Tissue angiotensin II

A matter of location

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Tissue angiotensin II: A matter of location

Thesis, Erasmus University, Rotterdam. With summary in Dutch.

ISBN 90-77017-88-7

An electronic version of this thesis is available in Adobe(R) PDF format on the following internet address: www.eur.nl/fgg/pharm/

Printed by [Optima] Grafische Communicatie, Rotterdam

Cover illustration: 'Swans reflecting elephants' by Salvador Dali.

Tissue angiotensin II: A matter of location

Weefsel angiotensine II: Een kwestie van lokatie

Proefschrift

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus
Prof. dr. ir. J.H. van Bemmel
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
donderdag 19 juni 2002 om 15.45 uur

door

Martin Paul Schuijt
geboren te Amersfoort

Promotiecommissie

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Financial support by the Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged.

Financial support by the following companies and foundations is gratefully acknowledged:
Novartis Pharma
AstraZeneca
Solvay Pharmaceuticals
Bristol-Meyers Squibb
J.E. Jurriaanse Stichting
Jacques H. de Jong Stichting
Dutch Kidney Foundation
Boehringer Ingelheim
Aurora Borealis
Merck Sharp & Dohme

Voor Marinke

...ich brauche keine Formel. Ich habe das Rezept in meiner Nase.

Das Parfum: Die Geschichte eines Mörderes

Patrick Süskind

Contents

Abbreviations

ACE	angiotensin-converting enzyme
Ang	angiotensin
ANP	atrial natriuretic peptide
AT_1	angiotensin II type 1
AT_2	angiotensin II type 2
CO	cardiac output
CRC	concentration response curve
EDA	end-diastolic cross-sectional area
EF	ejection fraction
ESA	end-systolic cross-sectional area
HPLC	high performance liquid chromatrography
HR	heart rate
LADCA	left anterior descending coronary artery
LCXCA	left circumflex coronary artery
LV	left ventricle/ventricular
MAP	mean arterial blood pressure
MI	myocardial infarction/infarcted
M6P/IGFII	mannose 6-phosphate/insulin-like growth factor II
MVC	myocardial vascular conductance
L-NAME	N^G-nitro-L-arginine methyl ester
NO	nitric oxide
NOS	nitric oxide synthase
RAS	renin-angiotensin system
RV	right ventricle/ventricular
RVC	renal vascular conductance

Chapter 1

General introduction

Based on: Cardiac angiotensin II: an intracrine hormone? *Am J Hypertens*. 2002; in press

Clinical and experimental data obtained with renin-angiotensin system (RAS) blockers show that their beneficial effects on cardiac structure and function are independent,[1,2] at least in part, of their blood pressure lowering effects. It has therefore been suggested that angiotensin (Ang) II is synthesized locally in the heart, independently of its generation in the circulation. Such local synthesis may occur in the interstitial space, on the cell surface or within cardiac cells (Figure 1). It is still highly controversial to what degree all components required for local Ang II generation are actually produced in the heart. In this chapter we briefly describe the most important effects of Ang II in the heart and then focus on its synthesis at cardiac tissue sites, in order to unravel whether Ang II in the heart acts as an para-, auto- and/or intracrine hormone.

Cardiac angiotensin II: its function

Ang II is believed to be an important modulator of cardiac growth and function. It induces cell growth via stimulation of Ang II type 1 (AT_1) receptors.[3,4] In agreement with this concept, myocardial hypertrophy was observed in transgenic animals overexpressing AT_1 receptors on cardiomyocytes,[5-7] and diminished left ventricular remodeling occurred after myocardial infarction in AT_1 receptor knockout mice.[8] However, not all studies in transgenic animals agree on this matter. For instance, the pressure overload-induced cardiac hypertrophy in AT_1 receptor knockout mice was not different from that in wildtype mice,[9] indicating that in this model AT_1 receptor-mediated Ang II signaling is not essential for the development of hypertrophy. Furthermore, in mice overexpressing AT_1 receptors on cardiomyocytes, cardiac hypertrophy was either already present at birth, thereby resulting in prenatal lethality,[6] or started after 6 weeks only, and then led to early death due to development of heart failure.[5] A detailed analysis of the degree of AT_1 receptor stimulation (i.e., of the cardiac Ang II levels) in utero and after birth, and of the efficiency of the Ang II-induced activation of signaling pathways in the two mice strains that were applied in these overexpression studies will help to explain why AT_1 receptor-dependent cardiac hypertrophy occurs either at an early or a late stage.

Stimulation of Ang II type 2 (AT_2) receptors is believed to inhibit cell growth,[3,4,10-12] although a recent study in AT_2 receptor knockout mice demonstrated that AT_2 receptors are essential for the development of left ventricular hypertrophy in Ang II-induced hypertension.[13] Similarly conflicting data have been obtained with regard to apoptosis. In cultured vascular smooth muscle cells[14] and fibroblasts[15] AT_2 receptor activation results in apoptosis, whereas in

cardiomyocytes apoptosis is attributed to AT_1 receptors.[16,17] In support of the latter, apoptosis was unaltered in hearts of mice overexpressing AT_2 receptors on cardiomyocytes,[18] and AT_1 receptor blockade reduced the number of apoptotic cells in spontaneously hypertensive rats.[17] Thus, the respective roles of AT_1 and AT_2 receptors with regard to the effects of Ang II on cardiac growