of the endothelin system is involved in the sunitinib induced rise in MABP [10-12]. Apart from inducing vasoconstriction, ET-1 activates vascular NADPH-oxidase, leading to an increase in oxidative stress through enhanced reactive oxygen species (ROS) production [13, 14]. Since oxidative stress is generally increased in hypertension and plays a pathogenic role in the development and progression of cardiovascular disease, we hypothesized that enhanced formation of ROS, contributes to the sunitinib-induced cardiovascular side effects [15].

To further explore the cardiovascular side effects of sunitinib we performed detailed studies in chronically instrumented awake swine. Firstly, we studied the effects of angiogenesis inhibition on the systemic, pulmonary and coronary circulations, as well as on cardiac performance under resting conditions and during exercise. Secondly, we explored to what extent alterations in NO-mediated vasodilator tone, ET-1-mediated vasoconstriction and oxidative stress contribute to the cardiovascular side effects of angiogenesis inhibition.

Methods

Animals

Studies were performed in accordance with the American Physiological Society's "Guiding Principles in the Care and Use of Laboratory Animals" and with approval of the Animal Care Committee of Erasmus MC. Six crossbred Yorkshire × Landrace swine of either sex (2 to 3 months old; 21±1 kg at the time of surgery) were entered into the study.

Surgical procedures

Swine were sedated (20 mg/kg ketamine im + 1 mg/kg midazolam im), anesthetized (thiopental sodium 15 mg/kg iv), intubated and ventilated with a mixture of O_2 and N_2 (1:2) [16]. Anesthesia was maintained with midazolam (2 mg/kg + 1 mg kg^{-1} h^{-1} iv) and fentanyl (10 μg kg^{-1} h^{-1}, iv). Subsequently animals were instrumented under sterile conditions as previously described [17, 18]. Briefly, a thoracotomy was performed through the left fourth intercostal space. Subsequently, a polyvinylchloride catheter was inserted into the aortic arch, for the measurement of aortic pressure and blood sampling for the determination of PO_2, PCO_2, pH, O_2 saturation and hemoglobin concentration (ABL800, Radiometer). A high fidelity Konigsberg pressure transducer was inserted into the left ventricle (LV) via the apex for measurement of LV pressure and maximum rate of rise and fall of LV pressure ($LVdP/dt_{max}$, $LVdP/dt_{min}$) as indices for contractility and relaxation. Fluid-filled catheters were implanted for measurement of blood pressures in the LV, left atrium (LA) and pulmonary artery (PA). Flow probes were placed around the proximal left anterior descending coronary artery (LAD, 2.5-3 mm, Transonic Systems) for measurement of coronary blood flow (CBF) and around the aorta (16 mm, Transonic Systemic) for measurement of cardiac output and stroke volume (SV) [17, 18]. Electrical wires and catheters were tunneled subcutaneously to the back. The chest was closed and the animals were allowed to recover. Animals received analgesia (0.3 mg buprenorphine im) for 2 days and antibiotic prophylaxis (25 mg/kg amoxicillin and 5 mg/kg gentamycin iv) for 5 days.

Experimental protocols

Sunitinib

Studies were performed 1-4 weeks after surgery with swine resting and/or exercising on a motor-driven treadmill. Excellent reproducibility of exercise trials has been reported previously [17, 18]. After obtaining hemodynamic measurements with swine lying quietly on a treadmill, a five-stage (1-5 km/h) treadmill exercise protocol was started with each exercise stage lasting 2-3 min. Hemodynamic variables were continuously recorded and both arterial and mixed venous blood samples were collected during the last 60 s of each exercise stage for the determination of body oxygen consumption [18]. Sunitinib L-malate was given in a daily oral dose of 50 mg, i.e. the same dose as used in patients. Sunitinib was mixed with food and administered early in the morning. Resting hemodynamics were measured 4 hours after the first dose of sunitinib and after 1 week of daily sunitinib administration. After 1 week of sunitinib administration the exercise protocol was repeated to assess the effects of sunitinib on exercise-induced hemodynamic responses.

Acute pharmacological interventions

After measuring systemic, pulmonary and coronary hemodynamics at rest acute intravenous doses of an endothelial NO-synthase (NOS) inhibitor, a dual ET_A and ET_B receptor ($ET_{A/B}$) antagonist or a ROS scavenger cocktail were given on separate days in random order and

cardiovascular effects were assessed. Subsequently swine were exposed to sunitinib for 7-10 days and the pharmacological interventions were repeated, in random order on separate days, to assess the roles of NO, ET-1 and ROS in sunitinib-induced cardiovascular effects.

Pharmacological agents

The eNOS inhibitor N^{ω}-nitro-L-arginine (LNNA (Sigma)) was given as a single intravenous dose of 20 mg/kg [19]. The dual ET_A and ET_B receptor (ET_A/ET_B) antagonist tezosentan (a kind gift from Dr Clozel, Actelion Pharmaceuticals Ltd.) was intravenously administered over 10 min in a dose of 3 mg/kg, followed by a continuous infusion of 6 mg kg^{-1} h^{-1} iv [20]. For ROS scavenging, the superoxide dismutase (SOD)-mimetic 4-Hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPOL, 30 mg/kg iv), in combination with N-Acetyl-cysteine (NAC, 150 mg/kg iv), and N-Mercaptoproprionylglycine (MPG, 1 mg/kg/min iv) were infused in 10 minutes prior to the measurements [21-24].

Determination of ET

ET was assessed in plasma from 4 swine, obtained before and after sunitinib, using a chemiluminescent ELISA according to the manufacturer's protocol (QuantiGlo®, R&D Systems).

Data acquisition and analysis

Digital recording and off-line analysis of hemodynamic data and computation of body O_2 consumption (BVO_2) have been described in detail elsewhere [18]. To correct for growth of swine, cardiac index (CI) and stroke volume index (SVi) were calculated as cardiac output and stroke volume, respectively, divided by body weight. Systemic vascular resistance index (SVRi) was computed as MABP divided by CI. Pulmonary vascular resistance index (PVRi) was computed as mean pulmonary artery pressure (MPAP) minus mean left atrial pressure (MLAP) divided by CI. Finally, coronary vascular resistance (CVR) was calculated as MABP divided by CBF. Statistical analysis was performed using regression analysis with each animal as a dummy variable and with BVO_2, heart rate, as well as sunitinib as independent variables. Statistical significance was accepted at $P \leq 0.05$. Data are presented as mean±SEM.

Results

Effect of sunitinib at rest

Oral administration of the first dose of sunitinib resulted in an increase in MABP within 4 hours in 5 out of 6 animals but did not result in a change in plasma ET levels (Table 1). The increase in MABP was associated with an increase in LVSP, but $LVdP/dt_{max}$, $LVdP/dt_{min}$ and SVi did not change (Table 1). Also, no change in CBF occurred, while CVR slightly increased, likely reflecting an autoregulatory increase in coronary vasomotor tone in response to the increase in MABP. The first dose of sunitinib had no effect on either PAP or PVRi (Table 1).

Seven days of daily sunitinib administration resulted in sustained systemic vasoconstriction as evidenced by an increase in SVRi and MABP in all animals (Table 1). The increase in MABP was accompanied by a decrease in heart rate and CI and an increase in LVSP, while $LVdP/dt_{max}$, $LVdP/dt_{min}$ and SVi were unchanged. Repeated doses of sunitinib had no effect on the coronary or the pulmonary vasculature, as CBF, CVR, MPAP and PVRi were similar to values before sunitinib administration (Table 1). Moreover, plasma ET levels were not significantly altered following one week of sunitinib (Table 1).

Tabel 1. Resting hemodynamic responses prior to and 4 hours and 7 days after sunitinib administration.

Parameters	Baseline	4h Sunitinib	1wk Sunitinib
HR (beats/min)	112 ± 6	111 ± 10	105 ± 7*
MABP (mmHg)	83 ± 5	93 ± 5*	97 ± 6*
CI (ml min^{-1} kg^{-1})	155 ± 11	162 ± 17	132 ± 12*
SVRi (mmHg L^{-1} min kg)	547 ± 45	607 ± 69	780 ± 108*
LVSP (mmHg)	110 ± 6	115 ± 8	116 ± 10
SVi (ml kg^{-1})	1.38 ± 0.06	1.46 ± 0.06*	1.26 ± 0.10
LV dP/dt_{max} (mmHg s^{-1})	2800 ± 460	2870 ± 260	2620 ± 250
LV dP/dt_{min} (mmHg s^{-1})	-2220 ± 160	-2280 ± 130	-2320 ± 190
MPAP (mmHg)	15 ± 2	17 ± 2	14 ± 2
MLAP (mmHg)	5 ± 2	7 ± 2	4 ± 1
PVRi (mmHg L^{-1} min kg)	64 ± 12	59 ± 5	68 ± 6
CBF (ml/min)	48 ± 2	44 ± 2	47 ± 3
CVR (mmHg ml^{-1} min)	1.64 ± 0.14	1.90 ± 0.23	2.00 ± 0.17*
ET (pg/ml)	2.4 ± 0.6	2.5 ± 0.7	2.8 ± 1.0

*Values are mean±SEM. HR, heart rate; MABP, mean blood pressure; CI, cardiac index; SVRi, systemic vascular resistance index; LVSP, left ventricular systolic pressure; SVi, stroke volume index; LV dP/dt, left ventricular rate of change in pressure; MPAP, mean pulmonary artery pressure; MLAP, mean left atrial pressure; PVRi, pulmonary vascular resistance index; CBF, coronary bloodflow; CVR, coronary vascular resistance; ET, plasma ET levels. * P <0.05 vs. baseline.*

Effect of sunitinib on hemodynamic parameters during exercise

Sunitinib did not influence the exercise-induced increase in heart rate or CI, while the sunitinib-induced increase in MABP and SVRi decreased with increasing exercise intensity (Fig. 1), indicating that the vasoconstrictor effect of sunitinib diminished with increasing exercise intensity. Sunitinib also had no effect on MPAP, PVRi or CBF during exercise, while the sunitinib-induced increase in CVR decreased with exercise-intensity (Fig. 1), reflecting the waning effect of sunitinib on MABP during exercise.

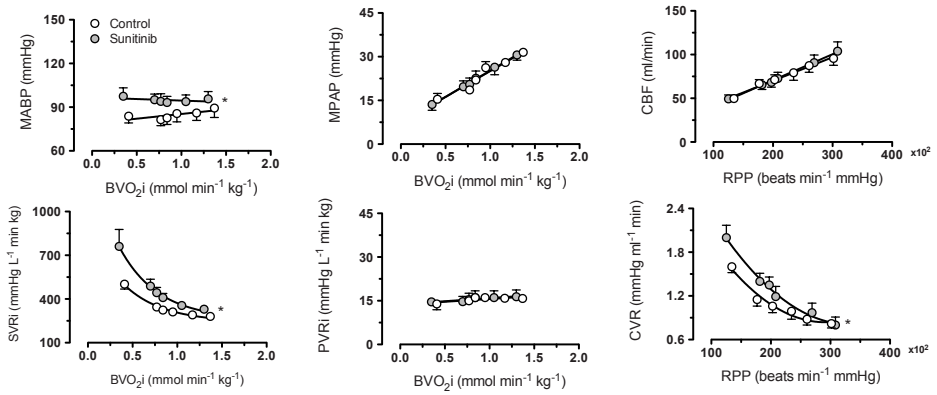

Fig. 1. *Effect of sunitinib on systemic, pulmonary and coronary hemodynamic parameters at rest and during graded treadmill exercise. BVO_2i: Body oxygen consumption; MABP: mean blood pressure; SVRi: systemic vascular resistance; MPAP: mean pulmonary artery pressure; PVRi: pulmonary vascular resistance; RPP: rate pressure product; CBF: coronary blood flow; CVR: coronary vascular resistance; * P<0.05 sunitinib vs. control.*

Effect of NO, ET and ROS inhibition on hemodynamic parameters during sunitinib

Administration of LNNA resulted in systemic vasoconstriction as evidenced by an increase in SVRi that resulted in increases in MABP and LVSP (Fig 2). The increase in MABP was associated with a, likely baroreflex-mediated decrease in heart rate and CI (Table 2). The effect of LNNA on systemic hemodynamics was slightly more pronounced after 1 week of sunitinib treatment (Fig. 2). Under baseline conditions, LNNA also increased PVRi, reflecting pulmonary vasoconstriction, while the effect of LNNA on PVRi after sunitinib treatment failed to reach statistical significance (P=0.13, Fig. 2). LNNA had no effect on CBF, but CVR increased. Sunitinib did not affect the LNNA-induced increase in CVR (Fig. 2).

Contrary to previous findings from our laboratory, in which $ET_{A/B}$ receptor blockade with tezosentan did result in a slight decrease in MABP, tezosentan had no effect on MABP or SVRi prior to sunitinib in the present study (Fig. 2). In contrast, in the presence of sunitinib, MABP and SVRi decreased in response to tezosentan administration (Fig. 2). The tezosentan-induced decrease in SVRi was identical to the increase in SVRi induced by sunitinib. In the absence of sunitinib, administration of tezosentan resulted in a decrease in PVR. This tezosentan-induced pulmonary vasodilation was not altered by administration of sunitinib (Fig. 2). Tezosentan had no effect on the coronary vasculature (Fig. 2) or on LVSP, $LVdP/dt_{max}$, $LVdP/dt_{min}$ and SVi in the absence of sunitinib, while SVi increased slightly during sunitinib administration (Table 2).

Table 2. Changes in hemodynamic parameters as a result of eNOS inhibition, $ET_{A/B}$ blockade and ROS scavenging in the absence and presence of sunitinib.

Parameter	Sunitinib	LNNA	Tezosentan	ROS
ΔHR	Control	-11 ± 7	6 ± 3	22 ± 8*
(beats/min)	Sunitinib	-24 ± 3*†	0 ± 5	22 ± 7*
ΔMABP	Control	24 ± 1*	0 ± 3	-13 ± 2*
(mmHg)	Sunitinib	32 ± 3*†	-12 ± 4*	-5 ± 2*†
ΔCI	Control	42 ± 8*	12 ± 5	35 ± 6*
(ml min^{-1} kg^{-1})	Sunitinib	47 ± 8*	13 ± 4*	22 ± 4*
ΔLVSP	Control	18 ± 5*	4 ± 3	-9 ± 5
(mmHg)	Sunitinib	24 ± 7*	-9 ± 6	-10 ± 3*
ΔSVi	Control	-0.23 ± 0.10	0.02 ± 0.04	0.05 ± 0.06
(ml/kg)	Sunitinib	-0.16 ± 0.07	0.10 ± 0.03*	-0.03 ± 0.06
Δ$LV\ dP/dt_{max}$	Control	-123 ± 216	-20 ± 123	158 ± 152
(mmHg/s)	Sunitinib	-580 ± 342	217 ± 69*	118 ± 129
Δ$LV\ dP/dt_{min}$	Control	-185 ± 46*	-27 ± 135	22 ± 78
(mmHg/s)	Sunitinib	-202 ± 70*	82 ± 170	156 ± 69†

*HR, heart rate; MABP, mean blood pressure; CI, cardiac index; LVSP, left ventricular systolic pressure; SVi, stroke volume index; LVdP/dt, left ventricular rate of change in pressure; LNNA, N^{ω}-nitro-L-arginine; ROS, reactive oxygen species scavenging. * P<0.05 effect of LNNA, Tezosentan or ROS scavenging vs. corresponding baseline; † P<0.05 effect of LNNA, Tezosentan or ROS scavenging altered as a result of sunitinib.*

Prior to sunitinib, ROS scavenging resulted in systemic vasodilation as evidenced by the decreases in MABP and SVRi (Fig. 2). The effect of ROS scavenging on MABP but not SVRi was reduced following sunitinib (Fig. 2). In the pulmonary circulation, ROS scavenging resulted in a decrease in PAP prior to sunitinib, while it had no effect on PAP in the presence of sunitinib. Moreover, ROS scavenging did not affect PVRi either in the absence or presence of sunitinib (Fig. 2). ROS scavenging had no effect on CBF either before or after sunitinib. Finally, ROS scavenging did not affect dP/dt_{max}, dP/dt_{min} or SVi either before or after sunitinib (Table 2).

Discussion

The main findings of the present study in chronically instrumented awake swine are (*i*) administration of the RTKI sunitinib induces a rapid rise in systemic BP due to an increase in systemic vascular resistance, without affecting the pulmonary or coronary circulation; (*ii*) the vasoconstrictor effect of sunitinib on the systemic circulation waned with increasing exercise intensities; (*iii*) sunitinib had no adverse effects on cardiac function either at rest or during exercise; (*iv*) the systemic hemodynamic effects of sunitinib were fully reversed upon administration of the ET_A/ET_B receptor blocker tezosentan, confirming previous findings that an increase in endogenous ET-mediated vasoconstrictor tone is involved in the sunitinib-induced rise in MABP; and finally, (*v*) no evidence was found that a decrease in NO-bioavailability or an increase in oxidative stress contributed to the systemic vasoconstriction produced by sunitinib [10-12].

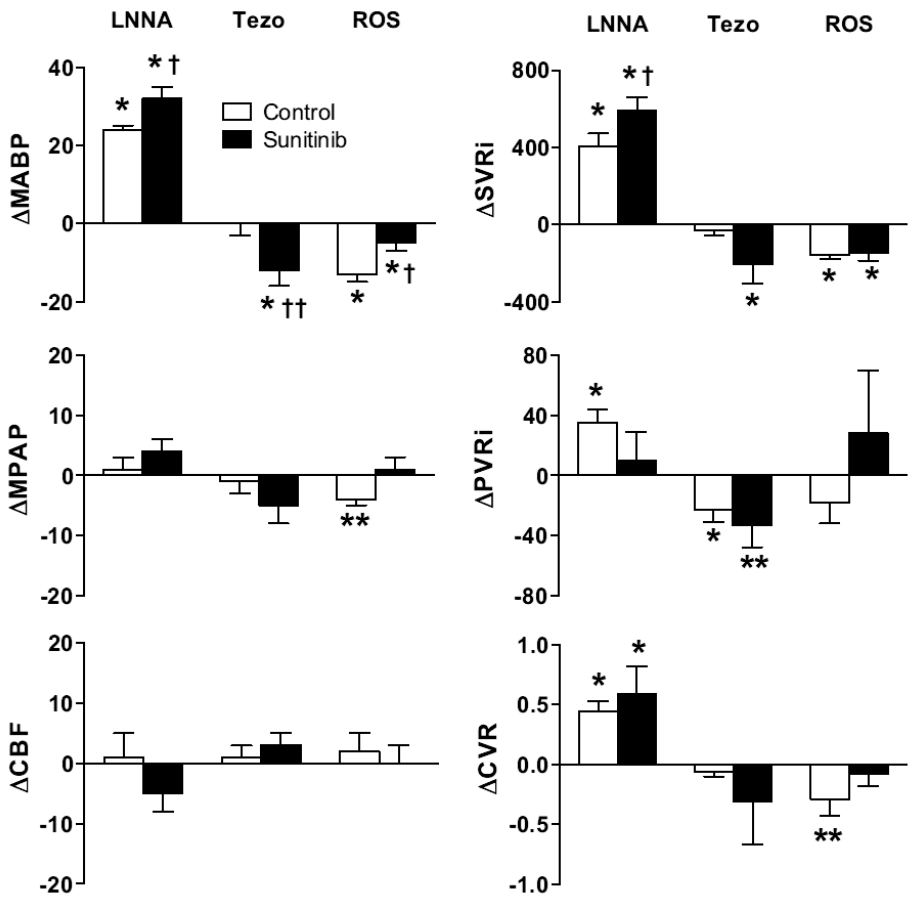

Fig. 2 *Effect of inhibition of eNOS with LNNA, $ET_{A/B}$ receptor blockade with tezosentan (tezo) and ROS scavenging on systemic, pulmonary and coronary hemodynamic parameters in the absence and presence of sunitinib. Data are shown as changes from corresponding baseline. MABP: mean blood pressure; SVRi: systemic vascular resistance; MPAP: mean pulmonary artery pressure; PVRi: pulmonary vascular resistance; CBF: coronary blood flow; CVR: coronary vascular resistance; * P<0.05; ** P<0.10 vs. baseline; † P<0.05, †† P<0.10, effect after sunitinib vs. effect in control.*

In accordance with data in both rats and patients, administration of sunitinib induced a rapid and sustained increase in systemic MABP in swine [10-12]. Since VEGF is not only important for the formation, but also for the remodeling of blood vessels, a sunitinib-induced decrease in microvessel density per unit of volume has been proposed as a potential mechanism underlying the MABP rise during anti-angiogenic therapy [5]. Indeed, prolonged treatment with the RTKIs sunitinib and telatinib has been reported to be associated with capillary rarefaction [9, 25, 26]. To increase vascular resistance in a particular vascular bed by 5%, it has been estimated that 40% rarefaction of fourth order vessels is required [27]. It is inconceivable that such an extensive degree of systemic rarefaction can occur within 4 h after initiation of angiogenesis inhibition. In addition, the current observations that the sunitinib-induced systemic vasoconstriction diminished with increasing levels of exercise and the immediate normalization of MABP and systemic vascular resistance with subsequent ET_A/ET_B blockade, further argue against rarefaction as a mechanism underlying the MABP rise associated with angiogenesis inhibition (Fig. 2). Therefore, we consider vasoconstriction the most important mechanism involved in the MABP rise during anti-angiogenesis therapy.

To explore potential mechanisms underlying the sunitinib-induced rise in vascular resistance and MABP we investigated the acute effects of ET_A/ET_B receptor blockade, eNOS inhibition and ROS scavenging prior and following one week of sunitinib treatment. We have previously shown that administration of sunitinib in patients and rats is associated with an increase in circulating ET-1 levels [2]. In the present study, sunitinib did not significantly alter plasma ET-levels although an increase in plasma ET was observed in 3 out of 4 animals. Since ET-secretion is predominantly abluminal [28], its plasma level does not adequately reflect its effect on vascular tone. This effect should therefore preferentially be assessed using an ET receptor antagonist, as performed in the present study. We and others have demonstrated in rats that co-administration of an ET-1 receptor antagonist with a RTKI can largely or completely prevent the rise in MABP, suggesting that activation of the endothelin pathway plays an important role in the sunitinib-induced MABP rise [11, 12]. These observations in rats are fully supported by the present findings in swine, as the sunitinib-induced increase in SVRi and MABP completely reversed to pre-sunitinib values in response to acute administration of $ET_{A/B}$ receptor blocker tezosentan.

ET-1 exerts part of its vasoconstrictor effect through activation of NADPH-oxidase and generation of ROS [29-31]. In addition, hypertension has been reported to be associated with an increase in ROS [15]. Since ROS can be derived from multiple sources within the circulation, including NADPH-oxidase, mitochondrial respiration, and neutrophils, we used a cocktail of antioxidants, including the SOD-mimetic TEMPOL, to achieve extensive ROS scavenging [24, 32]. Using this approach, the present study shows that the effect of ROS-scavenging on MABP was reduced, while the effect on SVRi was unchanged following 1 week of sunitinib treatment. The apparent discrepancy between the effect of ROS scavenging on MABP and SVRi is most likely due to the reduction in CI following sunitinib. As SVRi is calculated as the ratio of MABP and CI, a smaller change in blood pressure can result in a similar change in SVRi. Importantly, no evidence for an *increase* in sunitinib-induced ROS-mediated vasoconstriction was found. Of note, activation of NADPH-oxidase is also a critical step in the VEGF-receptor signaling cascade [33]. If this pathway is blocked following sunitinib administration, it is possible that activation of NADPH-oxidase via the ET-1 pathway is offset by a decreased activation of NADPH-oxidase due to VEGF-receptor inhibition.

Since VEGF is known to stimulate eNOS via the phosphatidylinositol 3-kinase-AKT pathway through activation of the VEGFR-2 receptor, the enhanced vasoconstrictor response to

acute eNOS inhibition after sunitinib administration observed in the present study was unexpected [6, 34, 35]. This finding implies that the sunitinib-induced rise in MABP is accompanied by an increase in NO-dependent vasodilator tone. Given the complex interaction between NO and ET, it is possible that the enhanced NO-mediated vasodilator tone is due to increased ET_B receptor stimulation while simultaneously limiting ET-induced vasoconstriction [36]. An alternative explanation may be the use of young, healthy swine in the present study, while patients with cancer are generally older and may have endothelial dysfunction. By comparing the response to eNOS inhibition before and after sunitinib we aimed to correct for age and potential underlying vascular disease. Nevertheless, it is possible that the increase in vascular tone in response to sunitinib resulted in an increase in shear stress, thereby enhancing NO production [37], and that this effect is more pronounced in young healthy swine as compared to patients with underlying cardiovascular disease. Indeed, sunitinib-treatment for 8 days was accompanied by a decreased urinary nitrate excretion in adult rats and in patients receiving various inhibitors of the VEGF-pathway and the RTKI vandetanib decreases systemic plasma nitrate/nitrite levels in treated patients [8, 12, 38]. Results of clinical studies using FMD of the brachial artery as an index of NO bioavailability are conflicting. In one study administration of the RTKI telatinib was associated with a decrease in FMD from 6.0% to 3.9%, while in another study administration of the RTKI vandetanib had no effect on FMD (12.0% before and 13.8% after vandetanib) [8, 9]. In the study reported by Steeghs *et al.* the vasodilation to nitroglycerin was also reduced (from 17.0% to 9.7%) to a comparable extent as the decrease in FMD, indicating a diminished response of the vascular smooth muscle cells to NO rather than a selective decrease in NO-bioavailability [9]. In the coronary microcirculation of rats exposed to sunitinib for 8 days we found, using the Langendorff heart model, that the vasodilator responses to both bradykinin (endothelium-dependent) and sodium-nitroprusside (endothelium-independent) were impaired [10]. Remarkably, in this model we also found an attenuated vasoconstrictor response to angiotensin II. These findings suggest generalized impairment of vascular smooth muscle function during sunitinib administration that is still unexplained. Part of the variable findings between different studies may be explained by the fact that the various RTKIs have different target receptors [1], thereby affecting the NO pathway to a greater or lesser extent. Altogether, until now evidence is lacking to conclude that a decrease in NO-bioavailability is a predominant factor in RTKI-induced vasoconstriction.

Our study is the first to investigate potential adverse effects of the RTKI sunitinib on the pulmonary vasculature. Contrary to its vasoconstrictor effect in the systemic circulation, sunitinib did not cause pulmonary vasoconstriction either at rest or during exercise. Variation in expression and function of ET_A and ET_B receptors between these two circulations may provide an explanation for this difference. The ET_A receptor predominates in the systemic and coronary vasculature, whereas the ET_B receptor predominates in the pulmonary microcirculation, where it is present on both the endothelium and the vascular smooth muscle cells and also functions as a clearance receptor [39]. In a previous study, using the same animal model as in the present study, we found that the dose of ET-1 required to induce vasoconstriction in the systemic circulation is lower than the dose to induce pulmonary vasoconstriction [20]. Apparently, the sunitinib-induced activation of the endothelin-pathway was not sufficient to induce pulmonary vasoconstriction thereby precluding adverse effects of RTKI on the pulmonary circulation.

In the present study administration of sunitinib was without adverse effects on stroke volume or LVdP/dtmax and LVdP/dtmin, indices of left ventricular systolic and diastolic function, either at rest or during exercise. Moreover, no evidence was found for coronary vasoconstriction as CBF did not change during sunitinib administration. In contrast, sunitinib

treatment of patients with imatinib-resistant, metastatic gastrointestinal stromal tumors was associated with the development of congestive heart failure in 8% of patients and a decrease in LVEF of at least 10% in 28% of patients [2]. This discrepancy might in part be explained by the difference in exposure time to sunitinib as well as the fact that our intervention was performed in young and healthy animals, whereas in the clinical study pre-existent coronary artery disease remained as the only statistically significant predictor for congestive heart failure in a multivariate logistic regression model.

Perspectives

Angiogenesis inhibition with sunitinib induces a marked rise in MABP, which is independent of changes in endogenous ROS and NO production, but completely reversed by endothelin receptor antagonism with tezosentan. These findings support previous observations that sunitinib-induced hypertension is endothelin-mediated, whereas decreased NO-bioavailability or enhanced oxidative stress do not appear to be involved. Since we recently showed that endothelin receptor antagonism could also prevent sunitinib-induced proteinuria and urinary ET-1 excretion (markers of renal injury), endothelin receptor antagonists appear logical candidates to counteract the sunitinib-induced cardiovascular and renal adverse effects [12]. In addition, ET-1 has been shown to promote angiogenesis in cancer and therefore ET-1 receptor antagonism may have complementary therapeutic effects [40, 41]. However, carefully conducted clinical trials are required to demonstrate whether indeed endothelin receptor antagonists may be the preferred agents to treat the cardiovascular and renal side effects associated with anti-angiogenesis therapy in patients with cancer.

Disclosures

None

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Chapter 3

Cytochrome P450 2C9 exerts a vasoconstrictor influence on coronary resistance vessels in swine at rest and during exercise

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Abstract

A significant endothelium-dependent vasodilation persists after inhibition of nitric oxide synthase (NOS) and cyclo-oxygenase (COX) in the coronary vasculature, which has been linked to the activation of cytochrome P450 (CYP) epoxygenases expressed in endothelial cells, and subsequent generation of vasodilator epoxyeicosatrienoic acids (EETs). Here, we investigated the contribution of CYP 2C9 metabolites to regulation of porcine coronary vasomotor tone in vivo and in vitro. Twenty-six swine were chronically instrumented. Inhibition of CYP 2C9 with sulfaphenazole (5 mg kg^{-1} iv) alone had no effect on bradykinin-induced endothelium-dependent coronary vasodilation in vivo, but slightly attenuated bradykinin-induced vasodilation in the presence of combined NOS/COX blockade with N^{ω}-Nitro-L-arginine (20 mg kg^{-1} iv) and indomethacin (10 mg kg^{-1} iv). Sulfaphenazole had minimal effects on coronary resistance vessel tone at rest or during exercise. Surprisingly, in the presence of combined NOS/COX blockade, a significant coronary vasodilator response to sulfaphenzole became apparent, both at rest and during exercise. Subsequently, we investigated in isolated porcine coronary small arteries (~250 μm) the possible involvement of reactive oxygen species (ROS) in the paradoxical vasoconstrictor influence of CYP 2C9 activity. The vasodilation by bradykinin in vitro in the presence of NOS/COX blockade was markedly potentiated by sulfaphenazole under control conditions, but not in the presence of the ROS scavenger N-(2-Mercaptoproprionyl)-glycine. In conclusion, CYP 2C9 can produce both vasoconstrictor and vasodilator metabolites. Production of these metabolites is enhanced by combined NOS/COX blockade, and is critically dependent on the experimental conditions. Thus, production of vasoconstrictors slightly outweighed the production of vasodilators at rest and during exercise. Pharmacological stimulation with bradykinin resulted in vasodilator CYP 2C9 metabolite production when administered in vivo, whereas vasoconstrictor CYP 2C9 metabolites, most likely ROS, were dominant when administered in vitro.

Introduction

Coronary blood flow is tightly regulated to maintain a consistently high level of myocardial oxygen extraction over a wide range of myocardial oxygen demands [1-3]. This tight regulation is dependent on a myriad of vasoconstrictor and vasodilator influences, exerted by the autonomic nervous system, myocardium, endothelium and blood [1, 2]. Nitric oxide (NO) and prostacyclin (PGI_2) are well-known endothelium-derived vasodilators that exert significant vasodilator influences in the coronary microcirculation of swine [4] and which are in part mediated via inhibition of endothelin, particularly during exercise [5]. In addition to NO and PGI_2, there is a variety of vasodilators, including S-nitrosothiols, K^+, H_2O_2 and cytochrome P450 (CYP450) metabolites [6-9], that produce vasodilation via opening of vascular smooth muscle K^+ channels and subsequent membrane hyperpolarization [10, 11]. The contribution of these so-called endothelium-derived hyperpolarizing factors (EDHFs) [12-14] to endothelium-dependent vasodilation varies, depending on the species and vascular bed studied [11, 15], as well as on the agonist used to stimulate the endothelium [11, 16].

One group of EDHFs is produced by CYP450 isoenzymes [17-19], of which there are two classes: (*i*) epoxygenases, which catalyze the formation of epoxyeicosatrienoic acids (EETs), and (*ii*) ω-hydroxylases that produce 20-hydroxyeicosatetraenoic acid (20-HETE) [19, 20]. Endothelial cells are capable of synthesizing the 4 regioisomeric EETs, 14,15-, 11,12-, 8,9-, and 5,6-EETs, with 11,12-EETs being the major metabolite produced by CYP 2C9 epoxygenase [6, 21-23]. A CYP 2C epoxygenase homologous to CYP 2C8/9 has been identified as a putative EDHF synthase in porcine coronary vasculature [24], and was shown to mediate the bradykinin-induced endothelium-dependent hyperpolarization of vascular smooth muscle cells, in the presence of NO synthase and cyclooxygenase blockade, in isolated porcine coronary large arteries [10]. In contrast, another study failed to observe an effect of CYP 2C9 inhibition on the bradykinin-mediated vasodilation of porcine coronary small arteries (~300 μm) in vitro [9]. However, most of the coronary vascular resistance resides in vessels smaller than 200 μm in diameter [25]. Furthermore, the role of CYP 2C9 metabolites in porcine coronary resistance vessel dilation produced by bradykinin in vivo has not been investigated to date. Consequently, the first aim of the present study was to study the contribution of CYP 2C9 metabolites to the coronary vasodilation produced by bradykinin in awake resting swine in the absence and presence of inhibition of NO synthase (NOS) and cyclo-oxygenase (COX).

The mechanisms that mediate exercise hyperemia in the heart remain incompletely understood [1, 2]. Since there is evidence that CYP 2C9 metabolites contribute to skeletal muscle exercise hyperemia and enhance oxygen uptake in the human leg [26], we hypothesized that CYP 2C9 metabolites play a similar role in exercise hyperemia in the heart. Consequently, the second aim of the present study was to determine the contribution of endogenous CYP 2C9 metabolites in the control of coronary resistance vessel tone in swine at rest and during exercise.

The results of these in vivo studies showed that, whereas CYP 2C9 metabolites contributed to bradykinin-induced coronary vasodilation, CYP 2C9 metabolites exerted a basal coronary vasoconstrictor influence. Since CYP 2C9 has been shown to produce reactive oxygen species (ROS) [6, 27], the third aim of our study was to further explore the contribution of CYP 2C9 metabolites to bradykinin-induced coronary vasodilation. Specifically, we tested in vitro the hypothesis that ROS scavenging would enhance the CYP 2C9-mediated vasodilation by bradykinin.

Methods

Animals

Studies were performed in accordance with the "Guiding Principles in the Care and Use of Laboratory Animals" as approved by the Council of the American Physiological Society, and with approval of the Animal Care Committee at Erasmus Medical Center Rotterdam. Twenty six crossbred Yorkshire × Landrace swine (2-3-month-old, 22±1 kg at the time of surgery) of either sex entered the study. Daily adaptation of animals to laboratory conditions started one week before surgery and continued during the first week after surgery.

Surgical procedure

Swine were sedated with ketamine (30 mg kg^{-1} iv), anaesthetized with thiopental (10 mg kg^{-1} iv), intubated and ventilated with a mixture of O_2 and N_2 (1:2) to which 0.2-1% (vol/vol) isoflurane was added [28-30]. Anesthesia was maintained with midazolam (2 mg kg^{-1} iv) and fentanyl (10 μg kg^{-1} per hour iv). Under sterile conditions, the chest was opened via the fourth left intercostal space and a fluid-filled polyvinylchloride catheter was inserted into the aortic arch for the measurement of mean aortic pressure and blood sampling for the determination of PO_2, PCO_2, pH, O_2 saturation and hemoglobin concentration. A microtipped high fidelity pressure-transducer ($P_{4.5}$, Konigsberg Instruments) was inserted into the left ventricle via the apex. A polyvinylchloride catheter was also inserted into the left ventricle to calibrate the Konigsberg transducer left ventricle pressure signal. Other polyvinylchloride catheters were inserted into the left atrium to measure pressure and into the pulmonary artery for the systemic administration of drugs. A Transonic flow probe was placed around the proximal left anterior descending coronary artery (LAD) for the measurement of coronary blood flow [31]. Finally, small angiocatheters were inserted into the anterior interventricular vein for coronary venous blood sampling [28], and into the left anterior descending coronary artery for intracoronary infusion of bradykinin. Electrical wires and catheters were tunneled subcutaneously to the back. Then, the chest was closed and animals were allowed to recover, receiving analgesia (0.3 mg buprenorphine im) for 2 days and antibiotic prophylaxis (25 mg kg^{-1} amoxicillin and 5 mg kg^{-1} gentamycin iv) for 5 days.

In vivo experimental protocols

In vivo studies were performed 1-3 weeks after surgery. In the first series of experiments we investigated the contribution of CYP 2C9 metabolites to bradykinin-induced coronary vasodilation in resting swine, while in the second series of experiments we investigated the contribution of CYP 2C9 metabolites to the coronary hyperemia during exercise. Since both NO and PGI_2 can act on CYP 2C9 enzymes and inhibit the formation of EDHFs [32, 33], studies were performed in the absence and presence of combined NOS and COX inhibition. All four protocols were performed on different days and in random order.

Contribution of CYP 2C9 metabolites to bradykinin-induced coronary vasodilation. Hemodynamic measurements, consisting of left atrial and aortic blood pressures, heart rate, and coronary blood flow were obtained in nine swine, while animals were resting quietly. Subsequently, the coronary blood flow responses to bradykinin were determined. Bradykinin was infused into the coronary artery in consecutive doses of 0.03, 0.1, 0.3, 1.0 and 3.0 μg min^{-1}, with each infusion step lasting 3 min. Hemodynamic measurements were recorded at baseline and throughout the entire infusion protocol. Then, the infusion was stopped and coronary blood flow was allowed to return to baseline values. 30 min later, the selective CYP 2C9 inhibitor

sulfaphenazole (inhibition constant Ki value of 0.3±0.1 μM for CYP 2C9), which results in >100 fold less inhibition of CYP 2C8 (Ki of 63 μM) or 2C18 (Ki of 29 μM), and no inhibition of CYP 1A1, 1A2, 3A4 and 2C19) [6, 34], was infused intravenously in a dose of 5 mg kg^{-1} over 10 min. 10 min after completion of administration, the bradykinin dose response protocol was repeated.

On a different day, four resting swine received systemic administration of the endothelial NOS inhibitor N^{ω}-Nitro-L-arginine (NLA) in a dose of 20 mg kg^{-1} followed by systemic administration of the COX inhibitor indomethacin in a dose of 10 mg kg^{-1} [35]. 10 min later, the intracoronary bradykinin dose response protocol, as described above, was performed. 30 min after completion of bradykinin infusion, 5 mg kg^{-1} indomethacin was intravenously infused [35]. Then, sulfaphenazole was infused intravenously, in a dose of 5 mg kg^{-1}, and 10 min later the bradykinin dose response protocol was repeated.

Contribution of CYP 2C9 metabolites to coronary resistance vessel tone at rest and during exercise. With fourteen swine lying quietly on the treadmill, resting hemodynamic measurements were obtained and arterial and coronary venous blood samples were collected. Hemodynamic measurements were repeated and rectal temperature was measured with animals standing on the treadmill. Subsequently, a five-stage exercise protocol (1-5 km h^{-1}) was begun with each stage lasting 3 min. Hemodynamic variables were continuously recorded and blood samples collected during the last 30 seconds of each exercise stage, at a time when hemodynamics had reached a steady state. At the conclusion of the exercise protocol, animals were allowed to rest for 90 min, resulting in a complete return of hemodynamic variables to baseline values. Subsequently, sulfaphenazole was intravenously administered in a dose of 5 mg kg^{-1}, and 10 min after completion of administration, the exercise protocol was repeated. We have previously observed excellent reproducibility of two consecutive exercise protocols [28].

On a different day, the NLA (20 mg kg^{-1}) and indomethacin (10 mg kg^{-1}) were infused intravenously in ten swine [35], and the exercise protocol as described above was performed. 90 min later, indomethacin was administered in a dose of 5 mg kg^{-1} (which, in combination with the long-lasting effect of NLA, results in similar hemodynamic conditions as observed after administration of NLA and indomethacin prior to the first exercise protocol [35]), followed by intravenous infusion of sulfaphenazole (5 mg kg^{-1}) and the exercise protocol was repeated.

In vitro experimental protocols

Myograph Studies. Swine hearts (n=16) were collected at a local slaughterhouse. Coronary small arteries (diameter ≈250 μm) were dissected out and stored overnight in cold, oxygenated Krebs bicarbonate solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4. The next day, coronary arteries were cut into segments of ≈2 mm length and mounted in microvascular myographs (Danish Myo Technology) with separated 6 ml organ baths containing Krebs bicarbonate solution aerated with 95% O_2/5% CO_2 and maintained at 37°C. Changes in contractile force were recorded with a Harvard isometric transducer. Following a 30 min stabilization period, the internal diameter was set to a tension equivalent to 0.9 times the estimated diameter at 100 mmHg effective transmural pressure. The vessels were then exposed to 30 mM KCl twice. Endothelial integrity was verified by observing dilation to 10 nM substance P after preconstriction with 100 nM of the stable thromboxane A_2 analogue U46619. Then vessels were subjected to 100 mM KCl to determine the maximal vascular contraction. Thereafter, vessels were allowed to equilibrate in fresh organ bath fluid for 30 min before initiating different experimental protocols [9]. Only one protocol was executed per vessel and each experimental protocol was performed in 5 to 8 vessels, each obtained from different animals.

Contribution of CYP 2C9 metabolites to bradykinin-induced coronary vasodilation in vitro. Following preconstruction with U46619 (100 nM), concentration-responses to bradykinin (10^{-10}-10^{-7} M) were conducted in control vessels and in vessels incubated with sulfaphenazole (10^{-5} M), N-(2-Mercaptoproprionyl)-glycine (MPG) (3×10^{-4} M), and combined sulfaphenazole and MPG. All experiments were performed in the absence and presence of N^{ω}-Nitro-L-arginine methyl ester HCl (LNAME) (10^{-4} M) and indomethacin (10^{-5} M), respectively.

Data Analysis

Digital recording and off-line analysis of hemodynamic data and computation of myocardial O_2 consumption (MVO_2) have been described in detail elsewhere [28, 36, 37]. Coronary vascular conductance (CVC) was calculated as coronary blood flow divided by mean aortic pressure minus left atrial pressure. Myocardial O_2 delivery (MDO_2) was computed as the product of LAD coronary blood flow and arterial blood O_2 content. MVO_2 in the region of myocardium perfused by the LAD was calculated as the product of coronary blood flow and the difference in O_2 content between arterial and coronary venous blood. MEO_2 was computed as the ratio of MVO_2 and MDO_2. Vascular relaxation responses to bradykinin were expressed as percentage of contraction to U46619.

Statistical Analysis

Statistical analysis of in vivo hemodynamic data was performed using two-way ANOVA for repeated measures, followed by post-hoc analysis using Scheffe test (for the effects of bradykinin and exercise) and t-test (for the effects of drugs NLA+Indo and Sulfa) when appropriate. To test the effects of drug treatment on the relation between MVO_2 and coronary venous O_2 tension ($cvPO_2$), coronary venous O_2 saturation ($cvSO_2$), regression analysis was performed using drug treatment and MVO_2, as well as their interaction as independent variables, and by assigning a dummy variable to each animal. The effects of drug treatment on the bradykinin responses in vitro were analyzed using two-way (bradykinin × drug) ANOVA. Statistical significance was accepted when $P<0.05$. Data are presented as mean±SEM.

Results

Contribution of CYP 2C9 metabolites to bradykinin-induced coronary vasodilation

Intracoronary infusion of bradykinin resulted in dose-dependent increases in coronary blood flow and in coronary vascular conductance up to 180 % (Fig. 1,Table 1). Inhibition of CYP 2C9 with sulfaphenazole had no effect on bradykinin-induced coronary vasodilation.

After blockade of NOS/COX pathways by NLA and indomethacin, the coronary blood flow increments in response to bradykinin were blunted at lower dosages (0.03 μg min^{-1}, 0.1 μg min^{-1}, and 0.3 μg min^{-1}) but enhanced at higher dosages (1 μg min^{-1} and 3 μg min^{-1}) (Table 1). The latter was likely due to an increase in coronary perfusion pressure as aortic blood pressure increased from ~80 mmHg to ~140 mmHg following NLA and indomethacin. Consequently, at lower dosage of bradykinin the increase in coronary vascular conductance was attenuated in the presence of blockade of NOS/COX pathways, whereas at higher dosages the bradykinin-mediated increments in coronary conductance were comparable to those observed in the absence of NOS/COX blockade (Fig. 1). These findings suggest that bradykinin-induced coronary vasodilation is principally dependent on NO and PGI_2 at lower dosages but at higher dosages appears independent of NO and PGI_2.

In the presence of NOS/COX inhibition, sulfaphenazole attenuated the bradykinin-mediated increases in coronary blood flow (Table 1) and coronary vascular conductance (Table 1, Fig. 1), particularly at higher dosages of bradykinin (both $P<0.01$ for bradykinin × sulfaphenazole). These findings indicate that CYP 2C9 metabolites contribute to bradykinin-induced coronary resistance vessel dilation, but only when NOS/COX pathways are blocked.

Table 1. Effects of CYP2C9 inhibition on hemodynamic response to bradykinin.

	Dosage (µg/min)					
	Baseline	0.03	0.1	0.3	1	3
Heart rate, beats/min						
Control	109 ± 4	119 ± 7*	114 ± 6	119 ± 8	117 ± 6	116 ± 4*
Sulfa	112 ± 7	112 ± 6	116 ± 7	115 ± 5	119 ± 6	122 ± 9
NLA+Indo	89 ± 12	83 ± 8†	86 ± 7†	99 ± 5†	96 ± 5	101 ± 4
NLA+Indo+Sulfa	94 ± 10	94 ± 7‡	96 ± 8‡	100 ± 8	99 ± 9*	95 ± 7
MAP, mmHg						
Control	82 ± 2	83 ± 2	83 ± 2	84 ± 2	84 ± 2	82 ± 4
Sulfa	84 ± 2	82 ± 3	82 ± 2	83 ± 2	86 ± 2	84 ± 2
NLA+Indo	144 ± 6†	141 ± 10†	142 ± 11†	136 ± 7†	145 ± 4†	136 ± 2†
NLA+Indo+Sulfa	132 ± 8	132 ± 8	125 ± 7	125 ± 4	123 ± 5	121 ± 3‡
CBF, ml/min						
Control	38 ± 3	46 ± 4*	53 ± 5*	73 ± 10*	89 ± 13*	107 ± 20*
Sulfa	38 ± 3	43 ± 3*	55 ± 7*	71 ± 8*	86 ± 18	111 ± 20*
NLA+Indo	37 ± 5	36 ± 4	39 ± 5	56 ± 6*†	110 ± 21†	136 ± 13*†
NLA+Indo+Sulfa	35 ± 2	39 ± 3	39 ± 3	47 ± 6	66 ± 7*‡	98 ± 8*‡
CVC, 10^{-2} ml/min/mmHg						
Control	49 ± 4	57 ± 6	66 ± 7	90 ± 11*	111 ± 20*	137 ± 31*
Sulfa	48 ± 5	55 ± 4	71 ± 10	91 ± 12*	113 ± 35*	147 ± 31*
NLA+Indo	30 ± 4†	29 ± 1†	32 ± 2†	48 ± 5†	87 ± 17*	117 ± 12*
NLA+Indo+Sulfa	31 ± 1	34 ± 2	36 ± 2	43 ± 5	62 ± 7*	95 ± 11*‡

*MAP: Mean aortic pressure; CBF: Coronary blood flow; Sulfa: Sulfaphenazole; NLA: N^{ω}-Nitro-L-arginine; Indo: Indomethacin. Values are mean±SEM, * $P<0.05$ vs. baseline; † $P<0.05$ effect of NLA and Indo; ‡ $P<0.05$ effect of Sulfa.*

Contribution of CYP 2C9 metabolites to coronary resistance vessel tone at rest and during exercise

Exercise produced graded increase in heart rate, LV systolic pressure, and the maximum rate of rise in left ventricular pressure with minimal effects on mean aortic blood pressure (Table 2). The consequent increase in MVO_2 during exercise was met by a similar increase in coronary blood flow and myocardial oxygen supply, so that oxygen extraction, $cvPO_2$ and $cvSO_2$ were maintained relatively constant during exercise (Table 3, Fig. 2).

Intravenous infusion of sulfaphenazole had no systemic hemodynamic effects (Table 2), but resulted in a slight increase in coronary vascular conductance (Table 3, $P<0.05$ by ANOVA) leading to an increase in myocardial O_2 supply that exceeded the O_2 demand, thereby resulting in small increases in $cvPO_2$ and $cvSO_2$ both at rest and during exercise (Fig. 2). The small increases in $cvPO_2$ (overall 0.9±0.2 mmHg) was, however, highly consistent and hence reached statistical significance ($P<0.05$), indicating that sulfaphenazole produced a small degree of coronary vasodilation that was similar at rest and during exercise.

In accordance with previous studies from our laboratory [4], administration of NLA and indomethacin resulted in a pronounced increase in aortic pressure that was accompanied by a,

probably baroreflex-mediated, decrease in heart rate; these effects waned with incremental exercise levels (Table 2). NLA and indomethacin also produced coronary vasoconstriction, as reflected in the marked reductions in $cvPO_2$ and $cvSO_2$, and which were well maintained during exercise (Fig. 2). Subsequent intravenous infusion of sulfaphenazole resulted in increases in $cvPO_2$ and $cvSO_2$ at rest that were maintained during exercise (Fig. 2), reflecting coronary vasodilation. Importantly, the increases in $cvPO_2$ and $cvSO_2$ by sulfaphenazole were enhanced by pretreatment with NOS/COX inhibition (Fig. 2). Taken together these findings suggest that endogenous CYP 2C9 metabolites exert a net vasoconstrictor influence on the coronary vasculature, which is blunted by NO and PGI_2.

Contribution of ROS to the CYP 2C9-mediated vasoconstriction

To investigate the role of ROS in opposing the bradykinin-induced vasodilation, we studied the effect of ROS scavenging with MPG on the vasodilator response to bradykinin in isolated coronary small arteries in vitro, both in the absence and presence of NOS and COX inhibition and/or CYP 2C9 inhibition. Bradykinin resulted in dose-dependent small artery relaxation (Fig. 3). MPG shifted the bradykinin concentration-response curve to the left, indicating that bradykinin-induced vasorelaxation was limited by simultaneous ROS production. However, sulfaphenazole had no effect on bradykinin-induced relaxation either in the presence or absence of MPG (Fig. 3), indicating that, similar to our in vivo observations, CYP 2C9 does not contribute to bradykinin-induced vasorelaxation in vitro. Blockade of NOS and COX by LNAME and indomethacin significantly attenuated bradykinin-induced vasorelaxation. The effect of subsequent ROS scavenging with MPG was enhanced, indicating that ROS production was increased following inhibition of NOS and COX. In contrast to our in vivo observations, administration of sulfaphenazole in the presence of LNAME and indomethacin enhanced the bradykinin-induced vasorelaxation in vitro (Fig. 3), indicating that production of vasoconstrictor metabolites by CYP 2C9 predominated in vitro. The observation that this effect of sulfaphenazole was no longer present in the presence of MPG suggests that indeed CYP 2C9 is a functionally significant source of ROS in isolated porcine coronary small arteries, but only when NOS and/or COX are blocked.

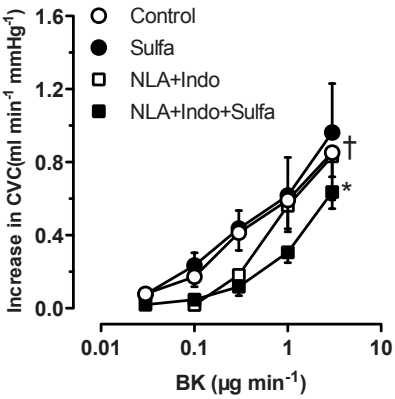

Fig. 1. *Effects of CYP 2C9 inhibition with sulfaphenazole on bradykinin (BK)-induced vasodilation in the coronary vasculature in the absence and presence of NOS/COX blockade with N^{ω}-Nitro-L-arginine (NLA) and indomethacin (Indo). Values are mean±SEM. CVC: Coronary vascular conductance. * P<0.05 effect of Sulfa in the presence of NLA+Indo; † P<0.05 effect of NLA+Indo.*

Discussion

The present study is the first to investigate the contribution of CYP 2C9 metabolites to the bradykinin-induced vasodilation in the coronary microcirculation in vivo and the contribution of CYP 2C9 metabolites to exercise hyperemia. The main findings were that (*i*) CYP 2C9 inhibition by sulfaphenazole did not affect bradykinin-induced coronary vasodilation under control conditions either in vivo or in vitro but (*ii*) in the presence of combined NOS/COX

blockade, CYP 2C9 inhibition blunted the bradykinin-induced coronary vasodilation in vivo, whereas *(iii)* it enhanced the bradykinin-induced coronary vasodilation in vitro; (*iv*) the potentiation by CYP 2C9 inhibition of bradykinin-induced vasodilation in vitro was abolished following ROS scavenging with MPG; *(v)* inhibition of CYP 2C9 produced slight but consistent coronary vasodilation in vivo at rest, that was maintained during exercise; (*vi*) the vasodilation by CYP 2C9 inhibition was enhanced in the presence of NOS/COX blockade. The implications of these findings will be discussed below.

Table 2. Hemodynamic effects of CYP 2C9 inhibition during exercise.

	Rest	Exercise level (km/h)				
	Lying	1	2	3	4	5
Heart rate, beats/min						
Control	122 ± 3	160 ± 4*	173 ± 5*	197 ± 6*	221 ± 8*	245 ± 8*
Sulfa	125 ± 5	156 ± 5*	168 ± 4*	188 ± 6*	217 ± 6*	239 ± 7*
NLA+Indo	78 ± 4†	105 ± 4†	113 ± 5*†	124 ± 5*†	149 ± 5*†	172 ± 3*†
NLA+Indo+Sulfa	86 ± 6	109 ± 4*	113 ± 4	128 ± 4	153 ± 4*	189 ± 4*
MAP, mmHg						
Control	98 ± 2	94 ± 3	92 ± 3*	94 ± 3	96 ± 3	97 ± 3
Sulfa	94 ± 3	89 ± 3	93 ± 3	94 ± 3	95 ± 3	96 ± 3
NLA+Indo	149 ± 8†	140 ± 7*†	137 ± 7*†	134 ± 7*†	128 ± 6*†	126 ± 5*†
NLA+Indo+Sulfa	137 ± 6	119 ± 4*‡	118 ± 4*‡	119 ± 4*‡	118 ± 4*‡	118 ± 4‡
LVSP, mmHg						
Control	113 ± 3	116 ± 4	117 ± 4	120 ± 4	124 ± 5*	132 ± 5*
Sulfa	111 ± 4	112 ± 4	117 ± 4	119 ± 5	124 ± 5*	131 ± 6*
NLA+Indo	155 ± 8†	150 ± 8*†	147 ± 8*†	140 ± 5*†	139 ± 5*	143 ± 5*
NLA+Indo+Sulfa	145 ± 8	128 ± 5*‡	126 ± 5*	128 ± 4*‡	130 ± 4*	133 ± 4*‡
LVdP/dt max, mmHg/s						
Control	2840 ± 190	3730 ± 240*	3940 ± 280*	4410 ± 310*	4950 ± 340*	5910 ± 490*
Sulfa	3070 ± 250†	3580 ± 250	3880 ± 250*	4310 ± 360*	4800 ± 320*	5770 ± 490*
NLA+Indo	2760 ± 240	3750 ± 360*	3830 ± 370*	3970 ± 290*	4530 ± 300*	5460 ± 260*
NLA+Indo+Sulfa	2690 ± 200	2990 ± 190	3120 ± 180*	3450 ± 210*	4230 ± 290*	5440 ± 400*
LAP, mmHg						
Control	6 ± 1	6 ± 1	7 ± 1	9 ± 1*	11 ± 1*	12 ± 1*
Sulfa	5 ± 1	5 ± 1	8 ± 1*	10 ± 1*	12 ± 1*	12 ± 1*
NLA+Indo	13 ± 4	17 ± 3†	16 ± 3†	14 ± 4	14 ± 3	10 ± 1
NLA+Indo+Sulfa	16 ± 3	13 ± 3	12 ± 3	13 ± 3	14 ± 3	11 ± 3

*MAP: Mean aortic pressure; LVSP: Left ventricular systolic pressure; LVdP/dtmax: Maximum rate of rise in left ventricular pressure; LAP: Mean left atrial pressure; Sulfa: Sulfaphenazole; NLA: N^{ω}-Nitro-L-arginine, Indo: Indomethacin. Values are mean±SEM; * P<0.05 vs. Rest lying; † P<0.05 effect of NLA and Indo; ‡ P<0.05 effect of Sulfa.*

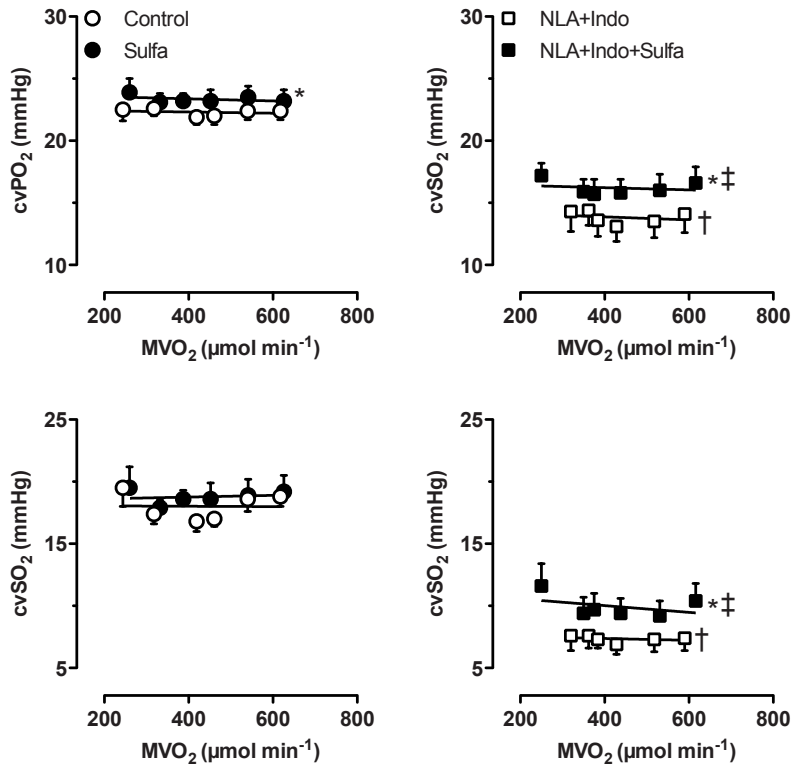

Fig. 2. *Effects of CYP 2C9 inhibition with sulfaphenazole on the relation of myocardial oxygen consumption (MVO_2) and coronary venous oxygen tension ($cvPO_2$) and saturation ($cvSO_2$) at rest and during exercise, in the absence (left) and presence (right) of NOS- and COX- blockade with N^{ω}-Nitro-L-arginine (NLA) and indomethacin (Indo). Values are mean±SEM; solid lines represent best fit regression lines of the mean data points. * P<0.05 effect of Sulfa; † P<0.05 effect of NLA+Indo; ‡P<0.05 effect of Sulfa different in the presence of NLA+Indo as compared to the effect of Sulfa under control conditions.*

Contribution of CYP 2C9 metabolites to bradykinin-induced coronary vasodilation

The endothelium plays a critical role in regulation of coronary vasomotor tone, not only by production of well-known factors like NO and PGI_2 but also through production of other factors that influence the membrane-potential of the vascular smooth muscle cells [38]. One of these EDHFs is 11,12-EET, a metabolite of CYP 2C9. The present study investigated the contributions of NO, prostanoids and CYP 2C9 metabolites on the bradykinin-induced coronary resistance vessel dilation in swine in vivo. We found that bradykinin-induced vasodilation of the coronary microcirculation was not altered by CYP 2C9 inhibition with sulfaphenazole, whereas inhibition of NOS and COX attenuated the vasodilator response to bradykinin. Subsequent administration of sulfaphenazole further attenuated the vasodilator response to bradykinin. Similarly, in vitro studies, in isolated porcine coronary large and small arteries, indicate that endothelium-dependent vasodilation produced by bradykinin is principally NO-mediated [6, 9, 39-41], while CYP 2C9 metabolites only contribute to bradykinin-induced endothelium-dependent coronary vasodilation in the absence of NO and PGI_2 [10, 39, 40]. In contrast, in the in vivo canine heart, bradykinin-induced dilation of both large (100-200 µm) and small (<100 µm) coronary

arterioles remained unaffected by NO synthase and COX inhibition, but was abolished by subsequent CYP 2C9 inhibition [42], suggesting an important role for CYP 2C9 vasodilator metabolites in the vasodilation produced by bradykinin, particularly in vivo.

Table 3. Effect of CYP 2C9 inhibition on myocardial O_2 balance during exercise.

	Rest	Exercise level (km/h)				
	Lying	1	2	3	4	5
CBF, ml/min						
Control	67 ± 7	99 ± 10*	107 ± 11*	123 ± 15*	140 ± 15*	157 ± 16*
Sulfa	74 ± 9	99 ± 12*	111 ± 13*	125 ± 15*	143 ± 16*	163 ± 14*
NLA+Indo	47 ± 9†	68 ± 11*†	73 ± 12*†	81 ± 13*†	95 ± 15*†	106 ± 14†
NLA+Indo+Sulfa	51 ± 8	75 ± 15*	79 ± 15*	90 ± 17*	105 ± 19*	120 ± 19
CVC, 10^{-2} ml/min/mmHg						
Control	76 ± 8	115 ± 13*	130 ± 15*	145 ± 18*	165 ± 20*	184 ± 21*
Sulfa	86 ± 12	123 ± 16*	135 ± 19*	152 ± 20*	174 ± 23*	196 ± 20*
NLA+Indo	39 ± 8†	62 ± 14*†	68 ± 16*†	77 ± 17*†	97 ± 23*†	110 ± 21†
NLA+Indo+Sulfa	47 ± 9	80 ± 22*	88 ± 24*	98 ± 27*	118 ± 30*	132 ± 28
Hb, g%						
Control	8.1 ± 0.3	8.3 ± 0.3	8.3 ± 0.2	8.5 ± 0.2*	8.8 ± 0.2*	9.0 ± 0.2*
Sulfa	8.0 ± 0.2	8.3 ± 0.2	8.3 ± 0.2	8.3 ± 0.2*	8.7 ± 0.2*	9.0 ± 0.2*
NLA+Indo	8.7 ± 0.2	8.5 ± 0.2	8.6 ± 0.2	8.7 ± 0.2	8.9 ± 0.1	9.3 ± 0.1*
NLA+Indo+Sulfa	7.9 ± 0.3	7.9 ± 0.2‡	7.9 ± 0.2‡	8.1 ± 0.2‡	8.4 ± 0.2*‡	8.7 ± 0.2*‡
$artPO_2$, mmHg						
Control	97 ± 2	93 ± 3	96 ± 3	95 ± 2	94 ± 3	92 ± 4
Sulfa	99 ± 2	98 ± 2	96 ± 4	99 ± 3	95 ± 3	93 ± 3*
NLA+Indo	114 ± 2	113 ± 2†	111 ± 3†	109 ± 3	105 ± 4*	103 ± 3*
NLA+Indo+Sulfa	106 ± 2	107 ± 2‡	105 ± 2	105 ± 2	100 ± 4‡	100 ± 4
$artSO_2$, %						
Control	95 ± 1	94 ± 1	95 ± 1	95 ± 1	95 ± 1	94 ± 1
Sulfa	95 ± 1	95 ± 1	94 ± 1	95 ± 1	95 ± 1	94 ± 1
NLA+Indo	97 ± 1	96 ± 1†	96 ± 1†	96 ± 1	95 ± 1	96 ± 1
NLA+Indo+Sulfa	96 ± 1	97 ± 1	97 ± 1	96 ± 1	96 ± 1	96 ± 1
MVO_2, µmol/min						
Control	244 ± 16	318 ± 37	419 ± 31*	460 ± 29*	537 ± 48*	615 ± 45*
Sulfa	260 ± 19	332 ± 35	387 ± 36*	452 ± 41*	541 ± 52*	626 ± 48*
NLA+Indo	320 ± 64	362 ± 66	384 ± 74	428 ± 81*	518 ± 100*	590 ± 98*
NLA+Indo+Sulfa	250 ± 45	350 ± 83	375 ± 91	438 ± 101*	531 ± 121*	616 ± 121*

*CBF: Coronary blood flow; CVC: Coronary vascular conductance; Hb: Hemoglobin; $artPO_2$: arterial oxygen pressure; $artSO_2$: Arterial oxygen saturation; MVO_2: Myocardial oxygen consumption; Sulfa: Sulfaphenazole; NLA: N^{ω}-Nitro-L-arginine; Indo: Indomethacin. Values are mean±SEM; * P<0.05 vs. Rest lying; † P<0.05 effect of NLA and Indo; ‡ P<0.05 effect of Sulfa.*

Recent studies indicate that NO, PGI_2 and CYP 2C9 metabolites are not the only substances produced in response to bradykinin, and indicate a role for hydrogen peroxide in bradykinin-induced vasodilation of coronary arterioles [43]. In contrast, we found in the present study that ROS scavenging with MPG enhanced the bradykinin-induced vasodilation of isolated coronary small arteries in vitro, suggesting release of vasoconstrictor ROS in response to bradykinin that limits the bradykinin induced vasodilation. To our surprise we observed that, following NOS and COX inhibition, sulfaphenazole enhanced, rather than reduced, bradykinin-induced vasodilation in vitro. Moreover, in the presence of MPG, this effect of sulfphenazole was no longer observed, suggesting that CYP 2C9 was at least in part responsible for the bradykinin-induced ROS production. This notion is in accordance with previous studies showing that CYP 2C9 can be a physiologically relevant source of ROS in the coronary vasculature [6, 27]. The divergent findings that, in response to bradykinin in vivo, CYP 2C9 exerts principally a vasodilator influence, whereas a vasoconstrictor influence predominates in vitro, is difficult to explain, but illustrates that the balance between CYP 2C9 derived vasodilator and vasoconstrictor metabolites depends on the experimental conditions. Finally, our findings in vivo as well as in vitro both support the concept that CYP 2C9 activity is suppressed under physiological conditions by NO and PGI_2 [32, 33, 42]. This likely involves a direct effect on CYP 2C9, or may be due to membrane depolarization following loss of NO and PGI_2, leading to a membrane potential that is more susceptible to the actions of EETs and ROS.

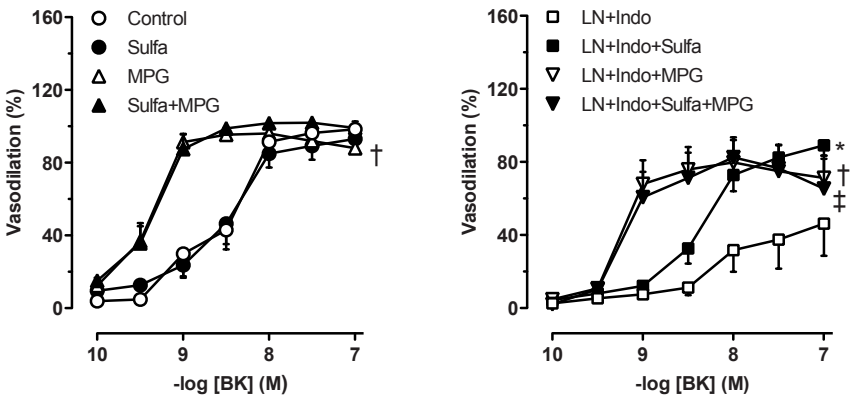

Fig. 3. *Effects of CYP 2C9 inhibition with sulfaphenazole (Sulfa) and reactive oxygen species scavenging with MPG on bradykinin (BK) dose response in isolated coronary small arteries in the absence (left) and presence (right) of LNAME and indomethacin. Values are mean±SEM. * P<0.05 effect of Sulfa; † P<0.05 effect of MPG; ‡ P<0.05 effect of Sulfa different in the presence of MPG.*

Contribution of CYP 2C9 metabolites to exercise hyperemia in the heart

Observations in the human leg indicate that CYP 2C9 metabolites contribute to exercise hyperemia when NOS is inhibited [26], suggesting that CYP 2C9 can also be activated by physiological stimuli such as exercise. We therefore investigated whether CYP 2C9 metabolites also contribute to coronary hyperemia during exercise. Since intravenous administration of inhibitors of vasomotor pathways often results in systemic hemodynamic changes, and hence myocardial metabolism, changes in coronary vasomotor tone during exercise cannot simply be assessed by changes in coronary blood flow or coronary vascular conductance. A sensitive way to

study the regulation of coronary resistance vessel tone during exercise is to examine the relation between coronary venous oxygen levels and myocardial oxygen consumption [2, 44]. Thus, an increase in vasomotor tone, produced by blockade of an endogenous vasodilator pathway, will result in a decrease in myocardial oxygen delivery at a given level of myocardial oxygen consumption, forcing the heart to increase its oxygen extraction to fulfill its oxygen requirement, thereby resulting in a decrease in coronary venous oxygen level. The coronary venous O_2 level therefore represents an index of myocardial tissue oxygenation, i.e. the balance between O_2 delivery and O_2 consumption that is determined by coronary vasomotor tone [2, 44].

Using the myocardial O_2 balance as an index of coronary vasomotor tone, we have previously shown that alterations in the balance between endothelial vasodilators, including NO and prostanoids, and endothelial vasoconstrictors contribute to exercise hyperemia in the porcine coronary vasculature [4, 5, 37]. In the present study, we investigated the vasomotor influence of CYP 2C9 activity in exercise hyperemia. Surprisingly, inhibition of CYP 2C9 with the selective inhibitor sulfaphenazole [6, 34] resulted in a parallel upward shift of the relations between myocardial O_2 consumption and $cvPO_2$ as well as $cvSO_2$, indicating that inhibition of CYP 2C9 resulted in a small degree of vasodilation both at rest and during exercise. These findings show that CYP 2C9 metabolites exerted a net vasoconstrictor influence rather than, or in excess of, a vasodilator influence. The coronary vasodilator effect of sulfaphenazole was enhanced by prior inhibition of NOS and COX, consistent with previous observations that CYP 2C9 activity is blunted by NO and PGI_2 [32, 33, 42].

The exact identity of the vasoconstrictor(s) produced by CYP 2C9 under basal conditions remains to be elucidated. However, the present study, as well as previous studies [6, 27], has shown that CYP 2C9 can be a physiologically relevant source of ROS in the porcine coronary vascular bed. Apparently, the production of ROS, which induce vasoconstriction, can occur simultaneously with the production of vasodilator EETs and can also be suppressed by sulfaphenazole [27]. The observation that CYP 2C9 inhibition reduced the vasodilator response to bradykinin in vivo, but enhanced it in vitro, while it also resulted in vasodilation at rest and during exercise, suggests that the balance of vasodilators and vasoconstrictors produced by CYP 2C9 varies based on the experimental conditions. To our knowledge, it is unknown which factors affect this balance. However, there may be a role for the endogenous antioxidants and hence the 'redox' status of the vasculature. Thus, under basal conditions, and particularly in situations where production of ROS is increased (such as during exercise or in the presence of endothelial dysfunction), when antioxidants are decreased (endothelial dysfunction) or in the presence of increased oxidative stress (high oxygen concentrations in vitro), the vasoconstriction due to production of ROS may predominate, whereas upon infusion of a NO-dependent vasodilator like bradykinin or acetylcholine, NO, ROS and EETs all increase. In this situation, part of the NO may be used to scavenge ROS, allowing the vasodilator influence of EETs to dominate. In accordance with this concept, sulfaphenazole enhances the NO-mediated component of bradykinin-induced vasodilation in healthy vessels [27], improved endothelium-dependent, NO-mediated, vasodilation to acetylcholine in the forearm of patients with coronary artery disease [22], and restored post-ischemia vascular dysfunction in rats [45]. In all cases, administration of sulfaphenazole was accompanied by a decrease in oxidative stress [22, 27, 45].

Conclusions

The present study demonstrates that CYP 2C9 can produce both vasoconstrictor and vasodilator metabolites. Production of these metabolites is enhanced by combined NOS/COX blockade, and is critically dependent on the experimental conditions. Thus, production of vasoconstrictor CYP

2C9 metabolites was dominant over production of vasodilator metabolites in swine at rest and during treadmill exercise. In contrast, pharmacological stimulation with bradykinin resulted in vasodilator CYP 2C9 metabolite production when studied in the intact coronary circulation in vivo, whereas vasoconstrictor CYP 2C9 metabolites, most likely ROS, were dominant when bradykinin was studied in isolated coronary resistance arteries in vitro. Future studies are warranted to further investigate the mechanisms that underlie these divergent influences of CYP 2C9 on coronary resistance vessel tone.

Acknowledgments

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Chapter 4

Phosphodiesterase 5 inhibition- induced coronary vasodilation is reduced after myocardial infarction

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Abstract

The balance between production and removal of cGMP in coronary vascular smooth muscle is of critical importance in determination of coronary vasomotor tone, and thereby in regulating coronary blood flow. cGMP production by soluble guanylyl cyclase is activated by nitric oxide, whereas cGMP breakdown occurs through phosphodiesterase 5 (PDE5). We hypothesized that myocardial infarction (MI) alters the balance between production and removal of cGMP in the coronary vasculature and thereby alters control of coronary vasomotor tone. Chronically instrumented swine, with and without a 2-wk old MI, were exercised on a treadmill in the absence and presence of the PDE5 inhibitor EMD360527 (300 μg/kg/min iv). Inhibition of PDE5 produced coronary resistance vessel dilation, which was more pronounced at rest than during exercise in normal swine. PDE5 gene expression was markedly reduced in coronary resistance vessels isolated from the remote myocardium of MI swine, which was accompanied by a similarly marked attenuation of coronary vasodilation by PDE5 inhibition in MI swine. The coronary vasoconstriction produced by inhibition of NO synthesis with N^{ω}-nitro-L-Arginine (20 mg/kg iv) was only slightly smaller in swine with MI. Interestingly, inhibition of NO synthesis reduced the vasodilator response to subsequent PDE5 inhibition in normal but not in MI swine. Conversely, PDE5 inhibition enhanced the coronary vasoconstriction produced by NO synthesis inhibition in normal but not in MI swine, suggesting that downregulation of PDE5 mitigated the loss of NO vasodilator influence. In conclusion, the expression and vasoconstrictor influence of PDE5 is markedly attenuated in the coronary resistance vessels in remote myocardium after MI, which may serve as a compensatory mechanism to mitigate the loss of NO vasodilator influence.

Introduction

Left ventricular (LV) function depends critically on an adequate oxygenation of the myocardium. Since myocardial oxygen extraction is already high under resting conditions, any substantial increase in myocardial oxygen demand must be met by an increase in oxygen supply and hence an increase in coronary blood flow (CBF) [1, 2]. This tight matching of CBF to myocardial metabolism is the result of a delicate balance between a myriad of vasomotor signals stemming from cardiomyocytes, autonomic nerve terminals and the endothelium [1-4].

The endothelium-derived vasodilator nitric oxide (NO) produces coronary vasodilation through activation of soluble guanylyl cyclase (sGC) and formation of cGMP in vascular smooth muscle [5]. cGMP signaling is terminated through breakdown of cGMP by phosphodiesterase 5 (PDE5) and to a lesser extent by PDE1 [6]. The contribution of PDE5 in regulating coronary vascular tone at rest and during exercise in the normal heart is incompletely understood. Thus, PDE5 inhibition resulted in an increase in diameter of the large coronary arteries in awake resting swine [7], whereas it had no effect on coronary resistance vessel tone in dogs either at rest or during exercise [8]. One explanation for these divergent observations could be that the vasoconstrictor influence of PDE5 depends on the degree of endogenous cGMP production [5], which is supported by observations in isolated vessels showing that vasodilation produced by PDE5 inhibition was blunted by NO synthase (NOS) inhibition [9, 10]. Importantly, NOS inhibition resulted in coronary resistance vessel constriction in awake swine [11-13] and humans [14-16], but had little [17] or no effect on coronary resistance vessel tone in awake dogs [18, 19]. These observations suggest that the contribution of the NO-cGMP pathway to regulation of coronary vasomotor tone varies between species and hence that, in contrast to dogs, PDE5 could potentially exert a significant coronary vasoconstrictor influence in the porcine and human coronary microvasculature. Therefore, the first aim of the present study was to determine the effects of PDE5 inhibition on the coronary circulation of awake, healthy swine at rest and during exercise, and to study its dependency on NOS activity.

Endothelial dysfunction, characterized by a lower NO bioavailability, is typically observed in patients with congestive heart failure [20]. Similarly, in swine with LV dysfunction secondary to a 2-3 week old myocardial infarction (MI), the contribution of NO to coronary resistance vessel tone in the remote myocardium at rest and during exercise [21] as well as the NO dependent vasodilator response to agonists [12] were blunted. Loss of NO bioavailability and hence decreased production of cGMP could result in a reduced vasoconstrictor influence of PDE5. Consequently, the second aim of the present study was to study the effect of PDE5 inhibition on coronary resistance vessel tone in the remote myocardium after MI, and the contribution of blunted NOS activity in this process.

Methods

Studies were performed in accordance with the “Guiding Principles in the Care and Use of Vertebrate Animals in Research and Training” as approved by the Council of the American Physiological Society, and with approval of the Animal Care Committee of the Erasmus MC Rotterdam. A total of 56 crossbred Landrace × Yorkshire swine of either sex (2-3 months old) entered the study.

Surgical procedures

Swine (22±1 kg at the time of surgery) were sedated (20 mg/kg ketamine and 1 mg/kg midazolam, im), anesthetized (thiopental 15 mg/kg, iv), intubated and ventilated with a mixture of O_2 and N_2 (1:2) to which 0.2-1.0% (v/v) isoflurane was added [11]. Anesthesia was maintained with midazolam (2 mg/kg + 1 mg/kg/h, iv;) and fentanyl (10 µg/kg/h, iv). Swine were instrumented under sterile conditions as previously described [11, 22]. Briefly, a thoracotomy was performed in the fourth left intercostal space. Subsequently, a polyvinylchloride catheter was inserted into the aortic arch, for the measurement of mean aortic pressure and blood sampling for the determination of P_{O_2}, P_{CO_2}, pH (ABL 505, Radiometer), O_2-saturation and hemoglobin concentration (OSM3, Radiometer). A fluid filled catheter and a high fidelity Konigsberg pressure transducer were inserted into the left ventricle (LV) via the apex. Fluid filled catheters were also implanted into the left atrium for pressure measurements and in the pulmonary artery for infusion of drugs. A small angiocatheter was inserted into the anterior interventricular vein for coronary venous blood sampling [11]. Finally, a transit-time flow probe (Transonic Systems) was placed around the left anterior descending coronary artery (LAD) for measurement of coronary blood flow [23].

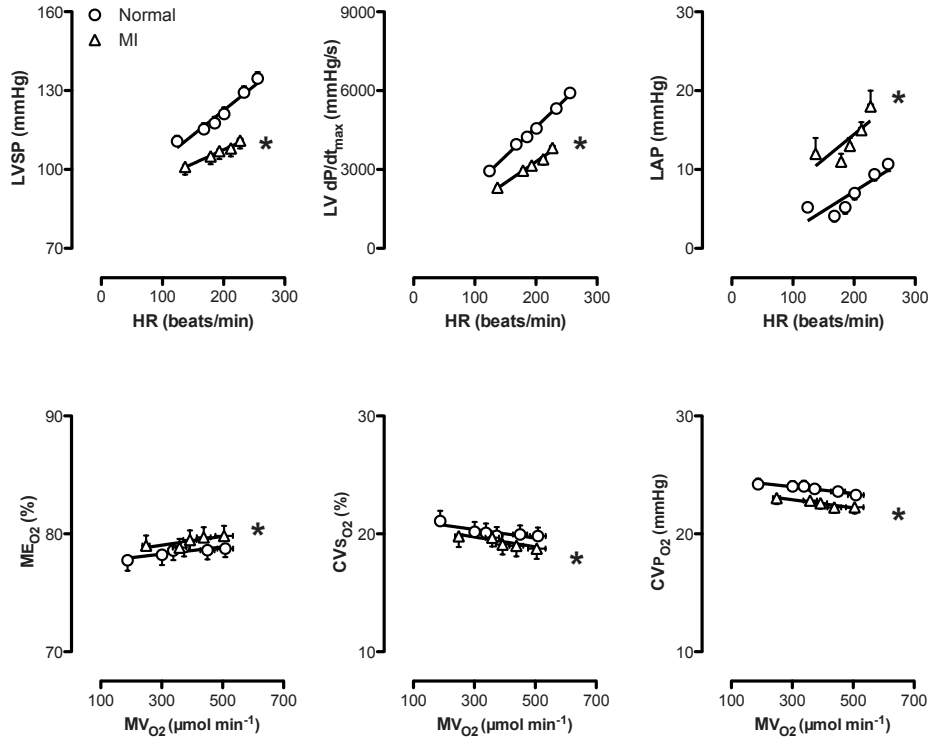

Fig. 1. *Left ventricular function and myocardial oxygen balance during graded treadmill exercise in 30 normal swine (circles) and 20 MI swine (triangles). Data are means±SEM; HR: heart rate; LVSP: left ventricular systolic pressure; LV dP/dtmax maximum rate of rise of left ventricular pressure; LAP: left atrial pressure; myocardial O_2 consumption (MVO_2) and myocardial O_2 extraction (MEO_2), coronary venous O_2 saturation ($CVSO_2$) and coronary venous O_2 tension ($CVPO_2$) * P<0.05 Up- or downward shift of the relation in MI vs. Normal swine.*

In all 56 swine the proximal part of the left coronary circumflex artery (LCx) was exposed, but only in 26 animals the LCx was permanently occluded during surgery with a silk suture to produce a MI [12, 24]. Three MI swine died during surgery due to recurrent fibrillation. Electrical wires and catheters were tunneled subcutaneously to the back, the chest was closed and animals were allowed to recover. Animals received analgesia (0.3 mg buprenorphine im, for 2 days) and antibiotic prophylaxis (25 mg/kg amoxicillin and 5 mg/kg gentamycin iv, for 5 days). Three MI swine died overnight following surgery. All catheters were flushed daily with heparinized saline (1000-5000IU/ml saline) to prevent clotting of the catheters.

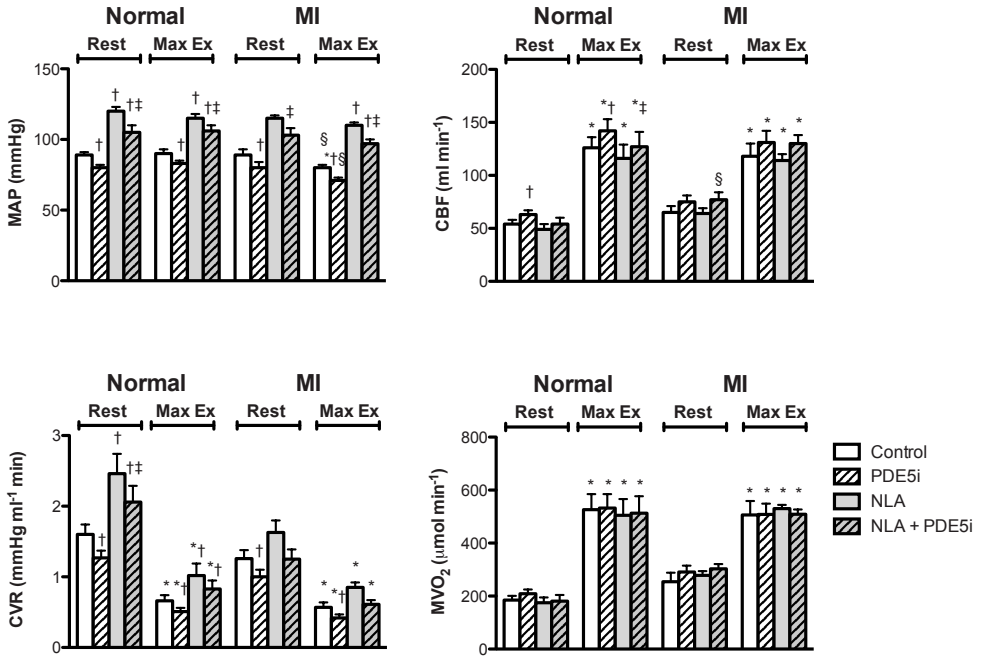

Fig. 2. *Effect of phosphodiesterase 5 inhibition (PDE5i) with EMD360527 (300 µg/kg/min iv) and/or eNOS inhibition with NLA (20 mg/kg iv) on key hemodynamic variables, mean arterial pressure (MAP), coronary blood flow (CBF), coronary vascular resistance (CVR) and myocardial O_2 consumption (MVO_2). Data are means±SEM; * P<0.05 vs rest, † P<0.05 vs corresponding control, ‡ P<0.05 NLA+PDE5i vs. NLA alone, § P<0.05, MI vs. normal*

Experimental protocols

Studies were performed approximately 2 weeks after surgery, with animals exercising on a motor-driven treadmill. We have previously shown excellent reproducibility of the hemodynamic response in consecutive bouts of exercise in both normal [11] and MI swine [12].

PDE5 inhibition. After hemodynamic measurements (lying and standing), blood samples (lying), and rectal temperature (standing) had been obtained, swine were subjected to an exercise protocol on a motor driven treadmill (1-5 km/h for normal swine and 1-4 km/h for swine with MI). Hemodynamic variables were continuously recorded and blood samples collected during the last 60 s of each 3 min exercise stage, at a time when hemodynamics had reached a steady state. After 90 minutes of rest, the PDE5 inhibitor EMD360527 (a gift from Merck KgA), was administered to twelve normal and eight MI swine by an infusion of 300 µg/kg/min iv and the exercise protocol was repeated. We have previously demonstrated that this dose of EMD360527 produces significant systemic and pulmonary vasodilation [25].

EMD360527 demonstrates at least 45-fold selectivity for PDE5 (IC_{50}=0.007 μM) compared to PDE6 (IC_{50}=0.32 μM), 94-fold selectivity for PDE1 (IC_{50}=0.66 μM), 137-fold selectivity for PDE10 (IC_{50}=0.96 μM), and > 1400-fold selectivity for PDE2, PDE3, PDE4 and PDE7 (all IC_{50}>10 μM) [25]. In these assays PDE1-4 was from guinea pig heart muscle, PDE5 from human thrombocytes, PDE6 from bovine retina, while PDE7 and PDE10 were obtained by expression of cDNA in COS-7 cells and E. Coli respectively.

Table 1: Changes in hemodynamic parameters at rest and during maximal exercise in response to PDE5 inhibition in the absence and presence of eNOS inhibition.

		Normal swine		Swine with MI	
	Treatment	Rest	Max exercise	Rest	Max exercise
HR	Control	124 ± 1	261 ± 7*	139 ± 4§	229 ± 6*
(bpm)	PDE5i	144 ± 5†	268 ± 7*†	148 ± 6	237 ± 6*
	NLA	91 ± 4†	234 ± 7*†	104 ± 4§	196 ± 6*†
	NLA+PDE5i	105 ± 8	240 ± 7*‡	121 ± 5‡	208 ± 9
MAP	Control	89 ± 2	90 ± 3	89 ± 4	80 ± 2§
(mmHg)	PDE5i	80 ± 2†	83 ± 2†	80 ± 4†	71 ± 2*†§
	NLA	120 ± 3†	115 ± 3†	115 ± 2†	110 ± 2†
	NLA+PDE5i	105 ± 5†‡	106 ± 4†‡	103 ± 5‡	97 ± 3†‡
LVSP	Control	106 ± 1	134 ± 0*	96 ± 3	110 ± 4*
(mmHg)	PDE5i	107 ± 1	130 ± 0*†	96 ± 3	106 ± 4*
	NLA	127 ± 5†	144 ± 2*†	127 ± 6	133 ± 3
	NLA+PDE5i	110 ± 4‡	139 ± 3*†‡	116 ± 3	126 ± 1
LV dP/dt_{max}	Control	2610 ± 130	5280 ± 310*	2250 ± 240	3570 ± 200*§
(mmHg/s)	PDE5i	3130 ± 160†	5050 ± 320*	2530 ± 200†	3430 ± 330*§
	NLA	2110 ± 110†	6080 ± 440*	2170 ± 190	3750 ± 250§
	NLA+PDE5i	2890 ± 250	5800 ± 420*	2470 ± 280	3670 ± 10§
LAP	Control	6 ± 1	13 ± 1*	11 ± 2§	18 ± 2*§
(mmHg)	PDE5i	4 ± 1	13 ± 1*	10 ± 2§	19 ± 2*§
	NLA	8 ± 1	11 ± 1*	15 ± 3§	16 ± 1§
	NLA+PDE5i	6 ± 1	12 ± 1*	11 ± 2§	18 ± 2†
CBF	Control	54 ± 4	126 ± 10*	65 ± 6	118 ± 12*
(ml/min)	PDE5i	63 ± 4†	142 ± 11*†	75 ± 6	131 ± 11*
	NLA	49 ± 5	116 ± 13*	64 ± 5	114 ± 6*
	NLA+PDE5i	54 ± 6	127 ± 14*‡	77 ± 7§	130 ± 8*
CVR	Control	1.6 ± 0.1	0.7 ± 0.1*	1.3 ± 0.1	0.6 ± 0.1*
(mmHg/ml/min)	PDE5i	1.3 ± 0.1†	0.5 ± 0.1*†	1.0 ± 0.1†	0.4 ± 0.1*†
	NLA	2.5 ± 0.3†	1.2 ± 0.2*†	1.6 ± 0.2	0.8 ± 0.1*
	NLA+PDE5i	2.1 ± 0.2†‡	1.0 ± 0.1*†	1.3 ± 0.1	0.6 ± 0.1*

*HR: heart rate; MAP: mean arterial pressure; LVSP: left ventricular systolic pressure; LV dP/dt_{max}: maximal rate of rise of left ventricular pressure; LAP: left atrial pressure; CBF: coronary blood flow; CVR: coronary vascular resistance; PDE5i: PDE5 inhibition with EMD360527; NLA: eNOS inhibition with N^{ω}-Nitro-L-arginine Data are mean±SEM; * P<0.05 vs rest; † P<0.05 vs corresponding control; ‡ P<0.05 NLA +PDE5i vs. NLA alone; § P<0.05 MI vs. normal swine*

eNOS inhibition. On a different day, a control exercise protocol was performed as described above. After 90 minutes of rest, the NOS-inhibitor N^{ω}-nitro-L-Arginine (NLA) was administered in a dose of 20 mg/kg iv to 30 normal and 20 MI swine and the exercise protocol was repeated [11, 12]. This large group of swine also contains historical data of swine with and without MI used in other studies in which NLA was administered [11, 12, 21, 26].

PDE5 inhibition in the presence of eNOS inhibition. Nine normal and six MI swine that had undergone a control exercise protocol and an exercise protocol in the presence of NLA, were allowed to rest for another 90 min after which they received the PDE5 inhibitor EMD360527 and were subjected to a third exercise protocol.

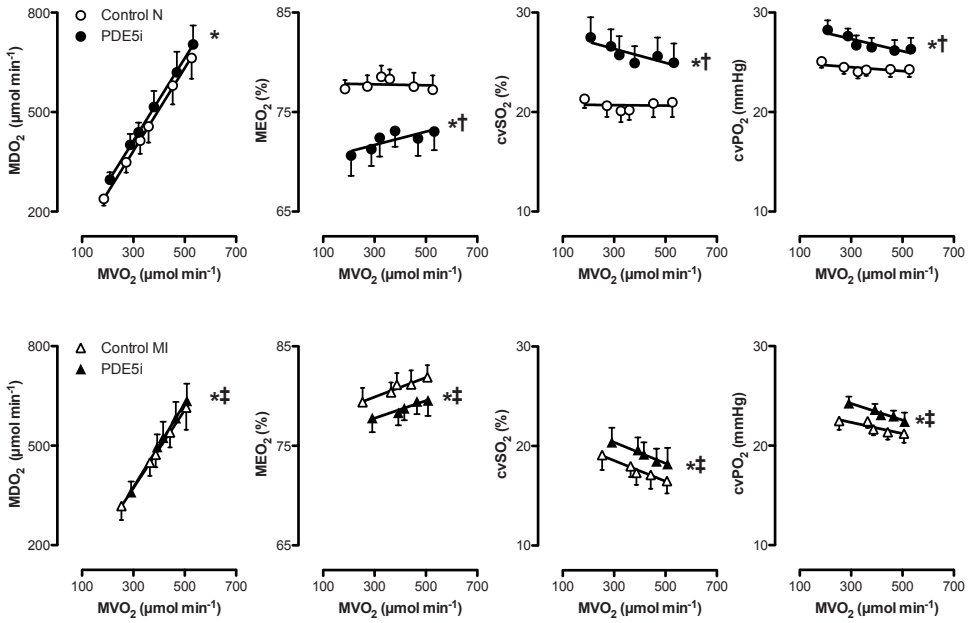

Fig. 3. *Effect of phosphodiesterase 5 inhibition (PDE5i) with EMD360527 (300 µg/kg/min iv) on the relation between myocardial O_2 consumption (MVO_2) and myocardial O_2 delivery (MDO_2), myocardial O_2 extraction (MEO_2), coronary venous O_2 saturation ($CVSO_2$) and coronary venous O_2 tension ($CVPO_2$) in 12 normal swine (top panels) and 8 swine with myocardial infarction (bottom panels). Data are means±SEM; To improve legibility, MVO_2 error bars have been omitted, which can be found in Table 2. * P<0.05 up- or downward shift of the relation in the presence of PDE5i vs corresponding control relation, † P<0.05, rotation of the relation in the presence of PDE5i vs. corresponding control relation, ‡ P<0.05 effect of PDE5i different in MI vs. Normal animals.*

Quantitative real-time PCR analysis

Coronary small arteries isolated from the LAD perfusion territory of 6 normal swine and of 4 swine in which MI had been induced 5 weeks prior to sacrifice, were used for detection of PDE5 mRNA (sense: 5'-TGGTGAACCCTTGAACATCA-3', antisense: 5'-GTGAATGTCCCACCATTTCC-3'). RNA was extracted from 4-6 frozen vessel samples per group using a Qiagen RNA kit. cDNA was synthesized from 100 ng of total RNA with iScript Reverse Transcriptase (Bio-Rad). Quantitative real-time PCR (MyIQ, Bio-Rad) was performed with SYBR Green (Bio-Rad) [27]. Target gene mRNA levels was expressed relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control [28].

Data analysis

Digital recording and off-line analysis of hemodynamic data and computation of myocardial VO_2 have been described in detail elsewhere [11]. Myocardial O_2 delivery (MDO_2) to the region of myocardium perfused by the LAD was computed as the product of CBF and arterial blood O_2 content. Myocardial O_2 consumption (MVO_2) was calculated as the product of CBF and the

difference in O_2 content between arterial and coronary venous blood. Myocardial O_2 extraction (MEO_2) was computed as the ratio of MVO_2 and MDO_2 [11].

Statistical analysis

Statistical analysis of hemodynamic data was performed using three-way (MI, drug treatment and exercise) or two-way (MI and exercise) analysis of variance (ANOVA) for repeated measures, as appropriate. When significant effects were detected, post-hoc testing for the effects of exercise, drug treatment and MI was performed using Scheffe's test. To test for the effects of MI and drug treatment on the relation between MVO_2 and MDO_2, coronary venous O_2 tension ($CVPO_2$), saturation ($CVSO_2$) or MEO_2, regression analysis was performed with animals as a dummy variable, and MI, drug treatment and MVO_2 as independent variables. Interaction terms (drug treatment and/or MI × MVO_2) were used to interpret alterations in the effect of drug treatment and/or MI during exercise. Linearity of the relations was confirmed by plotting the predicted value of the dependent variable against the residual value (measured value-predicted value). In all cases, residuals were scattered around zero over the entire range of predicted values.

An unpaired t-test was used to compare mRNA between normal and MI swine. Statistical significance was accepted when $P \leq 0.05$. Data are presented as mean±SEM.

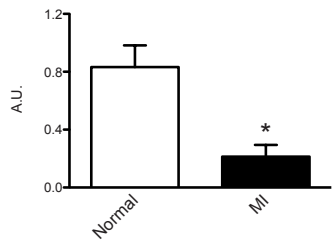

Fig. 4. *Phosphodiesterase 5 (PDE5) mRNA expression normalized to mRNA expression of GAPDH in coronary small arteries from the LAD territory. Vessels were isolated 5 weeks after induction of MI or Sham operation. * P<0.05 MI vs. Normal*

Results

Myocardial O_2 balance in normal and MI swine

In normal swine, exercise resulted in an increase in heart rate and LV contractility, which was accompanied by a parallel increase in MVO_2 (Fig. 1). The latter was met by an equivalent increase in CBF and hence MDO_2, so that MEO_2, $CVPO_2$ and $CVSO_2$ remained essentially unchanged (Fig. 1). MI of the LCx region increases the workload of the surviving myocardium [24, 29], which together with the increase in heart rate, produced a trend towards an increase in resting MVO_2 in the LAD area (Fig. 1). The increased myocardial oxygen demand in MI swine was principally met by an increase in LAD flow (Table 1, Fig. 2) but also by a small increase in MEO_2, resulting in slightly lower levels of $CVPO_2$ and $CVSO_2$ in MI compared to normal swine, both at rest and during exercise (Fig. 1).

Vasoconstrictor influence of PDE5 in coronary resistance vessels

PDE5 inhibition resulted in a decrease in blood pressure (Fig. 2), reflecting systemic vasodilation that was accompanied by a, probably baroreceptor-reflex mediated increase in heart rate (Table 1). In normal swine, PDE5 inhibition resulted in coronary vasodilation as evidenced by a decrease in coronary vascular resistance (Fig. 2, Table 1). This vasodilation resulted in an increase in CBF and MDO_2, so that MEO_2 decreased, resulting in an increased $CVPO_2$ and $CVSO_2$ (Fig. 3). The increase in MDO_2 in response to PDE5 inhibition was similar at rest and during exercise, whereas the increases in $CVPO_2$ and $CVSO_2$ were progressively attenuated during incremental levels of exercise (Fig. 3).

The hemodynamic responses to PDE5 inhibition in MI swine were similar to those in normal swine. Thus, PDE5 inhibition produced a decrease in mean aortic blood pressure (Fig.

2), which was accompanied by an elevation in heart rate (Table 1). Yet, the decrease in MEO_2 as well as the increases in MDO_2, $CVPO_2$ and $CVSO_2$ in response to PDE5 inhibition was significantly smaller in MI swine as compared to normal swine (Fig. 3). Moreover, in contrast to normal swine, these responses to PDE5 inhibition in MI swine were maintained during exercise.

Consistent with the reduced vasodilator response to PDE5 inhibition, mRNA for PDE5 was markedly reduced in coronary small arteries isolated from the remote territory of swine with MI as compared to normal swine (Fig. 4).

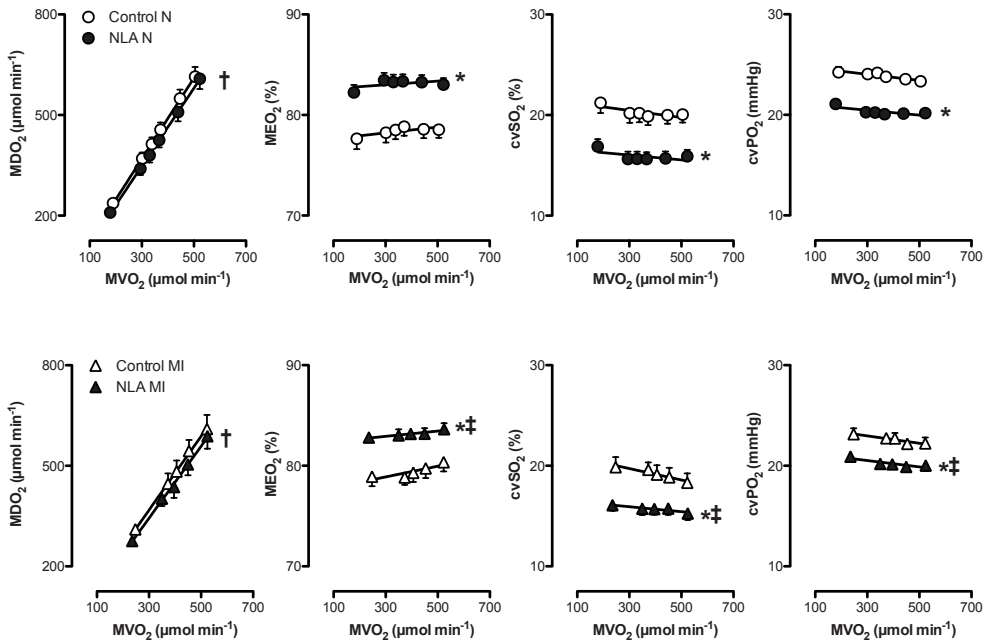

Fig. 5. *Effect of eNOS inhibition with NLA (20 mg/kg iv) on the relation between myocardial O_2 consumption (MVO_2) and myocardial O_2 delivery (MDO_2), myocardial O_2 extraction (MEO_2), coronary venous O_2 saturation ($CVSO_2$) and coronary venous O_2 tension ($CVPO_2$) in 28 normal swine (top panels) and 20 swine with myocardial infarction (bottom panels). Data are means±SEM. To improve legibility, MVO_2 error bars have been omitted. * P<0.05 up- or downward shift of the relation in the presence of NLA vs. corresponding control relation, † P<0.05, rotation of the relation in the presence of NLA vs. corresponding control relation, ‡ P<0.05 effect of NLA different in MI vs. Normal animals.*

Integrated control of coronary resistance vessel tone by eNOS and PDE5

In agreement with recent observations in our laboratory [21], the increase in MEO_2 and the decreases in MDO_2, $CVPO_2$ and $CVSO_2$ produced by eNOS inhibition were slightly but significantly smaller in MI as compared to normal animals (Fig. 5). Thus, coronary vasoconstriction in response to eNOS inhibition was blunted in MI swine, consistent with a reduction in NO bioavailability after MI. To investigate the integrated control of coronary resistance vessel tone by eNOS and PDE5, we studied the effects of PDE5 inhibition in a subgroup of animals after prior inhibition of eNOS with NLA. The vasodilator response to PDE5 inhibition was significantly attenuated by NLA in normal swine, as evidenced by blunted increases in MDO_2, $CVPO_2$ and $CVSO_2$, as well as a reduced decrease in MEO_2 as compared to its effect under control conditions (comparison Fig. 3 and Fig. 6). These results indicate that NO is an important source

of cGMP in normal swine. In contrast, the coronary vasodilation in response to PDE5 inhibition was minimally affected by pretreatment with NLA in MI swine (comparison Fig. 3 and Fig. 6), indicating that other factors are principally responsible for GC activation in MI swine.

To enable comparison of the effects of NLA in the absence and presence of PDE5 inhibition, we plotted in Fig. 7 the relation between MDO_2 and $CVSO_2$ during PDE5 inhibition (from Fig. 3) and during NLA+PDE5 inhibition (from Fig. 6) and compared it to relations during Control and NLA (both from Fig. 5). Fig. 7 illustrates that the shift in the relations between MVO_2 to MDO_2, MEO_2, $CVPO_2$ and $CVSO_2$ in response to NLA was markedly enhanced by pretreatment with PDE5 inhibition in normal but not in MI swine, indicating that PDE5 activity limited the vasodilator influence of NO in normal swine, but not in MI swine. Taken together these observations suggest that downregulation of PDE5 in resistance vessels within the remote myocardium of MI swine served to blunt the loss of NO vasodilator influence.

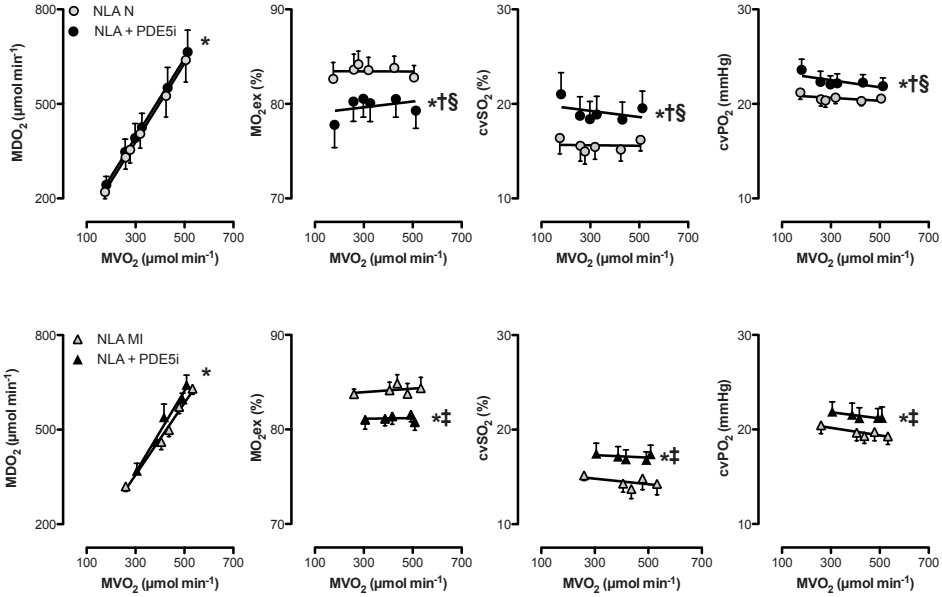

Fig. 6. *Effect of phosphodiesterase 5 inhibition (PDE5i) with EMD360527 (300 µg/kg/min iv) after eNOS inhibition with NLA (20 mg/kg iv) on the relation between myocardial O_2 consumption (MVO_2) and myocardial O_2 delivery (MDO_2), myocardial O_2 extraction (MEO_2), coronary venous O_2 saturation ($CVSO_2$) and coronary venous O_2 tension ($CVPO_2$) in 9 normal swine (top panels) and 6 swine with myocardial infarction (bottom panels). Data are means±SEM; To improve legibility, MVO_2 error bars have been omitted, which can be found in Table 2. * P<0.05 up- or downward shift of the relation in the presence of NLA+PDE5i versus corresponding NLA relation, † P<0.05, rotation of the relation in the presence of NLA+PDE5i vs. corresponding NLA relation, ‡ P<0.05 effect of PDE5i different in MI vs. Normal animals, § P<0.05 effect of PDE5i different in in the presence and absence of NLA.*

Discussion

The main findings of the present study are that (*i*) PDE5 inhibition resulted in coronary resistance vessel dilation; (*ii*) after MI, the coronary resistance vessel dilation in the remote myocardium produced by PDE5 inhibition was attenuated; (*iii*) PDE5 gene expression was reduced in coronary small arteries isolated from the remote myocardium after MI; (*iv*)

Inhibition of eNOS produced coronary vasoconstriction that was only slightly reduced in MI compared to normal swine; (*v*) PDE5-inhibition-induced coronary vasodilation was markedly reduced after prior inhibition of nitric oxide synthesis in normal swine, demonstrating that NO is a major activator of cGMP production; (*vi*) in contrast, in MI swine, PDE5 inhibition was not reduced by prior inhibition of eNOS, suggesting that other factors are responsible for cGMP production. The implications of these findings will be discussed below.

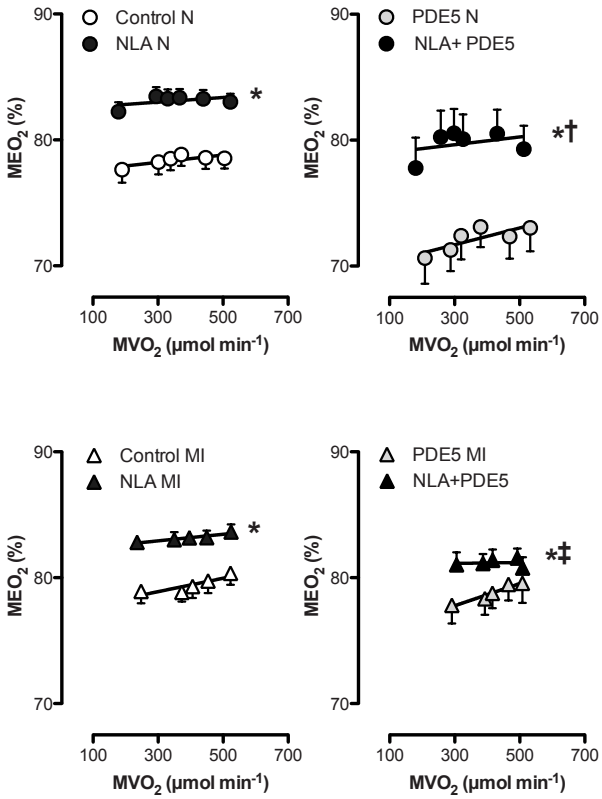

Fig. 7. *Effect of NO synthase inhibition (NLA) in the absence (left) and presence (right) of PDE5 inhibition (PDE5i) on the relation between myocardial O_2 consumption (MVO_2) and myocardial O_2 extraction (MEO_2) in normal swine (top panels) and MI swine (bottom panels). Data are means±SEM; To improve legibility, MVO_2 error bars have been omitted, which can be found in Table 2. * P<0.05 effect of NLA vs. corresponding control, † P<0.05 effect of NLA in presence of PDE5i vs. NLA alone, ‡ P<0.05 interaction between NLA and PDE5i different in MI vs. Normal animals.*

Methodological considerations

To interrogate the regulation of coronary resistance vessel tone, we employed systemic administration of pharmacological agents. As a consequence of the agents' systemic effects, perfusion pressure, oxygen carrying capacity of the blood and myocardial oxygen demand may change, all of which have indirect effects on coronary resistance vessel tone, thereby potentially confounding the direct effects of these agents and making interpretation of the results difficult. For example, eNOS inhibition resulted in systemic vasoconstriction, thereby increasing arterial

pressure and resulting in a, probably baroreceptor-mediated, decrease in heart rate and contractility. Conversely, PDE5 inhibition caused mild systemic vasodilation, thereby decreasing arterial pressure, and resulting in an increase in heart rate and contractility. The effects of changes in pressure and heart rate counterbalanced each other, and hence the overall influence of the systemic effects of eNOS- and PDE5-inhibition on MVO_2 did not reach statistical significance. In contrast, the alterations in pressure did result in an autoregulatory response, acting to maintain coronary blood flow commensurate with myocardial oxygen demand and contributing at least in part to the observed changes in calculated coronary vascular resistance. In addition, eNOS- and PDE5-inhibition produced changes in hemoglobin, particularly during exercise, requiring adjustments in coronary blood flow to maintain myocardial oxygen delivery commensurate with myocardial oxygen demand. These examples illustrate the difficulty in interpreting coronary blood flow and coronary vascular resistance data, when studying the mechanisms of coronary resistance vessel tone control in awake animals over a wide range of myocardial metabolic activities. To circumvent these confounding influences, and to separate direct from indirect effects of pharmacological agents on vascular tone, the so-called myocardial oxygen balance is used, i.e. the effect of a pharmacological agent on the relation between MVO_2 and MDO_2, $cvPO_2$, $cvSO_2$, or cv O_2 content is determined. For example, a direct decrease in coronary resistance vessel tone produced by PDE5 inhibition will increase coronary blood flow and hence MDO_2 at a given level of MVO_2. The increase in MDO_2 relative to the MVO_2 will permit a decrease in MEO_2 and results in an increase in $cvPO_2$ and $cvSO_2$ at a given level of MVO_2. The coronary venous oxygen content represents the most sensitive index of myocardial tissue oxygenation (i.e. the balance between MDO_2 and MVO_2) and is therefore commonly used to reflect coronary resistance vessel tone [1, 3, 4]. A parallel shift in these relations represents a vasomotor influence that is similar at rest and during exercise, whereas a rotation represents an increased or decreased effect during exercise [1, 3, 4]. Note that during exercise, when CBF and MDO_2 are higher than at rest, the *absolute* increase in MDO_2 required to obtain a similar upward shift in the relation between MVO_2 and coronary venous oxygen levels, is proportionally larger. Hence, the parallel upward shift in the relation between MVO_2 and MDO_2 in response to PDE5 inhibition is consistent with the rotation of the relation between MVO_2 and coronary venous oxygen levels and reflects a reduced vasoconstrictor influence of endogenous PDE5 activity during exercise as compared to rest in normal healthy swine.

Although the myocardial oxygen balance is regularly employed by us [4] as well as many other coronary physiologists [17, 30-36] in the assessment of coronary resistance vessel tone, other investigators [37] have expressed concern and pointed out several potential limitations. First, plotting MVO_2 versus $cvPO_2$ or $cvSO_2$ can be considered inappropriate because $cvPO_2$ and $cvSO_2$ are part of the formula to calculate MVO_2, so that MVO_2 is not an independent variable. Importantly, another index of myocardial work, the rate-pressure product (RPP), is linearly related to MVO_2, and substitution of RPP for MVO_2 as an independent variable yields virtually identical results (data not shown). However, since the heart principally employs aerobic metabolism as its source of energy, MVO_2 would appear to be a more appropriate index of myocardial work. A second concern is that the oxygen dissociation curve is influenced by various factors, including pH, pCO_2 and NO. This can affect the calculation of MVO_2, particularly when $cvSO_2$ is estimated from $cvPO_2$, using an assumed oxygen-dissociation curve [30, 38]. Importantly, in the present study MVO_2 was determined using highly accurate and direct measurements of not only $cvPO_2$ but also of Hb concentration and $cvSO_2$, thereby accounting for any potential changes in the oxygen dissociation curve. Thirdly, a linear relation between MVO_2 and cvPO2 or cvSO2 is often assumed, even when this is not apparent from visual inspection [39].

In the present study, we validated the use of linear regression analysis by statistically confirming linearity of all relations between MVO_2 and coronary venous oxygen levels, MEO_2 as well as MDO_2.

Table 2. Changes in blood gas parameters at rest and during maximal exercise in response to PDE5 inhibition in the absence and presence of eNOS inhibition.

		Normal swine		Swine with MI	
	Treatment	Rest	Max exercise	Rest	Max exercise
Hbart	Control	7.6 ± 0.3	9.1 ± 0.2*	8.2 ± 0.4	9.1 ± 0.3*
(g%)	PDE5i	8.0 ± 0.2	8.6 ± 0.2*†	8.1 ± 0.2	8.7 ± 0.2*†
	NLA	7.7 ± 0.3	9.6 ± 0.2*†	8.3 ± 0.3	9.5 ± 0.2*
	NLA+PDE5i	7.8 ± 0.3	9.4 ± 0.2*†‡	8.1 ± 0.2	8.8 ± 0.2*‡
SO_2art	Control	94 ± 1	92 ± 1	93 ± 1	91 ± 1
(%)	PDE5i	94 ± 1	91 ± 1*	92 ± 1	89 ± 2*
	NLA	96 ± 0	95 ± 1	95 ± 1	93 ± 1*
	NLA+PDE5i	95 ± 0	94 ± 1	93 ± 1‡	92 ± 1‡
PO_2art	Control	99 ± 2	86 ± 4*	96 ± 3	85 ± 3*
(mmHg)	PDE5i	94 ± 2	83 ± 4*	92 ± 3	78 ± 4*
	NLA	94 ± 3	84 ± 4*	92 ± 5	80 ± 5*
	NLA+PDE5i	93 ± 2	87 ± 4‡	84 ± 5	81 ± 5
MVO_2	Control	185 ± 16	526 ± 59*	254 ± 34	506 ± 53*
(μmol/min)	PDE5i	209 ± 16	532 ± 53*	291 ± 24	508 ± 41*
	NLA	175 ± 20	505 ± 61*	278 ± 16	530 ± 14*
	NLA+PDE5i	181 ± 23	513 ± 64*†	303 ± 18	509 ± 18*

*Hbart: arterial hemoglobin concentration; SO_2art: arterial oxygen saturation; PO_2art: arterial oxygen tension; MVO_2: myocardial oxygen consumption; PDE5i: PDE5 inhibition with EMD360527; NLA: eNOS inhibition with N^{ω}-Nitro-L-arginine Data are mean±SEM; *P<0.05 vs. rest; † P<0.05 vs. corresponding control; ‡ P<0.05 NLA+PDE5i vs. NLA alone; §P<0.05 MI vs. normal swine.*

Another potential methodological consideration concerns the selectivity of EMD360527 for PDE5. The balance between production and breakdown of cGMP contributes to the regulation of resistance vessel tone. cGMP in vascular smooth muscle is produced by soluble GC (sGC), which is primarily activated by NO and carbon monoxide (CO), as well as by membrane bound or particulate GC (pGC), which are activated upon ligand-binding to natriuretic peptide receptors, intestinal peptide receptors and orphan receptors [40]. In vascular smooth muscle, the enzymes responsible for degradation of the majority of cGMP are PDE1 and particularly PDE5 [6]. These are members of the family of phosphodiesterases, whose function is to remove cAMP and cGMP from various cell types [20, 41]. PDE5 is predominantly present in vascular smooth muscle and skeletal muscle [42, 43], although there are some reports of its presence in cardiomyocytes [44] and endothelial cells [45]. PDE5 removes cGMP by hydrolyzing it to 5' GMP [6]. EMD360527 demonstrates at least 45-fold selectivity for PDE5 as compared to other PDEs. If EMD360527 had inhibited cAMP degrading phosphodiesterases, this would have increased cAMP in vascular smooth muscle as well as cardiac muscle. We have previously shown that an increase in cAMP through inhibition of the cAMP-degrading enzyme PDE3 results in a small increase in heart rate and a large increase in cardiac contractility as measured through an increase in $LVdP/dt_{max}$ [46]. In the present study, administration of incremental dosages of EMD360527 resulted in a similar increase in heart rate, but a minimal increase in dP/dt_{max} (Fig. 8), suggesting that the effects of EMD360527 observed in the present study are indeed mediated through increases in cGMP.

Role of PDE5 regulating coronary resistance vessel tone at rest

Inhibition of PDE5 resulted in coronary vasodilation in both normal and MI swine. Thus, increasing the amount of cGMP in vascular smooth muscle by inhibiting its degradation reduces smooth muscle tone in the coronary microvasculature as evidenced by an increase in CBF as well as increases in $CVPO_2$ and $CVSO_2$. Moreover, the increase in CBF and hence in MDO_2 of ~10% in response to PDE5 inhibition corresponded well with the parallel ~10% decrease in MEO_2 measured in our experiments.

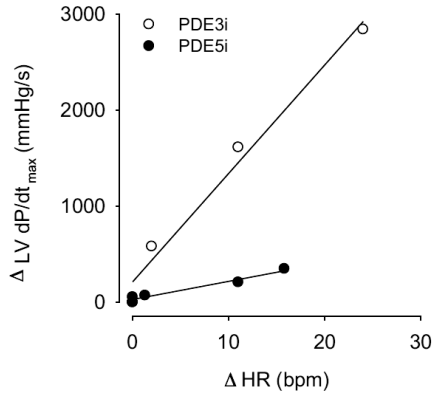

Fig. 8. *Effect of infusion of incremental dosages of phosphodiesterase 3 inhibition (PDE3i) with pimobendan (10, 25, 50 μg/kg/min iv [46]) and phosphodiesterase 5 inhibition (PDE5i) with EMD360527 (10, 30, 100 and 300 μg/kg/min iv) on the relation between changes in heart rate and changes in dP/dt$_{max}$. This figure clearly shows different effects of increasing cAMP (PDE3i) and cGMP (PDE5i) on cardiac function.*

Our findings in awake swine are in accordance with observations in anesthetized dogs, showing an increase in CBF in response to PDE5 inhibition with sildenafil [47]. Yet, a study in awake dogs showed that, although CBF increased, hemoglobin decreased, resulting in maintenance of MDO_2 [8]. In contrast, hemoglobin concentrations were not affected by PDE5 inhibition in resting swine in the present study (Table 2), so that myocardial oxygen delivery did increase in parallel with the increase in CBF in response to PDE5 inhibition. The diameter of the large coronary arteries did increase in response to PDE5 inhibition in both humans [48] and swine [7]. Yet, human studies fail to show an increase in coronary blood flow (as measured with positron emission tomography or with flow wire) in response to PDE5 inhibition under resting conditions [48-51], although PDE5 inhibition did augment adenosine-induced increases in coronary blood flow [49]. Importantly, however, all human studies involved patients with (severe) coronary artery disease (CAD), and even though the measurements were performed distal to non-diseased segments, it is likely that these patients had some degree of endothelial dysfunction in the coronary microcirculation [52], that may have limited the response to PDE5 inhibition. Hence, the lower levels of cGMP production could potentially explain the lack of effect of PDE5 inhibition in the coronary microvasculature of CAD patients. In support of this concept, it was shown that even in patients with mild CAD that showed little effect of PDE5-inhibition under basal conditions, PDE5 inhibition enhanced the, NO-mediated, vasodilation by acetylcholine of both the large epicardial coronary arteries and the coronary microcirculation [48]. Altogether, PDE5 inhibition has been shown to induce coronary vasodilation in the healthy coronary microcirculation under resting conditions, provided there is a sufficient production of NO and hence cGMP.

Role of PDE5 in regulating coronary resistance vessel tone during exercise

In contrast to a study in humans, in which the vasodilator effect of PDE5 inhibition increased during increased myocardial work [50] as well as a study in dogs, in which the vasodilator effect tended to increase during exercise [8], the coronary vasodilator effect of PDE5 inhibition slightly decreased during exercise in our porcine model. This finding may be interpreted to suggest that cGMP production decreased during exercise. However, in accordance with previous studies

from our laboratory [11, 12], the effect of eNOS inhibition on coronary vasomotor tone was well maintained during exercise, suggesting that NO-dependent activation of sGC was not altered. Moreover, we have previously shown that plasma levels of atrial natriuretic peptide increased during exercise [24], making it unlikely that activation of pGC decreased during exercise. A possible explanation for the unexpected observation that the effect of PDE5 inhibition decreases with incremental levels of exercise may be that the exercise-induced reduction in coronary resistance vessel tone blunts the sensitivity of vascular smooth muscle to cGMP. This notion is supported by unpublished observations from our laboratory that exogenous infusion of 8BrcGMP in a dose of 30 μg/kg/min for 10 minutes had no significant coronary vasodilator effect under control conditions, but did result in significant vasodilation, as evidenced by an increase in $CVPO_2$ (from 21.1±1.3 to 22.9±1.0 mmHg) and $CVSO_2$ (from 11.3±1.0 to 13.1±1.1%; both $P<0.05$), in the presence of NOS blockade. Another potential mechanism behind the reduction of the effect of PDE5 inhibition during exercise may be through interfering with sympathetic signaling [53]. Thus, cGMP has been shown to blunt norepinephrine release from sympathetic nerve endings in canine vascular smooth muscle [54], and in failing human hearts [55]. In the porcine coronary resistance vessels, β-adrenergic vasodilation predominates [56, 57], with little effect of either α-blockade or α-stimulation on coronary vascular tone [56, 58, 59]. During exercise, when sympathetic activity is high, particularly in the heart [60], PDE5 inhibition could thus have acted to suppress β-adrenergic vasodilation, thereby counteracting the direct vasodilator effect of PDE5 inhibition.

Importance of NO bioavailability for vasoconstrictor influence of PDE5

NO is an important stimulus for cGMP production through activation of sGC, so that the vasodilator effect of PDE5 inhibition is thought to be dependent on endogenous NO production by eNOS. This is also illustrated by the observation that the coronary vasodilation by acetylcholine, which is largely NO-mediated, is potentiated by PDE5 inhibition [8, 48].

A decreased NO bioavailability, such as occurs with endothelial dysfunction, is therefore likely to reduce the effect of PDE5 inhibition. To mimic endothelial dysfunction in the present study, we investigated the effect of PDE5 inhibition after prior NOS inhibition with NLA. In agreement with previous studies from our laboratory [11, 21], inhibition of NOS caused coronary vasoconstriction at rest and during exercise. Subsequent PDE5 inhibition resulted in coronary vasodilation that was markedly reduced as compared to the vasodilation to PDE5 inhibition under control conditions, indicating that NO is indeed an important activator of cGMP synthesis in the coronary circulation of normal swine. Nevertheless, even in the absence of NO, a significant portion of the coronary vasodilation in response to PDE5 inhibition was still present, suggesting that other factors, such as carbon monoxide and atrial natriuretic peptide, contribute to the generation of cGMP through activation of sGC and pGC, respectively, when NO synthesis is inhibited [40]. Interestingly, following eNOS inhibition, the vasodilation in response to PDE5 inhibition was maintained during exercise, suggesting that NO-independent cGMP levels are not influenced by exercise. This is surprising given our previous observation that ANP levels do increase at the highest exercise intensity [24].

cGMP in regulating coronary resistance vessel tone after MI

In the present study, the PDE5 mRNA expression in coronary small arteries from the remote non-infarcted myocardium was reduced after MI. Although mRNA expression cannot be equated to PDE5 protein level or activity, this finding is consistent with our in vivo observation of a reduced vasoconstrictor influence of PDE5 in remote myocardium. This reduced

vasoconstrictor influence of PDE5 may act to prolong the coronary vasodilator influence of cGMP. This finding has important implications for the interpretation of data on the vasoconstrictor effects of inhibition of NO production. In previous studies from our laboratory, we found that inhibition of NOS resulted in coronary vasoconstriction that was similar [12] or slightly decreased [21] in swine with MI. In the present study, involving a large number of normal and MI swine, we confirmed that coronary vasoconstriction following NOS inhibition was slightly less in swine with MI than in normal swine, suggesting that NO vasodilator influence was mildly reduced in the remote coronary vasculature after MI. The present study demonstrates that the reduced vasodilator influence of NO was not due to increased PDE5 activity. Rather, the reduced PDE5 gene expression and the blunted vasoconstrictor influence of PDE5 after MI, suggest that PDE5 is downregulated to prolong the biological availability of cGMP in an attempt to maintain the vasodilator influence of NO. This implies that the amount of NO required for this vasodilator influence is reduced. This interpretation is also supported by the observation that the vasoconstrictor responses to eNOS inhibition were enhanced by prior PDE5 inhibition in normal, but not MI, swine. The mechanism of loss of NO bioavailability was not determined in the present study. However, our recent observation that eNOS uncoupling is present in the remote zone after MI [61], suggests that both loss of NO production and reduced half life of NO, due to scavenging by superoxide, may contribute.

Finally, our observation that the vasodilator effect of PDE5 inhibition in MI swine was maintained in the presence of eNOS inhibition, indicates that factors other than NO must have contributed to the generation of cGMP in coronary resistance vessels of MI swine. Indeed, we have shown that plasma levels of ANP are increased in swine with MI [24], so that the contribution of particulate GC to the generation of cGMP is likely to be elevated in MI swine. Moreover, our data suggest that, although inhibition of eNOS does result in vasoconstriction in swine with MI, the main vasodilator effect of NO is not mediated by increasing cGMP, as the effect of eNOS inhibition was similar in the presence and absence of PDE5 inhibition. It is possible that the principal vasodilator effect of NO in the remote coronary circulation of swine with MI is mediated through limiting the vasoconstrictor effects of endothelin [26].

Conclusions

In healthy swine, PDE5 contributes to basal resting coronary resistance vessel tone but does not limit exercise-induced coronary vasodilation. The vasodilator effect of PDE5 inhibition is partially NO dependent. In contrast, in MI swine, the vasodilator effect of PDE5 inhibition in the remote surviving myocardium is NO-independent. Furthermore, PDE5 expression is attenuated compared to normal swine, which acts to maintain cGMP-mediated vasodilation in the coronary resistance vessels. In combination with previous data from our laboratory showing attenuation of ET_A- and AT_1-mediated vasoconstriction in the remote myocardium after MI [22, 60, 62], the present study suggests that attenuation of vasoconstrictor pathways serves to maintain coronary perfusion in remodeled myocardium after MI.

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Chapter 5

Phosphodiesterase 5 activity exerts a coronary vasoconstrictor influence in awake swine that is mediated in part via an increase in endothelin production

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Abstract

Nitric oxide (NO)-induced coronary vasodilation is mediated through production of cyclic guanosine monophosphate (cGMP), and through inhibition of the endothelin-1 (ET) system. We previously demonstrated that phosphodiesterase 5 (PDE5)-mediated cGMP breakdown and ET each exert a vasoconstrictor influence on coronary resistance vessels. However, little is known about the integrated control of coronary resistance vessel tone by these two vasoconstrictor mechanisms. In the present study, we investigated the contribution of PDE5 and ET to the regulation of coronary resistance vessel tone in swine at rest and during graded treadmill exercise. ET_A/ET_B receptor blockade with tezosentan (3 mg kg^{-1} iv) and PDE5 inhibition with EMD360527 (300 μg min kg^{-1} iv) each produced coronary vasodilation at rest and during exercise. In contrast, tezosentan failed to produce further coronary vasodilation in the presence of EMD360527. Importantly, EMD360527 (3 μM) had no significant effect on ET-induced contractions of isolated porcine coronary small arteries, but blunted the contractions produced by Big ET, suggesting that PDE5 inhibition limited ET production, while leaving ET responsiveness unperturbed. In conclusion, PDE5 activity exerts a vasoconstrictor influence on coronary resistance vessels that is mediated, in part, via an increase in ET production.

Introduction

Coronary blood flow is tightly coupled to myocardial oxygen demand to maintain a consistently high level of myocardial oxygen extraction [1-3]. This tight coupling has been proposed to depend on a myriad of vasodilators and vasoconstrictors, including nitric oxide (NO) and endothelin-1 (ET). NO causes coronary vasodilation by stimulating guanylyl cyclase in vascular smooth muscle to generate cyclic guanosine monophosphate (cGMP), which activates cGMP-dependent protein kinase G (PKG) [4]. Levels of cGMP in vascular smooth muscle are tightly regulated by several cyclic nucleotide phosphodiesterases (PDEs) that hydrolyze cGMP and terminate its vasodilator effect [5]. PDE5 is present in vascular smooth muscle cells in the coronary vascular bed, and thus has the potential to regulate coronary blood flow by exerting a vasoconstrictor influence. Indeed, PDE5 inhibition not only produced relaxation of coronary conduit arteries in swine in vitro [6] and in vivo [7], but also dilated coronary resistance vessels thereby increasing coronary blood flow in man [8] and swine [9].

NO not only induces coronary vasodilation through cGMP-mediated activation of vascular smooth muscle K^+ channels [2], but also produces vasodilation by inhibiting the coronary vasoconstrictor influence of ET during exercise [10]. We subsequently showed that this inhibition of ET by NO occurs principally through a reduced production of ET from Big ET [10, 11], and is likely cGMP-mediated [12-14]. Consequently, we hypothesized that PDE5 exerts a vasoconstrictor influence in the coronary microcirculation, in part, by abrogating the cGMP-mediated inhibition of ET. To test this hypothesis, we investigated the coronary vasodilator effects of ET receptor blockade in the absence and presence of PDE5 inhibition in awake swine at rest and during graded treadmill exercise. Since we observed that the vasodilation produced by ET receptor blockade was lost in the presence of PDE5 inhibition, we further evaluated the interaction between the two vasoconstrictor mechanisms in isolated coronary small arteries. For this purpose, we determined the responsiveness of the isolated coronary small arteries to ET and its precursor Big ET, in the absence and presence of PDE5 inhibition.

Methods

Experimental animals

Studies were performed in accordance with the "Guiding Principles in the Care and Use of Laboratory Animals" as approved by the Council of the American Physiological Society, and with approval of the Animal Care Committee at Erasmus University Medical Center Rotterdam. Sixteen crossbred Yorkshire × Landrace swine (2-3-month-old, 22±1 kg at the time of surgery) of either sex were entered into the in vivo study. Daily adaptation of animals to laboratory conditions started 1 week before surgery and continued during the first week after surgery. In vitro studies were performed in coronary small arteries isolated from the hearts of eight slaughterhouse pigs.

Surgical procedures

Swine were sedated with ketamine (20 mg kg^{-1} iv) and midazolam (1 mg kg^{-1} im), anaesthetized with thiopental (10 mg kg^{-1} iv), intubated and ventilated with a mixture of O_2 and N_2 (1:2) to which 0.2-1% (vol/vol) isoflurane was added [15, 16]. Anesthesia was maintained with midazolam (2 mg kg^{-1} iv) and fentanyl (10 μg kg^{-1} per hour iv). Under sterile conditions, the chest was opened via the fourth left intercostal space. A fluid-filled polyvinylchloride catheter was inserted into the aortic arch for the measurement of mean arterial pressure, and blood sampling for

determination of PO_2, PCO_2 and pH (ABL-600, Radiometer), and O_2 saturation and hemoglobin concentration (OSM3, Radiometer). A microtipped high-fidelity pressure-transducer ($P_{4.5}$, Konigsberg Instruments) was inserted into the left ventricle via the apex. Polyvinylchloride catheters were also inserted into the left ventricle to calibrate the Konigsberg transducer left ventricular pressure signal and into the pulmonary artery for administration of drugs. A Transonic flow probe was placed around the proximal left anterior descending coronary artery (LAD) for measurement of coronary blood flow. Finally, two small angiocatheters were inserted into the anterior inter-ventricular vein for coronary venous blood sampling [15]. Electrical wires and catheters were tunneled subcutaneously to exit at the back. Then, the chest was closed and animals were allowed to recover, receiving analgesia (0.3 mg buprenorphine im) and a slow release fentanyl patch (12 μg h^{-1}) for 3 days and antibiotic prophylaxis (25 mg kg^{-1} amoxicillin and 5 mg kg^{-1} gentamycin iv) for 5 days.

Table 1. Systemic hemodynamics and left ventricular function.

	Rest	Exercise (km/h)				
	Lying	1	2	3	4	5
HR, beats/min						
Control	136 ± 5	174 ± 5*	186 ± 7*	202 ± 8*	228 ± 9*	263 ± 9*
Tezo	145 ± 5	181 ± 8*	194 ± 10*	214 ± 9*	236 ± 10*	258 ± 9*
Control	110 ± 8	152 ± 12*	168 ± 11*	180 ± 11*	213 ± 11*	249 ± 8*
EMD	136 ± 8†	190 ± 12*†	196 ± 10*†	222 ± 8*†	243 ± 7*†	259 ± 7*
EMD+Tezo	147 ± 5†‡	208 ± 9*†‡	214 ± 8*†	237 ± 7*†‡	256 ± 6*†‡	270 ± 5*†
MAP, mmHg						
Control	93 ± 3	84 ± 3*	85 ± 4*	85 ± 4*	87 ± 4*	90 ± 6
Tezo	88 ± 6	75 ± 3*†	75 ± 4*†	79 ± 5	82 ± 4	81 ± 4†
Control	92 ± 5	83 ± 3	83 ± 3	84 ± 3	86 ± 3	89 ± 4
EMD	75 ± 4†	72 ± 4†	72 ± 4†	73 ± 3†	73 ± 4†	77 ± 4†
EMD+Tezo	70 ± 5†	67 ± 4†	65 ± 5†	67 ± 3†‡	67 ± 4†‡	72 ± 3†‡
LVSP, mmHg						
Control	110 ± 5	109 ± 4	112 ± 5	116 ± 5*	123 ± 6*	133 ± 8*
Tezo	106 ± 5	105 ± 4	107 ± 5	115 ± 6	122 ± 5*	126 ± 7*
Control	111 ± 4	102 ± 4	105 ± 3	106 ± 2	114 ± 4	123 ± 4*
EMD	98 ± 4	104 ± 3	105 ± 2	113 ± 4*	119 ± 5*	122 ± 5*
EMD+Tezo	96 ± 6	104 ± 4	109 ± 4	112 ± 5	120 ± 5*	124 ± 5*
LVdP/dtmax, mmHg/s						
Control	3010 ± 260	3560 ± 250*	3870 ± 330*	4100 ± 350*	4670 ± 600*	5470 ± 940*
Tezo	3160 ± 210	3600 ± 310	3870 ± 410*	4330 ± 470*	4770 ± 570*	5200 ± 990*
Control	2580 ± 210	3080 ± 220*	3250 ± 180*	3340 ± 150*	3820 ± 230*	4550 ± 300*
EMD	3010 ± 180	3670 ± 170*†	3740 ± 180*†	4090 ± 210*†	4370 ± 290*	4710 ± 320*
EMD+Tezo	2990 ± 190	3770 ± 240*†	3770 ± 150*†	4030 ± 160*†	4230 ± 160*	4520 ± 220*

*Values are mean±SEM. HR: Heart rate; MAP: Mean aortic pressure; LVSP: Left ventricular systolic pressure; LVdP/dtmax: Maximum rate of rise in left ventricular pressure. Tezo: Tezosentan; EMD: EMD360527. * P<0.05 vs. Rest lying; † P<0.05 vs. corresponding control; ‡ P<0.05, effect of tezo in the presence of EMD.*

Exercise studies

Studies were performed 1-2 weeks (11±1 days) after surgery with animals exercising on a motor-driven treadmill. Swine (n=16) were subjected to two different experimental protocols. In the first group, 9 swine were subjected to (*i*) control exercise and (*ii*) exercise in the presence of ET_A/ET_B receptor antagonist tezosentan (a gift from Actelion Pharmaceuticals, Allschwil, Switzerland). In the second group, 8 animals (one of which was subjected to both protocols)

were subjected to (*i*) control exercise, (*ii*) exercise in the presence of PDE5 inhibitor EMD360527 (a gift from Merck KGaA, Darmstadt, Germany) and (*iii*) exercise in the presence of combined EMD360527 and tezosentan.

Table 2. Myocardial oxygen balance.

	Rest	Exercise (km/h)				
	Lying	1	2	3	4	5
CBF, ml/min						
Control	63 ± 9	80 ± 11*	87 ± 12*	93 ± 13*	109 ± 17*	133 ± 24*
Tezo	66 ± 10	84 ± 14*	91 ± 16*	106 ± 17*	119 ± 18*	134 ± 21*
Control	58 ± 8	78 ± 13	82 ± 12*	86 ± 10*	111 ± 17*	132 ± 16*
EMD	66 ± 8†	98 ± 19†	105 ± 18*†	118 ± 16*†	133 ± 15*†	150 ± 15*†
EMD+Tezo	68 ± 9†	110 ± 19†	111 ± 17*†	125 ± 19*†	142 ± 16*†	154 ± 17*†
Hb, g%						
Control	7.9 ± 0.4	8.5 ± 0.4*	8.5 ± 0.4*	8.6 ± 0.4*	8.8 ± 0.4*	8.9 ± 0.4*
Tezo	8.4 ± 0.4	8.7 ± 0.3	8.6 ± 0.3	8.7 ± 0.3	8.8 ± 0.3*	8.8 ± 0.4*
Control	8.7 ± 0.2	9.0 ± 0.2	9.1 ± 0.2*	9.3 ± 0.1*	9.5 ± 0.2*	9.6 ± 0.2*
EMD	8.6 ± 0.3	8.8 ± 0.2	8.7 ± 0.2	8.6 ± 0.2†	8.8 ± 0.3†	8.8 ± 0.3†
EMD+Tezo	8.6 ± 0.3	8.4 ± 0.3	8.5 ± 0.3	8.5 ± 0.3†	8.6 ± 0.3†	8.8 ± 0.3†
$artPO_2$, mmHg						
Control	97 ± 4	98 ± 4	101 ± 3	100 ± 3	98 ± 4	98 ± 3
Tezo	99 ± 4	99 ± 7	97 ± 5	99 ± 5	98 ± 5	97 ± 5
Control	103 ± 3	98 ± 5	96 ± 5	99 ± 4	96 ± 5	92 ± 5
EMD	92 ± 3	95 ± 4	98 ± 5	97 ± 5	93 ± 5	89 ± 4
EMD+Tezo	96 ± 4	94 ± 5	97 ± 5	94 ± 4	93 ± 4	93 ± 3
$artSO_2$, %						
Control	95 ± 1	95 ± 1	96 ± 1	96 ± 1	95 ± 1	95 ± 1
Tezo	95 ± 1	95 ± 1	95 ± 1	95 ± 1	95 ± 1	95 ± 2
Control	97 ± 1	96 ± 1	95 ± 1	96 ± 1	95 ± 1	94 ± 2
EMD	95 ± 1	95 ± 1	95 ± 1	95 ± 1	95 ± 1	93 ± 2
EMD+Tezo	95 ± 1	95 ± 1	96 ± 1	95 ± 1	95 ± 1	95 ± 1
MDO_2, µmol/min						
Control	295 ± 35	402 ± 48*	438 ± 56*	476 ± 65*	573 ± 86*	707 ± 112*
Tezo	331 ± 48	429 ± 65*	466 ± 78*	551 ± 90*	617 ± 84*	701 ± 107*
Control	310 ± 37	417 ± 62*	447 ± 54*	486 ± 49*	623 ± 74*	738 ± 68*
EMD	342 ± 36	499 ± 64*†	529 ± 62*†	601 ± 57*†	696 ± 55*	780 ± 68*
EMD+Tezo	348 ± 35	546 ± 79*	561 ± 69*†	629 ± 68*†	730 ± 58*†	759 ± 70*
MVO_2, µmol/min						
Control	235 ± 29	320 ± 38*	354 ± 46*	384 ± 51*	459 ± 67*	556 ± 87*
Tezo	238 ± 30	339 ± 47*	368 ± 56*	435 ± 66*	484 ± 62*	550 ± 81*
Control	211 ± 16	294 ± 25*	326 ± 26*	371 ± 34*	469 ± 45*	571 ± 54*
EMD	237 ± 16	334 ± 28*	363 ± 30*	429 ± 36*	513 ± 36*	595 ± 61*
EMD+Tezo	239 ± 24	371 ± 48*	388 ± 38*	435 ± 35*	536 ± 37*	576 ± 48*

*Values are mean±SEM. CBF: Coronary blood flow; Hb: Hemoglobin; artPO2: Arterial oxygen tension; $artSO_2$: Aterial oxygen saturation; MDO_2: Myocardial oxygen delivery; MVO_2: Myocardial oxygen consumption. Tezo: Tezosentan; EMD: EMD360527. * P<0.05 vs. Rest lying; † P<0.05 vs. corresponding control.*

Effects of ET_A/ET_B receptor blockade. With swine lying quietly on the treadmill, resting hemodynamic measurements were obtained and arterial and coronary venous blood samples

were collected. Hemodynamic measurements were repeated and rectal temperature was measured with animals standing on the treadmill. Subsequently, a five-stage exercise protocol (1-5 km h^{-1}) was begun with each stage lasting 3 min. Hemodynamic variables were continuously recorded and blood samples collected during the last 30 seconds of each exercise stage, at a time when hemodynamics had reached a steady state. At the conclusion of the exercise trial, animals were allowed to rest for 90 min, resulting in a complete return of hemodynamic variables to baseline values. Subsequently, tezosentan was infused intravenously over 10 min in a dose of 3 mg kg^{-1}, followed by a continuous intravenous infusion of 6 mg kg^{-1} h^{-1} [10, 16] and the five-stage exercise trial was repeated. Tezosentan has a Ki of 10 nM for ET_B and a Ki of 20 nM for ET_A [17].

Effects of PDE5 inhibition and ET_A/ET_B receptor blockade. Ninety min after completing a control exercise trial as described above, swine received a continuous intravenous infusion of EMD360527 in a dose of 300 μg kg^{-1} min^{-1}. EMD360527 demonstrates at least 45-fold selectivity for PDE5 (IC_{50}=0.007 mM) compared to PDE6 (IC_{50}=0.32 mM), 94-fold selectivity for PDE1 (IC_{50}=0.66 mM), 137-fold selectivity for PDE10 (IC_{50}=0.96 mM), and >1400-fold selectivity for PDE2, PDE3, PDE4 and PDE7 (all IC_{50}>10 mM). Ten min after starting the infusion, the five-stage exercise trial was repeated, while infusion of EMD360527 was continued [9, 18]. Upon completion of the exercise trial, EMD360527 infusion was halted and animals were allowed to rest for another 90 min. Then, the EMD360527 infusion was continued while an intravenous infusion of tezosentan was started, in dose-regimen identical as described above for the individual infusions, and the five-stage exercise protocol was repeated. We have previously observed excellent reproducibility of consecutive exercise protocols [15].

Myograph studies of isolated coronary small arteries

Swine hearts (n=8) were collected at a local slaughterhouse. Coronary small arteries (diameter ≈250 μm) were dissected out and stored overnight in cold Krebs bicarbonate solution aerated with 95% O_2/5% CO_2 of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4 [19]. The next day, coronary arteries were cut into segments of ≈2 mm length and mounted in microvascular myographs (Danish Myo Technology) with separated 6 ml organ baths containing Krebs bicarbonate solution aerated with 95% O_2/5% CO_2 and maintained at 37°C. Changes in contractile force were recorded with a Harvard isometric transducer. Following a 30 min stabilization period, the internal diameter was set to a tension equivalent to 0.9 times the estimated diameter at 100 mmHg effective transmural pressure. The vessels were then exposed to 30 mM KCl twice. Endothelial integrity was verified by observing dilation to 10 nM substance P after preconstriction with 100 nM of the stable thromboxane-A_2 analogue 9,11-Dideoxy-11α, 9α epoxymethanoprostaglandin $F_{2\alpha}$ (U46619). Then vessels were subjected to 100 mM KCl to determine the maximal vascular contraction. Thereafter, vessels were allowed to equilibrate in fresh Krebs solution for 30 min before initiating different experimental protocols [20, 21]. Only one protocol was executed per vessel and each experimental protocol was performed in 7-8 vessels, each obtained from a different animal.

Effects of PDE5 inhibition on ET receptor sensitivity and ET production. Vessels were exposed to ET (10^{-9}-10^{-7} M) and Big ET (10^{-8}-10^{-6} M) in cumulative incubations (each dose present for 15 minutes) in the absence and presence of EMD360527 (3 μM) (7 vessels per group for ET response; 8 vessels per group for Big ET response). Based on in vivo [11] and in vitro [22, 23] observations, we selected a higher dose range of Big ET than ET to achieve similar vasoconstrictor responses. Big ET has no direct vasomotor effect [24], so that the Big ET-induced vasoconstriction reflects the conversion from Big ET to vasoactive ET [11].

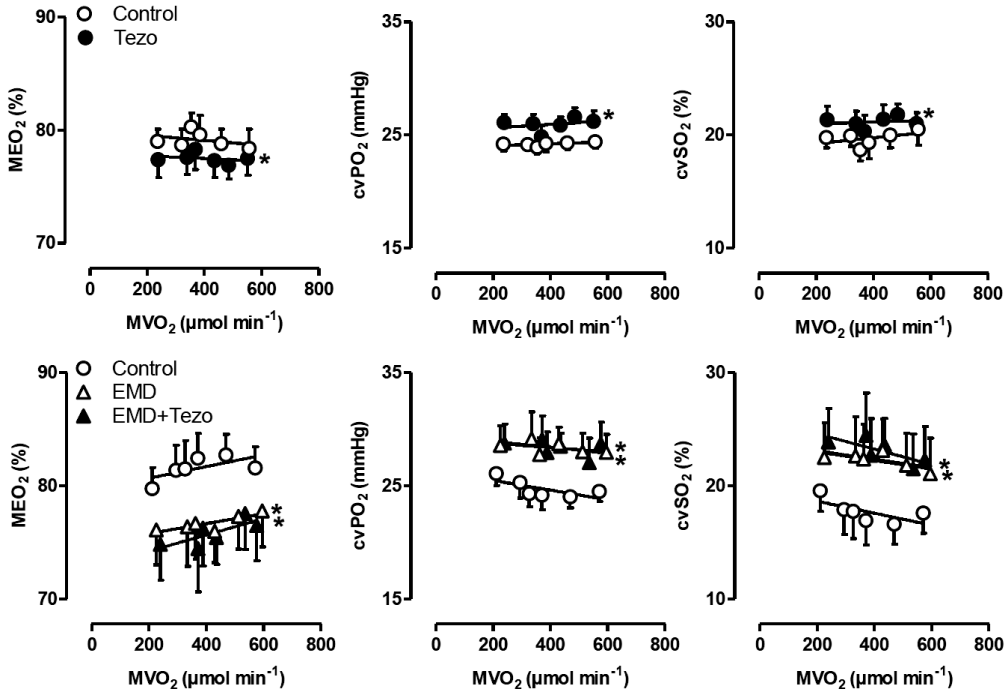

Fig. 1. *Effects of PDE5 inhibition and ET_A/ET_B receptor blockade on coronary resistance vessel tone in vivo. Shown are the effects of ET_A/ET_B receptor blocker tezosentan (Tezo), PDE5 inhibitor EMD360527 (EMD), and Tezo in the presence of EMD, on the relations between myocardial oxygen consumption (MVO_2), and myocardial oxygen extraction (MEO_2), coronary venous oxygen tension ($cvPO_2$) or saturation ($cvSO_2$) at rest and during graded treadmill exercise. Values are mean±SEM. * P<0.05, vs. corresponding Control.*

Data analysis and statistics

Digital recording and off-line analysis of hemodynamic data and computation of myocardial O_2 consumption (MVO_2) have been described in detail elsewhere [15, 16]. Myocardial O_2 delivery (MDO_2) was computed as the product of LAD coronary blood flow and arterial blood O_2 content. MVO_2 in the region of myocardium perfused by the LAD was calculated as the product of coronary blood flow and the difference in O_2 content between arterial and coronary venous blood. Myocardial oxygen extraction (MEO_2) was computed as the ratio of MVO_2 and MDO_2. In vitro vascular contraction responses to ET and Big ET were normalized to 100 mM KCl.

Statistical analysis of hemodynamic data was performed using two-way ANOVA for repeated measures followed by t-test analysis. To test the effects of drug treatment on the relation between MVO_2 and MEO_2, coronary venous O_2 tension ($cvPO_2$), coronary venous O_2 saturation ($cvSO_2$), regression analysis was performed using drug treatment and MVO_2, as well as their interaction as independent variables, and by assigning a dummy variable to each animal. Linearity of the relations was confirmed by plotting the predicted value of the dependent variable against the residual value (measured value-predicted value). In all cases, residuals were scattered around zero over the entire range of predicted values. The effects of EMD360527 on exogenous ET and Big ET response were assessed using two-way ANOVA. Statistical significance was accepted when $P<0.05$. Data are presented as mean±S.E.M.

Results

Integrated effects of EMD360527 and tezosentan in the coronary circulation

Graded treadmill exercise had minimal effects on mean aortic blood pressure, but produced marked increases in heart rate and $LVdP/dt_{max}$ (Table 1). The consequent increase in MVO_2 was met by a commensurate increase in coronary blood flow and MDO_2 (Table 2) so that MEO_2 and hence $cvSO_2$ and $cvPO_2$ remained relatively constant during exercise (Fig. 1).

In accordance with previous studies from our laboratory [9, 16], intravenous infusion of the ET_A/ET_B receptor blocker tezosentan or the PDE5 inhibitor EMD360527 alone, produced small decreases in mean aortic pressure, which were accompanied by, presumably baroreceptor reflex-mediated, increases in heart rate (Table 1). Combined infusion of EMD360527 and tezosentan further decreased mean aortic pressure compared to EMD360527 alone (Table 1).

Tezosentan tended to increase both coronary blood flow and haemoglobin, resulting in a small near-significant (P=0.09 by ANOVA) increase in MDO_2 (Table 2). As a result, the relation between MDO_2 and MVO_2 was shifted upwards, thereby allowing a small, but significant decrease in MEO_2 that resulted in a slight increase in $cvPO_2$ and $cvSO_2$, both at rest and during exercise (Fig. 1). EMD360527 resulted in a significant increase in coronary blood flow while haemoglobin was reduced (Table 2). The increase in coronary blood flow outweighed the decrease in haemoglobin resulting in an overall increase in MDO_2, thereby allowing a decrease in MEO_2, resulting in a marked increase in $cvPO_2$ as well as $cvSO_2$ (Fig. 1). Strikingly, in the presence of EMD360527, the effects of tezosentan on MEO_2, $cvSO_2$ and $cvPO_2$, were no longer observed, indicating that the vasoconstrictor influence of endogenous ET was abolished by PDE5 inhibition (Fig. 1).

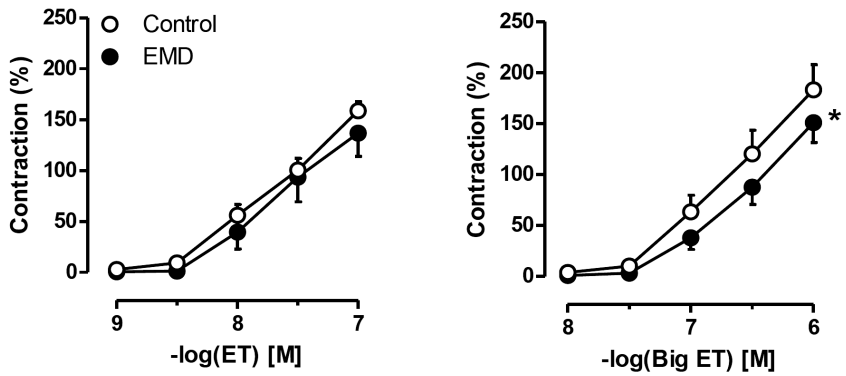

Fig. 2. *Effects of PDE5 inhibition on endothelin (ET) sensitivity and production from its precursor Big ET in coronary small arteries in vitro. Shown are effects of EMD360527 (EMD; 3 μM) on the vasoconstrictor responses to ET (n=7 with and without EMD) and Big ET (n=8 with and without EMD) in isolated coronary small arteries. Values are mean±SEM. * P<0.05, vs. corresponding Control.*

Effects of EMD360527 on ET receptor sensitivity and ET production

The lack of additional coronary vasodilation induced by combined ET and PDE5 inhibition as compared to PDE5 inhibition alone, suggested an interaction between the NO-cGMP and the ET systems. To further study this interaction, we conducted experiments in isolated coronary small arteries exposed to either ET or Big ET, both in the absence and presence of EMD360527. ET produced concentration-dependent contractions that were not affected by pretreatment with EMD360527 (Fig. 2). Big ET, in one order of magnitude higher dosages than ET, elicited similar

contractions compared to ET. Importantly, EMD360527 slighly, but significantly, attenuated the Big ET-induced vascular contraction (Fig. 2). These findings support our in vivo findings of an interaction between the NO-cGMP and the ET systems, and show that PDE5 inhibition limits the vasoconstrictor influence of ET by decreasing ET production from Big ET, while leaving ET responsiveness unperturbed.

Discussion

The main findings of the present study were that (*i*) PDE5 inhibition with EMD360527 and ET receptor blockade with tezosentan, each produced coronary vasodilation, with vasodilation by PDE5 inhibition being significantly greater than that produced by ET blockade; (*ii*) in the presence of PDE5 inhibition, ET receptor blockade failed to produce additional coronary vasodilation; (*iii*) PDE5 inhibition significantly attenuated Big ET- but not ET-induced contraction in isolated coronary small arteries. The implication of these findings will be discussed below.

Methodological considerations

Intravenous administration of blockers of vasoactive substances not only results in changes in coronary resistance vessel tone but can also produce pronounced changes in systemic hemodynamics. In the present study, PDE5 inhibition, ET_A/ET_B blockade and their combination resulted in decreases in mean aortic pressure, that are the result of systemic vasodilation [16, 18], and which require autoregulatory adjustments in coronary vascular resistance. In addition, the baroreceptor reflex-mediated increases in heart rate and contractility, together with the decrease in aortic blood pressure will result in changes in myocardial metabolism, requiring adjustments of myocardial oxygen supply and hence coronary blood flow. Finally, ET receptor blockade increased hemoglobin levels, while PDE5 inhibition produced a slight reduction in hemoglobin, which require adjustments in coronary blood flow to maintain myocardial oxygen delivery. Consequently, changes in coronary blood flow and/or coronary vascular resistance in response to PDE5 inhibition and ET blockade in vivo, not only reflect direct effects of these pharmacological interventions on coronary resistance vessel tone, but are also influenced by their effects on perfusion pressure, myocardial metabolic demand, and/or the blood's oxygen carrying capacity. Hence, interpretation of changes in coronary blood flow and coronary vascular resistance data is difficult when investigating the effects of pharmacological interventions on coronary resistance vessel tone in awake animals over a wide range of myocardial metabolic activities.

A sensitive way to study the regulation of coronary resistance vessel tone during exercise is to examine the relation between coronary venous oxygen levels and MVO_2 [3, 25]. Thus, a direct decrease in vasomotor tone by PDE5 inhibition, ET_A/ET_B blockade or their combination will increase MDO_2 at a given level of MVO_2. This increase in MDO_2 relative to MVO_2 will allow a decrease in MEO_2, thereby leading to increases in $cvPO_2$ and $cvSO_2$ and hence in an upward shift of the relation between MVO_2 and coronary venous oxygen levels. The coronary venous oxygen level thus represents a sensitive index of myocardial tissue oxygenation (i.e. the balance between MDO_2 and MVO_2) and is therefore commonly used to reflect coronary resistance vessel tone [3, 25].

Although we [3] and other coronary physiologists [26-33] often employ the myocardial oxygen balance to study the regulation of coronary resistance vessel tone, potential limitations of this approach have been pointed out [34]. For example, plotting MVO_2 versus $cvPO_2$ or $cvSO_2$ can be considered inappropriate because $cvPO_2$ and $cvSO_2$ are part of the formula to calculate MVO_2, so

that MVO_2 is not an independent variable. Importantly, another index of myocardial work, the rate pressure product (RPP), is linearly related to MVO_2, and substitution of RPP for MVO_2 as an independent variable yields virtually identical results (data not shown). However, since the heart principally employs aerobic metabolism as its source of energy, MVO_2 would appear to be a more appropriate index of myocardial work. Another potential concern is that the oxygen dissociation curve is influenced by various factors, including pH, pCO_2 and NO, which could affect computation of MVO_2, particularly when $cvSO_2$ is estimated from $cvPO_2$ using an assumed oxygen dissociation curve [26, 35]. In the present study, MVO_2 was determined using highly accurate and direct measurements of not only $cvPO_2$ but also of Hb concentration and $cvSO_2$, thereby accounting for any potential changes in the oxygen dissociation curve. Finally, a linear relation between MVO_2 and $cvPO_2$ or $cvSO_2$ is often assumed, even when this is not apparent from visual inspection [36]. In the present study, we validated the use of linear regression analysis by statistically confirming linearity of all relations between MVO_2 and MEO_2 as well as coronary venous oxygen levels.

Integrated control of coronary resistance vessel tone by ET and PDE5

In accordance with previous observations [10, 11, 25, 37, 38], we observed that ET receptor blockade resulted in coronary vasodilation, as evidenced by the decreased oxygen extraction and increased coronary venous oxygen content.

In agreement with a recent study from our laboratory [9], PDE5 inhibition with EMD360527 resulted in a downward shift of the relations between MVO_2 and MEO_2, and an upward shift of the relations between MVO_2 and either $cvSO_2$ or $cvPO_2$. These findings support the concept that PDE5 exerts a vasoconstrictor influence not only in large coronary conduit vessels [7], but also in coronary resistance vessels [8, 9], and show that the vasoconstrictor influence of PDE5 on coronary resistance vessels is also present during exercise. Similar to these observations in man and swine, administration of the PDE5 inhibitor sildenafil was reported to increase coronary blood flow in dogs [39, 40]. However, hemoglobin decreased in response to sildenafil and no shift of the relation between MVO_2 and MDO_2 [40]. Thus, it would appear that, in dogs, the increase in coronary blood flow was aimed at maintaining MDO_2 rather than the result of a direct effect of PDE5 inhibition on coronary resistance vessel tone.

The vasodilator effect produced by PDE5 inhibition was greater than that produced by ET blockade, indicating that the vasoconstrictor influence of PDE5 in coronary resistance vessels is greater than that of endogenous ET. Importantly, in the presence of PDE5 inhibition with EMD360527, ET_A/ET_B blockade with tezosentan failed to produce additional vasodilation of coronary resistance vessels beyond that produced by PDE5 inhibition alone. This observation is highly suggestive of a direct interaction between the NO-cGMP and ET systems. Indeed, we have previously shown that NO elicits coronary vasodilation in part via inhibition of ET-mediated vasoconstriction [37], which is likely mediated through reduced production of ET from Big ET [11]. Interestingly, experiments in the porcine aorta [12], the rat heart [13], and cultured pulmonary arterial endothelial cells [14] have shown that not only NO, but also the non-hydrolysable cGMP analogue 8Br-cGMP, can suppress ET production and release. Moreover, it has recently been shown that dibutyryl-cGMP, another cGMP analogue, reduced endothelin converting enzyme (ECE) mRNA expression, protein content and activity in bovine aortic endothelial cells, which was mediated through PKG [41]. Taken together, these studies indicate that inhibition of ET production by NO is mediated via downstream signaling through cGMP and PKG. Consistent with these observations, we found that PDE5 inhibition significantly attenuated the vasoconstrictor response of isolated coronary small arteries to Big ET, but not to

ET, further supporting the concept that interaction between the NO-cGMP system and the ET system occurs via inhibition of ECE by cGMP in the coronary circulation.

Conclusions

The present study demonstrated that PDE5 inhibition blunted the vasoconstrictor influence of ET in porcine coronary resistance vessels, which appeared principally the result of inhibition of ET production, with no change in ET responsiveness. These findings indicate that PDE5 exerts a vasoconstrictor influence in the porcine coronary circulation that is mediated in part, via enhancing ET production.

Acknowledgements

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Chapter 6

Pulmonary vasoconstrictor influence of endothelin in exercising swine depends critically on phosphodiesterase 5 activity

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Am J Physiol Lung Cell Mol Physiol *Under Revision*

Abstract

Both phosphodiesterase 5 (PDE5) inhibition and endothelin (ET) receptor blockade have been shown to induce pulmonary vasodilation. However, little is known about the effect of combined blockade of these two vasoconstrictor pathways. Since nitric oxide (NO) exerts its pulmonary vasodilator influence via production of cyclic guanosine monophosphate (cGMP) as well as through inhibition of ET, we hypothesized that interaction between the respective signalling pathways precludes an additive vasodilator effect. We tested this hypothesis in chronically instrumented swine running on the treadmill by comparing the vasodilator effect of PDE5 inhibitor EMD360527 (300 μg min kg^{-1}), ET_A/ET_B antagonist tezosentan (3 mg kg^{-1}) and combined administration of EMD360527 and tezosentan. In the systemic circulation, vasodilation by tezosentan and EMD360527 was additive, resulting in a marked drop in blood pressure (17±2 mmHg). In the pulmonary circulation, both EMD360527 and tezosentan produced vasodilation. However, tezosentan had no pulmonary vasodilator effect in the presence of EMD360527. Moreover, in isolated preconstricted porcine pulmonary small arteries (~300 μm) EMD360527 (1 nM-10 μM) induced dose-dependent vasodilation. Tezosentan (1 nM-10 μM) failed to have a vasodilator effect both in the absence and presence of EMD360527. EMD360527 (3 μM) blunted Big ET-induced pulmonary small artery contraction significantly more (78±8%) than the contraction in vessels produced by ET (32±12%). In conclusion, in healthy swine, PDE5-inhibition produces pulmonary vasodilation that is mediated in part through inhibition of the ET-pathway, thereby precluding an additive vasodilator effect of ET_A/ET_B receptor blockade in the presence of PDE5 inhibition.

Introduction

The pulmonary circulation is a low-pressure, low-resistance system with little vascular tone [1, 2]. As a result, it has little capacity for additional vasodilation, which is especially notable during exercise, when an increased cardiac output induces a dramatic increase in pulmonary artery pressure [1, 2]. The increase in pulmonary artery pressure during exercise is limited by an increase in pulmonary vascular conductance (PVC), through passive pulmonary distension and active pulmonary vasodilation. Nevertheless, the work load of the right ventricle increases during exercise, and has been shown to be an important determinant of exercise capacity [3]. Consequently, small changes in pulmonary vascular tone and PVC, which have an obvious impact on pulmonary pressure, will influence exercise capacity.

Pulmonary vascular tone is determined by an interplay between vasodilators and vasoconstrictors, such as nitric oxide (NO) and endothelin (ET). Thus, exercise-induced pulmonary vasodilation is largely NO-mediated [1, 4], and can be enhanced by prolonging the half-life of its second messenger cGMP through inhibition of phosphodiesterase 5 (PDE5) [5]. Since PDE5 is abundantly expressed in pulmonary vascular smooth muscle in particular [6, 7], PDE5 inhibition has clinically been used to selectively increase PVC without inducing systemic hypotension in patients with pulmonary hypertension [8-10]. NO not only induces pulmonary vasodilation through direct effects on vascular smooth muscle, but also indirectly by blunting ET-mediated pulmonary vasoconstriction in swine [11, 12]. Nevertheless, ET exerts a vasoconstrictor influence on the pulmonary vasculature [13, 14], that is relatively small under basal resting conditions, but, surprisingly, becomes more pronounced during exercise, thereby limiting the exercise-induced pulmonary vasodilation [11, 15]. Thus, both PDE5 inhibition and ET receptor blockade increase PVC, particularly during exercise, and combined inhibition of both pathways may result in an additive increase in PVC and may therefore synergistically improve exercise capacity in patients with pulmonary hypertension. However, since NO blunts ET-mediated pulmonary vasoconstriction [11] and since ET can enhance NO production via ET_B receptor stimulation [1], it is also possible that interaction between the respective signalling pathways precludes such additive vasodilator effect. Therefore, the aim of the present study was to evaluate the vasodilator effect of ET receptor blockade on the pulmonary vasculature in vivo in the presence of PDE5 inhibition. Since we found no additive pulmonary vasodilator effect of ET receptor blockade following PDE5 inhibition, we further investigated whether this lack of effect was the result of a direct interaction between the NO-cGMP and the ET pathway, or due to a lack of residual tone in the pulmonary vasculature following PDE5 inhibition, using isolated pulmonary small arteries. For this purpose, we investigated if PDE5 inhibition and ET_A/ET_B receptor blockade could act synergistically when pulmonary tone was increased with the stable thromboxane A_2 analogue U46619, and we evaluated the responsiveness of the isolated pulmonary small arteries to ET and its precursor Big ET, in the absence and presence of PDE5 inhibition.

Methods

In vivo studies

Animals

Studies were performed in accordance with the Council of Europe Convention (ETS123)/ Directive) (86/609/EEC) for the protection of vertebrate animals used for experimental and other scientific purposes, and with approval of the Animal Care Committee of the Erasmus MC

Rotterdam. Thirteen crossbred Yorkshire × Landrace swine (2-3 months old, 22±1 kg at the time of surgery) of either sex entered the study. Daily adaptation of animals to laboratory conditions started 1 week before surgery and continued during the first week after surgery.

Surgical procedures

Swine were sedated with ketamine (20 mg kg^{-1} iv), and midazolam (1 mg kg^{-1} im), anesthetised with thiopental (10 mg kg^{-1} iv), intubated and ventilated with a mixture of O_2 and N_2 (1:2) to which 0.2-1% (vol/vol) isoflurane was added.[11, 16, 17] Anesthesia was maintained with midazolam (1 mg kg^{-1} per hour iv) and fentanyl (10 μg kg^{-1} per hour iv). Under sterile conditions, the chest was opened via the fourth left intercostal space and fluid-filled polyvinylchloride catheters were directly inserted into the aortic arch, left atrium and pulmonary artery by puncture of these structures for blood sampling and blood pressure measurement (Combitrans® pressure transducers, Braun, Melsungen, Germany). A Transonic flow probe (16 mm Transonic Systems) was positioned around the ascending aorta for measurement of cardiac output. Catheters were tunnelled to the back and animals were allowed to recover, receiving analgesia (0.3 mg buprenorphine im) for 2 days and antibiotic prophylaxis (25 mg kg^{-1} amoxicillin and 5 mg kg^{-1} gentamycin iv) for 5 days.

Exercise protocols

Experimental design.

Studies were performed 1-2 weeks (11±1 days) after surgery with animals exercising on a motor-driven treadmill. Swine (n=13) were subjected to two different experimental protocols. In the first group, animals (n=7) performed *(i)* control exercise and *(ii)* exercise in the presence of ET_A/ET_B antagonist tezosentan (a gift from Actelion Pharmaceuticals, Allschwil, Switzerland). In the second group, animals (n=7, one animal overlapping with the fist group) performed *(i)* control exercise, *(ii)* exercise in the presence of PDE5 inhibitor EMD360527 (a gift from Merck KGaA, Darmstadt, Germany) and *(iii)* exercise in the presence of combined EMD360527 and tezosentan.

Effects of ET_A/ET_B receptor blockade during exercise. With swine standing quietly on the treadmill, resting hemodynamic measurements consisting of left atrial, aortic and pulmonary artery blood pressures, heart rate and cardiac output were obtained, arterial and mixed venous blood samples were collected and rectal temperature measured. Subsequently, a five-stage exercise protocol (1, 2, 3, 4 and 5 km h^{-1}) was started with each stage lasting 2-3 min. Hemodynamic variables were continuously recorded and blood samples collected during the last 30 seconds of each exercise stage, at a time when hemodynamics had reached a steady state. Following the exercise trial, animals were allowed to rest for 90 min, resulting in a complete return of hemodynamic variables to baseline values [4]. In the second exercise protocol, tezosentan was infused intravenously over 10 min in a dose of 3 mg kg^{-1}, followed by a continuous intravenous infusion of tezosentan in a dose of 6 mg kg^{-1} h^{-1} and the five-stage exercise protocol was repeated. We have previously shown that this dose of tezosentan abolishes the increase in blood pressure in response to endothelin [15]. Tezosentan has a pA_2 of 9.5 for ET_A and a pA_2 of 7.7 for ET_B receptors, indicating only a 63-fold selectivity for ET_A receptors compared with ET_B receptors [18, 19].

Effects of PDE5 inhibition during exercise. Animals from the second group underwent the same exercise protocol as those in the first group, but consisting of three consecutive exercise trials. First, a control exercise trial was performed as described above. Following the exercise trial, animals were allowed to rest for 90 min, resulting in a complete return of hemodynamic

Table 1. Hemodynamic effects of ET antagonism, PDE5 inhibition and the combination.

	Rest	Exercise (km/h)				
	Standing	1	2	3	4	5
HR, beats/min						
Control	144 ± 4	168 ± 4*	177 ± 6*	194 ± 8*	223 ± 10*	256 ± 8*
Tezo	158 ± 6†	177 ± 6*†	188 ± 10*	207 ± 9*	233 ± 10*†	257 ± 8*
Control	135 ± 14	152 ± 14*	167 ± 13*	181 ± 12*	216 ± 12*	249 ± 9*
EMD	159 ± 7	195 ± 12*†	199 ± 11*†	223 ± 9*†	243 ± 8*	259 ± 8*
EMD+Tezo	177 ± 9†‡	207 ± 11*†‡	214 ± 9*†	236 ± 8*†	257 ± 7*†	270 ± 6*†
MAP, mmHg						
Control	86 ± 6	81 ± 4	83 ± 5	85 ± 5	87 ± 5	91 ± 7*
Tezo	71 ± 6†	73 ± 4†	74 ± 4†	76 ± 6†	78 ± 4†	82 ± 5†
Control	83 ± 3	83 ± 4	83 ± 3	84 ± 3	86 ± 4	89 ± 4
EMD	75 ± 4	73 ± 5†	73 ± 4†	74 ± 3†	73 ± 4†	76 ± 5†
EMD+Tezo	68 ± 4†	68 ± 5†	65 ± 6†	67 ± 3†‡	67 ± 4†	72 ± 4†
PAP, mmHg						
Control	12 ± 2	15 ± 1	18 ± 1	20 ± 1*	24 ± 2*	28 ± 2*
Tezo	8 ± 1†	12 ± 1	15 ± 1†	17 ± 1†	21 ± 1*	26 ± 1*
Control	12 ± 1	16 ± 2	17 ± 2	19 ± 2*	24 ± 3*	27 ± 3*
EMD	11 ± 1	13 ± 2†	15 ± 2†	17 ± 2*†	19 ± 2*†	22 ± 3*†
EMD+Tezo	12 ± 1	14 ± 1	15 ± 1	17 ± 2†	20 ± 2*	24 ± 3*
LAP, mmHg						
Control	-0.4 ± 1.2	1.0 ± 0.8	3.9 ± 0.4*	4.8 ± 0.8*	6.4 ± 1.3*	8.7 ± 1.4*
Tezo	-2.2 ± 0.4†	1.3 ± 0.7	3.3 ± 0.9*	4.1 ± 1.3*	7.0 ± 1.5*	10.0 ± 1.6*
Control	-2.3 ± 1.8	1.2 ± 0.9	1.8 ± 1.1	3.5 ± 1.1*	5.6 ± 2.0*	5.9 ± 2.2*
EMD	1.1 ± 2.1	0.8 ± 1.4	2.8 ± 1.2	3.9 ± 1.2	5.4 ± 2.1*	6.6 ± 2.5*
EMD+Tezo	0.2 ± 1.4	1.6 ± 1.0	3.2 ± 1.3	4.1 ± 1.5*	6.6 ± 2.1*	9.1 ± 2.6*
CO, l/min						
Control	5.2 ± 0.5	6.0 ± 0.3*	6.4 ± 0.5*	7.1 ± 0.5*	7.9 ± 0.5*	8.5 ± 0.7*
Tezo	5.3 ± 0.4	6.2 ± 0.4*	6.7 ± 0.5*	7.5 ± 0.6*	8.3 ± 0.6*†	9.1 ± 0.8*†
Control	5.7 ± 0.7	6.5 ± 0.7*	7.1 ± 0.7*	7.7 ± 0.8*	8.8 ± 0.7*	9.8 ± 0.7*
EMD	6.2 ± 0.6	7.7 ± 0.8*†	8.2 ± 0.8*†	9.0 ± 0.7*†	9.8 ± 0.6*†	10.4 ± 0.7*†
EMD+Tezo	6.9 ± 0.7‡	8.1 ± 0.9*†	8.6 ± 0.7*†	9.2 ± 0.6*†	10.2 ± 0.7*†	10.8 ± 0.6*†
SV, ml						
Control	36 ± 3	36 ± 2	37 ± 2	37 ± 2	35 ± 2	34 ± 3
Tezo	34 ± 2	36 ± 2	37 ± 2*	38 ± 2*	37 ± 3*	36 ± 3
Control	42 ± 4	43 ± 4	43 ± 4	43 ± 4	41 ± 3	40 ± 3
EMD	39 ± 4	39 ± 3	41 ± 4	40 ± 3	40 ± 2	40 ± 3
EMD+Tezo	39 ± 4	39 ± 3	40 ± 3	39 ± 3	40 ± 2	40 ± 2

*HR: Heart rate; MAP: Mean aortic pressure; LAP: Left atrial pressure; CO: Cardiac output; SV: Stroke volume; Tezo: Tezosentan; EMD: EMD360527. Values are mean±SEM. * P<0.05 vs. Standing; † P<0.05 effect of tezo; ‡ P<0.05 effect of tezo in the presence of EMD360527.*

variables to baseline values. Subsequently, the EMD360527 was infused continuously in a dose of 300 μg min kg^{-1} intravenously and 10 min after starting the infusion, the five-stage exercise protocol was repeated, while the infusion was continued [20]. EMD360527 demonstrates at least 45-fold selectivity for PDE5 (IC_{50}=0.007 mM) compared to PDE6 (IC_{50}=0.32 mM), 94-fold selectivity for PDE1 (IC_{50}=0.66 mM), 137-fold selectivity for PDE10 (IC_{50}=0.96 mM), and > 1400-fold selectivity for PDE2, PDE3, PDE4 and PDE7 (all IC_{50}>10 mM). In these assays PDE1-4 was from guinea pig heart muscle, PDE5 from human thrombocytes, PDE6 from bovine retina, while PDE7 and PDE10 were obtained by expression of cDNA in COS-7 cells and E. Coli

respectively. Subsequently, the EMD infusion was stopped and the animals were allowed to rest for another 90 min.

Effects of ET_A/ET_B receptor blockade in the presence of PDE5 inhibition during exercise. Then animals received an intravenous infusion of EMD360527 together with the ET_A/ET_B antagonist tezosentan, in dosages identical as described above for the individual infusions, and the five-stage exercise protocol was repeated. We have previously observed excellent reproducibility of consecutive exercise protocols [17].

In vitro studies

Tissues

Pig lungs (n=16) were collected at a local slaughterhouse. Pulmonary small arteries (diameter ~300 μm) were removed and stored overnight in cold, oxygenated Krebs bicarbonate solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4.

The next day, pulmonary small arteries were cut into segments of ~2 mm length and mounted in microvascular myographs (Danish MyoTechnology) with separated 6 ml organ baths containing Krebs bicarbonate solution aerated with 95% O_2/5% CO_2 and maintained at 37°C. Changes in contractile force were recorded with a Harvard isometric transducer. Following a 30 min stabilization period, the internal diameter was set to a tension equivalent to 0.9 times the estimated diameter at 20 mmHg effective transmural pressure. Vessels were then exposed to 30 mM KCl twice. Endothelial integrity was verified by observing dilation to 10 nM substance P after preconstriction with 100 nM of the thromboxane A_2 analogue U46619. Then vessels were subjected to 100 mM KCl to determine the maximal vascular contraction. Thereafter, vessels were allowed to equilibrate in fresh organ bath fluid for 30 min before initiating different experimental protocols [21].

Effects of PDE5 inhibition and ET_A/ET_B blockade. Pulmonary small arteries were preconstricted with 100 nM U46619, and concentration-response curves were constructed to EMD360527 (1 nM-10 μM) (n=7), tezosentan (1 nM-10 μM [22]) (n=7), and combined EMD360527 and tezosentan (n=7).

Effects of PDE inhibition on ET receptor sensitivity and ET production. The concentration-response to ET (3-100 nM [23], n=9) and Big ET (3-100 nM, n=7) were conducted in control vessels and vessels in the presence of EMD360527 (3 μM). Big ET has no direct vasomotor effects, therefore Big ET-induced vasoconstriction is used as an index of Big ET conversion to vasoactive ET.

Data analysis and statistics

Digital recording and off-line analysis of hemodynamic data have been described in detail elsewhere [16, 17]. Pulmonary vascular conductance (PVC) and systemic vascular conductance (SVC) were calculated as cardiac output divided by pulmonary artery pressure minus left atrial pressure and cardiac output divided by mean aortic pressure, respectively. PVC and SVC were used as indices for pulmonary and systemic vascular tone [24]. Total pulmonary resistance (TPR) was calculated as pulmonary artery pressure divided by cardiac output [25]. To accommodate for the varying weights between animals and groups, cardiac output, PVC, SVC and TPR were indexed to body weight. Pulmonary distensibility (α) was estimated using the formula described by Linehan and Reeves [26, 27] by minimizing the difference between predicted and measured PAP using Solver in Excel.

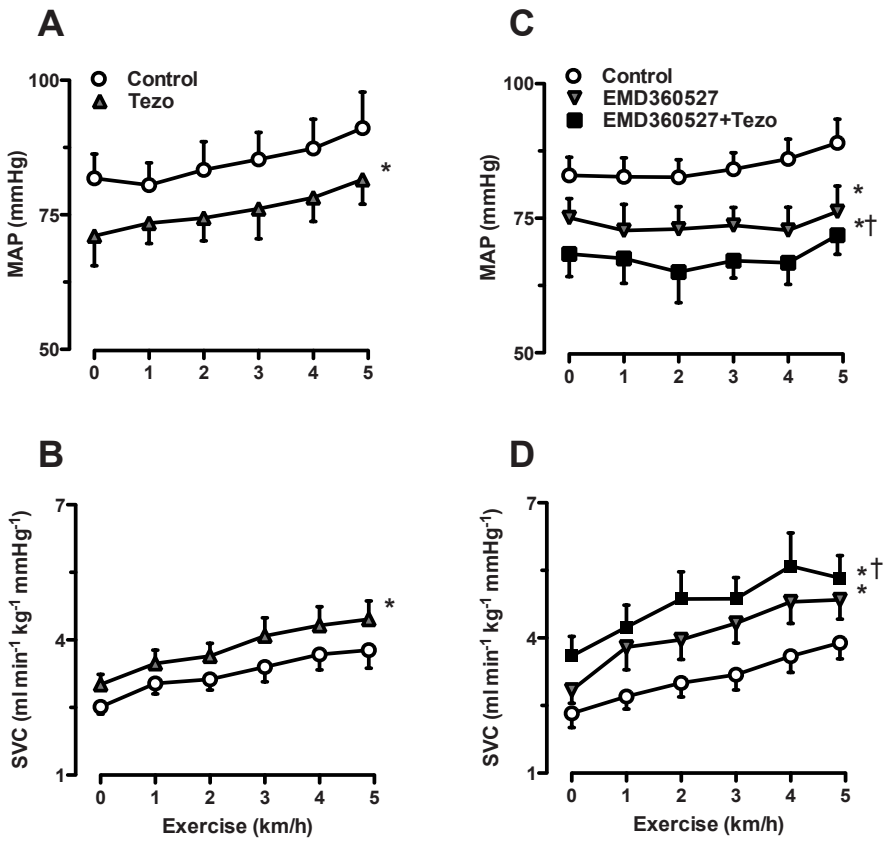

Fig. 1. *Effects of PDE5 inhibition and ET_A/ET_B receptor blockade on the systemic vasculature in vivo. Shown are the effects of the ET_A/ET_B receptor blocker tezosentan (Tezo), the PDE5 inhibitor EMD360527, and Tezo in the presence of EMD360527 on mean aortic pressure (MAP) and systemic vascular conductance (SVC) at rest and during exercise. Values are means±SEM. * P<0.05, drug effects vs. corresponding control; † P<0.05, tezosentan in the presence of EMD360527 vs. EMD360527 alone.*

$$PAP = \frac{[(1 + \alpha * LAP)^5 + 5 * \alpha * R_0 * CO]^{\frac{1}{5}} - 1}{\alpha}$$

In this formula R_0 is assumed to be PVR measured at rest.

Vascular relaxant responses in vitro to EMD360527, tezosentan and combined EMD360527 and tezosentan were expressed as percentage of contraction to U46619, and to ET and Big ET were normalised to 100 mM KCl. The effects of EMD360527 and tezosentan during exercise were analyzed using two-way ANOVA for repeated measures. The effect of EMD360527 on the responses to tezosentan during exercise, were tested using three-way ANOVA for repeated measures. The effect of EMD360527 and tezosentan on relation between the transpulmonary pressure gradient and cardiac index was analysezed using linear regression. The effects of EMD360527, tezosentan and combined EMD360527 and tezosentan on the preconstricted isolated pulmonary arteries were assessed using two-way ANOVA for repeated measures. The effect of EMD360527 on exogenous ET and Big ET response was assessed using two-way

ANOVA. Moreover, the EMD360527-induced reduction in contractile response to the highest dose of ET and Big ET was compared by a t-test using paired vessel segments from the same lung. In all ANOVAs post-hoc testing was performed using Scheffe's test. Statistical significance was accepted when $P<0.05$ (two-tailed). Data are presented as mean±SEM.

Results

Integrated effects of EMD360527 and tezosentan in the systemic circulation in vivo

Graded treadmill exercise up to 5 km h^{-1} resulted in a doubling of cardiac output, which was principally due to an increase in heart rate, as stroke volume was minimally affected (Table 1). Mean aortic pressure was maintained constant (Fig. 1 a, c), as the increase in cardiac output was balanced by a ~67% increase in SVC (Fig. 1b, d).

Infusion of either ET_A/ET_B receptor antagonist tezosentan or PDE5 inhibitor EMD360527 alone induced systemic vasodilation as evidenced by an increase in SVC (Fig 1b, d), resulting in a decrease in mean aortic pressure, despite concomitant, probably baroreceptor reflex mediated, increases in heart rate and cardiac output (Table 1, Fig 1a, c).

Infusion of the ET_A/ET_B receptor antagonist tezosentan following EMD360527 resulted in a further decrease in mean aortic pressure and increase in SVC, as well as a further increase in heart rate and cardiac output (Table 1, Fig. 1a, b). Hence, the effect of ET antagonism on the systemic vasculature was not affected by prior PDE5 inhibition and the integrated vasodilator effect of PDE5 inhibition and ET antagonism on the systemic vasculature was larger than the effect of PDE5 inhibition or ET antagonism alone.

Integrated effects of EMD360527 and tezosentan in the pulmonary circulation in vivo

Exercise resulted in a significant increase in pulmonary arterial pressure, which was principally the result of increase in cardiac output and to a lesser extent of increase in left atrial pressure (that is transmitted backwards into the pulmonary vasculature) (Table 1 and Fig. 2a, d). However, the transpulmonary pressure gradient (pulmonary artery pressure minus left atrial pressure) increased slightly less than cardiac output indicating that exercise-induced pulmonary vasodilation occurred as evidenced by an increase in PVC (Table 1 and Fig. 2b, e). The increase in PVC in the control exercise trials could be explained based on a distensibility ranging from 0.2 to 1.1 %/mmHg for the individual animals. TPR increased (Fig. 2c, f), reflecting an increase in right ventricular afterload.

In accordance with previous studies from our laboratory [11, 15], ET_A/ET_B blockade with tezosentan resulted in a decreased pulmonary artery pressure (Fig. 2a), with minimal effect on left atrial pressure (Table 1). Tezosentan had little effect on cardiac output at rest and low levels of exercise, but increased cardiac output significantly at 4 and 5 km/h compared to control exercise (Table 1). Tezosentan resulted in a downward rotation of the relation between cardiac output and transpulmonary pressure-gradient (Fig. 3a), reflecting a significant increase in PVC and decrease in TPR (Fig. 2b, c). Distensibility of the pulmonary vasculature was not significantly altered by tezosentan.

Similar to previous observations from our laboratory [20], infusion of PDE5 inhibitor EMD360527 resulted in a decreased pulmonary artery pressure, with minimal effect on left

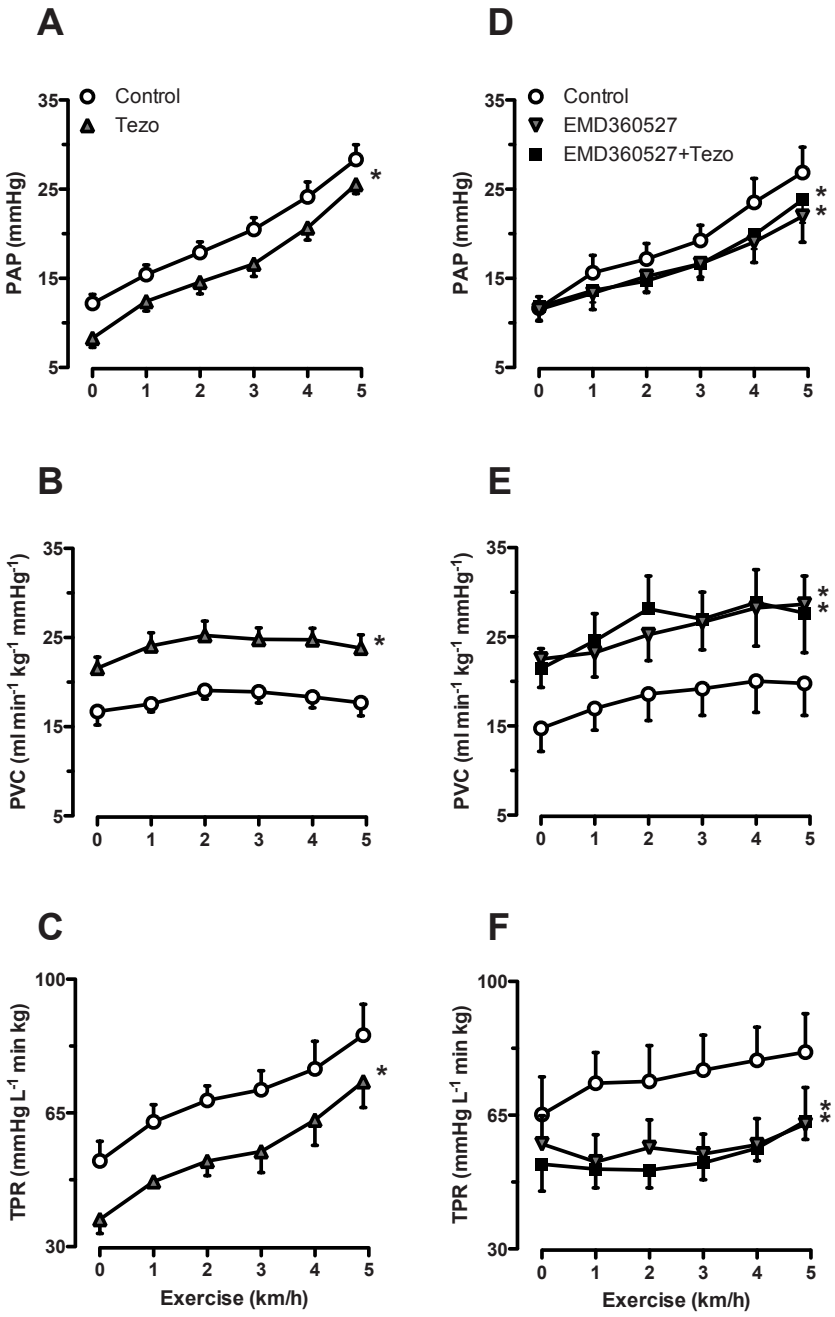

Fig. 2. *Effects of PDE5-inhibition and ET_A/ET_B receptor blockade on the pulmonary vasculature in vivo. Shown are the effects of the ET_A/ET_B receptor blocker tezosentan (Tezo), the PDE5 inhibitor EMD360527, and Tezo in the presence of EMD360527 on pulmonary artery pressure (PAP), pulmonary vascular conductance (PVC) and total pulmonary resistance (TPR) at rest and during exercise. Values are means±SEM. * P<0.05, drug effects vs. corresponding control.*

atrial pressure, whereas cardiac output increased significantly at all levels of exercise (Table 1 and Fig. 2d). EMD360527 caused a downward rotation of the relation between cardiac output and transpulmonary pressure-gradient (Fig. 3b), reflecting a significant increase in PVC (Fig. 2e) and decrease in TPR (Fig. 2f). Distensibility of the pulmonary vasculature was not significantly altered by EMD360527.

Infusion of tezosentan following PDE5 inhibition with EMD360527 did not result in further changes in pulmonary artery pressure, PVC, TPR (Fig. 2d, e, f) nor in the relation between the transpulmonary pressure-gradient and cardiac output (Fig. 3b), indicating that in the presence of PDE5 inhibition, ET_A/ET_B receptor blockade had no vasodilator effect on the pulmonary vasculature.

Interaction between the NO-cGMP and the ET pathways in isolated pulmonary small arteries

The lack of additive pulmonary vasodilation with tezosentan in the presence of EMD360527 could either be due to an interaction between the ET and cGMP pathways or to the fact that EMD360527 alone was sufficient to obtain maximal pulmonary vasodilation. Since these two scenarios are difficult to study in vivo, we performed dose responses of EMD360527, tezosentan and combined EMD360527 and tezosentan in isolated pulmonary small arteries preconstricted with U46619. EMD360527 caused dose-dependent vasodilation of the preconstricted vessel segments (Fig. 4). Tezosentan failed to induce relaxation in vitro either in the absence or presence of EMD360527 (Fig. 4).

To further investigate whether the lack of vasodilator effect of tezosentan in the presence of EMD360527 was the result of a direct suppression of the ET pathway by the NO-cGMP pathway, we measured constriction to ET and Big ET in the absence and presence of EMD360527 in isolated pulmonary small arteries. In the absence of EMD360527, both ET and Big ET induced dose-dependent vessel segment contraction (Fig. 5a, b). In the presence of EMD360527, the contraction to both ET and Big ET was significantly blunted (Fig. 5a, b), while the response to Big ET was blunted more than that of ET (Fig. 5c). These data indicate that PDE5 inhibition decreases the sensitivity of the pulmonary vasculature to ET and blunts the conversion of Big ET to ET in the pulmonary vasculature.

Discussion

The main findings of the present study were that 1) both ET_A/ET_B receptor blockade with tezosentan and PDE5 inhibition with EMD360527 resulted in systemic and pulmonary vasodilation, 2) ET_A/ET_B receptor blockade resulted in further vasodilation in the presence of PDE5 inhibition in the systemic circulation. 3) However, in the presence of PDE5 inhibition, ET_A/ET_B receptor blockade failed to produce additional vasodilation in the pulmonary circulation in vivo or in isolated preconstricted pulmonary small arteries in vitro, 4) PDE5 inhibition blunted ET-induced pulmonary small artery contraction in vitro, 5) Big ET-induced pulmonary small artery contraction was blunted more by PDE5 inhibition than ET-induced reduction. The implication of these findings will be discussed below.

The increase in PVC during exercise in the present study could represent passive distension as well as vasodilation due to a decrease in pulmonary vascular tone. Calculation of the distensibility coefficient α indicates that the increase in PVC during exercise could be explained based on distensibility alone. However, the increase in PVC in response to PDE5 inhibition or ET_A/ET_B receptor blockade must be the result of a reduction in pulmonary vascular tone, as the

increase in PVC is accompanied by a reduction in pulmonary artery pressure, that would passively decrease PVC.

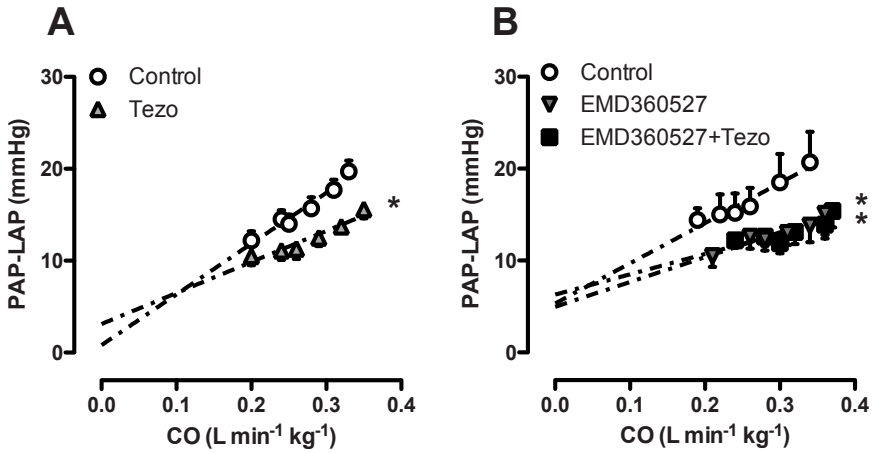

Fig. 3. *Effects of PDE5 inhibition and ET_A/ET_B receptor blockade on the pulmonary pressure-flow relationships. Shown are the effects of the ET_A/ET_B receptor blocker tezosentan (Tezo), the PDE5 inhibitor EMD360527, and Tezo in the presence of EMD360527 on the pressure-flow relationship at rest and during exercise. CI: cardiac index (cardiac output normalized by body weight). Values are means±SEM. * P<0.05, drug effects vs. corresponding control.*

The magnitude of these individual vasodilator effects of PDE5 inhibition and ET_A/ET_B receptor blockade in the systemic and pulmonary vascular beds are in good agreement with their respective effects in previous studies from our laboratory [11, 15, 20, 24]. Thus, PDE5 inhibition resulted in pulmonary and systemic vasodilation that was similar in magnitude at rest and during exercise. Pulmonary artery pressure decreased in response to ET_A/ET_B receptor blockade at rest, the increase in PVC in response to ET_A/ET_B receptor blockade under resting conditions was non-significant (P=0.29), whereas the increase in PVC was significant at all levels of exercise. Consistent with a recent study in isolated pulmonary arteries from fetal lambs [22] as well as with the lack effect of ET_A/ET_B receptor blockade on PVC under resting conditions in vivo [11, 15], tezosentan failed to induce vasodilation in isolated pulmonary small arteries, indicating that there is little ET synthesised in the pulmonary vasculature under basal resting conditions. Moreover, pulmonary vasodilation induced by ET_A/ET_B receptor blockade during exercise was lost in the presence of PDE5 inhibition.

In contrast to the observations in the pulmonary vasculature, we found that the systemic vasodilation produced by ET_A/ET_B receptor blockade was not influenced by prior PDE5 inhibition. In fact, the systemic vasodilation induced by combined treatment resulted in an average reduction in blood pressure of 17±2 mmHg, with average systolic blood pressure being 90±6 mmHg and average diastolic blood pressure being 49±3 mmHg following treatment, indicating marked hypotension. One additional pig, which was excluded from the analyses, was even unable to perform treadmill exercise following the combination treatment. In contrast to the findings in the present study, a recent study showed that the combination of ET_A/ET_B blockade by bosentan and PDE5 inhibition by sildenafil, at dosages that were ineffective in single treatments, did result in pulmonary vasodilation in rats without the occurrence of systemic hypotension [28]. The lack of systemic hypotension observed in that study is likely to be

due to the smaller increase in SVC (~47%) in combination with the larger increase in cardiac output (~60%) that was observed in the rats [28] as compared to the larger increase in SVC (55±14%) and the smaller increase in cardiac output (23±9%) in the present study. This difference in increase of cardiac output was most likely due to the decreased cardiac output at baseline in the rats with pulmonary hypertension, which was normalised following the reduction in afterload of the right ventricle. Alternatively, the prolonged duration of the treatment [28] may have resulted in recruitment of compensatory long-term blood pressure regulation mechanisms, *i.e.* activation of the renin-angiotensin-aldosterone system, that resulted in peripheral vasoconstriction (and hence limited the increase in SVC), thereby contributing to restoration of systemic pressure.

Multiple explanations could be forwarded for the different results of the combination treatment between the systemic and pulmonary vasculature in the present study. First, pulmonary vasomotor tone is lower as compared to systemic vasomotor tone, while the vasodilator effect of PDE5 inhibition on the pulmonary is larger than that on the systemic vasculature. Thus, maximal vasodilation may have been reached by PDE5 inhibition in the pulmonary circulation, while vasodilator reserve was still present in the systemic vasculature. However, even when tone was artificially increased in isolated pulmonary small arteries, a vasoconstrictor influence of ET either in the absence or presence of PDE5 inhibition was not uncovered, suggesting that the low pulmonary vascular tone is not a critical factor in explaining the different interaction between PDE5 inhibition and ET receptor blockade in the systemic versus the pulmonary vascular bed. Second, since the systemic vasculature is comprised of different vascular beds in parallel, it is possible that vasodilation in response to ET_A/ET_B receptor blockade occurred in a different vascular bed as vasodilation in response to PDE5 inhibition. This also precludes analysis of isolated systemic small arteries, as it is unclear which vascular bed should be chosen. Third, different receptors are involved in ET-induced vasoconstriction in the systemic and pulmonary vascular beds. Thus, the ET_B receptor is the main receptor involved in ET-induced vasoconstriction in the healthy porcine pulmonary vasculature, whereas the ET_A receptor is the predominant vasoconstrictor receptor in the systemic vasculature [15].

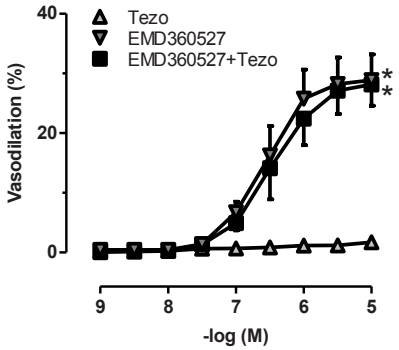

Fig. 4. *Effects of PDE5 inhibition and ET_A/ET_B receptor blockade on pulmonary small arteries in vitro. Shown are the effects of the PDE5 inhibitor EMD360527, the ET_A/ET_B receptor blocker tezosentan (Tezo) and Tezo in the presence of EMD360527 on isolated pulmonary arteries preconstricted with U46619 (100 nM). Each dose-response curve was obtained in 7 vessel rings (7 swine). Values are means±SEM. * P<0.05, EMD360527 or EMD360527+tezosentan vs. tezosentan.*

We have previously shown that endogenous NO acts to suppress the pulmonary vasoconstrictor influence of endogenous ET [11], particularly during exercise, which together with our results in isolated pulmonary small arteries points towards a direct interaction between the NO-cGMP system and the ET system. NO has been shown to directly modulate binding of ET to the ET_A receptor [29]. In addition to such direct effect of NO that would not be enhanced by PDE5 inhibition, experiments in porcine aorta, rat hearts and cultured pulmonary arterial endothelial cells, show that an increase in cGMP, induced by either NO or the non-hydrolysable cGMP analogue 8Br-cGMP suppresses ET production and release [30-32]. Moreover, plasma ET levels

were lower in rats with pulmonary hypertension treated with the PDE5 inhibitor sildenafil [33]. Although plasma ET levels do not always adequately reflect tissue ET levels, these data are consistent with a reduced ET production following PDE5 inhibition. These observations are further corroborated by our experiments in isolated vessels, showing that elevation of cGMP levels through PDE5 inhibition attenuated the vasoconstrictor response to Big ET to a larger extent than the vasoconstrictor response to ET, indicating that the interaction between the NO-cGMP system and the ET system occurs both at the level of the ET receptor and at the level of endothelin converting enzyme (ECE), the main enzyme that catalyses the conversion of Big ET to ET. Such interaction between cGMP and ECE requires presence of either cGMP in the endothelial cells or the presence of ECE in pulmonary vascular smooth muscle cells. Indeed, the presence of ECE in pulmonary smooth muscle cells has been confirmed in several studies [34, 35], while both guanylyl cyclase [36] and PDE5 [37] have been shown to be present in endothelial cells.

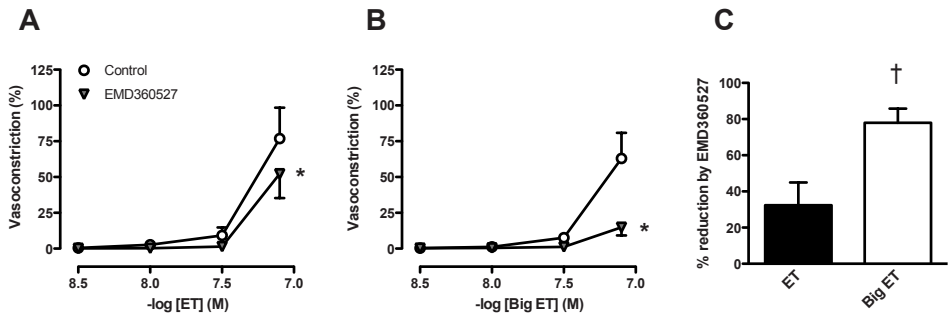

Fig. 5. *Effects of PDE5 inhibition on endothelin (ET) sensitivity and production from its precursor Big ET in pulmonary small arteries in vitro. Shown are effects of EMD360527 on vasoconstriction to ET (panel A (n=9 with and without EMD360527)) and Big ET (panel B (n=7 with and without EMD360527)) in isolated pulmonary small arteries. Effect of EMD360527 on vasoconstriction induced by the highest dose of ET was relatively smaller than its effect on the vasoconstriction induced by the highest dose of Big ET. Values are means±SEM. * P<0.05, control vs. EMD360527; † P<0.05 effect of EMD360527 different in ET- vs. Big ET-induced constriction.*

Conclusions and implications

The present data show that the interactions between the NO-cGMP system and the ET system in the pulmonary vasculature occurred at the level of ET receptor(s) as well as ECE and prevented an additive vasodilator effect of PDE5 inhibition and ET_A/ET_B receptor blockade in the healthy pulmonary vasculature.

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Chapter 7

Uridine adenosine tetraphosphate is a novel vasodilator in the coronary microcirculation which acts through purinergic P1 but not P2 receptors

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Pharmacol Res 67 (2013) 10-17

Abstract

Uridine adenosine tetraphosphate (Up_4A) has been identified as an endothelium-derived contracting factor, which acts through purinergic P2X and P2Y receptors. Since the coronary vascular actions of Up_4A are unknown, we investigated the vasoactive profile of Up_4A in coronary microvessels, and studied the involvement of purinergic receptor subtypes. Studies were performed in isolated porcine coronary small arteries (diameter ~250 μm), with and without endothelial denudation, mounted on a Mulvany wire myograph. Purinergic receptor expression was assessed by real-time PCR. Up_4A (10^{-9}-10^{-5} M) failed to induce contraction at basal tone, but produced concentration-dependent vasorelaxation in precontracted microvessels. Up_4A was slightly less potent than adenosine, ATP, and ADP in producing vasorelaxation, but significantly more potent than UTP and UDP. mRNA expression of $P2X_4$, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and A_{2A}, but not $P2X_1$, receptors was observed. However, Up_4A-induced vasodilation was unaffected by non-selective P2 receptor antagonist PPADS, $P2X_1$ antagonist MRS2159, $P2Y_1$ antagonist MRS2179 and $P2Y_6$ antagonist MRS2578, but was markedly attenuated by non-selective P1 receptor antagonist 8PT and A_{2A} antagonist SCH58261. Up_4A-induced vasodilation was not affected by ectonucleotidase inhibitor ARL67156, suggesting that A_{2A} stimulation was not the result of Up_4A breakdown to adenosine. Up_4A-induced vasodilation was blunted in denuded vessels; additional A_{2A} receptor blockade further attenuated Up_4A-induced vasodilation, suggesting that A_{2A} receptor mediated vasodilation is only partly endothelium-dependent. In conclusion, Up_4A exerts a vasodilator rather than a vasoconstrictor influence in coronary microvessels, which is mediated via A_{2A} receptors and is partly endothelium-dependent.

Abbreviations

8PT, 8-phenyltheophylline
COX, cycloxygenase
CYP 2C9, cytochrome P450 2C9
DMSO, dimethyl sulphoxide;
EDHF, endothelium-derived hyperpolarizing factor
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
LNAME, N^{ω}-nitro-L-arginine methyl ester HCL
NOS, nitric oxide synthase
PPADS, pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid
U46619, 9,11-Dideoxy-11α,9α epoxymethanoprostaglandin $F_{2\alpha}$
Up_4A, uridine adenosine tetraphosphate

Introduction

Increasing evidence suggests that extracellular nucleotides contribute to the regulation of cardiovascular tone [1-3]. In blood vessels, nucleotides, such as ATP and UTP, can be released from adventitial nerves or from platelets and endothelial cells to induce either vasoconstriction or vasodilation through purinergic receptors. In addition to these mononucleotides, dinucleotides exist [4, 5]. Uridine adenosine tetraphosphate (Up_4A) has been recently identified as the first dinucleotide found in living organisms that contains both purine and pyrimidine moieties, which acts as a novel potent endothelium-derived contracting factor [6]. This factor, isolated from the supernatant of stimulated human endothelium, increases mean arterial pressure in rat in vivo and causes vasoconstriction in isolated kidney of rats [6]. Since plasma concentrations of Up_4A detected in healthy subjects are in the vasoactive range, a role for Up_4A has been proposed in the regulation of vascular tone, and possibly in the pathogenesis of hypertension [6].

All purine and pyrimidine mononucleotides and dinucleotides exert their effects through purinergic receptors. Purinergic receptors have been classified into two subypes: P1 and P2 receptors, based on their pharmacological properties and molecular clonings [7, 8]. Four subtypes of P1 receptors (also termed adenosine receptors), all metabotropic, have been cloned, namely A_1, A_{2A}, A_{2B} and A_3 [9]. The P2 receptors belong to two major families: ionotropic P2X receptors and metabotropic P2Y receptors [10-12]. At least 7 P2X and 8 P2Y receptors have been cloned to date [13, 14]. Several studies demonstrated that at least some of these purinergic receptors are expressed in the porcine coronary vasculature [15-17]. However, the involvement of these purinergic receptors in regulation of coronary vascular tone remains unclear, because most of the purinergic receptors are capable of mediating responses to several nucleotides and have overlapping ligand preferences, while different purinergic receptors have opposite effects on vascular tone [9, 11].

Several in vitro studies have indicated that Up_4A induces vascular contraction through purinergic receptors. Thus, vasoconstriction was observed in rat renal artery through $P2X_1$ receptor [6], in rat aorta through P1 and P2X receptors [18], and in rat pulmonary arteries through P2Y receptors [19]. Furthermore, vasoconstriction was also observed in mouse renal arterioles [20] and in mouse aorta [21]. Up_4A-induced vascular contraction in rat aorta was potentiated by inhibition of nitric oxide synthase (NOS) [18], implying a modulatory role of nitric oxide (NO) and possibly other endothelium-derived vasodilator pathways in Up_4A-regulated vascular tone. There is also evidence that Up_4A produces vasodilation in isolated aortic rings of rats [18] and induces hypotension in conscious rats [21].

To date, the vasomotor actions of Up_4A in the coronary microcirculation have not been explored. Consequently, we investigated the vascular effects of Up_4A in coronary arterial microvessels, and determined the putative purinergic receptors involved in the vascular actions of Up_4A in coronary small arteries. In addition, we investigated the role of endothelium-derived relaxing substances in modulating the coronary vascular effects of Up_4A. Our findings indicate that Up_4A is a potent vasodilator in porcine coronary small arteries. This vasodilation is mediated via P1 (A_{2A}), but not P2 receptors, and is partly endothelium-dependent.

Materials and Methods

Drugs and solutions

Adenosine, ATP, ADP, UTP, UDP, 8PT (8-phenyltheophylline), SCH58261, PPADS (pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid), MRS2159, MRS2179, MRS2578,

LNAME ($N^{(0)}$-nitro-L-arginine methyl ester HCl), indomethacin, sulfaphenazole, U46619 (9,11-Dideoxy-11α, 9α epoxymethanoprostaglandin $F_{2\alpha}$), and substance P were all purchased from Sigma-Aldrich (Zwijndrecht, The Nederlands). Up_4A was obtained from Biolog Life Science (Bremen, Germany). ARL67156 was obtained from R&D (Abingdon, UK). MRS2579 and indomethacin were firstly dissolved in DMSO. Sulfaphenazole was dissolved in ethanol. All subsequent dilutions (at least 1000 fold) and other drugs were obtained with distilled water. PPADS and MRS2159 were protected from light.

Table 1. Primer information

Genes	Sequences		Sizes
	Sense	Antisense	
A_{2A}	5'-ATGTTGGGCTGGAATAGCTG-3'	5'-CACGGAGTTGGTGTGAGAGA-3'	426 bp
$P2X_1a$	5'-CGCCTTCTTCTTCGAGTACG-3'	5'-TACCTTGGGCCTTCCTTTCT-3'	401 bp
$P2X_1b$	5'-CGCCTTCTTCTTCGAGTACG-3'	5'-CAGCCAGACCCTTGAGTTTC-3'	194 bp
$P2X_1c$	5'-CGCCTTCTTCTTCGAGTACG-3'	5'-AGCCAGACCCTTGAGTTTCA-3'	190 bp
$P2X_4$	5'-TGTCCCCAGGCTACAATTTC-3'	5'-GGCAGCTTTTTCTCCCTTCT-3'	373 bp
$P2Y_1$	5'-TTCCTGACTTGCATCAGTGC-3'	5'-CAGTGCCCGAGTAGAAGAGG-3'	157 bp
$P2Y_2$	5'-GTGGCCTACAGCTTGGTCAT-3'	5'-GCGTGCGGAAGGAGTAGTAG-3'	235 bp
$P2Y_4$	5'-GACTGCCGGTTTAATGAGGA-3'	5'-AGGAAAAGGACGCTGCAGTA-3'	302 bp
$P2Y_6$	5'-CTGCTCTTGCCACCTGTGTA-3'	5'-AGGTTGGCGTAGAACAGGAA-3'	251 bp
GAPDH	5'-TCGGAGTGAACGGATTTG-3'	5'-CCTGGAAGATGGTGATGG-3'	219 bp

3 sets of $P2X_1$ primers a, b, and c were tested.

Myograph studies

Swine hearts (n=107) were collected from a local slaughterhouse. Small coronary arteries (diameter≈250 μm) were dissected out and stored overnight in cold, oxygenated Krebs bicarbonate solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4 [22]. The next day, coronary small arteries were cut into segments of ~2 mm length. In a subset of vessels the endothelium was removed with a single hair by gently rolling it back and forward. Subsequently, vessels were mounted in microvascular myographs (Danish Myo Technology) with separated 6 ml organ baths containing Krebs bicarbonate solution aerated with 95% O_2/5% CO_2 and maintained at 37°C. Changes in contractile force were recorded with a Harvard isometric transducer. Following a 30 min stabilization period, the internal diameter was set to a tension equivalent to 0.9 times the estimated diameter at 100 mmHg effective transmural pressure. The normalized vessels were exposed to 30 mM KCl twice. Endothelial integrity was verified by observing dilation to 10 nM substance P after preconstriction with 100 nM of the stable thromboxane A_2 analogue U46619. Then vessels were subjected to 100 mM KCl to determine the maximal vascular contraction. Thereafter, vessels were allowed to equilibrate in fresh organ bath fluid for 30 min before initiating different experimental protocols. Only one protocol was executed per vessel and within one protocol all vessels were obtained from different animals [23].

Experimental protocols

Coronary small arteries were subjected to Up_4A (10^{-9}-10^{-5} M) in the absence or presence of preconstriction with U46619 (100 nM) to test the vascular effect of Up_4A. To compare the vasodilator effects produced by other nucleotides, preconstricted vessels were exposed to incremental concentrations of adenosine (10^{-9}-10^{-5} M), ATP (10^{-9}-10^{-5} M), ADP (10^{-9}-10^{-5} M), UTP (10^{-6}-3×10^{-3} M), and UDP (10^{-7}-10^{-3} M).

To investigate the involvement of the different purinergic receptors, preconstricted vessels were subjected to in the absence and presence of P1 receptor antagonist 8PT (10 μM) [24],

adenosine A_{2A} receptor antagonist SCH58261 (100 nM), P2 receptor antagonist PPADS (10 μM) [19], $P2X_1$ receptor antagonist MRS2159 (30 μM) [25], $P2Y_1$ receptor antagonist MRS2179 (1 μM) [15] and $P2Y_6$ receptor antagonist MRS2578 (10 μM) [26]. To test whether $P2X_1$ receptors are functional, we studied concentration-responses to ATP in the absence and presence of $P2X_1$ antagonist MRS2159 (30 μM). Although ATP is not a selective $P2X_1$ receptor agonist, ATP is a ligand for most P2X and many P2Y receptors, and is capable of activating $P2X_1$ receptors [11]. To test whether $P2Y_1$ receptors are functional, we studied ADP concentration-responses in the absence and presence of $P2Y_1$ antagonist MRS2179 (1 μM).

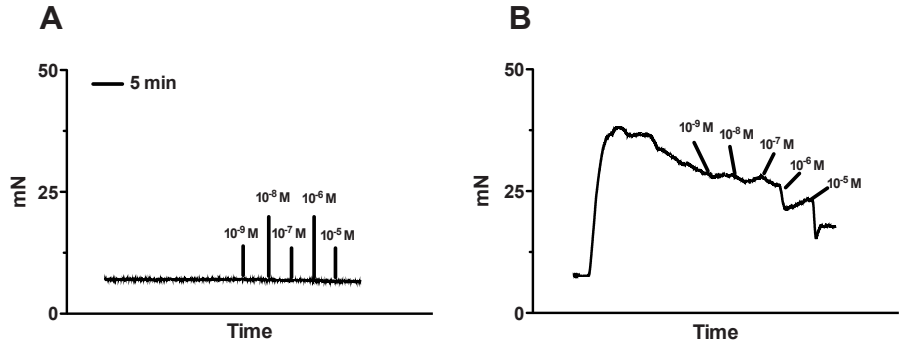

Fig. 1. *Up_4A induced vasodilation but not vasoconstriction in porcine coronary small artery. Shown are the representative recordings where Up_4A (10^{-9}-10^{-5} M) failed to produce any vasoconstrictor effect (A), but induced concentration-dependent vasodilation upon preconstriction with U46619 (100 nM) (B).*

To investigate whether vasodilation by Up_4A occurred, at least in part, through its degradation to adenosine or other nucleotides [5, 27-29], one group of vessels was exposed to Up_4A in the presence of ectonucleotidase inhibitor ARL67156 (10 μM) [24].

In addition, in another group of vessels, vasorelaxation responses to adenosine with and without 8PT (10 μM), SCH58261 (100 nM), PPADS (10 μM), MRS2159 (30 μM), MRS2179 (1 μM) and MRS2578 (10 μM) were compared to those produced by Up_4A.

To investigate the possible endothelial involvement in Up_4A-induced coronary vasorelaxation, concentration-responses to Up_4A (10^{-9}-10^{-5} M) were first studied in endothelium-intact and -denuded vessels. Subsequently, endothelium-intact vessels were incubated with NOS inhibitor LNAME (100 μM), cyclooxygenase (COX) inhibitor indomethacin (10 μM), and combined LNAME and indomethacin, and concentration-responses to Up_4A studied. Since cytochrome P450 (CYP) 2C9 metabolites have been proposed as putative EDHFs [30-32], concentration-responses to Up_4A were also studied in the vessels with CYP 2C9 selective inhibitor sulfaphenazole (10 μM) [23].

A significant part of the Up_4A-induced vasorelaxation remained after removing the endothelium, which implies the involvement of endothelium-independent vasodilation in response to Up_4A. Consequently, we also studied Up_4A responses in endothelium-denuded vessels in the absence and presence of 8PT (10 μM) and SCH58261 (100 nM), respectively, and compared to the vasodilator effect in endothelium-intact vessels.

Quantitative real-time PCR analysis

Endothelium-intact coronary small arteries were used for detection of A_{2A}, $P2X_1$, $P2X_4$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ mRNA. RNA was extracted from 5-7 frozen vessel samples per group using a Qiagen RNA kit. cDNA was synthesized from 100 ng of total RNA with iScript Reverse Transcriptase (Bio-Rad). Quantitative real-time PCR (MyIQ, Bio-Rad) was performed with SYBR Green (Bio-Rad). Target gene mRNA levels were expressed relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control [33]. Primer sequences are shown in the Table 1.

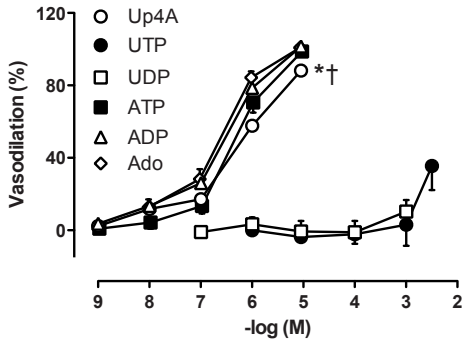

Fig. 2. *Vasodilator potency of mononucleotides compared to Up_4A. Shown are concentration-responses to Up_4A (10^{-9}-10^{-5} M) (n=48), UTP (10^{-6}-3×10^{-3} M) (n=8), UDP (10^{-7}-10^{-3} M) (n=6), ATP (10^{-9}-10^{-5} M) (n=6), ADP (10^{-9}-10^{-5} M) (n=5), and adenosine (Ado) (10^{-9}-10^{-5} M) (n=13) in coronary small arteries. Values are means±SEM. * P<0.05, effect of Up_4A vs. effect of Ado; † P<0.05, effect of Up_4A vs. effect of UTP or UDP.*

Data analysis and statistics

Vascular relaxation responses to Up_4A and other nucleotides were expressed as percentage of contraction to U46619 [22]. The effects of drug treatment on the Up_4A, ATP, ADP and adenosine responses were analyzed using two-way ANOVA (repeated measurement). The relative potency of Up_4A and other nucleotide responses was determined by comparing -log EC50. Statistical significance was accepted when $P<0.05$ (two-tailed). Data are presented as means±SEM.

Results

Vascular actions of Up_4A in coronary small arteries

Up_4A in concentrations of up to 10^{-5} M failed to produce an appreciable vasoconstrictor response in coronary small arteries at basal tone (Fig. 1A). In contrast, in coronary small arteries preconstricted with U46619, Up_4A resulted in concentration-dependent vasodilation (Fig. 1B).

Vasodilator potency of Up_4A in comparison with other nucleotides

To investigate the relative potency of Up_4A, we compared the vasorelaxation response produced by Up_4A to that produced by other nucleotides. Adenosine (-log EC50: 6.53) produced coronary vasodilation up to ~100 %, which was similar to ADP (-log EC50: 6.42), ATP (-log EC50: 6.27), and was slightly more potent than Up_4A (-log EC50: 6.16, P <0.05) (up to ~90 %). All purine-containing mononucleotides (ATP, ADP, adenosine) were more potent in their vasorelaxing actions than pyrimidine-containing mononucleotides (UTP, UDP). In fact, UTP and UDP produced minimal vasodilation even in doses of up to 10^{-3} M (Fig. 2.).

Involvement of purinergic receptors in Up_4A-induced vasodilation

Most previous studies investigating the role of Up_4A in the regulation of vascular tone showed a dominant involvement of P2 receptors [6, 19, 34], although one study showed the involvement of P1 receptor in response to Up_4A [18]. Therefore, we first studied the vasorelaxation responses to Up_4A incubated in coronary small arteries with selective and non-selective P2 receptor antagonists. Surprisingly, we observed that Up_4A-induced vasorelaxation was not affected by non-selective

P2 receptor antagonist PPADS (Fig. 3B), $P2X_1$ antagonist MRS2159, $P2Y_1$ antagonist MRS2179, or $P2Y_6$ antagonist MRS2578 (Fig. 3D). There are currently no selective antagonists for $P2Y_2$ and $P2Y_4$ receptors available. However, the observation that the non-selective P2 receptor antagonist PPADS had no effect on Up_4A-induced vasodilation, together with the observation that the $P2Y_2$ and $P2Y_4$ agonist UTP produced only minimal vasorelaxation (Fig. 2), indicate that $P2Y_2$ and $P2Y_4$ receptors are unlikely contributors to Up_4A-induced coronary small artery dilation. In contrast to the lack of effect of P2 receptor antagonists on the Up_4A-induced vasodilation, the non-selective P1 receptor antagonist 8PT did attenuate Up_4A-induced vasorelaxation (Fig. 3A). This effect was likely A_{2A} mediated, as the selective A_{2A} receptor antagonist SCH58261 similarly attenuated the Up_4A-induced vasorelaxation (Fig. 3C).

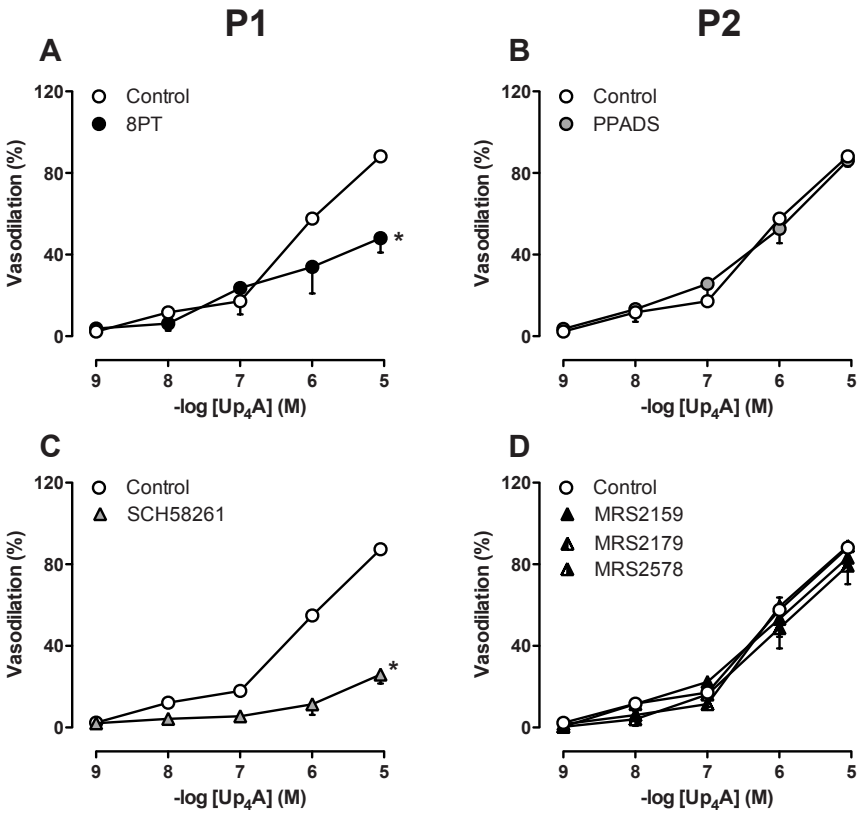

Fig. 3. *Effects of non-selective and selective P1 and P2 receptor blockade on Up_4A-induced vasodilation. Shown are concentration-responses to Up_4A (10^{-9}-10^{-5} M) in porcine small coronary arteries in the absence (n=48) and presence of 10 μM of the non-selective P1 receptor antagonist 8PT (n=6; panel A), 10 μM of the non-selective P2 receptor antagonist PPADS (n=6; panel B), 100 nM of the A_{2A} receptor antagonist SCH58261 (n=6; panel C), and 30 μM of the $P2X_1$ receptor antagonist MRS2159 (n=9), 1 μM of the $P2Y_1$ receptor antagonist MRS2179 (n=15), and 10 μM of the $P2Y_6$ receptor antagonist MRS2578 (n=5) (panel D). Values are means±SEM. * P<0.05, effect of drug vs. control.*

It has been proposed that $P2X_1$, $P2X_4$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ are the main P2 receptors that contribute to vascular tone regulation [6, 35]. Although non-selective P2 receptor and other P2 subtype blockade did not affect Up_4A-induced vasodilation, we still performed real-time PCR of

various P2 receptor subtypes, as well as P1 receptor subtype A_{2A} to illustrate the purinergic receptor expression profile. Despite the use of 3 different primer pairs, we failed to detect expression of $P2X_1$. In contrast, the other P2 receptor subtypes as well as A_{2A} receptor were expressed in the porcine coronary vasculature, particularly the $P2Y_1$ receptor (Fig. 4).

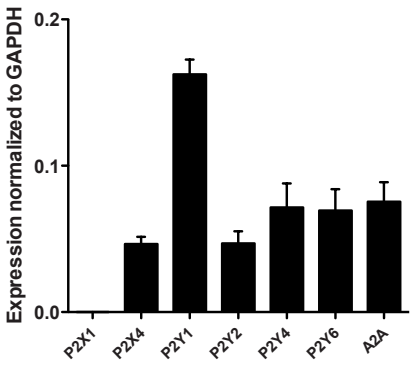

Fig. 4. *Expression profile of purinergic receptors in the coronary vasculature. Shown is expression profile of purinergic receptors by real-time PCR in coronary small arteries.*

To determine whether functional $P2X_1$ and $P2Y_1$ receptors are present in porcine coronary small arteries, we studied ATP and ADP concentration-responses, with and without $P2X_1$ and $P2Y_1$ antagonists, MRS2159 and MRS2179, respectively. MRS2179 significantly attenuated ADP-induced vasorelaxation (Fig. 5B), while MRS2159 did not affect the vasorelaxation produced by ATP (Fig. 5A). These functional observations are consistent with $P2X_1$ and $P2Y_1$ expression profiles, showing lack of $P2X_1$ receptor expression, whereas $P2Y_1$ receptors were abundantly expressed. Taken together, these findings suggest that, although most P2 receptor subtypes are expressed (some of which we showed to be functional in the porcine coronary microcirculation), these do not appear to be involved in the vasodilator responses to Up_4A.

In view of the involvement of P1 receptors in the Up_4A responses we compared its vasodilator profile to that of adenosine. Similar to Up_4A, we found that adenosine-induced vasodilation was not affected by PPADS, MRS2159, MRS2179, or MRS2578 (Fig. 6B, D), but was significantly attenuated by 8PT and SCH58261 (Fig. 6A, C).

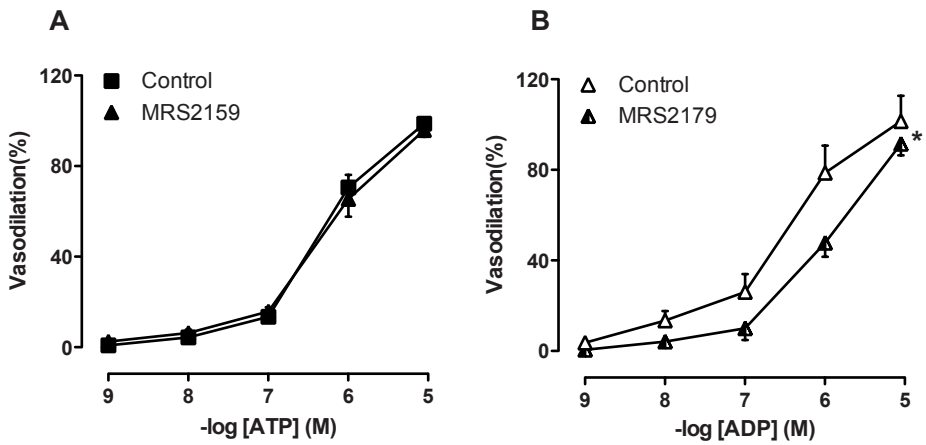

Fig. 5*. Vasodilator response to ATP and ADP. Shown are concentration-responses to ATP (10^{-9}-10^{-5} M) in the absence (n=6) and presence of MRS2159 ($P2X_1$) (30 μM) (n=6) (A); and concentration-responses to ADP (10^{-9}-10^{-5} M) in the absence (n=5) and presence of MRS2179 ($P2Y_1$) (1 μM) (n=6) (B). Values are means±SEM. * P<0.05, effect of drug vs. control.*

In view of the similarity of purinergic receptor involvement in the vasodilation by both adenosine and Up_4A, we investigated whether the Up_4A-induced coronary vasodilation could have been the result of possible degradation to adenosine. We exposed vessels to Up_4A in the absence and presence of the ectonucleotidase inhibitor ARL67156, and observed that Up_4A-induced vasodilation was unmitigated by ARL67156 (Fig. 7), indicating that Up_4A likely acts directly on P1 receptors.

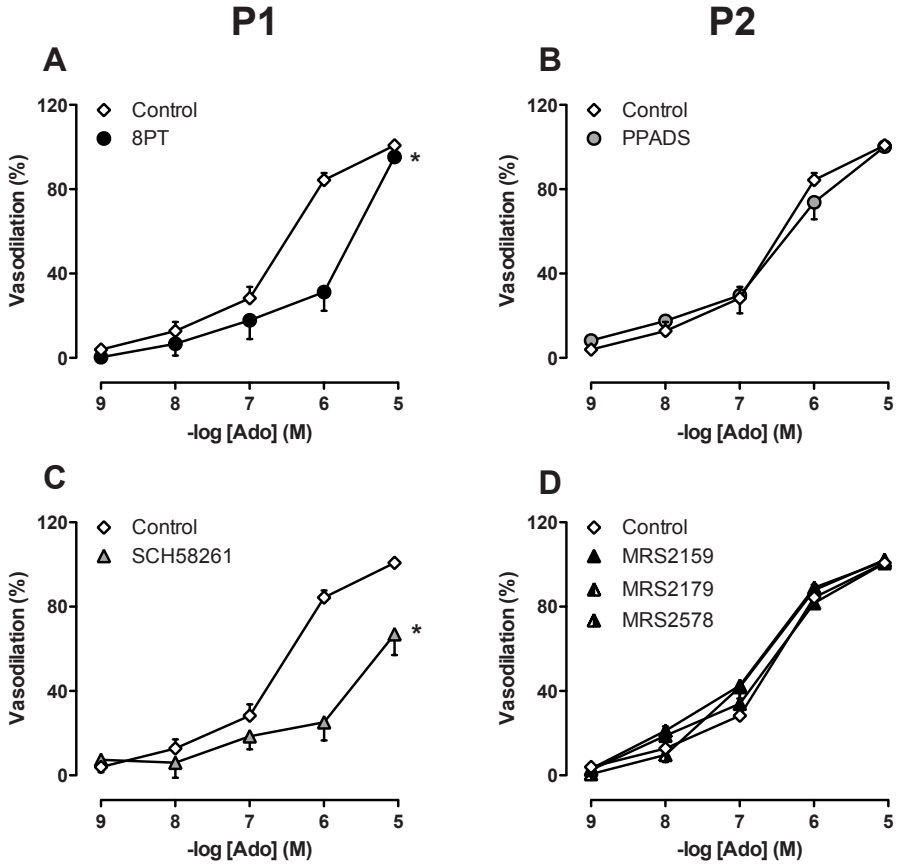

Fig. 6. *Effects of non-selective and selective P1 and P2 receptor blockade on vasodilation produced by adenosine (Ado). Shown are concentration-responses to adenosine (10^{-9}-10^{-5} M) in porcine small coronary arteries in the absence (n=13) and presence of 10 μM of the non-selective P1 receptor antagonist 8PT (n=5; panel A), 10 μM of the non-selective P2 receptor antagonist PPADS (n=6; panel B), 100 nM of the A_{2A} receptor antagonist SCH58261 (n=6; panel C), and 30 μM of the $P2X_1$ receptor antagonist MRS2159 (n=6), 1 μM of the $P2Y_1$ receptor antagonist MRS2179 (n=6), and 10 μM of the $P2Y_6$ receptor antagonist MRS2578 (n=6) (panel D). Values are means±SEM. * P<0.05, effect of drug vs. control.*

Endothelium-dependency of vasorelaxation produced by Up_4A

The Up_4A-induced vasodilation was attenuated, to a similar extent, by NOS inhibitor LNAME or COX inhibitor indomethacin, but not by the CYP 2C9 inhibitor sulfaphenazole (Fig. 8A). The reduction of Up_4A-induced vasodilation by the combination of LNAME and indomethacin was similar in magnitude to that obtained by endothelium denudation (Fig. 8B), indicating that

Up_4A-induced endothelium-dependent coronary vasodilation is principally mediated through NO and prostacyclin (PGI_2).

The Up_4A-induced vasodilation in endothelium-denuded vessels was further reduced by non-selective P1 blockade with 8PT or selective A_{2A} blockade with SCH58261 (Fig. 9), indicating that a large part of the (A_{2A}-mediated) Up_4A vasodilation was endothelium-independent.

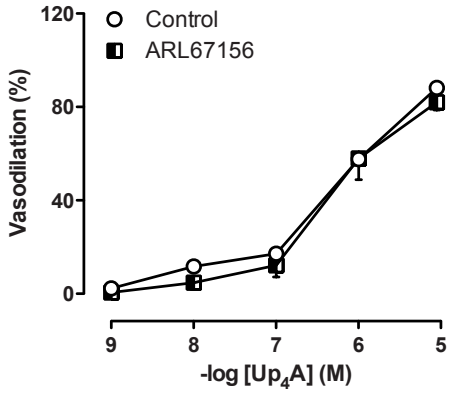

Fig. 7. *Effect of ectonucleotidase inhibition on Up_4A-induced vasodilation. Shown is concentration-responses to Up_4A (10^{-9}-10^{-5} M) in vessels in the absence (n=48) and presence of ARL67156 (10 μM) (n=6). Values are means±SEM.*

Discussion

Up_4A was first identified as a potent endothelium-derived vasoconstrictor in rat perfused kidney [6]. Several subsequent studies have confirmed that Up_4A produces vasoconstriction in rat renal artery [6], aorta [18], and pulmonary arteries [19]. Furthermore, vasoconstriction was observed in mouse renal arterioles [20] and aorta [21]. Conversely, there is evidence that Up_4A can produce vasodilation in isolated aortic rings of rats preconstricted with phenylephrine [18] and induce hypotension in conscious rats [21]. Notably, dual vasoactive actions of Up_4A have been observed in isolated perfused kidney with Up_4A acting as a vasoconstrictor via $P2X_1$ and $P2Y_1$ receptors and resulting in vasodilation via $P2Y_1$ and $P2Y_2$ receptors [36]. Here we show for the first time that Up_4A produced vasodilation rather than vasoconstriction in porcine coronary small arteries. The lack of any vasoconstrictor activity of Up_4A in the coronary microvessels is not readily explained. However, while purinergic receptors are present throughout the vascular tree their distribution can very markedly, depending on the regional vascular bed [9, 35, 37], vascular cell type, i.e. endothelial cell versus vascular smooth muscle cell [38-40], and animal species [41]. Thus, it is likely that the variable vascular responses to Up_4A reported previously and found in the present study depend on the specific purinergic receptor subtype distribution.

The dinucleotide Up_4A contains both purine and pyrimidine moieties, which can activate purinergic receptors to exert its vascular action. Activation of P1 receptors has been shown to be a potent vasodilator stimulus in the coronary vasculature. Thus, adenosine, ATP, ADP, which are all known to activate the P1 receptors, are among the most potent coronary vasodilators [15, 42]. In the present study, Up_4A-induced vasorelaxation, which was similar in magnitude compared to adenosine, ATP and ADP, was attenuated by non-selective blockade of P1 receptors. ATP and ADP can also produce vasodilation through $P2X_1$ (ATP) and/or $P2Y_1$ (ATP and ADP) receptors [43-45]. In contrast, P2 receptors have been shown to contribute mainly to vasoconstriction in response to Up_4A. Thus, Up_4A leads to vasoconstriction via P2Y receptors in rat pulmonary

artery [19]. Up_4A-induced vasoconstriction in perfused rat kidney depends not only on the activation of $P2X_1$ receptor [6] but also on the activation of $P2Y_2$ receptor [36]. In the present study, Up_4A-induced vasodilation was neither affected by non-selective blockade of P2 receptors nor selective blockade of $P2X_1$, $P2Y_1$, and $P2Y_6$ receptors. The involvement of $P2Y_2$ and $P2Y_4$, for which no selective antagonists are available, is unlikely in Up_4A-induced vasodilation, since the $P2Y_2/P2Y_4$ receptor agonist UTP did not have a vasodilator effect, which was consistent with a study in which UTP could not produce vasodilation in isolated canine coronary arteries [46]. The expression profile of purinergic receptors $P2X_4$, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and A_{2A}, which have been proposed to contribute to the nucleotide-induced vasodilation [6, 35], shows that these receptors were present in the coronary small arteries, with $P2Y_1$ most abundantly expressed. However, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors were not involved in Up_4A-induced vasodilation. Importantly, $P2Y_1$ was shown to be involved in ADP-induced vasodilation, indicating that the $P2Y_1$ receptor is indeed functional. We were not able to detect the presence of $P2X_1$ receptor in the coronary small arteries, which has been shown to contribute to Up_4A-induced vasoconstriction in isolated rat perfused kidney and ATP-induced vasodilation in isolated rat mesenteric arteries [6, 36, 45]. However, our expression data are consistent with the lack of effect of $P2X_1$ blockade on both Up_4A- and ATP-induced vasodilation, and the evidence that $P2X_1$ receptor could not be detected in rat large coronary arteries by immunohistochemistry [47].

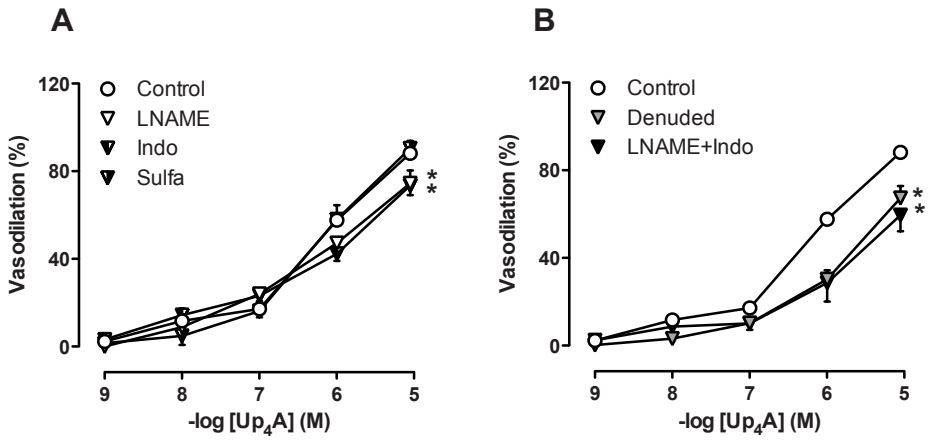

Fig. 8. *Endothelium-dependency of vasodilation induced by Up_4A. Shown are concentration-responses to Up_4A (10^{-9}-10^{-5} M) in endothelium-intact vessels in the absence (n=48) and presence of 100 μM of the NOS inhibitor LNAME (n=6), 10 μM of the COX inhibitor indomethacin (Indo; n=6), and 10 μM of the CYP 2C9 inhibitor sulfaphenazole (Sulfa; n=6) (panel A); and in endothelium-denuded vessels (n=13) as well as in the endothelium-intact vessels with and without combined LNAME and Indo (n=6) (B). Values are means±SEM. * P<0.05, effect of drug or denudation vs. control.*

It has been suggested that the vasodilator response to ATP is in part due to its degradation by ectonucleotidase to ADP, AMP and adenosine [5, 27-29]. Similarly, the vasodilator response of Up_4A can be caused by ectonucleotidase-mediated degradation to ATP and/or UTP [6]. In the present study, Up_4A was not likely degraded to ADP, as $P2Y_1$ blockade attenuated ADP-but not Up_4A-induced vasodilation. However, P1 receptors but not P2 receptors contributed to Up_4A-induced vasodilation, while the vasodilator potency of Up_4A was similar to that of adenosine. It is therefore possible that Up_4A-induced vasodilation was not a direct P1 effect but resulted from

its degradation to AMP and/or adenosine or other nucleotides by ectonucleotidase. To investigate this possibility, we compared the vasodilator response of adenosine and Up_4A, and how this response was affected by various purinergic receptor antagonists. In addition, we tested whether the vasodilator response to Up_4A was affected by ectonucleotidase inhibition. We observed a similar involvement of purinergic receptor subtypes in the vasodilator response to adenosine compared to Up_4A in that adenosine-induced vasodilation was similarly attenuated by non-selective blockade of P1 receptors and selective A_{2A}-receptor blockade, but not by $P2X_1$, $P2Y_1$ and $P2Y_6$ receptor antagonists. However, Up_4A-induced vasodilation was not attenuated by ectonucleotidase inhibition. Taken together, these findings indicate that Up_4A exerts a direct vasodilator effect mainly via P1 (A_{2A}) receptors.

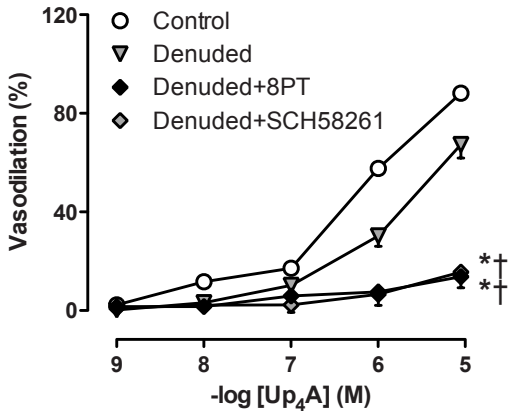

Fig. 9. *Endothelium-independent vasodilation induced by Up_4A. Shown are concentration-responses to Up_4A (10^{-9}-10^{-5} M) in endothelium-intact vessels (n=48), in endothelium-denuded vessels (n=13), and denuded vessels in the presence of P1 receptor antagonist 8PT (n=7) or A_{2A} receptor antagonist SCH58261 (n=7). Values are means±SEM. * P<0.05, effect of denudation vs. control; † P<0.05, effect of drugs after denudation.*

Stimulation of endothelial $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors induced by various extracellular nucleotides is known to cause NO-, PGI_2-, and EDHF-mediated vasodilation [48, 49]. Thus, Up_4A-induced activation of $P2Y_1$ and $P2Y_2$ receptor on endothelial cells was shown to enhance release of NO [36]. Similarly, we observed that vasodilation produced by Up_4A was significantly attenuated in endothelium-denuded vessels in the present study. The attenuation of Up_4A-induced vasodilation by endothelial denudation was similar in magnitude as the attenuation achieved by combined inhibition of NOS and COX. In contrast, although CYP 2C9 has been reported to generate an EDHF in the coronary vasculature [30-32], inhibition of CYP2 C9 did not affect Up_4A-induced vasodilation. These data are consistent with a recent study from our laboratory, in which CYP 2C9 inhibition alone did not affect vasodilation of isolated coronary small arteries to bradykinin [23].

Endothelial denudation significantly attenuated the Up_4A-induced vasodilation. However, Up_4A still produced over 60% vasodilation at the dose of 10^{-5} M. Non-selective blockade of P1 and selective blockade of A_{2A} receptors in denuded vessels produced significant further blunting of the Up_4A-induced vasodilation. This is consistent with the observation that P1 receptors located both on endothelial cells and smooth muscle cells contribute to vasodilator function in porcine coronary arterioles [40, 43, 44], and indicates that Up_4A-induced A_{2A} receptor-mediated vasodilation was both endothelium-dependent and -independent.

Conclusions

The present study indicates that contrary to observations in most arterial segments and vascular beds in rodents, Up_4A is a potent vasodilator in the porcine coronary microvasculature. Up_4A acts directly via activation of P1 rather than P2 receptors, and is partially endothelium-dependent resulting in the production of NO and PGI_2.

Acknowledgments

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Chapter 8

Blunted coronary vasodilator response to uridine adenosine tetraphosphate in post-infarct remodeled myocardium is due to reduced P1 receptor activation

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Abstract

We previously demonstrated that uridine adenosine tetraphosphate (Up_4A) exerts a potent vasodilator effect in the healthy porcine coronary vasculature. Since the coronary microvascular effects of Up_4A after myocardial infarction (MI) are unknown, the present study investigated the response to Up_4A in coronary microvessels from post-MI remodeled porcine myocardium, and the involvement of purinergic receptor subtypes. Coronary small arteries (diameter ~150 μm) were dissected from the apex of Sham-operated swine and swine in which MI had been produced 5 weeks earlier by transient (2 hr) occlusion of the left circumflex coronary artery, and mounted on Mulvany wire myographs. Up_4A (10^{-9}-10^{-5} M) produced coronary vasodilation that was reduced in MI as compared to Sham-operated swine. Up_4A-induced vasodilation was reduced by P1 blockade with 8-phenyltheophylline in Sham-operated swine and to a lesser extent in MI, while the attenuation by the A_{2A} receptor blocker SCH58261 was similar in Sham-operated and MI swine. Up_4A-induced vasodilation remained unaffected by non-selective P2 receptor antagonist PPADS, but was attenuated by selective $P2X_1$ and $P2Y_1$ receptor antagonists MRS2159 and MRS2179, albeit to a similar extent in Sham-operated and MI swine. These responses were paralleled by similar mRNA expression levels of A_{2A}, $P2X_1$ and $P2Y_1$ receptors in MI compared to slaughterhouse control swine. Finally, attenuation of Up_4A-induced coronary vasodilation by nitric oxide synthase inhibition was not attenuated in MI as compared to Sham-operated swine. In conclusion, blunted coronary vasodilation in response to Up_4A in MI swine is most likely due to reduced activation of P1, rather than P2, receptors and does not involve a loss of NO bioavailability.

Introduction

The endothelium releases a variety of vasodilators, such as nitric oxide (NO) and prostacyclin, and vasoconstrictors, such as endothelin and reactive oxygen species [1]. A novel endothelium-derived vasoactive factor named uridine adenosine tetraphosphate (Up_4A) has been recently identified [2]. Up_4A contains one purine and one pyrimidine moiety and therefore can exert its vasoactive effects through the purinergic P1 as well as P2 receptors [2-5]. These receptors are also involved in the vasoactive effects of purines such as adenosine, ATP and ADP as well as pyrimidines such as UTP and UDP. In contrast to the vasoconstrictor action of Up_4A in various vascular beds in rodents [3], we have recently shown that Up_4A exerts a potent vasodilator effect in the healthy porcine coronary vasculature that is largely mediated through P1 (A_{2A}) receptors [4] and partly involves the release of nitric oxide and prostacyclin from the endothelium. These observations suggest that Up_4A represents a novel vasodilator in the coronary microcirculation. Furthermore, the 10-fold elevation in plasma level of Up_4A observed in juvenile hypertensive subjects [6], as well as the observation that the vasoconstriction to Up_4A is potentiated in DOCA-salt hypertensive rats [7, 8], suggest that contribution of Up_4A to regulation of vasomotor tone may be altered in cardiovascular disease.

Following myocardial infarction (MI), the balance between endothelium-derived vasoactive factors is altered, even in the remote coronary vasculature; endothelial nitric oxide synthase (eNOS) uncoupling results in a decreased NO production, while the production of reactive oxygen species (ROS) is increased [9-14]. Moreover, we have previously shown that the coronary vasodilator response to ATP, which activates P1 as well as P2 receptors [5, 15] and involves the activation of eNOS [16], is blunted following MI [17]. These observations suggest that purinergic receptor expression and/or signaling is altered following MI, and hence that the response of the coronary vasculature to Up_4A may be altered after MI.

Consequently, the present study aimed to determine whether the coronary vascular response to Up_4A is altered after MI, and to study possible changes in the contribution of purinergic receptor subtypes to the Up_4A responses in the coronary microcirculation following MI.

Methods

Animals

Studies were performed in accordance with approval of the Animal Care Committee at Erasmus Medical Center Rotterdam (NL). Fourteen Crossbred Yorkshire × Landrace swine (2-3-month-old, 25±1 kg at the time of surgery) of either sex entered the study. After one week of daily adaptation to laboratory conditions, swine were sedated with an intramuscular injection of Zoletil (Tiletamine/Zolazepam; 5 mg/kg), Xylazine (2.25 mg/kg) and Atropine (1 mg), a small catheter was placed in an ear vein for subsequent administration of anesthesia and fluid. Swine were intubated and ventilated with a mixture of oxygen and nitrogen (1:2 v/v). Anesthesia was induced and maintained using fentanyl (10 μg/kg/h, iv) [18]. Heart rate was monitored and pain reflexes were checked regularly and when necessary, additional anesthesia was administered. A thoracotomy was performed in the fourth left intercostal space. A catheter was inserted into the aorta. Subsequently, the proximal part of the LCx was exposed and occluded for 2 hr followed by reperfusion to induce MI (n=7), whereas no occlusion was performed in Sham (n=7). Heparin (5000 IU) was administered just prior to occlusion and 1 hour into occlusion to prevent clotting in the distal coronary vasculature. The aortic catheter was tunneled subcutaneously to the back

and the thorax was closed in layers. Post-operative analgesia was by administration of Temgesic (Buprenorfine; 0.015 mg/kg im) in combination with a fentanyl slow-release patch (12 μg/h) for 72 h. The catheters were flushed three times per week with 5000 IU/ml heparine.

In vivo measurements

Five weeks after induction of MI or Sham operation, all animals were sedated with Zoletil (5 mg/kg), Xylazine (2.25 mg/kg) and Atropine (1 mg), anesthetized with pentobarbital (20 mg/kg/h iv) and artificially ventilated. A Millar catheter was inserted via the carotid artery for measurement of left ventricular pressure and a Swan Ganz catheter was inserted via the jugular vein for measurement of pulmonary artery pressure and of cardiac output via thermodilution [19]. Following thoracotomy, hearts were arrested and immediately excised and placed in cold, oxygenated Krebs bicarbonate buffer solution.

In vitro myograph studies

Coronary small arteries (diameter≈150 μm) were dissected out from the apex (perfusion territory of the left anterior descending coronary artery in swine) of swine with MI and Sham-operated swine, as well as from swine hearts obtained at a local slaughterhouse and stored overnight in cold, oxygenated Krebs bicarbonate solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4. The next day, arteries were cut into segments of ≈2 mm length and mounted in microvascular myographs (Danish Myo Technology) with separated 6 ml organ baths containing Krebs bicarbonate solution aerated with 95% O_2/5% CO_2 and maintained at 37°C. Changes in contractile force were recorded with a Harvard isometric transducer. Following a 30 min stabilization period, the internal diameter was set to a tension equivalent to 0.9 times the estimated diameter at 100 mmHg effective transmural pressure. The vessels were then exposed to 30 mM KCl twice. Endothelial integrity of coronary arteries was verified by observing dilation to 10 nM substance P, after preconstriction with 100 nM of the stable thromboxane A_2 analogue 9,11-Dideoxy-11α,9α epoxymethanoprostaglandin $F_{2\alpha}$ (U46619). Then vessels were subjected to 100 mM KCl to determine the maximal vascular contraction. Thereafter, vessels were allowed to equilibrate in fresh Krebs solution for 30 min before initiating different experimental protocols [4, 20]. Only one protocol was executed per vessel and within one protocol, all vessels were obtained from different animals.

Table 1. Primer information.

Genes	Sequences		Sizes
	Sense	Antisense	
A2A	5'-ATGTTGGGCTGGAATAGCTG-3'	5'-CACGGAGTTGGTGTGAGAGA-3'	426 bp
P2X1	5'-TTGAACCCCATTTCTTCCTG-3'	5'-AGTGCACCACACATCTGCTC-3'	248 bp
P2X4	5'-TGTCCCCAGGCTACAATTTC-3'	5'-GGCAGCTTTTTCTCCCTTCT-3'	373 bp
P2Y1	5'-TTCCTGACTTGCATCAGTGC-3'	5'-CAGTGCCCGAGTAGAAGAGG-3'	157 bp
P2Y2	5'-GTGGCCTACAGCTTGGTCAT-3'	5'-GCGTGCGGAAGGAGTAGTAG-3'	235 bp
P2Y4	5'-GACTGCCGGTTTAATGAGGA-3'	5'-AGGAAAAGGACGCTGCAGTA-3'	302 bp
P2Y6	5'-CTGCTCTTGCCACCTGTGTA-3'	5'-AGGTTGGCGTAGAACAGGAA-3'	251 bp
eNOS	5'-CTCTCCTGTTGGCCTGACCA-3'	5'-CCGGTTACTCAGACCCAAGG-3'	151 bp
GAPDH	5'-TCGGAGTGAACGGATTTG-3'	5'-CCTGGAAGATGGTGATGG-3'	219 bp

eNOS: endothelial nitric oxide synthase.

Experimental protocols

Coronary small arteries from both Sham-operated, slaughterhouse controls (SH-Controls) and MI swine were subjected to Up_4A (Biolog Life Science, Bremen, Germany) in incremental dosages ranging from 10^{-9} to 10^{-5} M in the absence and presence of preconstriction with U46619. Contrary to previous observations in various vascular beds in rodents [3], but similar to our previous study in isolated porcine coronary arteries, Up_4A failed to produce vasoconstriction. To assess whether the lack of vasoconstriction was due to our experimental conditions, we subjected rat mesenteric arteries (diameter≈100 μm) to the same dosages of Up_4A. Up_4A did produce vasoconstriction in these vessels (Fig. 1) that was similar in magnitude as in previous studies [8].

To assess the involvement of different purinergic receptors in the vasodilator response to Up_4A, coronary small arteries from Sham-operated and MI swine were pre-incubated with non-selective P1 receptor antagonist 8-phenyltheophylline (8PT, 10^{-5} M), non-selective P2 receptor antagonist pyridoxalphosphate-6-azophenyl-2', 4'-disulfonic acid (PPADS, 10^{-5} M) adenosine A_{2A} receptor antagonist SCH58261 (10^{-7} M), $P2X_1$ receptor antagonist MRS2159 (3×10^{-5} M), and $P2Y_1$ receptor antagonist MRS2179 (10^{-6} M) [4] followed by preconstriction with U46619 (100 nM). To investigate if the role of NO in the vasodilator response to Up_4A was altered after MI, vessels from both Sham-operated and MI swine were exposed to Up_4A (10^{-9}-10^{-5} M) in the absence and presence of nitric oxide synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methyl ester HCl (LNAME, 10^{-4} M) [4]. Unless otherwise mentioned, all antagonists and inhibitors were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Table 2. Hemodynamic and anatomic characteristics.

	Control n=7	MI n=7
Anatomic data		
BW (kg)	45.1 ± 0.2	39.6 ± 0.3*
LVW(g)	105.1 ± 0.5	132.0 ± 0.7*
LVW/BW (g/kg)	2.3 ± 0.1	3.3 ± 0.2*
Awake state		
HR (beat/min)	137 ± 8	143 ± 8
MAP (mmHg)	92 ± 1	93 ± 3
Anesthesie state		
HR (beat/min)	114 ± 4	84 ± 3*
MAP (mmHg)	100 ± 5	101 ± 5
CO (l/min)	4.2 ± 0.2	3.6 ± 0.4
PAP (mmHg)	20 ± 1	24 ± 3

*MI: Myocardial infarction; BW: Body weight; LVW: Left ventricular weight; LV/BW: Left ventricular weight to body weight ratio; HR: Heart rate; MAP: Mean arterial pressure; CO: Cardiac output; PAP: Pulmonary arterial pressure. Values are mean±SEM. * P<0.05 vs. Control.*

Quantitative real-time PCR analysis

For detection of A_{2A}, $P2X_1$, $P2X_4$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ mRNA, coronary small arteries (diameter≈150 μm) were snap-frozen in liquid nitrogen following dissection. For the mRNA expression, coronary small arteries from the apex of swine with MI were compared to those from SH-Controls. Total RNA was extracted from 4-7 frozen samples per group using a Qiagen RNA kit. cDNA was synthesized from 100 ng of total RNA with iScript Reverse Transcriptase (Bio-Rad). Quantitative real-time PCR (MyIQ, Bio-Rad) was performed with

SYBR Green (Bio-Rad) [4]. Target gene mRNA levels were expressed relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control [21]. Primer sequences are shown in Table 1 and are identical to the primers used in our previous study [4] with the exception of the $P2X_1$-primer pair.

Data analysis and statistics

Statistical analysis of hemodynamic data was performed using an un-paired t-test. Vascular contraction response to Up_4A was expressed as percentage of contraction to 100 mM KCl; vascular relaxation response to Up_4A was expressed as percentage of contraction to U46619. The effects of drug treatment on the Up_4A response were assessed using two-way RM-ANOVA. Statistical significance was accepted when $P \leq 0.05$ (two-tailed). Data are presented as mean±SEM.

Results

Effect of MI on hemodynamics and left ventricular hypertrophy

MI had little effect on heart rate and mean aortic blood pressure in the awake state (Table 2). Under anesthesia, just prior to sacrifice, cardiac output tended to be lower and pulmonary artery pressure tended to be higher in MI as compared to Sham-operated swine (Table 2). Left ventricular weight to body weight ratio was significantly increased in MI as compared to Sham-operated swine, indicating left ventricular hypertrophy in MI swine (Table 2).

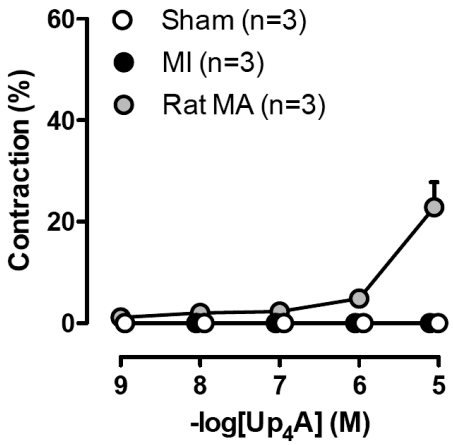

Fig. 1. *Up_4A concentration-response (10^{-9}-10^{-5} M) in coronary small arteries from Sham-operated swine (Sham) and swine with a 5-week old myocardial infarction (MI) and rat mesenteric arteries (MA) under basal tone. Values are mean±SEM.*

Up_4A-induced coronary vasodilation is reduced after MI

Cumulative concentrations of Up_4A failed to induce vasoconstriction in isolated porcine coronary small arteries from either Sham-operated or MI swine during spontaneous tone (Fig. 1). In contrast, Up_4A produced a concentration-dependent vasodilation up to 100% in precontracted coronary small arteries (Fig. 2). This vasodilator response to Up_4A was reduced in coronary small arteries from MI as compared to Sham-operated swine as well as SH-Controls (Fig. 2).

Involvement of purinergic receptor subtypes in response to Up_4A after MI

Up_4A-induced coronary vasodilation was significantly attenuated by the non-selective P1 receptor blocker 8PT as well as the A_{2A} blocker SCH58261 in both Sham-operated (Fig. 3A and 3C) and MI swine (Fig. 3B and 3D). Notably, the attenuation of the vasodilator response by 8PT, but not SCH58261, was reduced in MI as compared with Sham-operated swine. Non-selective P2 receptor blockade with PPADS failed to attenuate Up_4A-induced coronary vasodilation both in Sham-operated and MI swine (Fig. 4A and 4B). Since the P2 receptor family mediates both vasodilator and vasoconstrictor influences depending on the type of receptor subtypes as well as its location on endothelial cells or smooth muscle cells [5] and since PPADS blocks most, but not all P2 receptor subtypes [22], we further incubated coronary small arteries with specific receptor blockers for $P2X_1$ and $P2Y_1$ receptors. Both $P2X_1$ and $P2Y_1$ antagonists MRS2159 and MRS2179 significantly attenuated Up_4A-induced coronary vasodilation to the same extent in Sham-operated and MI swine (Fig. 4C, 4D, 4E and 4F).

Since selective blockers are not available for each individual purinergic receptor, the presence of mRNA for purinergic receptors (A_{2A}, $P2X_1$, $P2X_4$, $P2Y_7$, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$), known to be involved in vascular responses to purines and/or pyrimidines [22] was present in coronary small arteries. Only the mRNA expression level of $P2X_4$ and $P2X_7$ was significantly higher in MI than in SH-Controls, while $P2Y_4$ expression was only present in one vessel from the MI group (Fig. 5).

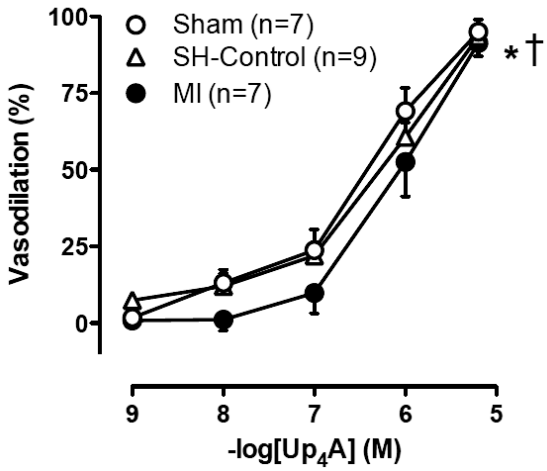

Fig. 2. *Up_4A concentration-responses (10^{-9}-10^{-5} M) in coronary small arteries from Sham, SH-Control and MI swine, preconstricted with U46619 (100 nM). Values are mean±SEM. * P≤0.05 vs. Sham, † P≤0.05 vs. SH-Control.*

Involvement of NO in response to Up_4A after MI

Up_4A-induced vasodilation in healthy porcine coronary small arteries is mediated in part by the NO pathway [4]. Since the contribution of endogenous NO to coronary resistance vessel tone in conscious swine was diminished after MI [12, 14, 23], we investigated whether the contribution of the NO-pathway to Up_4A induced coronary vasodilation was also altered in coronary small arteries from MI. The vasodilator response to substance P was similar (P=0.52) in coronary small arteries from Sham-operated (95±2%) and MI swine (93±2%). The NOS inhibitor LNAME, in a dose that significantly attenuated the vasodilation to substance P (to 63±10%), significantly

attenuated Up_4A-induced coronary vasodilation both in Sham-operated and MI swine. The effect of LNAME on Up_4A-induced vasodilation tended to be larger in coronary small arteries from MI than from Sham-operated swine, although this failed to reach statistical significance (P=0.08) (Fig. 6A and 6B).

Discussion

The main findings of the present study are that 1) Up_4A-induced coronary vasodilation was reduced in MI; 2) non-selective P1 receptor blockade markedly attenuated Up_4A-induced vasodilation while this attenuation was less in MI as compared to Sham-operated swine; 3) the attenuation of the vasodilator response to Up_4A by A_{2A} blockade was similar in MI and Sham-operated swine; 4) non-selective P2 receptor blockade did not attenuate Up_4A-induced coronary

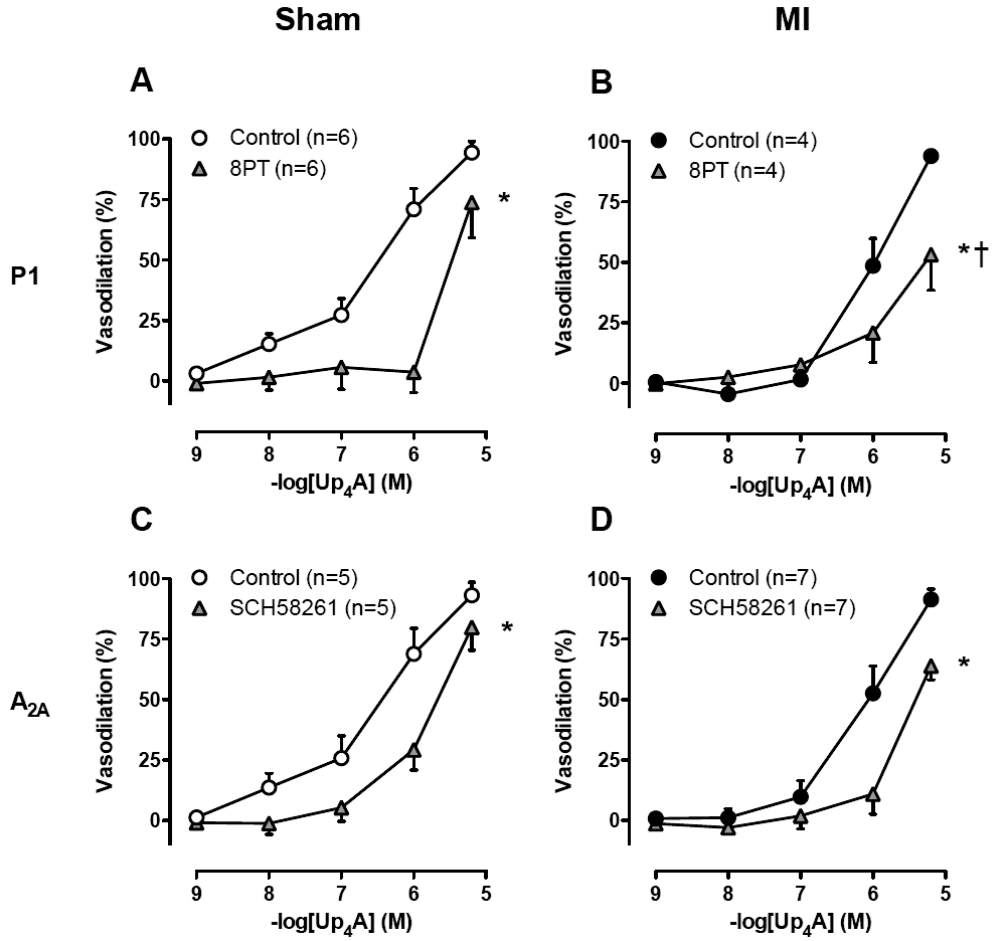

Fig. 3. *Effects of non-selective P1 (panels A&B) and selective A_{2A} (panels C&D) receptor blockade on Up_4A-induced vasodilation. Shown are concentration-responses to Up_4A (10^{-9}-10^{-5} M) in coronary small arteries of Sham-operated swine and swine with a 5-week old myocardial infarction in the absence and presence of 10 μM of non-selective P1 receptor antagonist 8PT, or 100 nM of A_{2A} receptor antagonist SCH5826. Values are mean±SEM. * P≤0.05 vs. corresponding vessels without blocker, † P≤0.05 MI vs. Sham.*

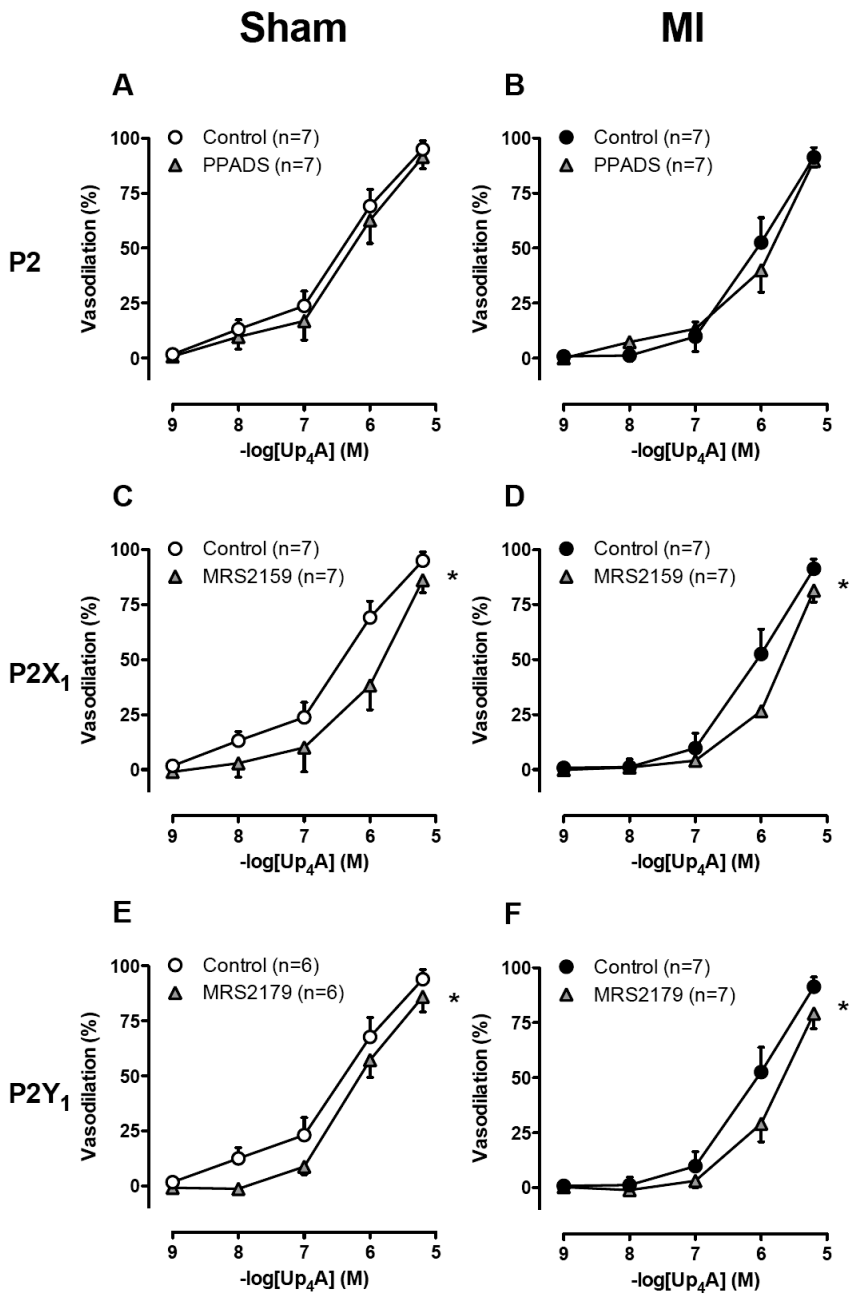

Fig. 4. *Effects of non-selective P2 and selective $P2X_1$ and $P2Y_1$ receptor blockade on Up_4A-induced vasodilation. Shown are concentration-responses to Up_4A (10^{-9}-10^{-5} M) in coronary small arteries of Sham-operated swine and swine with a 5-week old myocardial infarction; in the absence and presence of 10 μM of non-selective P2 receptor antagonist PPADS, 30 μM of $P2X_1$ receptor antagonist MRS2159 or 1 μM of $P2Y_1$ receptor antagonist MRS2179. Values are mean±SEM. * P≤0.05 vs. corresponding vessels without blocker.*

vasodilation, whereas selective blockade of $P2X_1$ and $P2Y_1$ significantly attenuated Up_4A-induced vasodilation to a similar extent in Sham-operated and MI swine; 5) these functional responses were paralleled by similar mRNA expression levels of A_{2A}, $P2X_1$ and $P2Y_1$ receptors in MI compared to SH-Control swine; 6) inhibition of NO synthase significantly attenuated Up_4A-induced coronary vasodilation, but to a similar extent in MI compared to Sham-operated swine. The implications of the present findings are discussed below.

Up_4A has been identified as a novel endothelium-derived vasoactive factor. Circulating levels of Up_4A in healthy subjects are ~4 nM, and are well within its vasoactive range [2]. In rats, Up_4A produces potent vasoconstriction in isolated perfused kidneys and increases blood pressure [2]. Moreover, Up_4A induces vasoconstriction in rat aorta [24] and pulmonary arteries [25].

Conversely, in the presence of increased vascular tone, Up_4A has been reported to be able to produce modest vasodilation in isolated aortic rings of rats [24] and in isolated perfused rat kidneys [26], and to elicit marked vasorelaxation of porcine coronary small arteries [4]. Similarly, in the present study Up_4A produced potent vasodilation in coronary small arteries of slightly smaller (~150 μm) size as compared to our previous study, in which small arteries of ~250 μm were studied [4], with no evidence of any vasoconstrictor response in coronary small arteries under basal tone. Apparently, the porcine coronary vasculature responds differently to Up_4A as compared to several other vascular regions in the rat. This difference was not due to the experimental conditions, as Up_4A did produce vasoconstriction in rat mesenteric arteries in our setup as well. Taken together, these findings suggest that the vasoactive influence induced by Up_4A depends on vascular beds and possibly species studied [3].

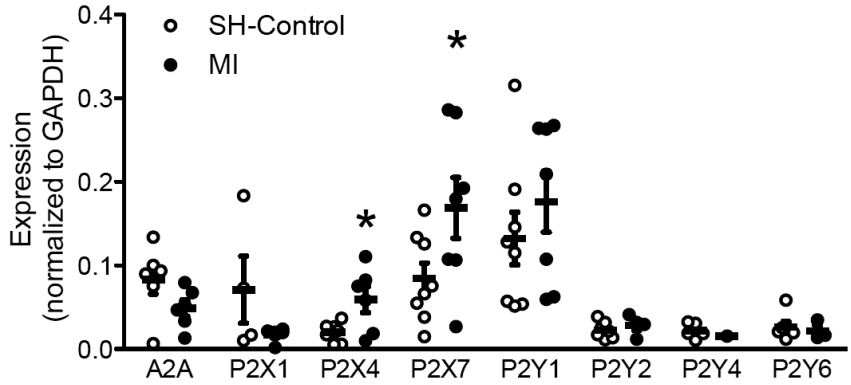

Fig. 5. *Expression profile of purinergic receptors in the coronary vasculature. Shown is the expression profile of A_{2A}, $P2X_1$, $P2X_4$, $P2X_7$, $P2Y_1$, $P2Y_2$ $P2Y_4$ and $P2Y_6$ normalized to GAPDH in intact coronary small arteries from both SH-Control and MI. Values are mean±SEM. * P≤0.05 vs. SH-Control.*

The role of Up_4A in regulation of vascular tone may be altered in cardiovascular disease. For instance, the vasoconstrictor response to Up_4A is potentiated in basilar, femoral and renal arteries of hypertensive rats [7, 8]. In the present study, the Up_4A dose response curve in coronary small arteries was shifted rightwards in MI compared to Sham-operated swine, with no change in maximal vasodilator response, indicating reduced sensitivity of the coronary microvasculature to Up_4A after MI. The reduced sensitivity to Up_4A after MI is consistent with our previous in vivo observation, that the coronary vasodilator response to ATP, that is also mediated via purinergic receptors and also involves nitric oxide production, was reduced after MI [17]. Apparently, the coronary vasculature exhibits a reduced response to purinergic receptor activation following MI. This reduced response can be due to altered contributions of purinergic

receptor subtypes [8, 27] and/or altered downstream signaling pathways [12], potentially due to endothelial dysfunction in the coronary vasculature after MI [12, 17, 23].

In the coronary vasculature, the purinergic receptors primarily involved in Up_4A-induced vasodilation are the P1 receptors [4]. Thus, non-selective P1 receptor blockade markedly attenuated Up_4A-induced coronary vasodilation, whereas non-selective P2 receptor blockade failed to influence the Up_4A dose response curves in either Sham-operated or MI swine. Notably, the attenuation by non-selective P1 receptor blockade in response to Up_4A in MI was significantly less than that in Sham-operated swine. Moreover, selective A_{2A} receptor blockade resulted in a similar attenuation of coronary vasodilation in response to Up_4A in Sham-operated and MI swine, which was consistent with the comparable expression levels of A_{2A} receptors in MI compared to SH-Control. These findings demonstrate that reduced activation of P1 receptors, rather than P2 receptors, was responsible for the reduced response to Up_4A after MI. Furthermore, although we cannot entirely exclude that the differences in response to 8PT and SCH58261 between MI and Sham vessels may be influenced by different degrees A_{2A} receptor blockade between these two antagonists, our observations could also be interpreted to suggest that a reduced activation of P1 receptor subtypes other than A_{2A} receptors is involved. Thus, several studies indicate that the A_{2B} receptor is also involved in coronary vasodilation in rats [28] and mice [29, 30]. Although A_{2B} receptors have also been shown to be present in the porcine coronary arteries [27, 31], measurements of flow in anesthetized miniswine with dyslipidemia [27], and diameter measurements in isolated porcine coronary small arteries [32] failed to show involvement of P1 receptor subtypes other than A_{2A} in adenosine-mediated coronary vasodilation. Nevertheless, we cannot exclude the possibility of A_{2B} involvement in response to Up_4A after MI.

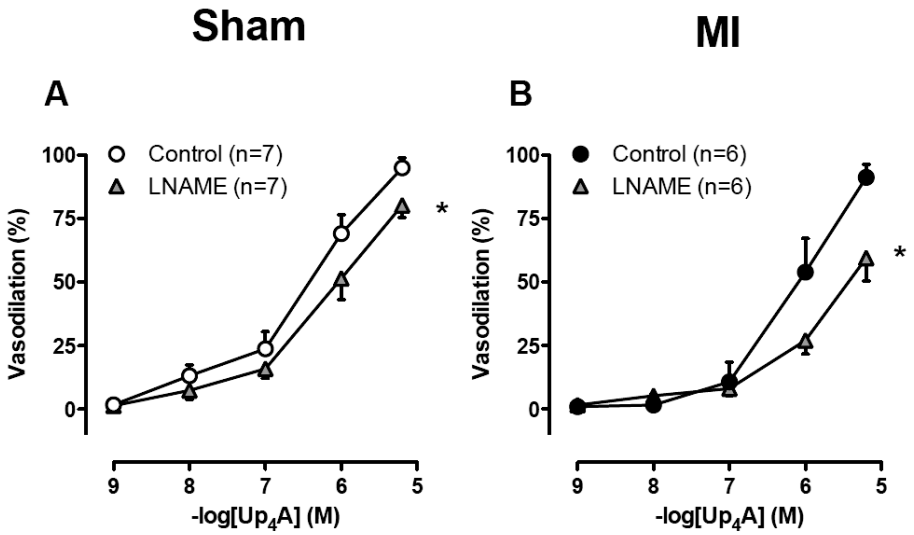

Fig. 6. *Effects of eNOS inhibition on Up_4A-induced vasodilation. Shown are concentration-responses to Up_4A (10^{-9}-10^{-5} M) in coronary small arteries of Sham-operated swine and swine with a 5-week old myocardial infarction in the absence and presence of 100 μM of the eNOS inhibitor LNAME. Values are mean±SEM. * P≤0.05 vs. corresponding vessels without blocker.*

In addition to activation of P1 receptors, the dinucleotide Up_4A is able to activate P2 receptors. P2 receptors in the vasculature are present on endothelial as well as vascular smooth

muscle cells. Activation of $P2Y_1$, $P2Y_2$ and sometimes $P2Y_4$, $P2Y_{11}$, $P2X_1$, $P2X_2$, $P2X_3$ and $P2X_4$ receptors on endothelial cells, leads to the production of nitric oxide (NO) and subsequent vasodilation [5]. Although the $P2X_7$ receptor is generally thought to be involved in inflammatory responses [5], the $P2X_7$ receptor has also been suggested to facilitate flow-dependent dilation [33]. Activation of $P2X_1$ receptors and, in some vessels, $P2X_2$, $P2X_4$ and $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors on smooth muscle cells, generally results in vasoconstriction although vasodilation is also sometimes observed [5]. In the present study, we did not distinguish between expression in endothelial and vascular smooth muscle cells, but we observed expression of $P2X_1$, $P2X_4$, $P2X_7$, $P2Y_1$, $P2Y_2$ $P2Y_4$ and $P2Y_6$.

Up_4A-induced vasodilation was partially blocked by $P2X_1$ as well as $P2Y_1$ receptor blockade. In our previous study in slightly larger vessels [4], we did not observe attenuation of the Up_4A response with either $P2X_1$ or $P2Y_1$ blockade, suggesting that purinergic signaling is dependent on vessel size. Our finding that we did not observe either expression or functional involvement of $P2X_1$ receptors in coronary arteries with diameter of ~250 μm [4] but did observe both expression and functional involvement of $P2X_1$ receptors in vessels with diameter of ~150 μm in the present study, is consistent with a study showing that P2X receptor expression is higher in smaller vessels [34]. This attenuation of the Up_4A response by $P2X_1$ and $P2Y_1$ blockade was similar in vessels from Sham-operated and MI swine, which is consistent with the expression of $P2X_1$ and $P2Y_1$ that was also similar between SH-Controls and swine with MI. The observation that vasodilation is reduced by $P2X_1$ and $P2Y_1$ receptor blockade suggests that these receptors are predominantly expressed on endothelial cells.

The involvement of P2 receptors other than $P2X_1$ and $P2Y_1$ in the response to Up_4A is more difficult to elucidate due to a lack of selective receptor blockers at the time of our study. Interestingly, the non-selective P2 receptor antagonist PPADS had no effect on the response to Up_4A. PPADS blocks most of P2 receptor subtypes, including $P2X_2$, $P2X_3$, $P2X_5$ [15], $P2X_7$ [35], $P2Y_1$ [36], $P2Y_2$, $P2Y_4$ [37] and $P2Y_6$ [38]. Thus, the difference between the non-selective P2 receptor antagonist PPADS and the more selective $P2X_1$ antagonist may be due the fact that PPADS does not block the $P2X_1$ receptor. Indeed, $P2X_1$-mediated renal vasoconstriction in response to Up_4A was not blocked by PPADS but was blocked by another non-selective P2 receptor antagonist suramin [26]. Since PPADS has been shown to block the $P2Y_1$ receptors [36], the observed difference between effect of PPADS and $P2Y_1$ receptor antagonist MRS2179 must be due to altered involvement of other P2 receptors that are blocked by PPADS and negate the blockade of the vasodilator $P2Y_1$ receptors. Thus, the loss of (vasodilator) endothelial $P2Y_4$ receptors [15] in vessels from MI may have contributed to the reduced response to Up_4A. Alternative candidates may be $P2Y_2$ or $P2Y_6$ receptors on vascular smooth muscle cells that have been shown to be present in large [37] and small porcine coronary arteries [4] as well as in human coronary vasculature [39] and mediate UTP- and UDP-induced vasoconstriction in the large coronary arteries [37]. However, expression of $P2Y_2$ or $P2Y_6$ receptors was not altered after MI. Moreover, although the expression of $P2X_4$ and $P2X_7$ receptors was increased in MI, it is unlikely that this difference in receptor expression explains the reduced response to Up_4A between vessels from Sham-operated and MI swine, since both $P2X_4$ and $P2X_7$ are most abundantly expressed on endothelial cells and are thought to induce vasodilation [22, 33]. Therefore, the increased expression of $P2X_4$ and $P2X_7$ receptors may explain the observation that eNOS inhibition attenuated the vasodilator response to Up_4A similarly in vessels from MI and normal swine. The maintained contribution of eNOS to the Up_4A-induced vasodilation is also consistent with the maintained, largely NO-dependent, vasodilator response to substance P in MI compared to Sham-operated swine. However these findings are somewhat surprising in view of previous

observations from our laboratory demonstrating partial uncoupling of eNOS [14], and increased levels of superoxide [10], which explain the blunted coronary vasodilator responses to ATP in post-MI remodeled myocardium in vivo [16]. Interestingly, we recently found that phosphodiesterase 5 expression levels and coronary vasoconstrictor influences are reduced in post-MI remodeled myocardium [12], acting to enhance the vasodilator influence of NO. This may explain why the vasodilator response to NO donor nitroprusside was maintained, despite higher ROS levels, which would act to reduce NO half-life. Although the disparate effects of MI on Up_4A, substance P and ATP are not readily explained, it is likely that the relative degrees of dependency on eNOS- and cGMP-mediated vasodilation of these three endothelium-dependent vasodilators contributed to the variable outcomes. Future studies are required to further determine how the different endothelium-dependent signal transduction pathways in coronary small arteries are modified in the post-infarct remodeled heart.

Methodological considerations

In order to test the involvement of the different purinergic receptors, we used pharmacological blockers of various receptor subtypes. A potential pitfall in such studies is that while *selectivity*, i.e. the capability of blocking a particular receptor as compared to related receptors, is usually well-defined, *specificity*, i.e. the potential for discriminating between negative and positive interactions and the cross-reactivity with other (unrelated) targets is less well-established, particularly when novel receptors and targets are still being discovered [40]. However, in the present study, within one species, it is unlikely that selectivity and specificity of the receptor blockers are altered by the presence of MI.

Another potential limitation of our study is that we used vessels from Sham-operated swine in the functional studies on the role of purinergic receptors in the response to Up_4A, whereas we used SH-Controls for receptor expression. However, since the response to Up_4A is similar between Sham and SH-Controls, receptor expression is likely to be similar as well.

Conclusions

The present study demonstrates that Up_4A-induced coronary vasodilation is reduced after MI. Although the A_{2A}-, $P2X_1$-, $P2Y_1$-receptors were all shown to be involved in Up_4A-induced vasodilation in coronary small arteries from both Sham-operated and MI swine, none of these receptors contributed to the blunted coronary vasodilation in MI. The observation that the reduction in the Up_4A-response by 8PT was less in vessels from MI as compared to Sham-operated swine suggests that P1- (adenosine) receptor subtypes other than A_{2A} may account for this blunted Up_4A-induced coronary vasodilation in MI.

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Chapter 9

Uridine adenosine tetraphosphate-induced coronary vasodilation is blunted in swine with pressure-overload: a role for vasoconstrictor prostanoids?

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Abstract

The potent vasoconstrictor influence of uridine adenosine tetraphosphate (Up_4A) observed in various vascular beds and the elevated plasma level of Up_4A detected in hypertensive patients suggest a role for Up_4A in the development of hypertension. Since prolonged high blood pressure results in maladaptive cardiac hypertrophy that predisposes to myocardial dysfunction and abnormalities in coronary perfusion, we investigated the effects of Up_4A on coronary vascular tone in swine with prolonged pressure overload of the left ventricle (LV) by aortic banding (AoB). After 8-week of LV pressure-overload, LV weight to body weight ratio and coronary wall-to-lumen ratio were increased, indicating myocardial and coronary medial hypertrophy. Up_4A failed to produce vasoconstriction in isolated coronary small arteries from Sham and AoB swine, but produced a potent vasodilation in preconstricted coronary vessels, which was significantly blunted in AoB compared to Sham swine. Blockade of purinergic P1, $P2X_1$, and $P2Y_1$ receptors attenuated Up_4A-induced vasodilation in Sham and AoB to a similar degree, which was also paralleled with a similar mRNA expression of A_{2A}, $P2X_1$ and $P2Y_1$ receptors in Sham and AoB. However, non-selective blockade of P2 receptors had no effect in Sham but attenuated Up_4A-induced vasodilation in AoB swine. The contribution of nitric oxide (NO) to Up_4A-induced vasodilation was similar in Sham and AoB, which additional blockade of cyclooxygenase (COX) enhanced Up_4A-induced vasodilation in AoB but not in Sham swine. In conclusion, Up_4A-induced coronary vasodilation is blunted in AoB, which appears to be due to the production of a vasoconstrictor prostanoid.

Introduction

Hypertension and aortic stenosis result in chronic pressure-overload of the left ventricle and are major risk factors for the development of heart failure [1]. Initially, pressure overload induces an increase cardiac contractility to sustain cardiac output [2]. However, prolonged pressure overload results in concentric myocardial remodeling, i.e. an increased cardiac myocyte size and thickening of the ventricular wall, which impairs ventricular relaxation and left ventricular filling [3]. This so-called diastolic dysfunction is an early step towards the development of heart failure [4]. Prolonged pressure overload has also been shown to cause structural and functional adaptations in the coronary vasculature, as evidenced by a decrease in coronary flow reserve and an increase in minimal coronary resistance [5-7]. The increase in minimal coronary resistance is attributed to increased extravascular compression of the arterioles, a decrease in capillary density and vascular remodeling [6, 8-11], while arteriolar density has been shown to be maintained [12, 13]. Functional changes in the coronary vasculature mainly result from an increase in oxidative stress and endothelial dysfunction [14-16] resulting in a disbalance between endothelium-derived vasodilators such as nitric oxide and prostacyclin and endothelium-derived vasoconstrictors like endothelin and reactive oxygen species [17].

A novel endothelium-derived vasoactive factor, uridine adenosine tetraphosphate (Up_4A), has recently been identified. Up_4A contains both purine and pyrimidine moieties and, like other extracellular nucleotides, exerts its vasoactive influence by binding to purinergic receptors [18, 19], a class of receptors comprising P1 (adenosine receptors) and P2 receptors, that can be further subdivided into P2X and P2Y subtypes [20]. A role for Up_4A in the pathogenesis of hypertension has been suggested by the observation that Up_4A-induced vasoconstriction in the renal, femoral and basilar artery is potentiated in hypertension [21, 22], while the Up_4A plasma concentration in hypertensive subjects is significantly higher as compared to that in healthy subjects and correlates with blood pressure [23].

The vasoactive effect of Up_4A is dependent on the vascular bed studied. Recent observations from our laboratory demonstrated that Up_4A produces potent vasodilation rather than vasoconstriction in the healthy porcine coronary vasculature [19]. This vasodilation is attenuated in the remote coronary vasculature after myocardial infarction [24], indicating that the role of Up_4A in the coronary vasculature changes in cardiovascular disease. Since endothelial dysfunction has been shown to be present in pressure-overload [25-27], the present study was carried out in order to characterize whether prolonged pressure overload alters the response to Up_4A in isolated coronary small arteries from swine and to determine the receptors and signaling pathway(s) mediating this altered vascular response to Up_4A.

Methods

Animals

Studies were performed in accordance with the "Guiding Principles in the Care and Use of Laboratory Animals" as approved by the Council of the American Physiological Society, and with approval of the Animal Care Committee at Erasmus Medical Center Rotterdam. Fifteen Crossbred Yorkshire × Landrace swine (2-3-month-old, 18.5±0.3 kg at the time of surgery) of either sex entered the study. After one week of daily adaptation to laboratory conditions, animals underwent either aortic banding (AoB; n=7) or a sham procedure (Sham; n=8).

Surgery

Swine were sedated with an intramuscular injection of Zoletil (Tiletamine/Zolazepam; 5 mg/kg), Xylazine (2.25 mg/kg) and Atropine (1 mg), and a small catheter was placed in an ear vein for subsequent administration of anesthesia and fluid. Swine were intubated and ventilated with a mixture of oxygen and nitrogen (1:2 vol/vol), to which 2% (vol/vol) isoflurane was added to maintain anesthesia [28, 29]. Under sterile conditions, the chest was opened via the fourth left intercostal space and fluid-filled polyvinylchloride catheters were inserted into the left ventricle (LV) and aortic arch (Ao) for the measurement of the pressure and blood sampling for the determination of PO_2, PCO_2, pH, O_2 saturation and hemoglobin concentration. The ascending aorta was exposed in all swine, a sterile plastic band was placed around it in AoB animals and gradually tightened until the systolic pressure gradient between distal Ao and proximal LV catheters reached approximately 60 mmHg. Then, the chest was closed and animals were allowed to recover, receiving analgesia (0.3 mg buprenorphine im) for 2 days and antibiotic prophylaxis (25 mg kg^{-1} amoxicillin and 5 mg kg^{-1} gentamycin iv) for 5 days. Pressure in the proximal and distal catheters were recorded at the time of surgery, as well as 1 week and 3 weeks after surgery, and the systolic pressure gradient was calculated. Due to malfunctioning of the catheters in a number of animals, pressures could not be obtained 8 weeks after initial surgery.

Eight weeks after initial Sham and AoB surgery, animals were re-anesthetized, intubated and ventilated as described above. Pentobarbital (20 mg/kg/h) was infused to induce and maintain anesthesia. A catheter was introduced via the femoral artery into the descending aorta for measurement of mean arterial pressure. A Swan Ganz catheter was inserted via the jugular vein and advanced into the pulmonary artery for measurement of pulmonary arterial pressure and cardiac output (via thermodilution) [30, 31]. Following thoracotomy, hearts were arrested and immediately excised and placed in cold, oxygenated Krebs bicarbonate buffer solution.

Table 1. Primer information.

Genes	Sequence		Size
	Sense	Antisense	
A_{2A}	5'-ATGTTGGGCTGGAATAGCTG-3'	5'-CACGGAGTTGGTGTGAGAGA-3'	426 bp
$P2X_1$	5'-TTGAACCCCATTTCTTCCTG-3'	5'-AGTGCACCACACATCTGCTC-3'	248 bp
$P2X_4$	5'-TGTCCCCAGGCTACAATTTC-3'	5'-GGCAGCTTTTTCTCCCTTCT-3'	373 bp
$P2X_7$	5'-CTTTTGCACCTTGAGCTTCC-3'	5'-TCCATGCTAAGGGATTCTGG-3'	152 bp
$P2Y_1$	5'-TTCCTGACTTGCATCAGTGC-3'	5'-CAGTGCCCGAGTAGAAGAGG-3'	157 bp
$P2Y_2$	5'-GTGGCCTACAGCTTGGTCAT-3'	5'-GCGTGCGGAAGGAGTAGTAG-3'	235 bp
$P2Y_4$	5'-GACTGCCGGTTTAATGAGGA-3'	5'-AGGAAAAGGACGCTGCAGTA-3'	302 bp
$P2Y_6$	5'-CTGCTCTTGCCACCTGTGTA-3'	5'-AGGTTGGCGTAGAACAGGAA-3'	251 bp
eNOS	5'-CTCTCCTGTTGGCCTGACCA-3'	5'-CCGGTTACTCAGACCCAAGG-3'	151 bp
GAPDH	5'-TCGGAGTGAACGGATTT-3'	5'-CCTGGAAGATGGTGATGG-3'	219 bp

eNOS: endothelial nitric oxide synthase.

Myograph studies

Coronary small arteries (diameter ~150 μm) were dissected out from the apex of 8 Sham-operated and 7 AoB swine and stored overnight in cold, oxygenated Krebs bicarbonate solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4. The next day, coronary small arteries were cut into segments of ~2 mm length and mounted in microvascular myographs (Danish Myo Technology) with separated 6 ml organ baths containing Krebs bicarbonate solution aerated with 95% O_2/5% CO_2 and maintained at 37°C [32]. Changes in contractile force were recorded with a Harvard isometric transducer. Following a 30 min stabilization period, the internal diameter was set to a tension equivalent to 0.9 times the estimated diameter at 100 mmHg effective transmural

pressure [32]. The normalized vessels were exposed to 30 mM KCl twice. Endothelial integrity was verified by observing dilation to 10 nM substance P after preconstriction with 100 nM of the stable thromboxane A_2 analogue 9,11-Dideoxy-11α,9α epoxymethanoprostaglandin $F_{2\alpha}$ (U46619). Then vessels were subjected to 100 mM KCl to determine maximal vascular contraction. Thereafter, vessels were allowed to equilibrate in fresh organ bath fluid for 30 min before initiating different experimental protocols [19, 32]. Only one protocol was executed per vessel and, within one protocol, all vessels were obtained from different animals.

Experimental protocols

Coronary small arteries from both Sham and AoB swine were subjected to Up_4A in incremental concentrations ranging from 10^{-9} to 10^{-5} M in the absence and presence of preconstriction with U46619 [19]. To assess the involvement of different purinergic receptors in the vasodilator response to Up_4A (10^{-9}-10^{-5} M), coronary small arteries from Sham and AoB swine were pre-incubated with non-selective P1 receptor antagonist 8-phenyltheophylline (8PT, 10^{-5} M), non-selective P2 receptor antagonist pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS, 10^{-5} M), $P2X_1$ receptor antagonist MRS2159 (3×10^{-5} M), and $P2Y_1$ receptor antagonist MRS2179 (10^{-6} M) followed by preconstriction with U46619 (100 nM) [19]. To investigate if the role of endothelium-derived factors (NO and prostanoids) in the vasodilator response to Up_4A was altered after AoB, vessels from both Sham and AoB were exposed to Up_4A (10^{-9}-10^{-5} M) in the absence and presence of nitric oxide synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methyl ester HCl (LNAME, 10^{-4} M) alone or in combination with cyclooxygenase (COX) inhibitor indomethacin (10^{-5} M) [19]. Preconstricted coronary small arteries from both Sham and AoB were also exposed to Up_4A after pre-incubation with LNAME (10^{-4} M) and indomethacin (10^{-5} M) in the absence and presence of cytochrome P450 (CYP) 2C9 inhibitor sulfaphenazole (10^{-5} M) [19].

Table 2. Anatomic and hemodynamic variables.

		Sham	AoB
		n=8	n=7
MAP (mmHg)	Surgery	84 ± 6	56 ± 3*
	Week 1	92 ± 2	83 ± 2*
	Week 3	88 ± 2	90 ± 3
LVSP (mmHg)	Surgery	--- - ---	155 ± 5
	Week 1	--- - ---	176 ± 8
	Week 3	--- - ---	188 ± 14
SAP (mmHg)	Surgery	99 ± 6	67 ± 4*
	Week 1	112 ± 2	100 ± 2*
	Week 3	105 ± 2	104 ± 3
Pressure gradient	Surgery	--- - ---	88 ± 2
	Week 1	--- - ---	82 ± 8
	Week 3	--- - ---	98 ± 6
At sacrifice			
BW (kg)		45 ± 0.9	42 ± 1.1
LVW/BW (g/kg)		2.3 ± 0.1	3.6 ± 0.1*
HR (beats/min)		109 ± 3	108 ± 4
MAP (mmHg)		98 ± 5	104 ± 3
CO (l/min)		4.0 ± 0.2	4.2 ± 0.1
PAP (mmHg)		19 ± 1	23 ± 1*

*AoB: Aorta banding; MAP: Mean arterial pressure measured in the descending aorta; LVSP: Left ventricular systolic pressure measured proximal to the band; SAP: Systolic artery pressure measured in the descending aorta; BW: Body weight at sacrifice; LVW: Left ventricular weight at sacrifice; HR: Heart rate; CO: Cardiac output; PAP: Pulmonary arterial pressure. Those values were obtained at sacrifice. Values are mean±SEM; * P<0.05 vs. Sham.*

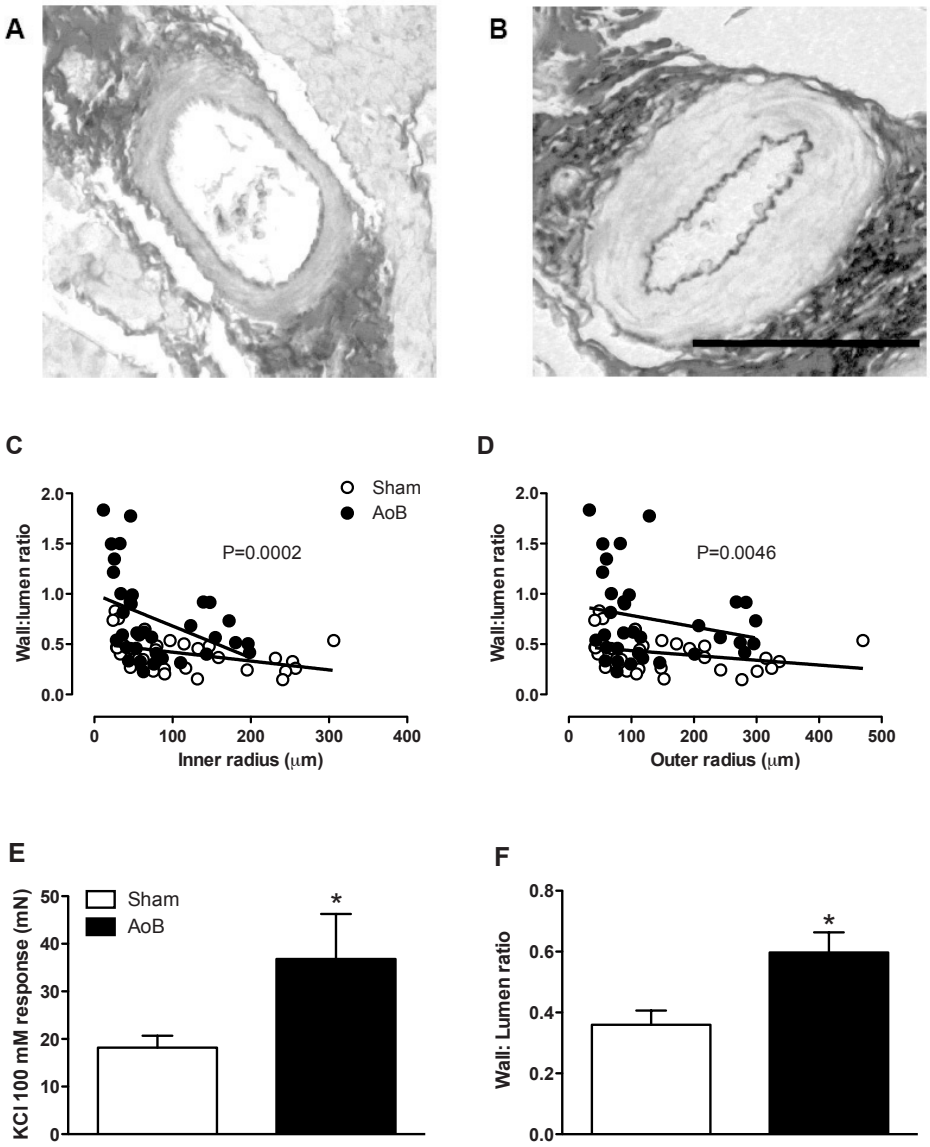

Fig. 1. *Typical examples of a coronary small artery within the myocardium of Sham-operated swine (panel A), swine with aorta banding (AoB) (panel B). The scale bar denotes 100 μm. The wall to lumen ratio both as a function of inner (panel C) and outer (panel D) was significantly increased in myocardium from swine with AoB. Shown are 34 vessels from 4 Sham-operated swine and 36 vessels from 5 AoB swine. The vasoconstrictor response to 100 mM KCl was significantly increased in vessels from AoB (n=6) as compared to Sham vessel (n=8), panel E. The wall to lumen ratio in vessels with diameters ranging from 100 μm to 200 μm was increased in AoB as compared to Sham (panel F). * P<0.05, AoB vs. Sham.*

Histology

Fresh sections of anterior wall of the left ventricle were fixed by 4% buffered formaldehyde and paraffin-embedded for histological analysis of remodeling of coronary small arteries. Sections were stained with resorcin-fuchsin, photographed and inner and outer area of the coronary small arteries was assessed by planimetry. Only transversely cut vessels with an inner radius below 200 μm were analyzed. Assuming circularity of the vessels, inner and outer radius were calculated as r= √(area/ π). Wall to lumen ratio was calculated as (outer-inner diameter)/ inner diameter.

Quantitative real-time PCR analysis

Following dissection, coronary small arteries (diameter ~150 μm) were snap-frozen in liquid nitrogen to be used for detection of purinergic receptor subtypes A_{2A}, $P2X_1$, $P2X_4$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$. mRNA. In addition, the expression of endothelial NOS (eNOS) was measured [33]. Total RNA was extracted from 5-7 frozen samples per group using a Qiagen RNA kit. cDNA was synthesized from 100 ng of total RNA with iScript Reverse Transcriptase (Bio-Rad). Quantitative real-time PCR (MyIQ, Bio-Rad) was performed with SYBR Green (Bio-Rad) [19]. Target gene mRNA levels were expressed relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control [34]. The primer sequences are shown in Table 1.

Data analysis and statistics

Hemodynamic data were averaged over a time period of at least 10 seconds. Vascular contractions were normalized to the response to 100 mM KCl, while vascular relaxation to Up_4A was expressed as percentage of contraction to U46619 [19]. Statistical comparison of hemodynamic data, purinergic receptor expression, vascular response to KCl and comparison of wall to lumen ratio of vessels <200 μm between Sham and AoB swine were performed using unpaired t-tests. The effect of AoB on wall to lumen ratio was also analyzed with ANCOVA, using inner or outer radius as covariant. The effects of AoB as well as drug treatment on the Up_4A response were assessed using two-way ANOVA for repeated measures. Statistical significance was accepted when $P \leq 0.05$ (two-tailed). Data are presented as means±SEM.

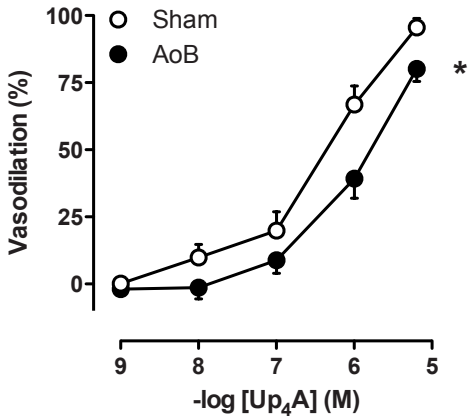

Fig. 2. *The vasodilator response to Up_4A was reduced in coronary small arteries from swine with AoB (n=7), as compared to Sham-operated swine (n=8). * P<0.05, AoB vs. Sham.*

Results

Characteristics of AoB animals

AoB of the ascending aorta resulted in a systolic pressure gradient of 88±2 mmHg as measured during surgery (Table 2). The systolic pressure gradient was stable over the time course of the experiment, as evidenced by a systolic pressure gradient of 82±8 and 98±6 mmHg at week 1 and week 3 after AoB (Table 2). Mean arterial pressure distal to the band was lower in AoB as compared to Sham during surgery and 1 week after surgery, but was similar to mean arterial pressure in Sham-operated swine 3 weeks after surgery. At sacrifice, the heart rate, mean arterial pressure, and cardiac output were comparable between Sham and AoB swine, while the pulmonary arterial pressure was slightly increased in AoB (Table 2). Eight weeks of AoB resulted in left ventricular hypertrophy as evidenced by a 43% increase in left ventricle weight to body weight ratio (Table 2). The wall to lumen ratio of coronary small arteries was also increased (Fig. 1A&B for typical examples, Fig 1C&D for all results, Fig 1F for vessels with diameter ranging from 100 to 200 μm) and the vascular response to 100 mM KCl in the coronary small arteries from AoB was significantly greater as compared to that from Sham (Fig. 1E). These observations indicate that during 8 weeks of AoB, swine develop left ventricular hypertrophy and as well as coronary vascular medial hypertrophy.

Up_4A-induced coronary vasodilation is blunted in AoB

Cumulative concentrations of Up_4A (10^{-9}-10^{-5} M) failed to induce vasoconstriction in coronary small arteries from either Sham or AoB swine (data not shown). In contrast, in vessels preconstricted with U46619, Up_4A produced dose-dependent vasodilation, but the vasodilation was significantly less in vessels form AoB as compared to Sham (Fig. 2).

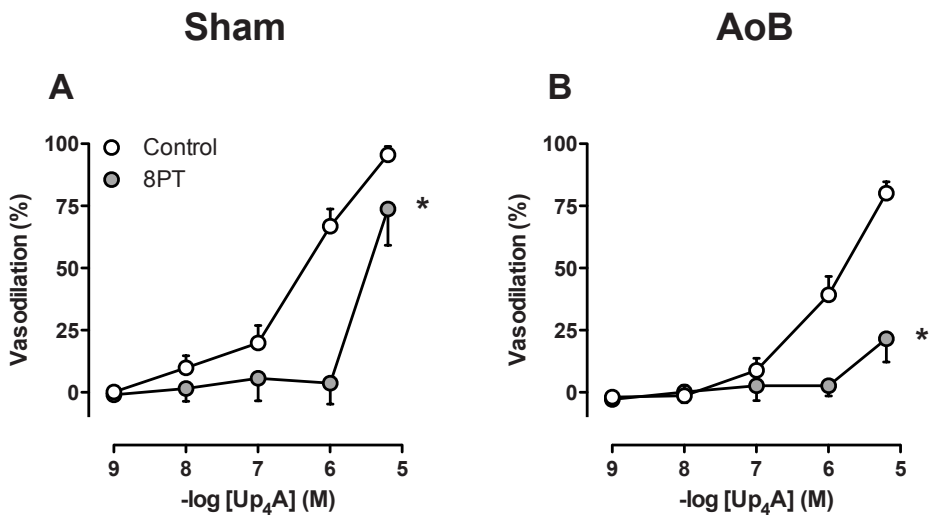

Fig. 3. *P1 receptor blockade with 8PT attenuated vasodilation to Up_4A to the same extent in coronary small arteries from Sham-operated swine (panel A, n=6) and from swine with AoB (panel B, n=6). * P<0.05 effect of 8PT vs. corresponding control.*

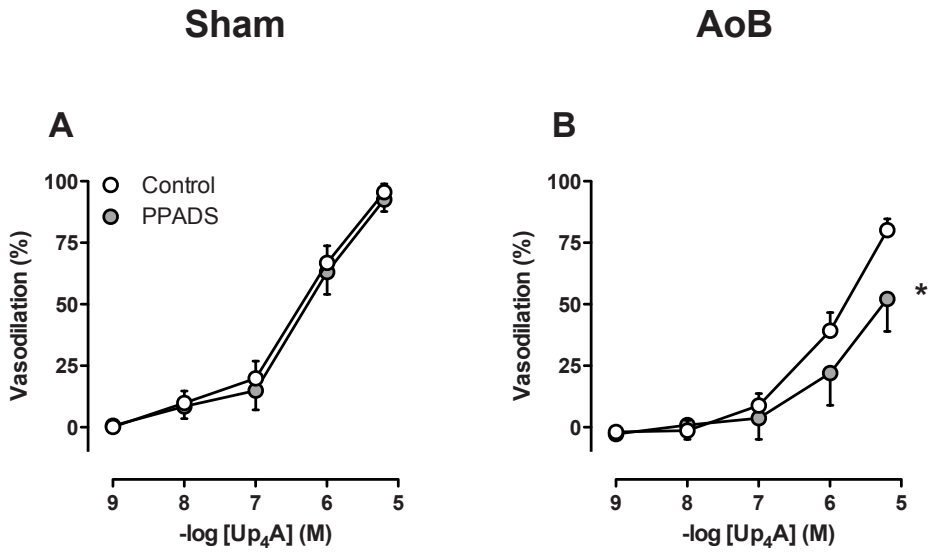

Fig. 4. *P2 receptor blockade with PPADS had no effect on vasodilation to Up_4A in coronary small arteries from Sham-operated swine (panel A, n=8) but attenuated vasodilation in coronary small arteries from swine with AoB (panel B, n=7). * P<0.05 effect of PPADS vs. corresponding control.*

Involvement of purinergic receptor subtypes

In accordance with the unaltered expression of the P1 receptor (Fig. 6), its blockade with the P1 receptor inhibitor 8PT attenuated Up_4A-induced vasodilation to the same extent in coronary small arteries from Sham (Fig. 3A) and AoB (Fig. 3B). Non-selective P2 receptor blockade with PPADS had no effect on Up_4A-induced vasodilation in coronary small arteries from Sham (Fig. 4A), but did attenuate Up_4A-induced vasodilation in vessels from AoB (Fig. 4B), suggesting alterations in the balance of vasodilator and vasoconstrictor P2 receptors. Selective $P2X_1$ and $P2Y_1$ receptor blockade, with MRS2159 and MRS2179 respectively, attenuated Up_4A-induced vasodilation in coronary small arteries from Sham swine (Fig. 5A, C). However, a similar attenuation was noted in vessels from AoB swine (Fig. 5B, D). A_{2A}, $P2X_1$, $P2X_7$, $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors were expressed, but no difference in expression in coronary small arteries between Sham and AoB swine were found. Expression of $P2X_4$ and $P2Y_4$ could not be detected (Fig. 6).

Involvement of endothelial factors in the response to Up_4A

To investigate the contribution of alterations in endothelial function to the attenuated response to Up_4A after AoB, we compared the effect of eNOS and COX inhibition on Up_4A-induced vasodilation. eNOS inhibition with LNAME attenuated Up_4A-induced vasodilation to the same extent in coronary small arteries from Sham and AoB swine (Fig. 7A, B), which was corroborated by similar eNOS expression level in vessels from AoB (0.16±0.04) and Sham (0.15±0.04). Hence, the attenuated vasodilation in AoB cannot be attributed to loss of NO.

In Sham-operated swine, combined inhibition of eNOS and COX (LNAME + indomethacin) attenuated Up_4A-induced vasodilation to the same extent as LNAME alone (Fig. 7C). In contrast, combined blockade of eNOS and COX in vessels from AoB swine significantly enhanced Up_4A-induced vasodilation compared to LNAME alone (Fig. 7D). These data suggest that

vasoconstrictor prostanoid(s) are produced in response to Up_4A in vessels of AoB, that attenuate vasodilation by Up_4A.

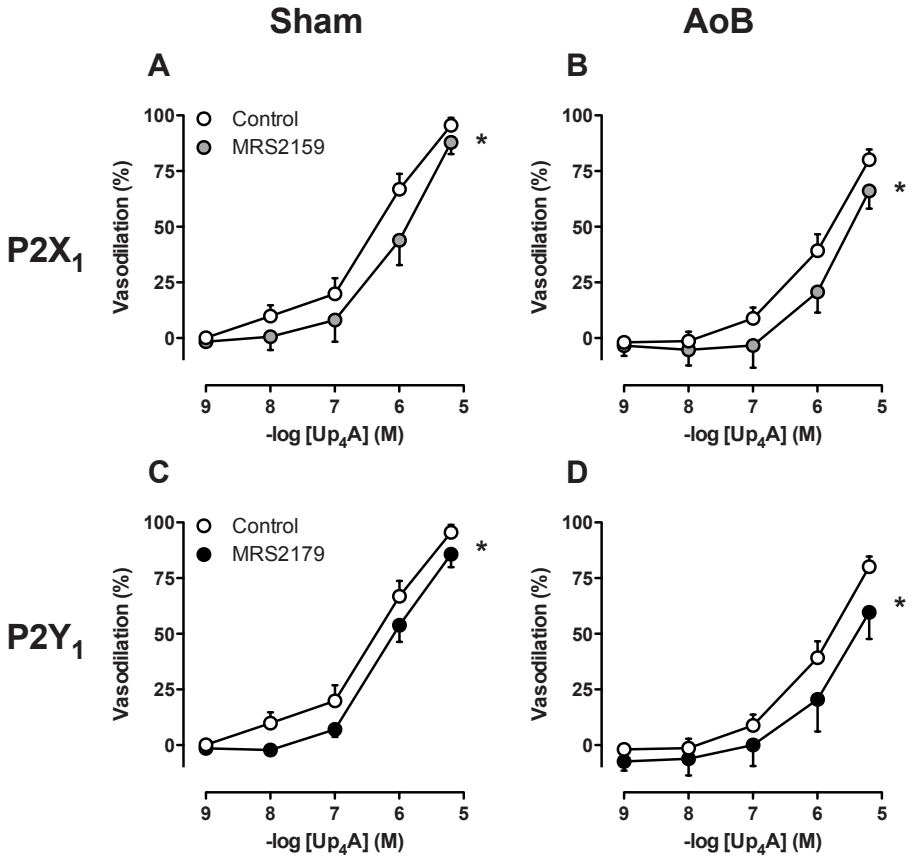

Fig. 5. *Both $P2X_1$ receptor blockade with MRS2159 and $P2Y_1$ receptor blockade with MRS2179 attenuated vasodilation to Up_4A to the same extent in coronary small arteries from Sham-operated swine (panel A and C, n=8 for MRS2159; n=7 for MRS2179) and from swine with AoB (panel B and D, n=7 for MRS2159 or MRS2179). * P<0.05 effect of MRS2159 or MRS2179 vs. corresponding control.*

As one of the sources of reactive oxygen species [36], cytochrome P450 2C9 (CYP 2C9) has been reported to produce vasoconstrictor metabolites in the coronary vasculature [35, 36]. To investigate whether the CYP 2C9 metabolites contribute to the blunted vasodilation in response to Up_4A in AoB swine, we tested the effect of CYP 2C9 inhibition in response to Up_4A with and without eNOS/COX blockade. In neither Sham nor AoB swine, CYP 2C9 inhibition with sulfaphenazole affected Up_4A-induced coronary vasodilation either in the absence or presence of eNOS/COX blockade (LNAME + indomethacin; data not shown), which is consistent with our recent study in which inhibition of CYP 2C9 alone had no effect on Up_4A-induced coronary vasodilation [19].

Discussion

The main findings of the present study were that (i) AoB resulted in medial hypertrophy of coronary small arteries as evidenced by an increase in wall to lumen ratio and by an increased KCl-induced contractile force. (ii) Up_4A-induced vasodilation was reduced in coronary small arteries from AoB as compared to Sham. (iii) Blockade of P1 receptors attenuated Up_4A-induced vasodilation to the same extent in Sham and AoB; (iv) while P2 receptor blockade with PPADS attenuated Up_4A-induced vasodilation in AoB but not Sham. (v) The response to selective $P2X_1$ and $P2Y_1$ blockade was similar in coronary small arteries from AoB and Sham. (vi) eNOS inhibition attenuated Up_4A-induced vasodilation to the same extent in Sham and AoB, whereas (vii) additional COX inhibition had no effect in Sham, but enhanced Up_4A-induced vasodilation in AoB. (viii) The expression of purinergic receptor subtypes involved in vascular tone regulation was not altered. The implications of these findings will be discussed below.

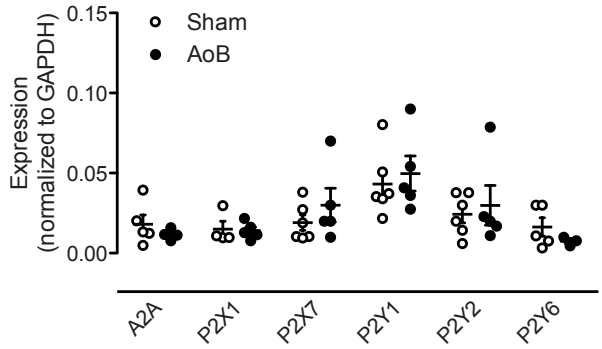

Fig. 6. *mRNA expression of various P receptor subtypes. mRNA expression was normalized to expression of GAPDH.*

Consistent with previous reports [25, 37], eight weeks of AoB resulted in left ventricular hypertrophy and coronary microvascular remodeling, as evidenced by a doubling of wall to lumen ratio of the coronary small arteries as well as a doubling of the contractile response to KCl. The vasodilator response to Up_4A was blunted in coronary small arteries from swine with AoB. Up_4A exerts its vasoactive effect through interaction with purinergic receptors. In accordance with previous studies from our laboratory [19], coronary vasodilation induced by Up_4A was mediated primarily by the P1 receptors. Moreover, neither the contribution of the P1 receptors nor the expression of the A_{2A} receptor, that is the most important vascular P1 receptor, was altered after AoB, indicating that, in contrast to our findings in the coronary vasculature after MI [24], the reduced response to Up_4A was not due to loss of P1 receptors.

The assessment of the contribution of the P2 receptors to the response to Up_4A is more difficult due to lack of selective antagonists of many of the P2 receptor subtypes. In general, activation of P2 receptors on endothelial cells is thought to result in vasodilation, whereas activation of P2 receptors on vascular smooth muscle cells results in vasoconstriction [19, 38]. The non-selective P2 antagonist PPADS attenuated Up_4A-induced vasodilation in coronary small arteries from swine with AoB, but not from Sham-operated swine, indicating that the contribution of P2 receptors blocked by PPADS is altered. PPADS has been shown to block $P2X_2$, $P2X_3$, $P2X_5$ [39], $P2X_7$ [40], $P2Y_1$ [41], $P2Y_2$, $P2Y_4$ [42] and $P2Y_6$ [43] receptors. In contrast to the altered effect of PPADS, selective blockade of $P2X_1$ as well as $P2Y_1$ receptors attenuated the vasodilator

response to Up_4A to the same extent in coronary small arteries from AoB and Sham-operated swine, which is in accordance with the unaltered expression of $P2X_1$ and $P2Y_1$ receptors. The observation that, in vessels from Sham-operated swine, $P2Y_1$ blockade attenuated the response to Up_4A, whereas PPADS, that has also been shown to block the $P2Y_1$ receptor, does not, suggests that PPADS also blocks a P2 vasoconstrictor receptor. Since the responses to $P2Y_1$ blockade and to PPADS are identical in vessels from AoB swine, activation of this unidentified P2 constrictor receptor seems to be reduced in AoB. The exact identity of this receptor remains to be elucidated.

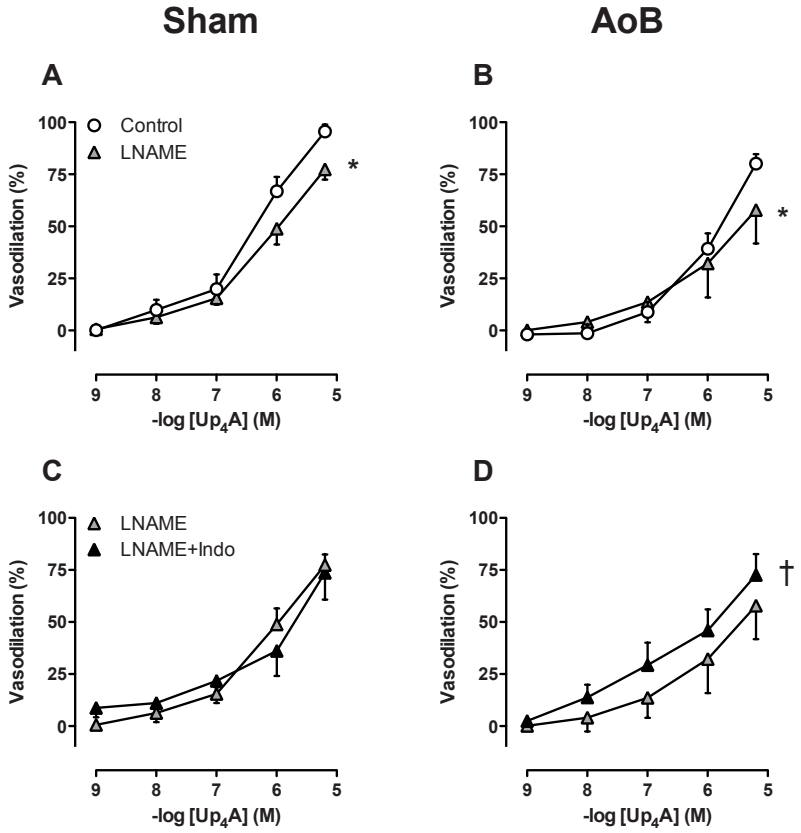

Fig. 7. *eNOS inhibition with LNAME attenuated the response to Up_4A to a similar extent in coronary small arteries from Sham-operated swine (panel A, n=8) and swine with AoB (Panel B, n=7). Subsequent inhibition of cyclooxygenase with indomethacin had no effect in vessels from Sham (panel C, n=7), but enhanced dilation to Up_4A in vessels from swine with AoB (panel D, n=6). * P<0.05 effect of LNAME vs. corresponding control; † P<0.05 effect of LNAME + indomethacin vs. LNAME alone.*

Since several studies have shown endothelial dysfunction in the porcine coronary vasculature following AoB [25, 27, 37, 44], we investigated whether the contribution of endothelial vasodilator pathways to Up_4A-induced vasodilation was altered. The blunted response to Up_4A was not due to a decreased contribution of nitric oxide, as both the effect of eNOS-inhibition with LNAME and eNOS expression were similar in coronary small arteries from Sham-operated swine and swine with AoB. The unaltered expression of eNOS is consistent with another study in

isolated coronary arteries from swine with AoB [27]. Also, the contribution of eNOS to bradykinin-induced vasodilation was maintained [37], despite the presence of eNOS uncoupling [27].

COX inhibition with indomethacin potentiated the vasodilator response to Up_4A in vessels from AoB, but not Sham-operated animals, suggesting that the reduced responsiveness to Up_4A was, at least in part, due to production of vasoconstrictor prostanoids. A shift in the balance from vasodilator prostanoids to vasoconstrictor prostanoids has been implicated in the pathogenesis of cardiovascular disease [45]. Thus, concentrations of vasoconstrictor prostanoids have been shown to be increased in the myocardium of hypercholesterolemic swine and selective COX2 inhibition reduced thromboxane production while leaving prostacyclin production unperturbed in hypercholesterolemic swine [46], suggesting that increased activity of COX2 may be involved in the production of vasoconstrictor prostanoids. This suggestion is supported by recent findings in the renal vasculature of diabetic rats showing that enhanced vasoconstriction in response to Up_4A is mediated through activation of COX2 and production of thromboxane [47]. The purinergic receptor responsible for the increased production of vasoconstrictor prostanoids remains to be identified. However, an increased production of vasoconstrictor prostanoids in response to agonist-induced receptor activation in AoB is not unique to Up_4A. Thus, vasodilation induced by bradykinin and serotonin was enhanced by COX inhibition, while endothelin-induced vasoconstriction was reduced [25]. Since both the B2- and 5HT1D-receptors, which were linked to the increased vasoconstrictor prostanoid production in a previous study [25], are G-protein coupled receptors, it is likely that the purinergic receptor involved also is a G-coupled receptor, *i.e.* a P2Y rather than a P2X receptor. $P2Y_2$ or $P2Y_6$ receptors have been shown to be present on vascular smooth muscle cells in large [42] and small porcine coronary arteries [19] as well as in human coronary vasculature [48] and mediate UTP- and UDP-induced vasoconstriction in the large coronary arteries [42]. However, to our knowledge, there is no evidence linking these receptors to prostanoid production. Future studies are required to further explore the role of various P2Y receptors, including the $P2Y_2$ and $P2Y_6$ receptors, in the production of vasoconstrictor prostanoid(s) in the coronary circulation of AoB swine.

Conclusions and perspectives

A role for Up_4A in the pathogenesis of hypertension has been suggested by the observation that Up_4A-induced vasoconstriction in the renal, femoral and basilar artery is potentiated in hypertension [21, 22], while the Up_4A plasma concentration in hypertensive subjects is significantly higher as compared to that in healthy subjects and correlates with blood pressure [23]. The present study demonstrates that pressure-overload also modulates the response of the coronary microvasculature to Up_4A. Thus, the Up_4A-induced vasodilation in the coronary microcirculation was significantly blunted in AoB, which likely resulted from altered P2 receptor activation and subsequent production of vasoconstrictor prostanoid(s). The exact nature of these vasoconstrictor prostanoids as well as P2 receptor subtypes involved in their production remains to be determined.

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Chapter 10

Altered purinergic signaling in uridine adenosine tetraphosphate-induced coronary vasodilation in swine with diabetes mellitus type 2

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Abstract

Uridine adenosine tetraphosphate (Up_4A) is a novel endothelium-derived vasoactive factor that produces vasodilation via purinergic receptors in the healthy porcine coronary microcirculation, which is partly endothelium-dependent. Given the deleterious effect of diabetes on endothelial function, we investigated the effects of diabetes on the coronary vasodilator response to Up_4A, as well as the role therein of purinergic receptor subtypes and endothelium-derived vasoactive factors. Coronary small arteries (~150 μm), dissected from the apex of normal healthy swine and swine 6 months after induction of type 2 diabetes with streptozotozin (50 mg/kg iv on three consecutive days) and fed a high fat diet, were mounted on wire myographs for functional studies of Up_4A (10^{-9}-10^{-5} M). Although Up_4A-induced vasodilation was identical in normal and diabetic swine, involvement of purinergic receptors and endothelial factors in the response was different. In diabetes, $P2X_7$ and NO-mediated vasodilator influences were blunted, while a thromboxane-mediated vasoconstrictor influence was unmasked. Conversely, an increased vasodilator influence via $P2Y_1$ and $P2Y_6$ receptors was observed, while CYP 2C9 switched from producing vasoconstrictor to vasodilator metabolites in diabetes. In conclusion, the coronary vasodilator response to Up_4A was maintained in diabetic swine, but involvement of purinergic receptor subtypes and endothelial factors was altered.

Introduction

Diabetes mellitus is the most common endocrine disorder in the world and constitutes an important risk factor for development of cardiovascular disease including coronary artery disease and diabetic heart disease. The latter is not only the consequence of proximal coronary artery disease but also of coronary microvascular disease [1]. Endothelial dysfunction is an important determinant of altered vascular reactivity and plays a major role in the etiology of diabetes-induced macrovascular and microvascular complications [2]. This endothelial dysfunction encompasses an imbalance between the secretion of endothelium-derived relaxing factors (such as NO and prostacyclin) and endothelium-derived constricting factors (such as endothelin and thromboxane) [3, 4].

Less than a decade ago, a novel endothelium-derived vasoactive factor, the dinucleotide uridine adenosine tetraphosphate (Up_4A), was identified [5]. Contrary to the vasoconstrictor effect of Up_4A in most vascular beds in rodents [5-9], Up_4A acts as a vasodilator in the coronary microcirculation in swine [10]. We have recently shown that the vasodilator response to Up_4A is reduced in the remote coronary vasculature after myocardial infarction (MI) [11], while in another study Up_4A-induced vasoconstriction was reported to be enhanced in renal arteries from rats with type 2 diabetes [12]. These findings suggest that Up_4A signaling may be altered in coronary artery disease and diabetes.

Like other extracellular nucleotides, Up_4A exerts its vasoactive influence by binding to purinergic receptors [10, 13]. The purinergic receptor family consists of P1 (adenosine receptors) and P2 receptor subtypes that can be further divided into P2X and P2Y receptors [14]. There is some evidence from rodent models and humans that vascular purinergic signaling is altered in diabetes [15]. Thus, the vasoconstrictor response to ATP, a putative P2 receptor agonist [16], was increased, while following preconstriction, the vasodilator response to ATP was decreased in mesenteric arteries from rats with diabetes [17, 18]. Similarly, the vasodilation to ATP, UTP and adenosine was impaired in femoral arteries of patients with type 2 diabetes [19].

Since there are no studies on the effects of Up_4A in large animal models of type 2 diabetes, the aim of the present study was to investigate the effects of type 2 diabetes in swine on the coronary microvascular vasodilator response to Up_4A, and the role therein of purinergic receptor subtypes and endothelium-derived vasoactive factors.

Methods

Animals

Studies were performed in accordance with the "Guiding Principles in the Care and Use of Laboratory Animals" as approved by the Animal Care Committee at Erasmus Medical Center Rotterdam. Seven female swine (2-3 month old, 25.1±1.2 kg) were used for the induction of diabetes. After one week of daily adaptation to laboratory conditions, diabetes was induced with intravenous injections of streptozotocin (50 mg/kg/day) over three days. This dose of streptozotocin resulted in a stable diabetes, as evidenced by glucose levels of 19.5±1.4 mmol/l (week 1), 21.6±2.9 mmol/l (week 3), 18.8±2.0 mmol/l (week 18) and 18.7±2.1 mmol/l (week 20). One to two weeks after diabetes induction, a saturated fat diet (25% of saturated fats and 1% of cholesterol) was gradually introduced [2]. Swine were housed in metabolic cages with unlimited access to food for 1 hour/meal, twice daily for the entire 6-month study duration. The diabetic status was continuously monitored by measurement of urine and blood levels of glucose and ketones. At sacrifice, diabetic swine weighed 104±8 kg. Another 5 healthy female Crossbred

Yorkshire × Landrace swine, purchased from the same breeder 2 weeks prior to sacrifice, and matched for age and weight of the diabetic swine (8-9 months, 119±5 kg) served as a control group.

Blood and tissue sampling

At sacrifice, the animals were sedated with an intramuscular injection of Zoletil (Tiletamine/Zolazepam; 5 mg/kg) and Xylazine (2.25 mg/kg), anesthetized with pentobarbital (20 mg/kg/h iv) and artificially ventilated. Catheters were inserted for arterial blood sampling [20]. The collected blood samples were stored for later determination of glucose and insulin levels. Following thoracotomy, hearts were arrested and immediately excised and placed in cold, oxygenated Krebs bicarbonate buffer solution.

Table 1. Primer information.

Genes	Sequence		Size
	Sense	Antisense	
A_{2A}	5'-ATGTTGGGCTGGAATAGCTG-3'	5'-CACGGAGTTGGTGTGAGAGA-3'	426 bp
$P2X_1$	5'-TTGAACCCCATTTCTTCCTG-3'	5'-AGTGCACCACACATCTGCTC-3'	248 bp
$P2X_4$	5'-TGTCCCCAGGCTACAATTTC-3'	5'-GGCAGCTTTTTCTCCCTTCT-3'	373 bp
$P2X_7$	5'-CTTTTGCACCTTGAGCTTCC-3'	5'-TCCATGCTAAGGGATTCTGG-3'	152 bp
$P2Y_1$	5'-TTCCTGACTTGCATCAGTGC-3'	5'-CAGTGCCCGAGTAGAAGAGG-3'	157 bp
$P2Y_2$	5'-GTGGCCTACAGCTTGGTCAT-3'	5'-GCGTGCGGAAGGAGTAGTAG-3'	235 bp
$P2Y_4$	5'-GACTGCCGGTTTAATGAGGA-3'	5'-AGGAAAAGGACGCTGCAGTA-3'	302 bp
$P2Y_6$	5'-CTGCTCTTGCCACCTGTGTA-3'	5'-AGGTTGGCGTAGAACAGGAA-3'	251 bp
eNOS	5'-CTCTCCTGTTGGCCTGACCA-3'	5'-CCGGTTACTCAGACCCAAGG-3'	151 bp
GAPDH	5'-TCGGAGTGAACGGATTTG-3'	5'-CCTGGAAGATGGTGATGG-3'	219 bp

eNOS: endothelial nitric oxide synthase.

Myograph studies

Left ventricular apices from diabetic swine (n=7) and normal (n=5) swine were collected at the time of sacrifice. Additional apices were collected from healthy swine hearts of either sex (n=5) obtained from a local slaughterhouse. Since the vascular response to Up_4A in the 5 normal animals did not differ from that in slaughterhouse animals (data not shown), data were pooled into a single control group (normal, n=10).

Coronary small arteries (diameter ~150 μm) were dissected out from the apex of 7 diabetic and 10 normal swine and stored overnight in cold, oxygenated Krebs bicarbonate solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4 [10, 21]. The next day, coronary arteries were cut into segments of ~2 mm length and mounted in microvascular myographs (Danish Myo Technology) with separate 6 ml organ baths containing Krebs bicarbonate solution aerated with 95% O_2/5% CO_2 and maintained at 37°C. Changes in contractile force were recorded with a Harvard isometric transducer. Following a 30 min stabilization period, the internal diameter was set to a tension equivalent to 0.9 times the estimated diameter at 100 mmHg effective transmural pressure [10, 21]. The normalized vessels were exposed to 30 mM KCl twice. Endothelial integrity was verified by observing dilation to 10 nM substance P after preconstriction with 100 nM of the stable thromboxane A_2 analogue 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619). Then vessels were subjected to 100 mM KCl to determine the maximal vascular contraction. Thereafter, vessels were allowed to equilibrate in fresh Krebs solution for 30 min before initiating different experimental protocols [10, 21]. Only one protocol was executed per vessel segment and, within one protocol, each vessel was obtained from a different animal.

Experimental protocols

The coronary small arteries from both normal and diabetic swine were subjected to increasing Up_4A concentrations (10^{-9}-10^{-5} M). Since no vasoconstrictor influence was observed in vessels from both normal and diabetic swine (data not shown), vessels were preconstricted with U46619 to study the vasodilator effect of Up_4A and the signaling pathways involved.

To investigate the involvement of purinergic receptors in Up_4A-induced vasodilation, preconstricted coronary small arteries from both normal and diabetic swine were exposed to increasing Up_4A concentrations (10^{-9}-10^{-5} M), in the absence and presence of the non-selective P1 receptor antagonist 8-phenyltheophylline (8PT, 10^{-5} M), the non-selective P2 receptor antagonist pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS, 10^{-5} M), and combined P1 and P2 blockade. To assess the role of specific purinergic receptor subtypes, vessels were incubated in the absence and presence of adenosine A_{2A} receptor antagonist SCH58261 (10^{-7} M), $P2X_1$ receptor antagonist MRS2159 (3×10^{-5} M), $P2X_7$ receptor antagonist A438079 (10^{-5} M) [22], $P2Y_1$ receptor antagonist MRS2179 (10^{-6} M), as well as $P2Y_6$ antagonist MRS2578 (10^{-5} M) [10].

A subset of coronary small arteries was de-endothelialized, preconstricted with U46619 and exposed to increasing Up_4A concentrations (10^{-9}-10^{-5} M); all other endothelium-intact coronary small arteries from both normal and diabetic swine were preconstricted with U46619 and exposed to increasing Up_4A concentrations in the absence and presence of the nitric oxide synthase (NOS) inhibitor LNAME (10^{-4} M), LNAME plus the cyclooxygenase inhibitor indomethacin (10^{-5} M) [10], or LNAME + indomethacin + the CYP 2C9 inhibitor sulfaphenazole (10^{-5} M). Finally, we studied the effect of thromboxane synthase inhibitor ozagrel (10^{-5} M) on the coronary vasodilator response to Up_4A.

Table 2. Plasma and lipid parameters of variables.

	Normal (n=5)	Diabetes (n=7)
Insulin (pg/ml)	8.79 ± 1.99	12.78 ± 5.42
Glucose (mmol/l)	7.16 ± 1.09	18.43 ± 0.98*
Cholesterol (mmol/l)	2.14 ± 0.27	17.66 ± 2.83*
HDL (mmol/l)	0.90 ± 0.09	3.20 ± 0.61*
LDL (mmol/l)	1.24 ± 0.25	15.58 ± 2.65*
Triglycerides (mmol/l)	0.19 ± 0.04	0.65 ± 0.16*

*HDL: High density lipoprotein; LDL: Low density lipoprotein. Values are mean±SEM; * P<0.05 vs. Normal.*

Quantitative real-time PCR analysis

Following dissection, endothelium-intact coronary small arteries were snap-frozen in liquid nitrogen to be used for detection of A_{2A}, $P2X_1$, $P2X_4$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors mRNA. In addition, the expression of endothelial NOS (eNOS) was measured [23]. Total RNA was extracted from 5-7 frozen samples per group using a Qiagen RNA kit. cDNA was synthesized from 100 ng of total RNA with iScript Reverse Transcriptase (Bio-Rad). Quantitative real-time PCR (MyIQ, Bio-Rad) was performed with SYBR Green (Bio-Rad) [10]. Target gene mRNA levels were expressed relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control [24]. Primer sequences are shown in Table 1.

Data analysis and statistics

Data are presented as mean±SEM. Statistical analysis of plasma lipid profile variables was performed using t-test (paired). Vascular relaxation responses to Up_4A were expressed as

percentage of contraction to U46619 [10]. The effects of drug treatment on Up_4A responses were analyzed using two-way ANOVA. Statistical significance was accepted when $P<0.05$ (two-tailed).

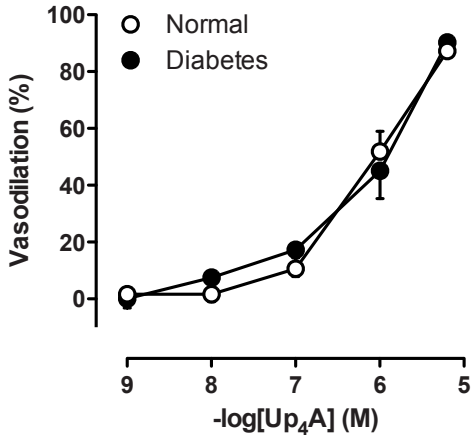

Fig. 1. *Vasodilator effects of Up_4A. Shown are concentration-responses to Up_4A (10^{-9}-10^{-5} M) in normal (n=10) and diabetic (n=7) coronary small arteries preconstricted with U46619 (100 nM). Values are mean±SEM. * P<0.05 vs. Normal.*

Results

Characteristics of diabetes

Six months after induction of diabetes with streptozotocin and start of the high fat diet, plasma levels of glucose, total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL) and triglycerides of diabetic swine were significantly elevated as compared to normal swine, while insulin levels were similar, consistent with type 2 diabetes (Table 2).

Vasoactive influence of Up_4A on coronary vasculature in swine with diabetes

Up_4A failed to elicit any vasoconstrictior response in coronary small arteries from both normal and diabetic swine (data not shown). In contrast, in coronary small arteries preconstricted with U46619, Up_4A produced concentration-dependent vasodilator responses of up to 100%, which were virtually identical in normal and diabetic swine (Fig. 1).

Effect of diabetes on expression and function of purinergic receptor subtypes

Non-selective P1 receptor blockade with 8PT and, to a lesser extent, non-selective P2 receptor blockade with PPADS, each attenuated the coronary vasodilator response to Up_4A (Fig. 2). These responses were however not different between normal and diabetic swine. In contrast, while the effect of combined administration of 8PT and PPADS was similar to the effect of 8PT alone in normal swine (Fig. 2A), the effect of 8PT and PPADS was significantly greater than the effect of 8PT alone in diabetic swine (Fig. 2B). Interestingly, in coronary vessels of diabetic swine incubated with 8PT and PPADS, Up_4A even produced a mild vasoconstrictor response (Fig. 2B). These findings suggest that the maintained coronary vasodilator response to Up_4A in diabetic swine is at least in part the result of an increased purinergic receptor-mediated vasodilator influence.

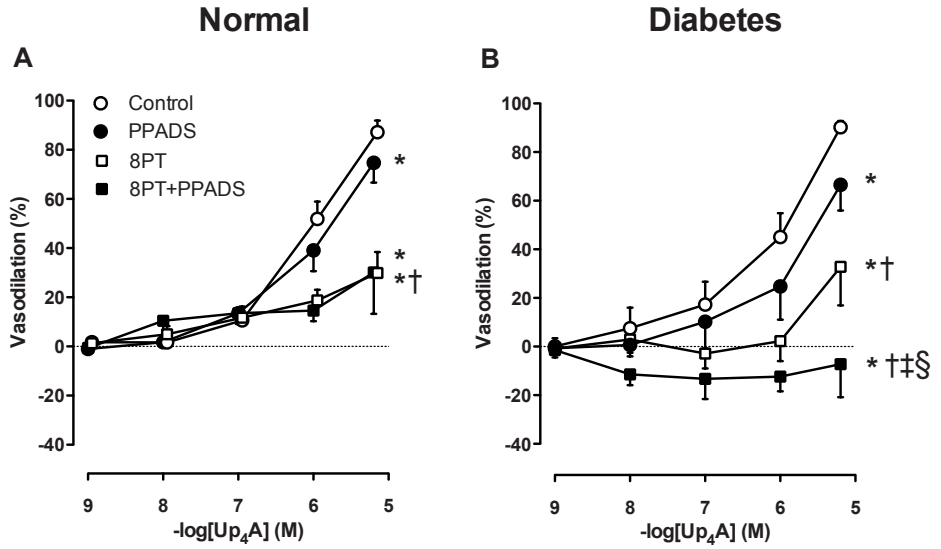

Fig. 2. *Effects of non-selective purinergic receptor blockade on Up_4A-induced vasodilation. Shown are concentration-responses to Up_4A (10^{-9}-10^{-5} M) in porcine coronary small arteries in the control vessels (n=10 in Normal; n=7 in diabetic swine) and in the presence of 10 μM non-selective P1 receptor antagonist 8PT (n=7 in Normal; n=6 in diabetic swine), in presence of 10 μM non-selective P2 receptor antagonist PPADS (n=10 in Normal; n=7 in diabetic swine) as well as in the presence of combined 8PT and PPADS (n=5 in Normal; n=4 in diabetic swine). Values are mean±SEM. * P<0.05 vs. Control; † P<0.05 vs. PPADS; ‡ P<0.05 vs. 8PT; § P<0.05 effect of 8PT+PPADS in diabetic swine differs from that in Normal.*

A_{2A} receptor blockade resulted in attenuation of the response to Up_4A that was comparable with the attenuation by 8PT and was not different between coronary small arteries from normal and diabetic swine (Fig. 3, panels A&F), despite slightly lower A_{2A} receptor mRNA expression levels (Fig. 4). $P2X_1$ receptor blockade with MRS2159 attenuated Up_4A-induced vasodilation to a similar extent in coronary small arteries from normal swine and swine with diabetes (Fig. 3, panels B&G), which was consistent with the unaltered mRNA expression between the two groups (Fig. 4). In contrast, $P2X_7$ receptor blockade with A438079 attenuated the vasodilator response to Up_4A in coronary small arteries from normal swine (Fig. 3C), but not from diabetic swine (Fig. 3H), which was consistent with the reduced $P2X_7$ mRNA expression in swine with diabetes (Fig. 4). The attenuation of the Up_4A-induced vasodilation in response to $P2Y_1$ blockade with MRS2179 was larger in coronary small arteries from diabetic (Fig. 3I) as compared to normal swine (Fig. 3D), despite the unaltered mRNA expression (Fig. 4). Finally, although mRNA for $P2Y_6$ was present in coronary small arteries from both normal and diabetic swine, $P2Y_6$ blockade with MRS2578 attenuated Up_4A-induced vasodilation only in diabetic (Fig. 3J) but not normal swine (Fig. 3E).

Role of endothelium in Up_4A-induced coronary vasodilation

Endothelial denudation, as confirmed by the absence of a vasodilator response to sub P (data not shown), attenuated the coronary vasodilator response to Up_4A in coronary small arteries

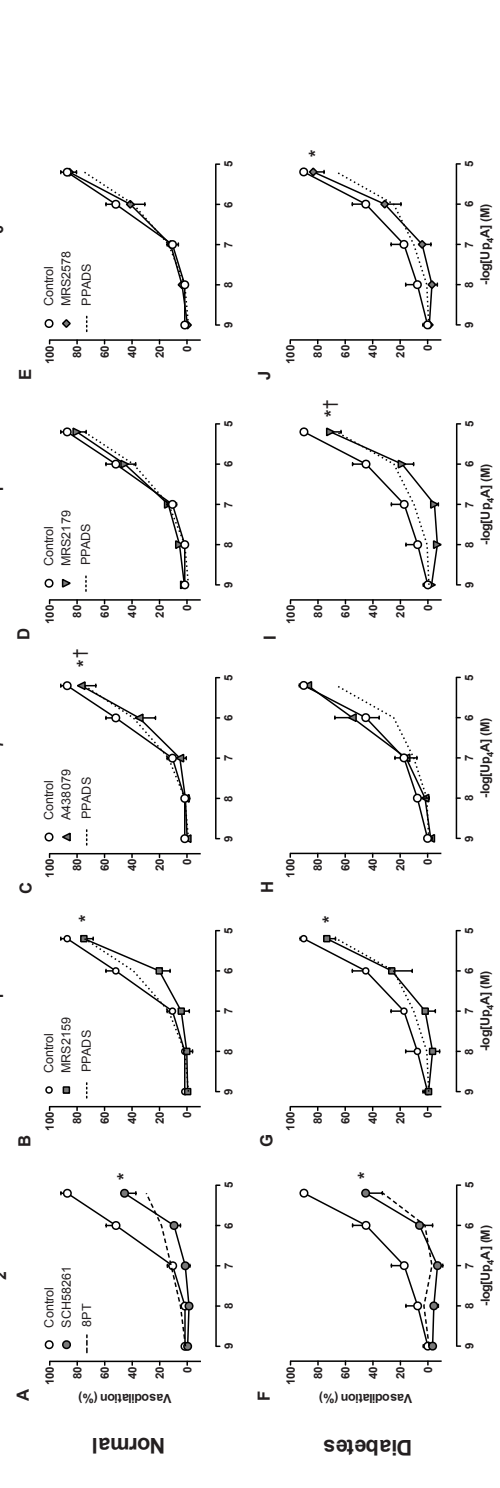

Fig. 3. *Involvement of specific purinergic receptor subtypes in Up_4A-induced vasodilation. Shown are concentration-responses to Up_4A (10^{-9}-10^{-5} M) in porcine coronary small arteries in control vessels (n=10 in Normal; n=7 in diabetic swine) and in the presence of 100 nM A_{2A} receptor antagonist SCH58261 (n=8 in Normal (panel A); n=7 in diabetic swine (panel F)); in the presence of 30 μM $P2X_1$ receptor antagonist MRS2159 (n=9 in Normal (panel B); n=7 in diabetic swine (panel G)); in the presence of 10 μM $P2X_7$ receptor antagonist A438079 (n=9 in Normal (panel C); n=6 in diabetic swine (panel H)); in the presence of 1 μM $P2Y_1$ receptor antagonist MRS2179 (n=9 in Normal (panel D) ; n=7 in diabetic swine (panel I)); and in the presence of 10 μM $P2Y_6$ receptor antagonist MRS2578 (n=9 in Normal (panel E); n=7 in diabetic swine (panel J)). For the sake of comparison, non-selective P1 blockade with 8PT is shown as dotted lines in panels A&F, while non-selective P2 blockade with PPADS is shown as dotted lines in panels B, C, D, E, F, G, H, I and J). Values are mean±SEM. * P<0.05 vs. Control; † P<0.05 effect of A438079 and MRS2179 in diabetic swine differs from those in Normal.*

from normal (Fig. 5A) but not from diabetic swine (Fig. 5E). Similarly, eNOS inhibition with LNAME attenuated the response to Up_4A in vessels from normal (Fig. 5A), but not from diabetic swine (Fig. 5E), which was consistent with its reduced expression in swine with diabetes (from 0.52±0.14 in normal to 0.19±0.03 in diabetes, $P<0.05$). Additional COX inhibition with indomethacin had no effect in coronary small arteries from normal swine (Fig. 5B) but tended to enhance the dilation in response to Up_4A in vessels from diabetic swine (Fig. 5F, $P=0.08$), suggesting the production of vasoconstrictor prostanoid(s), e.g. thromboxane. In the presence of LNAME, the thromboxane-synthase inhibitor ozagrel had no effect on Up_4A-induced vasodilation in coronary small arteries from normal swine (Fig. 5C), but enhanced the vasodilator response to Up_4A in vessels from diabetic swine (Fig. 5G). Moreover, this effect of ozagrel was significantly greater than that of indomethacin (Fig. 5, panels F&G), suggesting that, in addition to production of the vasoconstrictor prostanoid thromboxane, there was also production of a vasodilator prostanoid. In the presence of eNOS+COX blockade, inhibition of CYP 2C9 with sulfaphenazole enhanced the vasodilation to Up_4A in coronary small arteries from normal swine (Fig. 5D), whereas in vessels from diabetic swine, sulfaphenazole attenuated the vasodilation to Up_4A (Fig. 5H). These findings suggest that CYP 2C9 switches from the production of vasoconstrictor metabolites in normal swine to vasodilator metabolites in diabetic swine.

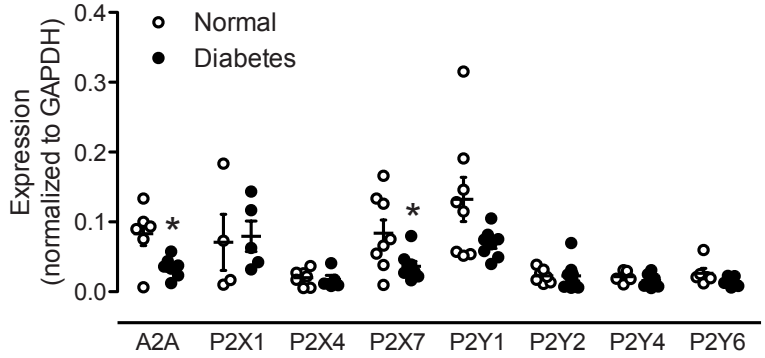

Fig. 4. *Expression profile of various purinergic receptor subtypes in Normal and diabetic swine. Shown are mRNA levels of each gene normalized to GAPDH. Values are mean±SEM. * P<0.05 vs. Normal.*

Discussion

The present study is the first to investigate the coronary vascular effects of the dinucleotide Up_4A in a large animal model of type 2 diabetes. The main finding was that Up_4A-induced vasodilation was not altered in coronary small arteries isolated from diabetic as compared to normal swine. However, the involvement of the different purinergic receptors in response to Up_4A was markedly different between diabetic and normal swine in that P1 and P2 blockade had an additive effect in diabetic, but not in normal swine. Purinergic $P2X_7$ receptor blockade attenuated Up_4A-induced vasodilation in normal but not in diabetic swine, while $P2Y_1$ and $P2Y_6$ blockade had no effect in normal swine while significantly attenuating Up_4A-induced vasodilation in diabetic swine. Moreover, the contribution of endothelium-derived vasoactive factors was markedly different between diabetic and normal swine, in that endothelial denudation as well as eNOS inhibition attenuated the response to Up_4A in normal but not

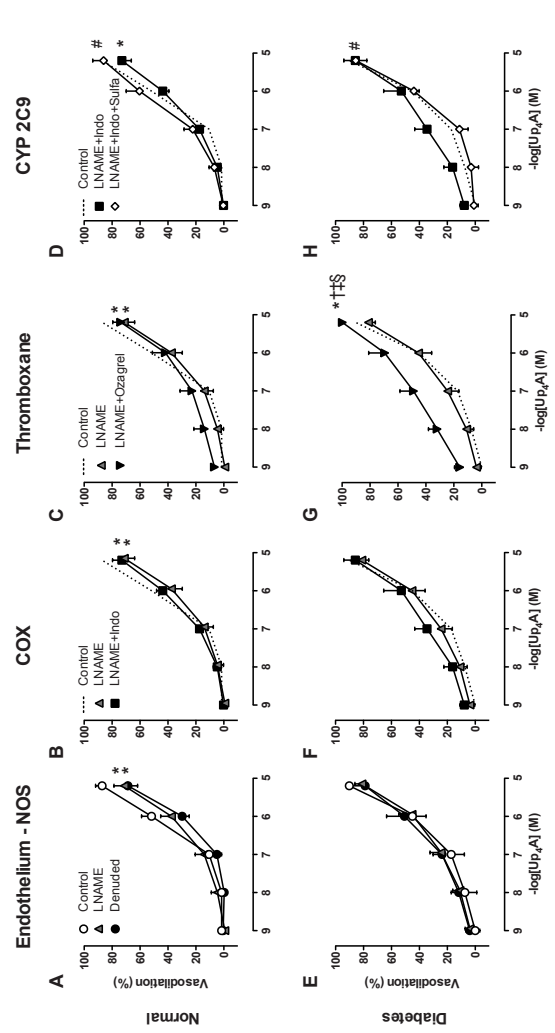

Fig. 5. *Involvement of the endothelium and endothelium-derived vasoactive factors in Up_4A-induced vasodilation. Shown are concentration-responses to Up_4A (10^{-9}-10^{-5} M) in porcine coronary small arteries in the absence and presence of endothelium (n=10 in Normal (panel A); n=7 in diabetic swine (panel E)); in the presence of 100 μM of the NOS inhibitor LNAME (n=10 in Normal (panel A); n=7 in diabetic swine (panel E)); in the presence of combined LNAME and 10 μM of the COX inhibitor indomethacin (Indo, (n=9 in Normal (panel B); n=7 in diabetic swine (panel F)); in the presence of combined LNAME and 10 μM thromboxane synthase inhibitor ozagrel (n=9 in Normal (panel C); n=7 in diabetic swine (panel G)); and in the presence of combined LNAME, Indo and 10 μM CYP 2C9 inhibitor sulfaphenazole (Sulfa, (n=7 in Normal (panel D); n=4 in diabetic swine (panel H)). For the sake of comparison, the effect of Up_4A in the absence of any blockers is shown as dotted lines in panels B, C, D, F, G and H). Values are mean±SEM. * P<0.05 vs. Control; † P<0.05 effect of Ozagrel after LNAME; ‡ P<0.05 effect of Ozagrel in diabetic swine differs from its effect in Normal; § P<0.05 effect of Ozagrel differs from effect of Indo; # P<0.05 effect of Sulfa after LNAME + Indo.*

diabetic swine. Combined blockade of NOS and COX had no effect on Up_4A-induced vasodilation as compared to NOS blockade alone in normal swine, but tended to enhance Up_4A-induced vasodilation in diabetic swine. This effect of COX inhibition was mimicked by selective thromboxane synthase inhibition. Finally, we found that CYP 2C9 metabolites exerted a vasoconstrictor influence in response to Up_4A in normal swine, but exerted a vasodilator influence on the Up_4A responses in diabetic swine. The implications of these findings will be discussed below.

The dinucleotide Up_4A was identified as an endothelium-derived vasoactive factor less than a decade ago [5]. Subsequent studies demonstrated that Up_4A induces vasoconstriction in most vascular beds, including rat renal artery [5], mouse renal arterioles [25], rat aorta [26], rat mesenteric artery [8] and rat pulmonary artery [6]. In addition to its vasoconstrictor effects in most vascular beds under basal tone, Up_4A can act as a vasodilator when vascular tone is elevated in the rat aorta and rat perfused kidney [26, 27]. In contrast, we previously found that Up_4A is purely a vasodilator in the porcine coronary microcirculation, without any vasoconstrictor activity [10]. We

have recently shown that the vasodilator response to Up_4A is reduced in the remote coronary vasculature after MI [11], while in another study Up_4A-induced vasoconstriction was reported to be enhanced in renal arteries from rats with type 2 diabetes [12]. These findings suggest that Up_4A signaling may be altered in coronary artery disease and in diabetes.

The functional involvement of purinergic receptors in response to Up_4A was generally similar in vessels from normal swine compared to the Sham-operated swine used in our previous study [11]. Although expression of the A_{2A} receptor was slightly reduced in coronary small arteries from diabetic swine, the majority of the vasodilator effect of Up_4A was still exerted through the purinergic P1 subtype A_{2A} receptors. The effect of combined P1 and P2 receptor blockade was markedly enhanced in the coronary small arteries from diabetic swine, even unmasking a modest vasoconstrictor effect in response to Up_4A, suggesting the P2 receptor subtypes involved in the response to Up_4A may differ between normal and diabetic swine. In accordance with this concept, we found that both the expression and contribution of $P2X_7$ receptors to Up_4A-induced vasodilation was decreased, while the contribution of $P2Y_1$ and $P2Y_6$ was increased in coronary small arteries from diabetic swine. However, expression of the $P2Y_1$ and $P2Y_6$ receptors was not significantly altered. The apparent discrepancy between receptor expression and function is likely due to the fact that receptor mRNA does not equate to receptor function. In addition, expression was studied in whole vessels, so that we cannot distinguish between expression in endothelial versus that in vascular smooth muscle cells. P2 receptors in the vasculature are present on endothelial as well as vascular smooth muscle cells. Activation of $P2X_1$ receptors and, in some vascular beds, $P2X_2$, $P2X_4$ and $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors on smooth muscle cells, generally results in vasoconstriction although vasodilation is also sometimes observed [28]. Activation of $P2Y_1$, $P2Y_2$ and possibly also $P2Y_4$, $P2Y_{11}$, $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$ and $P2X_7$ receptors on endothelial cells, leads to the production of nitric oxide (NO) and subsequent vasodilation [28, 29]. It is therefore possible that a shift in expression of the $P2Y_1$ and the $P2Y_6$ receptors from vascular smooth muscle to endothelium may have resulted in an increased contribution of these receptors to the vasodilator effect of Up_4A.

Diabetes is accompanied by microvascular dysfunction and a shift in the balance between endothelium-derived vasodilators and vasoconstrictors [3, 4]. In the present study, endothelial denudation reduced Up_4A-induced vasodilation in coronary small arteries from normal but not diabetic swine. This effect of endothelial denudation was similar to the effect of eNOS inhibition with LNAME, indicating a reduced involvement of NO in the vasodilator response to Up_4A in diabetes. A reduced eNOS activity is a hallmark of coronary endothelial dysfunction in humans with diabetes [30] and was also observed in our recent study in coronary small arteries of diabetic swine [2], and is consistent with the reduced eNOS expression in diabetes observed in the present study. Endothelial dysfunction often results in a shift in production of endothelial vasoactive factors, i.e. from NO to prostanoids and EDHFs [31]. In the present study, we found that inhibition of COX had no effect in coronary small arteries from normal swine, but resulted in an enhanced vasodilator response to Up_4A in diabetic swine, suggesting that a vasoconstrictor prostanoid was produced in response to Up_4A in diabetes, most likely thromboxane. An increased contribution of thromboxane to vasoconstriction in response to Up_4A was also recently shown in a study in renal arteries from type 2 diabetic rats [12], although in that study the increased contribution of thromboxane appeared to be due to an increased sensitivity rather than an increased production of thromboxane. In the present study, the effect of ozagrel was more pronounced than the effect of indomethacin, suggesting that besides the vasoconstrictor prostanoid thromboxane, there was also some production of a vasodilator prostanoid. A shift from production of vasodilator to vasoconstrictor prostanoids has also been shown in the vasculature of streptozotocin-treated

diabetic mice and rats [32] and therefore appears to be a more generalized response in diabetes. A potential mechanism behind the increased thromboxane production may be that low-grade inflammation results in eNOS uncoupling in diabetes, which subsequently leads to the production of reactive nitrogen species such as peroxinitrite, that is capable of inactivating PGI_2-synthase and cause a shift in production from prostacyclin to thromboxane [32, 33].

EDHF has been demonstrated to compensate for the loss of NO in diabetes [34] and CYP 2C9 has been reported to generate EETs that can act as EDHF in the coronary vasculature [35]. In addition to these vasodilator EETs, CYP 2C9 can produce ROS [36], which act as vasoconstrictors in the porcine coronary vasculature [37]. Although we have previously shown in pre-adolescent swine that inhibition of CYP 2C9, either alone [10] or in the presence of inhibition of eNOS/COX (unpublished), does not affect Up_4A-induced vasodilation, we found in the present study in adult swine that inhibition of CYP 2C9, in the presence of inhibition of eNOS and COX, potentiated the vasodilator response to Up_4A in coronary small arteries from normal swine. These data are consistent with the notion that CYP 2C9 activity may be increased with age [38], but predominantly produces ROS in healthy porcine coronary small arteries [36]. In contrast, in coronary small arteries from swine with diabetes, inhibition of CYP 2C9 (also in the presence of inhibition of eNOS and COX), reduced the vasodilation in response to Up_4A, suggesting that CYP 2C9 switches from the production of vasoconstrictor to vasodilator metabolites in diabetes. Such production of vasodilator metabolites by CYP 2C9 in diabetes is consistent with other studies showing that CYP 2C9 metabolites can act as an EDHF [35] and that EDHF(s) act to compensate for the loss of NO in coronary vasculature in diabetes [34].

Conclusions

In conclusion, despite blunted $P2X_7$ and NO-mediated vasodilator influences as well as an enhanced TXA-mediated vasoconstrictor influence, the coronary vasodilator response to Up_4A was fully maintained in diabetic swine. The latter was likely the result of an increased vasodilator influence via $P2Y_1$ and $P2Y_6$ receptors and of CYP 2C9 switching from producing vasoconstrictor to vasodilator metabolites.

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Chapter 11

Summary and general discussion

Summary, discussion and future perspective

The endothelium plays a primary role in the regulation of vascular tone. By endocrine, autocrine and paracrine mechanisms, the endothelium releases various relaxing (e.g. VEGF, NO prostacyclin and EDHFs) and contracting (e.g. ET, ROS and Up_4A) factors [1]. These factors act through activation of their respective receptors located on the endothelium and/or smooth muscle cells and/or through activation of signaling pathways, resulting in alterations in vascular tone. The interplay of those substances released from the endothelium, is an important contributor to maintenance of vascular homeostasis [2]. On the other hand, an imbalance of the release of endothelium-derived relaxing factors (EDRFs) and endothelium-derived contracting factors (EDCFs) may contribute to the pathophysiology of a growing list of cardiovascular diseases, including diabetes mellitus, hypertension, atherosclerosis and coronary artery disease as well as heart failure [3]. Importantly, endothelial dysfunction, as characterized by a decreased vasodilator and an increased vasoconstrictor release, or a shift from vasodilator to vasoconstrictor state, is increasingly recognized as an early indicator of these disease states [4]. The studies described in this thesis therefore were aimed at investigating the role of various endothelium-derived vasodilators and vasoconstrictors in the regulation of tone in the healthy vasculature as well as a potential role for their (dys)regulation in various cardiovascular disease models.

Control of resistance vessel tone by vascular endothelial growth factor

In addition to the well-known biological role of VEGF in angiogenesis, wound healing and tumor growth [5, 6], endogenous VEGF plays a crucial role in regulating vascular tone by activating eNOS and subsequent NO production, thereby resulting in vasodilation [7, 8]. It, therefore, has been suggested that NO bioavailability is reduced during inhibition of the VEGF pathway in cancer patients receiving anti-VEGF treatment, resulting in vasoconstriction and the development of hypertension [9, 10]. Hypertension is the most important cause of discontinuation of therapy with sunitinib, an orally available tyrosine kinase inhibitor, used to treat renal cell cancer, imatinib-resistant gastrointestinal stromal tumors, as well as breast, colon and lung cancers [11, 12]. It has been proposed that the hypertension is caused by vasoconstriction of the systemic resistance vasculature, but the exact mechanisms behind this vasoconstriction were unknown. In addition, whether the anti-VEGF therapy also adversely affects the coronary and pulmonary circulations is largely unknown. Consequently, in **Chapter 2**, we studied the effect of sunitinib-treatment on the pulmonary, coronary and systemic circulations, and investigated to what extent a shift in EDRFs and EDCFs contributed to sunitinib-induced hypertension. The results obtained in chronically instrumented healthy swine, demonstrated that sunitinib produces vasoconstriction selectively in the systemic vascular bed, without affecting the pulmonary and coronary circulation. Contrary to our initial hypothesis, the sunitinib-mediated systemic hypertension was principally attributed to an increased vasoconstrictor influence of one of the EDCFs, i.e. ET, with no apparent contributions of a loss of NO bioavailability or increased oxidative stress, suggesting that endogenous VEGF signaling exerts a vasodilator effect to counteract the influence of ET in systemic vascular bed (Fig. 1). This study confirmed that vasodilation not only occurs as the result of the release of EDRFs, but also indirectly through inhibition of EDCFs.

A great number of studies have shown that anti-VEGF resulted in signaling alterations at cellular level of various cell types [13, 14]. At organism level of vascular systems, anti-VEGF has reported to mainly affect systemic circulation [10], although some studies demonstrated the effect of anti-VEGF on the coronary malformation at embryonic/postnatal stages [15, 16], as well as the

impairment of coronary collateral growth [17]. Consistent with these observations, the vasoconstrictor influence of anti-VEGF with sunitinib could be only observed in porcine systemic but not coronary and pulmonary circulations. Therefore, systemic blood pressure was elevated without affecting coronary and pulmonary systems. This selective effect induced by sunitinib may be due to the different origins of the endothelium in these vascular beds during embryonic development that resulted in distinct function of the endothelium in response to VEGF [18]. However, pulmonary toxicity does occur in patients with squamous cell lung cancer receiving anti-VEGF therapy, although the chance of side effect is rare [19]. This observation suggests that in patients with cancer, a potential vasoconstrictor effect of sunitinib on the coronary and pulmonary vasculature may be unmasked and needs to be explored.

As summarized above, the sunitinib-induced rise in systemic blood pressure in swine is mainly due to the activation of endothelin system. Although ET antagonism by blocking ET_A/ET_B receptors can largely reverse the increased blood pressure to its pre-sunitinib value, the exact mechanism causing the activation of endothelin system remains to be investigated but several explanations can be forwarded. VEGF plays a crucial role in regulating endothelial homeostasis. There is evidence showing the interaction between VEGF pathway and endothelin system [14, 20, 21], VEGF inhibition with sunitinib may thus induce endothelial cell activation resulting in production of endothelin. Future studies need to investigate this interaction in endothelial cells of various systemic vascular beds, such as renal and coronary endothelial cells. Enhanced activity of endothelin converting enzyme may account for the observed increased endothelin involvement by sunitinib. However, this is rather unlikely as some studies demonstrated that instead of anti-VEGF, VEGF induces the production of endothelin by increasing the mRNA level of endothelin converting enzyme in vascular endothelial cells [22].

Based on the observation that activation of endothelin system plays an important role in the sunitinib-induced systemic hypertension, endothelin receptor blockers can thus be applied to combat this side effect. Importantly, selective blockade of ET_A receptor has been shown to inhibit hypoxia-induced breast cancer cell invasion, which suggests endothelin axis as a promising target in treating breast cancer. Since sunitinib is tested in clinical trial in patients with breast cancer [23], endothelin antagonism may have a double role not only in alleviating side effect but also in treating the cancer. On the other hand, while ET receptor blockade is effective in treating hypertension, side effects like peripheral edema and fluid retention may occur and prohibit the use of ET receptor blocker to control systemic blood pressure [24]. Other anti-hypertensive drugs (calcium channel blocker, angiotensin-converting enzyme inhibitor, angiotensin II type 1 receptor blocker et al.) may therefore be a more promising alternative to alleviate the sunitinib-induced systemic hypertension.

Control of coronary resistance vessel tone by CYP 2C9 activity

The vasodilator influence of well-known EDRFs NO and PGI_2 has been extensively studied in various vascular beds [25-27]. For instance, in the systemic circulation, both endogenous NO and PGI_2 contribute to systemic vasodilation [28]. The vasodilator effect of PGI_2 was suppressed by NO, suggesting an interaction between these two EDRFs exists. Although NO and PGI_2 can be considered the most important EDRFs, [27] it should be noted that a significant endothelium-dependent vasodilation persists after inhibition of NO synthase (NOS) and cyclooxygenase (COX), suggesting the existence of other endothelium-dependent vasodilator pathways [29]. One such pathway is characterized by factor(s) capable of hyperpolarizing smooth muscle cells via opening K^+ channels, which have been termed EDHFs [29]. The role of cytochrome P450 2C9 (CYP 2C9) metabolites as an EDHF in the regulation of vasomotor tone have been well described, based on

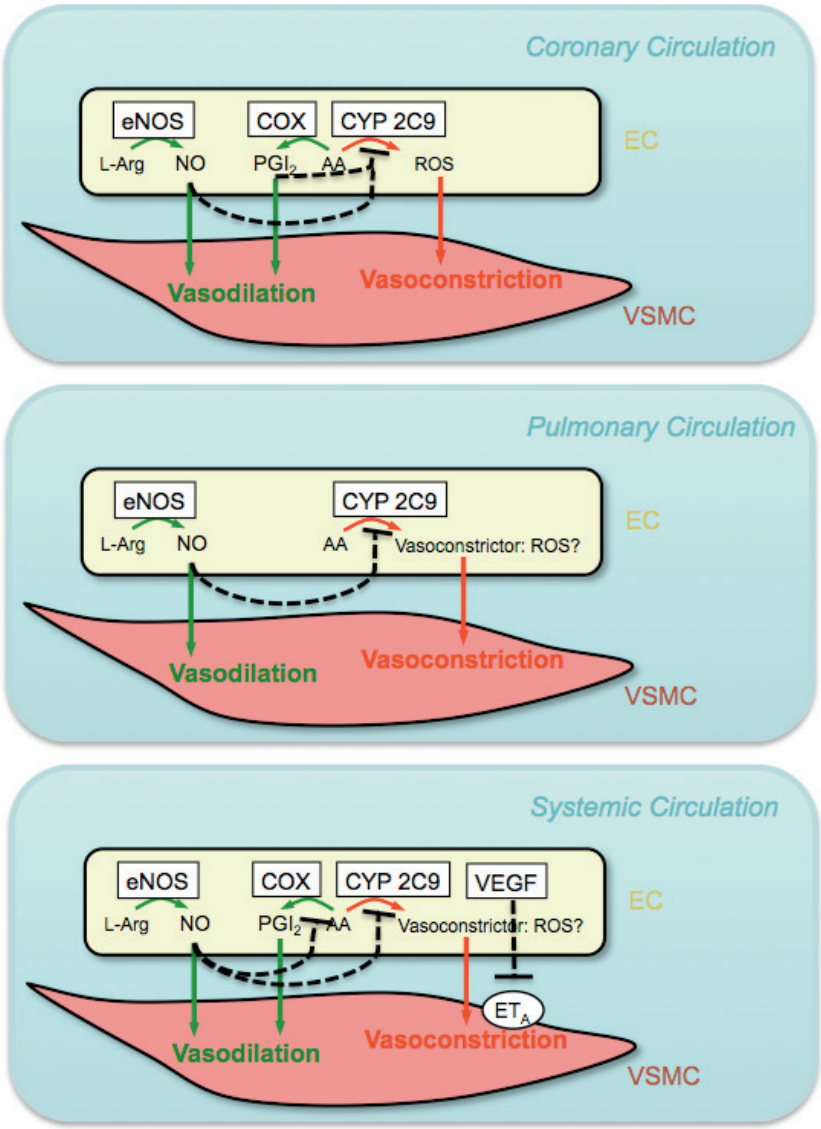

Fig. 1. *The vasoactive influence of various endothelium-derived relaxing factors (EDRFs) and endothelium-derived contracting factors (EDCFs) on the coronary, pulmonary and systemic circulations in vivo.*

the fact that CYP 2C9 is abundantly expressed in the vasculature, and has been reported to participate in agonist-induced vasodilation in many vascular beds (coronary, pulmonary, subcutaneous, and hepatic artery) [30, 31]. However, little is known about the endogenous role of CYP 2C9 metabolites in the regulation of coronary resistance vessel tone at rest or during exercise. Accordingly, in **Chapter 3** we investigated the endogenous vasomotor influence of CYP 2C9 metabolites in the regulation of coronary vascular tone in chronically instrumented swine model. Surprisingly, CYP 2C9 metabolites exerted a vasoconstrictor rather than EDHF-mediated vasodilator influence on the coronary resistance vessels both at rest and during exercise, which was enhanced in the presence of NOS/COX blockade. In contrast, pharmacological activation of

CYP 2C9 with bradykinin resulted in predominant production of vasodilator CYP 2C9 metabolites when studied in the intact coronary circulation in vivo. Subsequently, coronary small arteries were isolated to study the possible involvement of ROS in the paradoxical vasoconstrictor influence of CYP 2C9 activity. The results showed that vasoconstrictor CYP 2C9 metabolites, most likely ROS, were generated in vitro setup.

Due to the heterogeneity of the endothelium and the different characteristics of vascular beds [32-34], the role of CYP 2C9 metabolites in the coronary vasculature may differ from that in the pulmonary vasculature. Indeed, previous studies have shown that CYP 2C9 metabolites exerted an EDHF-mediated coronary vasodilation [30] but a vasoconstrictor influence in the lung [35]. Consistent with the observation in the lung [35], unpublished data from our laboratory indicated that CYP 2C9 metabolites endogenously contribute to pulmonary vasoconstriction at rest and during exercise, albeit only after blockade of NOS/COX. Moreover, the vasoconstrictor effects of CYP 2C9 metabolites have been enhanced in the coronary vasculature or unveiled in the pulmonary vasculature after depletion of NO/PGI_2, indicating an interaction between CYP 2C9 metabolites and those major EDRFs in both circulations (Fig. 1). In contrast to the in vitro study with coronary small arteries, we found that in isolated pulmonary small arteries CYP 2C9 metabolites produced mainly vasodilation in response to bradykinin (Fig. 2). Although little evidence has been shown for the role of CYP 2C9 metabolites in isolated porcine pulmonary arteries, several studies did show involvement of an EDHF in response to bradykinin in porcine pulmonary arteries [36, 37]. In the coronary small arteries, bradykinin has been proposed to produce ROS [38], and bradykinin B2 receptors seem to be involved in this process [39]. Whether ROS was also produced through this pathway in the pulmonary small arteries is largely unknown (Fig. 2), and deserves further investigation.

The identity of the CYP 2C9-derived vasoactive metabolites produced in our study remains to be elucidated. In additional to ROS, CYP 2C9 has been reported to mainly generate 11,12-epoxyeicosatrienoic acids (EETs) in the endothelial cells [40], which result in vasodilation by opening potassium channels [41]. Therefore, the vasoconstrictor influence by CYP 2C9 metabolites observed in the coronary vasculature (Chapter 3) might result from the predominant effect of vasoconstrictor ROS over vasodilator EETs, as the interaction between ROS and EETs has been proposed [42]. On the other hand, the amount of EETs generated by CYP 2C9 can be hydrolyzed by soluble epoxide hydrolase (sEH) [41]. The endogenous activity of sEH might be very high in both coronary and pulmonary circulations of our animal models; thereby a rapid degradation of EETs to a less vasoactive dihydroxyeicosatrienoic acids took place [43]. Together with a relatively constant amount of ROS, this resulted in an overall vasoconstrictor influence. It is of interest to further explore if and how cardiovascular diseases, such as coronary artery disease, pulmonary hypertension and heart failure, affect the activity of the enzymes that are involved in production and degradation of EETs, as well as their interaction with ROS.

Integrated control of resistance vessel tone by NO/cGMP and endothelin

NO-mediated vasodilation is mediated through the activation of downstream sGC, which subsequently produces cGMP. cGMP is short-lived, because it is hydrolyzed by phosphodiesterase (PDE) 5 [44]. The activity of PDE5 determines the rate of degradation of cGMP thereby regulating vascular tone. The balance between production and removal of cGMP in coronary vascular smooth muscle is of critical importance in determining coronary vasomotor tone. Since little is known about the vasoconstrictor role of PDE5 in the coronary circulation, particularly the alteration of PDE5 influence in myocardial infarction (MI), in **Chapter 4**, we investigated the endogenous contribution of PDE5 to the coronary vascular tone regulation in

healthy swine and swine after MI at rest and during exercise. In healthy swine, the results showed that PDE5 inhibition resulted in a pronounced coronary vasodilation at rest, which was progressively attenuated during exercise. The vasodilator effect by PDE5 inhibition was partially NO dependent. In MI swine, PDE5 inhibition-induced coronary vasodilation of remote myocardium was attenuated as compared to healthy swine, which was accompanied by a similarly marked reduced PDE5 gene expression. In contrast to healthy swine, the responses to PDE5 inhibition were maintained during exercise. The reduced vasodilator effect of PDE5 inhibition appeared to be NO independent (**Chapter 4**).

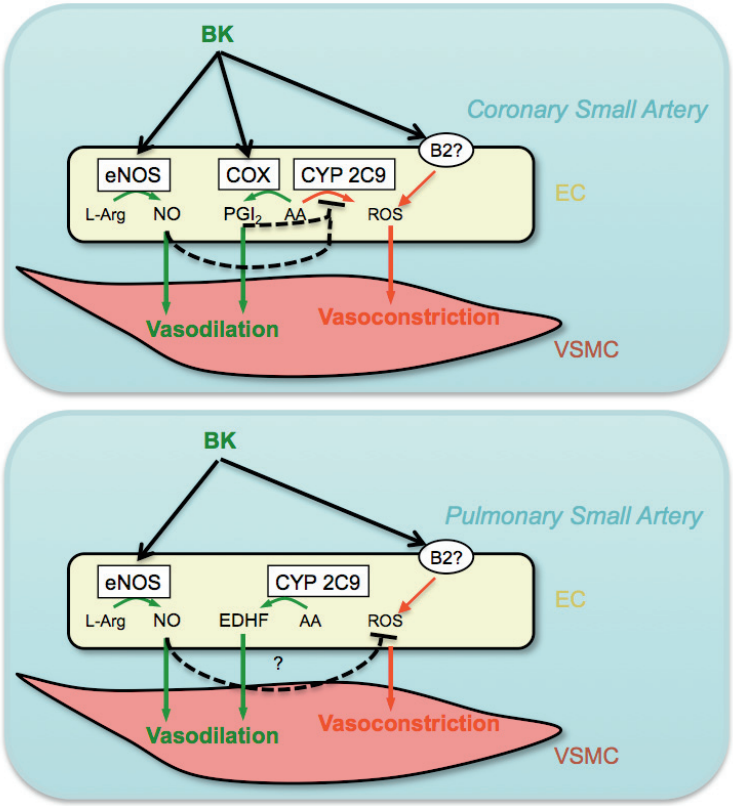

Fig. 2. *The vasoactive influence of cytochrome P450 2C9 metabolites on the isolated coronary and pulmonary small arteries in response to bradykinin.*

Previous studies from our laboratory demonstrated that even under healthy conditions, coronary vasodilation is mediated by the production of NO and inhibition of ET [45, 46]. The interaction between these two systems exists particularly during exercise [46]. Furthermore, we have shown that NO acts in concert with PGI_2 to suppress the coronary vasoconstrictor effect of ET [45]. Specifically, this exercise-induced attenuation of ET-mediated coronary vasoconstriction occurs through a reduced ET production rather than reduced ET receptor sensitivity [47], suggesting that cGMP/NO may in part acts through blunting of ET production. Based on these findings, we further investigated the effect of PDE5 inhibition on the endogenous ET vasoconstrictor influence in **Chapter 5**, and found that ET blockade failed to further dilate the coronary vasculature in the presence of PDE5 inhibition at rest and during exercise. This lack of

additional vasodilator effect was shown in isolated coronary small arteries to be the result of reduced ET production rather than reduced ET receptor sensitivity.

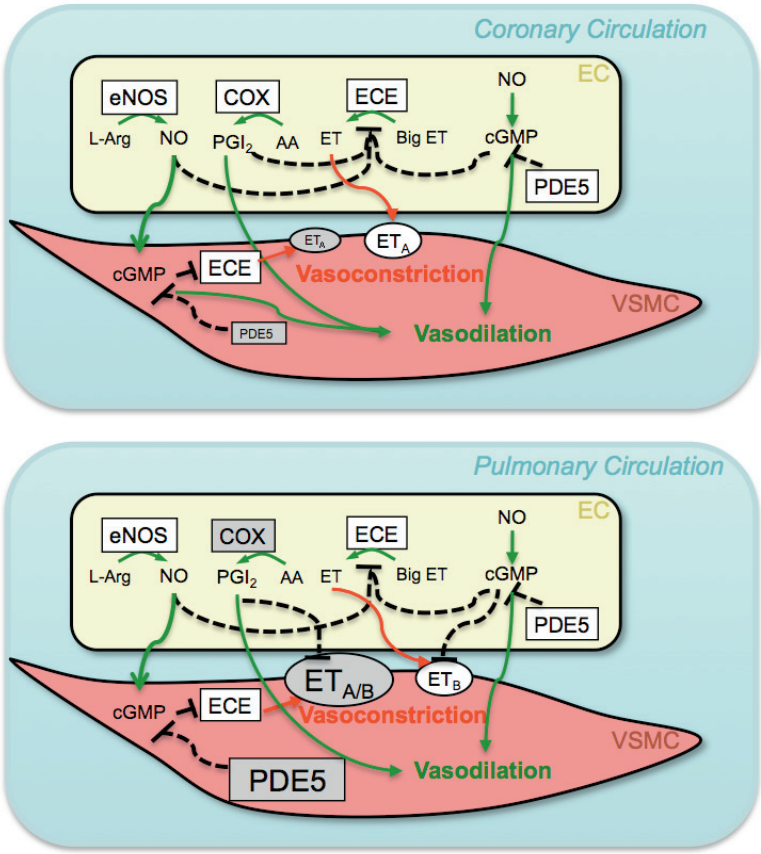

Fig. 3. *The overview of the vasoactive influence of various EDRFs and EDCFs as well as its interaction in health and disease on coronary and pulmonary circulations. The gray represents the findings observed in disease.*

In the pulmonary vasculature, many studies have shown that both PDE5 inhibitors such as sildenafil and ET receptor blockers such as bosentan result in vasodilation. These drugs have therefore been widely used as a therapeutic tool in treating patients with pulmonary hypertension [48-50]. However, little is known whether the combination of those two treatments has additional therapeutic benefits compared to each individual treatment alone. Moreover, the effects of the combination therapy in terms of long-term outcome of the prognosis, their optimal dosages and the safety remains to be controversial [49]. In light of these considerations, we further studied the interaction between PDE5 and endothelin in the pulmonary vasculature (**Chapter 6**). The results showed that similar to the observation in the coronary circulation, PDE5 inhibition attenuated vasoconstrictor influence of ET both in vivo and in vitro thereby preventing an additive vasodilator effect of PDE5 inhibition and ET receptor antagonism. Interestingly, and in contrast to the coronary circulation, the interaction between the NO-cGMP system and the ET system occurred at the level of ET receptor(s) as well as endothelial converting enzyme.

In accordance with previous studies [51, 52], endogenous PDE5 exerts a vasoconstrictor influence in both coronary and pulmonary circulations [53]. However, the effect of PDE5 inhibition

appeared slightly more pronounced (Chapter 4) or similar (Chapter 5) at rest than during exercise in the coronary circulation, whereas this effect was more pronounced during exercise than at rest in the pulmonary circulation [53]. In the presence of endothelial dysfunction induced by MI (Chapter 4), the expression and function of PDE5 was attenuated thereby resulting in a reduced vasoconstrictor influence of PDE5 in the coronary vasculature. Moreover, the coronary response to NO inhibition was reduced in MI, and the vasoconstrictor response to NO inhibition was not enhanced by prior to the PDE5 inhibition. These findings suggest that decreased PDE5 activity in the coronary vasculature may compensate for the loss of NO thereby endogenously participating in a protective progress in disease. In contrast, with endothelial dysfunction mimicked by reducing NO bioavailability, this vasoconstrictor effect is enhanced in the pulmonary circulation [53]. The enhanced PDE5 activity may exacerbate the effects of a loss of endothelial function, more specifically a loss of NO, and hence contribute to the pathogenesis of pulmonary hypertension [53].

The marked difference in endothelial control of resistance vessel tone between coronary and pulmonary circulations is in line with other reports from our group. Thus, whereas both NO and PGI_2 contribute to coronary vasodilation, only NO but not PGI_2 plays a vasodilator role in the pulmonary vasculature in healthy swine [28]. The different influence of NO and PGI_2 activates different downstream signaling (for instance cGMP and cAMP), which can affect ET system differently between coronary and pulmonary vasculatures [54, 55]. Figure 3 compares the main findings of Chapter 4, Chapter 5 and Chapter 6 together with some of our previous studies regarding the vasoactive difference between coronary and pulmonary circulations.

A limitation of our studies is that the tissue level of cGMP in the presence of PDE5 inhibition alone or the combination treatment with PDE5 inhibition and ET receptor blockade was not measured. Future studies focusing on this aspect will provide greater insight into the interaction of cGMP and ET systems. Moreover, the studies on the mechanism of the interaction between PDE5 and ET were conducted in isolated vessels, in which blood flow and blood cell components were lacking. Since the NO-cGMP axis can be activated by various local and circulating factors, such as shear stress, ATP that released from red blood cells [56], and since these influence may become more pronounced during exercises, we cannot exclude the effect of those factors on the vasoconstrictor influence of ET. Additional investigation on the interaction between other NO donors or cAMP axis and ET systems will specify the interaction between these signaling pathways. Indeed, our recent experiments (unpublished) showed that the cGMP analogue 8-bromo-cGMP attenuated Big ET-induced vasoconstriction in both coronary and pulmonary small arteries, whereas it only attenuated ET-induced vasoconstriction in pulmonary but not coronary small arteries. Moreover, the cAMP analogue 8-bromo-cAMP had no effect on the response to either ET or Big ET in pulmonary small arteries.

An average reduction in systemic blood pressure of 17±2 mmHg observed in Chapter 6 by combination treatment with PDE5 and ET inhibition was consistent with many other reports showing an induction of hypotension by combined PDE5 inhibition and other vasodilators in patients with pulmonary arterial hypertension [49, 57]. The optimal combination therapy remains a concern and future studies are needed to determine the right dosage based on safety and efficacy [49]. To lower the dosage used in the mono-therapy when combination therapy is applied may avoid this side effect and bring a promising outcome [58]. In contrast to the clinical trials where patients orally receiving PDE5 inhibitor such as sildenafil, PDE5 inhibitor was intravenously administered to the healthy animals in the present thesis. The route of administration of anti-pulmonary hypertension drugs also needs to be optimized, since through different routes, for instance oral sildenafil+oral bosentan, oral sildenafil+iv. epoprostenol or oral sildenafil+inhaled

treprostinil, these combination therapies may result in different outcomes in terms of efficacy as well as side-effects [59, 60].

Effects of Up_4A on coronary microvessels in health and disease

Up_4A has been identified as a novel potent EDCF [61]. This factor is endogenously released from endothelium and can be biosynthesized by VEGFR2 [62]. Like other nucleotides, Up_4A exerts its vasoactive influence by activating purinergic receptors [63]. Up_4A is the first dinucleotide found in living organisms that contains both purine and pyrimidine moieties [61]. Up_4A may potentially activate both P1 and P2 receptors to regulate vasomotor tone [63]. After the discovery of Up_4A, the vasoactive role of Up_4A in various vascular beds has been subsequently addressed, including in renal arteries [61], pulmonary arteries [64, 65], aorta [66, 67], mesenteric arteries [68]. In those studies, Up_4A exerted potent vasoconstrictor influences through purinergic P2 receptors [69], although Up_4A can also produce vasodilation in the rat aortic arteries [66] and isolated perfused kidney [70] when basal tone is elevated. Although a dominant vasoconstrictor response to Up_4A in those vascular beds is now well established, the effects of Up_4A on the coronary microcirculation had not been studied previously. Consequently, in **Chapter 7** we investigated the vasoactive role of Up_4A in healthy coronary microvessels in swine. Contrary to observations in most arterial segments and vascular beds in rodents, we found that Up_4A failed to induce coronary vasoconstriction, but rather induced potent vasodilation. Furthermore, we found that Up_4A acted mainly via activation of purinergic P1 (A_{2A}) but not P2 receptors. Moreover, Up_4A-induced potent coronary vasodilation was partially mediated by NO and PGI_2, while CYP 2C9 metabolites did not appear to play a role.

The potent vasodilator influence by Up_4A observed in healthy porcine coronary vasculature is contradictory to most arterial vascular beds studied in rodents [61, 64, 65, 68, 71], although a vasodilator effect of Up_4A has been reported for some other vascular beds including rat aorta [66], and mice systemic vascular beds [67]. Notably, dual vasoactive actions of Up_4A have been observed in isolated perfused kidney with Up_4A acting as a vasoconstrictor as well as vasodilator [70]. In the healthy coronary vasculature, Up_4A fails to produce any vasoconstriction. Yet in line with previous observations [68], we also observed vasoconstriction in rat mesenteric arteries (Chapter 8) and thoracic aorta (unpublished data from our laboratory) in response to Up_4A with the same dosage range used in preconstricted porcine vessels. This further confirms that Up_4A purely induces coronary vasodilation in our setup. Furthermore, based on the multi-subtype of purinergic receptors present through almost entire vascular tree, their distribution on different cell types [72, 73], and vascular segments within the same species [63, 74] can vary markedly. To test this variability, we have studied the effect of Up_4A on additional healthy vascular beds other than coronary small arteries in swine. The preliminary data showed that Up_4A has neither vasoconstrictor nor vasodilator effects in either the mammary arteries or pulmonary small arteries (unpublished); the latter contrasts with observations by Gui et al. [64] who showed that Up_4A produced a potent vasoconstriction but not vasodilation in rat pulmonary arteries. Similarly, Matsumoto et al. compared vasoactive effects of Up_4A on renal, pulmonary, basilar, femoral, thoracic and mesenteric arteries in rats. They found that Up_4A induced vasoconstriction in all of those vascular beds, but the potency of Up_4A-induced vasoconstriction in those vascular beds was very different with the highest contraction of 60% in pulmonary arteries and lowest contraction of 20% in femoral arteries [65, 68]. This suggests that the vasoactive effects of Up_4A depend not only on the vascular bed but also on the species studied (Table 1).

In the healthy coronary vasculature (Chapter 7), Up_4A produces vasodilation up to 100%, which is as potent as the effect by well-studied mono-nucleotide vasodilators such as adenosine, ATP and ADP [75, 76]. This vasodilator effect by Up_4A is not mediated by potential degradation to

adenosine, as it was not affected by inhibition of ectonucleotidase. A significant part of the vasodilator response to Up_4A is mediated by activation of A_{2A} receptors located on both endothelium and smooth muscle cells. In contrast to previous studies in rodents where Up_4A activates P2 receptors to exert potent vasoconstriction [61, 69], Up_4A-induced vasodilation in this thesis mainly involves P1 receptor activation rather than P2 receptors. In the different chapters (Chapter 7-10), we have used different control vessels in terms of vessel size and age of the animals, which may affect the different involvement of P receptors in the vasculature [77, 78]. In Chapter 7, we dissected the coronary small arteries with diameter of ~250 μm from slaughter house swine with an age of 6 months; in Chapter 8 and 9, coronary small arteries with diameter of ~150 μm from animals with age of 4-5 months were dissected, while in Chapter 10 vessels with diameter of ~150 μm from animals with an age of 9 months were studied. Indeed, in addition to the P1 receptor involvement (Chapter 7), some P2 receptor subtypes particularly $P2X_1$ and $P2Y_1$ receptors were found to functionally contribute to Up_4A-induced vasodilation in control vessels in Chapter 8 and 9, suggesting an increased involvement of purinergic receptors in smaller vessels [78]. On the other hand, only $P2X_1$ receptors but not $P2Y_1$ were involved in this process in Chapter 10, suggesting a decreased involvement of purinergic receptors during aging [77]. Taken together, in the healthy porcine coronary vasculature, in vessels with different diameters and/or vessels of animals with different ages, the involvement of purinergic receptor subtypes in vasodilation in response to Up_4A may differ. Of note, no difference in the potency by Up_4A was observed in control vessels from Chapter 7-10, despite different involvement of P receptors.

Table 1. Vasoactive effects of Up4A in various vascular beds in different species.

	Arterial segments	Diameter	Species		Receptors	Pathway	Delivery	Reference
In vitro	Coronary artery	~250 μm	Pig	Vasodilation	P1 (A2A)	NO		Chapter 7
		~150 μm		Vasodilation	A2A, P2X1, P2X7, P2Y1			Chapter 8, 9, 10
	Aorta		Rat	Vasoconstriction	P1, P2X	L-type Ca2+ channel Rho kinase		Linder
				Vasodilation		Superoxide NO		
			Mouse	Vasoconstriction Vasodilation		Prostanoid		Hansen
	Perfused kidney		Rat	Vasoconstriction Vasodilation	P2X1, P2Y2, P2Y4 P2Y1, P2Y2	NO		Jankowski, Tolle Tolle
	Renal artery		Rat	Vasoconstriction	P2Y2, P2Y4, P2Y6	ERK		Matsumoto
	Perfused afferent arterioles		Mouse	Vasoconstriction				Jankowski
	Pulmonary artery	1-1.5 mm	Rat	Vasoconstriction	P2Y	Ca2+ (extracellular/intracellular stores)		Gui
	Mesenteric artery	<100 μm	Rat	Vasoconstriction	P2X1			Matsumoto
	Basilar artery		Rat	Vasoconstriction	P2X1, P2Y			Matsumoto
	Femoral artery		Rat	Vasoconstriction	P2X1, P2Y			Matsumoto
In vivo	Aorta		Anesthetized rat	Hypertension			Intraaotical bolus	Jankowski
	Resistance artery	<100 μm	Conscious rat	Hypotension			iv. infusion	Hansen

NO: nitric oxide; ERK: extracellular-regulated kinase.

The plasma concentrations of Up_4A are increased in juvenile hypertensive humans compared with normotensive subjects, and Up_4A concentration significantly correlates with the left ventricular mass and intima/media wall thickness in hypertensive patients [79], suggesting a role of Up_4A in the development of cardiovascular diseases. Indeed, with DOCA-hypertensive rat model, Matsumoto et al. demonstrated that Up_4A-induced vasoconstriction is enhanced in renal, basilar and femoral arteries [65, 68]. Recently, the same group observed that Up_4A-induced renal vasoconstriction is enhanced in diabetes [80]. These studies further confirm the concept that abnormal Up_4A-induced vasoconstriction is associated with vascular dysfunction seen in cardiovascular diseases [69]. Consequently, we further investigated whether the response of the coronary vasculature to Up_4A was altered in a variety of models of cardiovascular disease including MI (**Chapter 8**), pressure overload-induced left ventricular dysfunction (**Chapter 9**) as well as diabetes (**Chapter 10**). In all the diseased conditions, Up_4A did not induce vasoconstriction, but still produced vasodilation, although the vasodilator influence was blunted

several weeks after induction of MI or aortic banding (AoB). In the remote coronary vasculature after MI (**Chapter 8**), Up_4A-induced vasodilation was attenuated. Although the A_{2A}, $P2X_1$, $P2Y_1$ receptors were all shown to be involved in Up_4A-induced vasodilation in both control and MI, none of these receptors contributed to the blunted coronary vasodilation in MI. However, the reduction in the Up_4A response by P1 receptor inhibition was less in MI as compared to control suggesting that P1 receptor subtypes other than A_{2A} may account for this blunted Up_4A-induced coronary vasodilation in MI. In the coronary vasculature of the pressure overloaded left ventricle (**Chapter 9**), Up_4A-induced vasodilation was also significantly blunted. The contribution of P1 receptors to Up_4A-induced vasodilation remained unaltered; hence the blunted vasodilation mainly resulted from altered activation of P2 receptors. Moreover, the production of a prostanoid-derived vasoconstrictor in response to Up_4A in AoB has been observed. This vasoconstrictor prostanoid is like to be thromboxane (TXA), since the expression of $P2Y_{12}$ receptor was elevated in AoB (unpublished). $P2Y_{12}$ has been proposed to activate the release of thromboxane thereby resulting in vasoconstriction [81, 82]. In **Chapter 10**, streptozotocin-induced diabetes (DM) resulted in a coronary endothelial dysfunction as evidenced by an attenuated response to bradykinin (Fig. 4.) [83]. Despite blunted $P2X_7$ and NO-mediated vasodilator influences as well as an enhanced TXA-mediated vasoconstrictor influence, the coronary vasodilator response to Up_4A was fully maintained in DM swine. The latter was likely the result of an increased vasodilator influence via $P2Y_1$ and $P2Y_6$ receptors and of CYP 2C9 switching from producing vasoconstrictor to vasodilator metabolites. Similarly, given the production of TXA, $P2Y_{12}$ receptors are likely to be activated by Up_4A in DM, as speculated in Chapter 9.

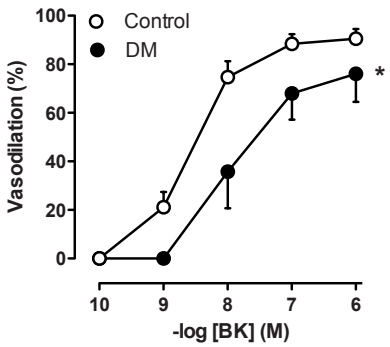

Fig. 4. *The concentration response of bradykinin in the isolated coronary small arteries in control and diabetic swine (DM). Values are mean±SEM. * P<0.05 vs. normal (unpublished).*

MI, AoB and DM are different types of diseases and their influence on the alteration of receptor involvement as well as post-receptor signaling in response to Up_4A may vary. Based on the observations from MI, AoB and DM (Chapter 8-10), different involvement of purinergic receptors and vasoactive signaling pathways has been clearly demonstrated. However, given the multiple expression of P receptor subtypes in the vasculature as well as the lack of specific receptor antagonist [74], we cannot exclude the involvement of other P receptor subtypes in the altered vascular response to Up_4A in disease. For instance, in MI, the increased mRNA expression of $P2X_4$ and $P2X_7$ may account for the maintained NO production. Moreover, the low mRNA expression of the (vasodilator) $P2Y_4$ receptor was only detected in one vessel out of seven tested vessels in MI as compared to the presence of $P2Y_4$ receptors in all five control vessels. The reduced $P2Y_4$ expression may contribute to the blunted Up_4A-mediated vasodilation. Similarly, in AoB, the results showed an altered P2 receptor subtypes involvement in blunted Up_4A-induced vasodilation. According to the unpublished data that mRNA level of $P2Y_{12}$ was elevated in AoB (0.69±0.2 in AoB vs. 0.09±0.1 in Sham), we speculate that $P2Y_{12}$ is likely to be one of those altered P2 receptor subtypes. At the post-receptor level, a vasoconstrictor prostanoid, mostly likely TXA, was involved in Up_4A-mediated vasodilation in both AoB and DM (Chapter 9 and Chapter 10). Since TXA is involved in inflammatory responses which have been shown to be present in the coronary vasculature both

after MI as well as in DM [84], it is possible that TXA observed in DM is also produced in MI and contributes to the blunted coronary vasodilation in response to Up_4A. However, this remains to be investigated in the future studies. In addition, we did not observe the involvement of CYP 2C9 metabolites in response to Up_4A in AoB, whereas vasodilator CYP 2C9 metabolites were generated in DM, suggesting an EDHF role of CYP 2C9 metabolites may appear in certain diseases to compensate for the loss of major vasodilator pathways [85]. Therefore, vasodilator CYP 2C9 metabolites may also be generated in response to Up_4A in MI contributing to the maintained NO influence, but this remains to be studied. Figure 5 summarizes an overview of the vasodilator effect of Up_4A on coronary vasomotor tone in health and disease, as well as the involvement of multiple purinergic receptor subtypes and post-receptor signaling in this process.

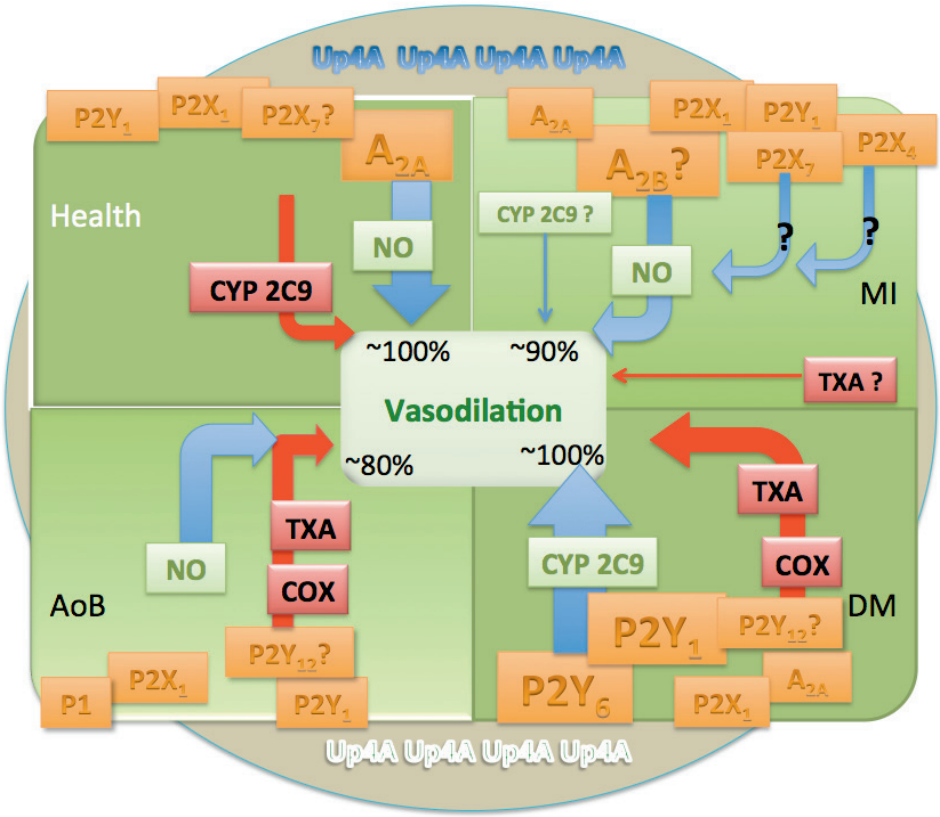

Fig. 5. *The overview of the vasodilator effect of Up_4A on the coronary microcirculation in health and disease. Blue arrow represents a vasodilator effect, whereas red arrow represents a vasoconstrictor effect.*

Our findings from Chapter 7-10 definitely yield new insights into the vasoactive influence of Up_4A on coronary vascular tone and its alterations in cardiovascular disease. However, many questions remain unresolved. For instance, what is the plasma concentration of Up_4A in swine in health and in various diseases, particularly the local Up_4A level in the heart, as can potentially be measured in coronary venous blood? What is the vasoactive effect of Up_4A in coronary circulation in vivo? Through which purinergic receptor does Up_4A activate NO, PGI_2 or CYP 2C9 in the coronary vasculature? In addition, some other research topics are of interest to investigate in the future to further elucidate the biological role of Up_4A in health and disease. For instance, which

ion channels on the smooth muscle cells are ultimately responsible for Up_4A-induced coronary vasodilation? Does Up_4A play a role in coronary hyperemia during exercise? How do human coronary arteries respond to Up_4A? Although Up_4A had no effect in healthy porcine pulmonary small arteries, it is unknown whether Up_4A can regulate pulmonary vascular tone in diseased condition, such as pulmonary hypertension. Since Up_4A can be synthesized by VEGFR2 [62] and another dinucleotide Up_4U has been recently found to be a novel endothelium-derived angiogenic factor [86], it remains to be investigated whether Up_4A is also involved in angiogenesis. Considering the fact that extracellular nucleotides are released in cancer, when the purinergic receptor expression is elevated [87], Up_4A may also play a role in cancer development. An understanding of the vascular effects of Up_4A in other cardiovascular diseases, such as atherosclerosis and stroke remains the topic of future studies.

Conclusions and clinical implications

By investigating various vasodilators and vasoconstrictors, including VEGF, CYP 2C9 metabolites, PDE5, ET and Up_4A, we found in this thesis that those vasoactive factors individually or interactively contribute to the regulation of microvascular tone in health and disease. Importantly, the study of the effect of those factors on the microvasculature in vivo and/or in vitro has brought us additional insights, which unveils a dual aspect of those factors. Thus, in Chapter 2 VEGF inhibition did not result in reduced NO bioavailability, but rather activated ET production, suggesting VEGF does not always result in vasodilator activation but may also suppress vasoconstrictor pathway. In Chapter 3 endogenous CYP 2C9 generated vasoconstrictor metabolites rather than EDHFs (as is generally assumed) in the healthy coronary microcirculation in vivo. On the other hand, depending on different experimental conditions CYP 2C9 was capable of producing both vasodilator and vasoconstrictor metabolites in response to agonist stimuli. In Chapter 4 instead of an enhancement, the coronary vasoconstrictor influence of PDE5 in the post-infarct remodeled myocardium was reduced, suggesting PDE5 inhibition may have a minimal therapeutic effect in patients with MI. In Chapter 5 and 6, ET blockade did not have an additive effect in the presence of PDE5 inhibition in both coronary and pulmonary circulations, demonstrating an inhibitory effect of cGMP on the ET system. In Chapter 7-10 Up_4A was found to be a potent vasodilator rather than vasoconstrictor in the coronary microcirculation both in health and various cardiovascular disease states. This potent vasodilator influence was mediated mainly by P1 and to a lesser extent by P2 receptors. In conclusion, a better understanding of the role of changes in endothelial function in the pathophysiology of microvascular dysfunction may help to elucidate the mechanisms underlying the development of cardiovascular disease and may help to develop new targets in the future to improve the cardiovascular health of humans.

The importance of microvascular dysfunction in the development of ischemic heart disease has been underestimated for decades. For instance, coronary macrovascular atherosclerosis has been considered the principal cause of ischemic heart disease, with clinical management dedicated to identifying and treating the coronary artery stenosis [88]. However, various lines of evidence have point towards an increasingly important role of the coronary microcirculation in the clinical presentation and prognosis of patients who have typical chest pain despite normal epicardial coronary arteries [89]. Therefore, a deep understanding of mechanisms regulating microvascular function is of critical importance in understanding its role in disease, especially because these regulatory mechanisms vary substantially across species, vascular beds and due to

comorbidities [90]. Future studies should therefore focus on the mechanisms of microvascular disease in various cardiovascular disease states, particularly in ischemic heart disease.

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Chapter 12

Samenvatting

Nederlandse samenvatting

Het endotheel speelt een belangrijke rol in de regulatie van vaattonus door het produceren van verschillende vaatverwijdende (bijv. VEGF, NO en prostacycline) en vaatvernauwende (bijv. ET, ROS en Up_4A) factoren[1]. Deze vasoactieve factoren activeren hun bijbehorende receptoren op het endotheel en/of in gladde spiercellen, die op hun beurt verschillende signaaltransductiewegen activeren. Dit leidt tot veranderingen in vaattonus en draagt bij aan de vasculaire homeostase [2].

Endotheeldysfunctie wordt gekenmerkt door een verstoring in de balans van vaatverwijdende (endothelium-derived relaxing factors, EDRFs) en vaatvernauwende factoren (endothelium-derived constricting factors, EDCFs). Endotheeldysfunctie kan bijdragen aan de pathofysiologie van diverse cardiovasculaire aandoeningen, zoals diabetes mellitus, hypertensie, atherosclerose, coronair vaatlijden en hartfalen [3]. De verstoorde balans tussen EDRFs en EDCFs wordt ook gezien als een vroege indicator voor het ontstaan van deze aandoeningen [4]. De studies beschreven in dit proefschrift zijn gericht op het onderzoeken van de dubbele rol van endotheel-afhankelijke vaatverwijders en vaatvernauwers in de regulatie van vaattonus in de gezonde vasculatuur en hun potentiële bijdrage aan (dys)regulatie in verschillende cardiovasculaire ziektemodellen.

VEGF, wat staat voor Vasculaire Endotheliale Groei Factor, is, zoals de naam aangeeft, betrokken bij vaatgroei, en speelt daarom een belangrijke rol in angiogenese (vaatnieuwvorming), en wondheling. [5, 6],Daarnaast activeert binding van VEGF aan zijn receptoren ook NO productie via eNOS en resulteert daarmee vaatverwijding [7, 8]. Een van de behandelingen van kanker bestaat uit het remmen van vaatnieuwvorming door anti-VEGF therapie, waardoor de tumor te weinig bloed krijgt, en daardoor niet kan groeien. Echter, een bijwerking van deze behandelingen in kankerpatiënten is het ontstaan van hypertensie, wat ook de belangrijkste reden is om de behandeling te onderbreken. De exacte mechanismen achter VEGF inhibitie geïnduceerde systemische hypertensie en de effecten van anti-VEGF therapie op het coronaire en pulmonale vaatbed zijn grotendeels onbekend. Een potentiële verklaring voor het optreden van hypertensie bij anti-VEGF-therapie zou kunnen zijn dat de productie van NO minder wordt[9]. Om meer inzicht te krijgen in de door Sunitinib, gebruikt in de behandeling van verschillende soorten kanker [10, 11], veroorzaakte functie veranderingen in het vaatbed hebben we in **Hoofdstuk 2** de effecten van sunitinib op het systemische, pulmonale en coronaire vaatbed onderzocht. Daarbij is vooral gekeken naar de invloed van sunitinib op de balans tussen EDRFs en EDCFs en hoe een veranderingen in deze balans kunnen bijdragen aan de ontwikkeling van hypertensie. In gezonde chronisch geïnstrumenteerde varkens zorgt sunitinib voor hypertensie door vaatvernauwing in het systemische vaatbed, zonder de vaattonus te veranderen in het pulmonale of coronaire vaatbed. In tegenstelling tot onze initiële hypothese, was sunitinib-gemedieerde systemische hypertensie voornamelijk het gevolg van een toegenomen vaatvernauwing door endotheline (ET), terwijl de invloed van zowel NO als zuurstofradicalen (ROS) onveranderd waren. Dit betekent dat de vaatverwijdende invloed van VEGF onder normale omstandigheden voornamelijk het gevolg is van het onderdrukken van ET-gemedieerde vaatvernauwing. Deze studie bevestigt dat vaatverwijding plaatsvindt als het directe gevolg van EDRF secretie, maar ook indirect door inhibitie van EDCFs.

De vaatverwijdende invloed van de bekende EDRFs NO en PGI_2 is uitgebreid bestudeerd in verschillende vaatbedden [12-14]. Zowel endogeen NO als endogeen PGI2 zorgen voor vaatverwijding in de systemische circulatie [15]. Het vaatverwijdende effect van PGI_2 wordt onderdrukt door NO, wat suggereert dat er een interactie tussen deze twee EDRFs bestaat. Hoewel NO en PGI_2 kunnen worden beschouwd als de belangrijkste EDRFs [14], blijft er na remming van deze twee systemen nog steeds endotheel-afhankelijke vaatverwijding aanwezig. Deze vaatverwijding suggereert het bestaan van andere endotheel-afhankelijke vaatverwijdende systemen [16]. Een aantal van deze systemen veroorzaakt opening van K^+ -kanalen in het membraan van de gladde spiercellen in de vaatwand. Deze opening van K^+-kanalen verandert de membraanpotentiaal van de gladde spiercellen, wat hyperpolarisatie van deze cellen veroorzaakt. Deze factoren worden dan ook endotheel-afhankelijke hyperpolariserende factoren (EDHFs) genoemd [16]. Een van deze EDHFs wordt geproduceerd door het enzym cytochroom P450 2C9 (CYP 2C9), in respons op activatie van bepaalde receptoren op de endotheel cellen [17, 18]. Er is echter niet bekend of deze CYP 2C9 metabolieten ook een rol spelen in de regulatie van coronaire vaatweerstand tijdens inspanning. In **Hoofdstuk 3** bestudeerden we de endogene invloeden van CYP 2C9 metabolieten op de coronaire vaatweerstand in ons chronisch geïnstrumenteerde varkensmodel en in geïsoleerde coronaire microvaatjes. In plaats van de verwachte vaatverwijding, veroorzaakte CYP 2C9 metabolieten vaatvernauwing in de coronairen van onze varkens zowel tijdens rust als inspanning. Deze vaatvernauwing werd versterkt door de gecombineerde blokkade van de productie van NO en PGI_2. Farmacologische activatie van CYP 2C9 door toedienen van bradykinine zorgde voor vaatverwijding *in vivo*, maar in CYP 2C9-gemedieerde vaatvernauwing geïsoleerde coronaire microvaatjes. Deze resultaten zijn een duidelijke illustratie van het duale gezicht van systemen die de vaattonus regelen en laten zien dat de productie van vasoactieve stoffen sterk afhankelijk is van de experimentele omstandigheden.

NO-gemedieerde vaatverwijding leidt tot activatie van het enzym soluable gunaylyl cyclase (sGC) in de gladde spiercellen. Dit enzym produceert cGMP, een signaalstof die leidt tot vaatverwijding. cGMP heeft een zeer korte levensduur, omdat het direct wordt gehydrolyseerd door het enzym phosphodiesterase (PDE) 5 [19]. De activiteit van PDE5 bepaalt de hoeveelheid cGMP en dus de hoeveelheid vaatverwijding. Het evenwicht tussen productie en afbraak van cGMP in coronaire vasculaire gladde spiercellen is een cruciale determinant van de coronaire vaattonus. Aangezien er weinig bekend is over de rol van PDE5 in het coronaire vaatbed, en hoe deze rol verandert na een myocard infarct (MI), bestudeerden wij in **Hoofdstuk 4** de rol van PDE5 in coronaire vaattonus regulatie in gezonde varkens en varkens met een MI, zowel tijdens rust als tijdens inspanning. In gezonde varkens resulteerde PDE5 inhibitie vaatverwijding tijdens rust en inspanning. In varkens met een MI was de PDE5 genexpressie en de vaatverwijding als gevolg van PDE5 blokkade sterk afgenomen in de coronaire vaten. In de coronairen van gezonde varkens werd de door PDE5 remming geinduceerde vaatverwijding minder na blokkade van de aanmaak van NO, terwijl dit niet het geval was in varkens met een myocard infarct (**Hoofdstuk 4**). Eerdere data uit ons laboratorium lieten ook zien dat vaatvernauwing door andere vaatvernauwende factoren (endotheline en angiotensine) is afgenomen in het geremodelleerde myocard na een MI [19-21]. Dit suggereert dat het laten afnemen van de invloed van vaatvernauwende systemen een mechanisme is om de coronaire perfusie te handhaven in het geremodelleerde myocard na een MI.

Eerdere studies van ons laboratorium toonden aan dat coronaire vaatverwijding tijdens inspanning voor een groot deel wordt gemedieerd door de productie van NO en de inhibitie van ET [19, 22]. Deze twee systemen staan niet op zichzelf, maar er is een interactie tussen deze twee systemen aanwezig tijdens inspanning [19]. Verder toonden onze studies aan dat NO, in samenspraak met PGI_2, het vaatvernauwende effect van ET remt tijdens inspanning. Dit verminderde effect van ET is vooral het gevolg van een verminderde productie, terwijl de ET receptor gevoeligheid niet lijkt te veranderen [23]. Het was echter niet duidelijk of het remmende effect van NO op ET gemedieerd werd door cGMP, en daarmee of dit effect eventueel versterkt zou kunnen worden door PDE5 blokkade. De interactie tussen PDE5 blokkade en het ET systeem in de coronaire circulatie werd verder onderzocht in **Hoofdstuk 5**. Zowel tijdens rust als inspanning zorgde ET blokkade niet voor extra vaatverwijding als PDE5 al geremd was. In kleine geïsoleerde coronaire arteriolen toonden we aan dat dit gebrek aan additionele vaatverwijding het gevolg was van een verlies in ET productie en niet vanwege verminderde ET receptor gevoeligheid.

Uit meerdere studies is gebleken dat PDE5 blokkers (bijv. Sildenafil) en ET receptor blokkers (bijv Bosentan) zorgen voor vaatverwijding [24-27]. Deze medicijnen zijn daarom ook een veel gebruikt therapeutisch hulpmiddel bij de behandeling van patiënten met hartfalen of pulmonaal hypertensie [25-27]. Er is weinig bekend over de mogelijke toegevoegde waarde van de combinatie van deze twee therapieën bij de behandeling van pulmonale hypertensie [25]. In **Hoofdstuk 6** onderzochten wij daarom de mogelijke interactie tussen PDE5 remming en endotheline receptor blokkade in het pulmonale vaatbed. Vergelijkbaar met de resultaten in het coronaire vaatbed, zorgde PDE5 blokkade voor een afname van het effect van ET blokkade zowel *in vivo* als *in vitro*. In tegenstelling tot het coronaire vaatbed, vindt de interactie tussen het NO-cGMP systeem en het ET systeem in het pulmonale vaatbed zowel op productie- als receptorniveau plaats.

In de vorige studies onderzochten wij de interacties tussen verschillende bekende EDRFs en EDCFs in verscheidene vaatbedden en onder zowel zieke als gezonde condities. In **Hoofdstukken 7-10** is gekeken naar een nieuwe vasoactieve factor met de naam Up_4A.

Up_4A is geïdentificeerd als een andere krachtige EDCF [28]. Deze factor kan worden gesynthetiseerd door VEGFR2 in de endotheelcellen [29]. Net als andere nucleotiden, oefent Up4A zijn vasoactieve invloed uit door het activeren van purinerge receptoren [30]. Omdat Up_4A zowel purine en pyrimidine groepen bevat kan deze stof zowel P1 als P2 receptoren activeren en zo vaattonus beïnvloeden [28, 30]. De rol van Up_4A is onderzocht in het verschillende vaatbedden, waaronder de nierslagaders [28], de pulmonaire slagaders [31, 32], de aorta [33, 34] en de mesenterische slagaders [35]. Uit deze studies werd geconcludeerd dat Up_4A een EDCF is, die vaatvernauwing veroorzaakt via activatie van de de P2 receptoren [36]. Hoewel Up_4A initieel beschreven werd anls een EDCF, kan deze stof ook vaatverwijding veroorzaken van zowel de aorta [33] als in de geïsoleerde geperfundeerde nier [37], maar alleen wanneer de basale vaattonus verhoogd was.
In deze studies was nog niet gekeken naar de effecten van Up_4A op het coronaire vaatbed. In **Hoofdstuk 7** onderzochten we de vasoactieve rol van Up_4A in gezonde coronaire bloedvaten van varkens. Up_4A resulteert in de coronairen van varkens voor potente vaatverwijding. Deze vaatverwijding werd veroorzaakt door activatie van purinerge P1 (A_{2A}) , en niet P2-receptoren. De vaatverwijding veroorzaakt door Up_4A is ten dele endotheel-afhankelijk en wordt gemedieerd door NO en PGI_2 terwijl CYP 2C9 metabolieten niet bijdragen aan het vaatverwijdende effect van Up_4A.

De plasmaconcentratie van Up_4A is verhoogd bij jonge mensen met een verhoogde bloeddruk en de Up_4A concentratie heeft een significante correlatie met de linker ventrikel massa en dikte van de vaatwand in deze patiënten [38]. Dit suggereert een rol voor Up_4A in de ontwikkeling van cardiovasculaire ziekten. Matsumoto et al. toonde dan ook aan dat Up_4A geïnduceerde vaatvernauwing wordt versterkt in nier , basilaire en femorale slagaders in een DOCA-hypertensief rat model [32, 35]. Dezelfde groep ontdekte recent dat Up_4A geïnduceerde renale vaatvernauwing versterkt is bij een diermodel voor diabetes [39]. Deze studies bevestigden dat abnormale Up_4A geïnduceerde vaatvernauwing kan worden geassocieerd met vasculaire dysfunctie bij verschillende cardiovasculaire aandoeningen [36]. In dit proefschrift hebben we verder onderzocht of de respons van Up_4A in het coronaire vaatbed werd veranderd door verschillende modellen van cardiovasculaire ziekten, zoals MI (**Hoofdstuk 8**), drukoverbelasting-geïnduceerde linker ventrikel dysfunctie als gevolg van aortic banding (AoB) (**Hoofdstuk 9**) en diabetes (**Hoofdstuk 10**).
In deze drie verschillende varkensmodellen voor cardiovasculaire ziekten zorgde Up_4A nog steeds voor coronaire vaatverwijding, ook al was de vaatverwijdende invloed verminderd ten gevolge van MI of AoB. In **Hoofdstuk 8** hebben we onderzocht welke receptoren mogelijkerwijs betrokken zijn bij de veranderde respons op Up_4A. Hoewel we de verantwoordelijke receptor nit hebbn kunnen identificeren, hebben we uitgesloten dat vermindering van dit effect werd veroorzaakt door een verandering in de betrokkenheid van A_{2A} , $P2X_1$ of $P2Y_1$ receptoren.

In de coronaire vaten van varkens met AoB (**Hoofdstuk 9**), was Up_4A geïnduceerde vaatverwijding ook aanzienlijk verminderd. De bijdrage van P1 receptoren aan Up_4A-geïnduceerde vaatverwijding is ongewijzigd gebleven, vandaar dat de gereduceerde vaatverwijding vooral het gevolg moet zijn van veranderde activatie van P2-receptoren. In het AoB model werd in respons op Up_4A de productie van een prostanoide-afgeleide vaatvernauwer geobserveerd. Deze vaatvernauwer is waarschijnlijk thromboxaan (TXA), aangezien de expressie van $P2Y_{12}$ receptoren was toegenomen na AoB (ongepubliceerde data). IN de literatuur is er een verband aangetoond tussen activatie van $P2Y_{12}$ receptoren en de productie van TXA wat vervolgens leidt tot vaatvernauwing [40, 41].
Ook in het derde ziektemodel, streptozotocine-geïnduceerde diabetes (DM), werd gekeken naar de respons op Up_4A. In dit model is coronaire endotheel dysfunctie aanwezig, geïllustreerd door een verzwakte respons op bradykinine (**Hoofdstuk 10**). Hoewel de coronaire verwijding in respons op Up_4A niet veranderd was in DM varkens, bleek de betrokkenheid van de verschillende purinerge receptoren wel veranderd. De vaatverwijdende invloed van de $P2X_7$-receptor was verminderd, terwijl de vaatverwijdende invloed via $P2Y_1$ en $P2Y_6$ receptoren was toegenomen. Daarbij was ook de betrokkenheid van de verschillende EDRFs en EDCFs veranderd; NO was niet langer betrokken bij de vaatverwijding in respons op Up_4A in DM, terwijl CYP 2C9 vaatverwijdende metabolieten produceerde in DM en vaatvernauwende metabolieten in controle vaten. Daarnaast bleek dat Up_4A in varkens met DM resulteerde in productie van TXA, wat vasoconstrictie geeft.

Samen tonen de resultaten uit **dit proefschrift** dat vaattonusregulatie een samenspel is van vaatvernauwers en vaatverwijders. De scheiding tussen vaatvernauwers en vaatverwijders is niet zo strikt. Afhankelijk van de omstandigheden kunnen bepaalde enzymen zoals eNOS en CYP 2C9 zowel vaatverwijders als vaatvernauwers produceren. Daarnaast zijn er vasoactieve stoffen die afhankelijk van de omstandigheden en het vaatbed zowel vaatverwijding als vaatvernauwing kunnen geven. In dit proefschrift is gekeken naar zowel bekende als nieuwe vasoactieve

factoren, met een bijzonder focus op de coronaire microvasculatuur. Omdat vele cardiovasculaire ziekten vaattonus beïnvloeden hebben we het effect van belangrijke cardiovasculaire ziekten op de acties van deze vasoactieve factoren onderzocht. Onze studies laten zien dat resultaten verkregen in een bepaald vaatbed in een bepaalde diersoort niet zonder meer geëxtrapoleerd kunnen worden naar een andere diersoort, een ander vaatbed, of zelfs naar andere omstandigheden (*in vivo* vs *in vitro*, gezonde vaten vs zieke vaten). Dit proefschrift draagt bij aan het begrip over de complexe interacties tussen vaatvernauwers en vaatverwijders en hun controle van microvasculaire tonus tijdens gezondheid en ziekte. Het beschreven onderzoek zou kunnen bijdragen aan het identificeren en ontwikkelen van therapeutische targets om cardiovasculaire ziekten te kunnen bestrijden.

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PhD Portfolio

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Promotor: *Prof.dr. D.J.G.M. Duncker*
Supervisor: *Dr. D. Merkus*

PhD training	Year	ECTs
Academic Skill		
Experimental Animal Course	2010	3
In-Depth Courses		
NHS Course Vascular Biology	2011	2
NHS Course Cardiac Function and Adaptation	2010	2
COEUR Courses (6 courses)	2009-2011	9
Cardiac Anatomy Course	2010	0.2
Presentations		
International Symposium on Mechanisms of Vasodilation (MOVD)	2013	0.5
Invited Lecture at Luzhou Medical College	2013	0.5
American Heart Association Scientific Session	2012	0.5
International Conference of Physiological Science (ICPS)	2012	0.5
Joint Meeting of German Microcirculation and Vascular Biology (GfMVB)	2012	0.5
International Conferences		
MOVD, Zurich, Switzerland	2013	1
Experimental Biology, Boston, USA	2013	1
Experimental Biology, San Diego, USA	2012	1
AHA Scientific Session, Los Angeles, USA	2012	1
ICPS, Suzhou, China	2012	1
GfMVB, Mannheim, Germany	2012	1
Seminars and Symposiums		
COEUR Seminars	2009-2013	4.5
COEUR PhD Day	2010-2011	0.8
Erasmus Symposiums	2009-2012	2.6
Erasmus Lectures	2009-2012	0.6

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Zhou Z, de Wijs-Meijler D, Lankhuizen I, Jankwski J, Jankowski V, Danser AH, Duncker DJ, Merkus D. Blunted coronary vasodilator response to uridine adenosine tetraphosphate in post-infarct remodeled myocardium due to reduced P1 receptor activation. *Pharmacol Res*. 2013 Aug;77:22-29

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** Contributed equally*

Abstract

Zhou Z, Sorop O, de Beer VJ, Heinonen I, Danser AH, Merkus D, Duncker DJ. Altered coronary endothelial signalling in response to uridine adenosine tetraphosphate in diabetes. *Eur Heart J*. 2013 in press

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Curriculum Vitae

Zhichao Zhou (周稚超) was born on November 17th 1981 in Zigong, Sichuan province, China. From 2001 to 2007, he studied Medicine at North Sichuan Medical College in Nanchong, finalized with a one and a half year internship at The Second People' Hospital in Chengdu. He graduated with the honor of "Excellent Medical Graduate" in 2007. By comparing the clinical work during his internship at the hospital to basic research that he had performed at the early phase of medical study, Zhichao found that the latter one would be more interesting and may be more suitable for his future. With a high score on the entrance examination for the graduate study, he was selected by The Laboratory of Cardiovascular Diseases under the supervision of Prof.dr. Xin to study the estrogen-mediated protection in cardiac hypertrophy at Sichuan University in Chengdu in 2007. Awarded the national grant of "Government Graduate Student Overseas Study Program" in 2009, Zhichao joined the Experimental Cardiology Division, Department of Cardiology, at Erasmus MC in the Netherlands as a PhD candidate. Under the supervision of Prof.dr. Duncker and Dr. Merkus, he performed research on the topic of "Coronary Blood Flow Regulation in Health and Disease". In the later phase of his PhD study, he focused on the role of purinergic receptor in the regulation of coronary vasomotor tone in health and various cardiovascular diseases.

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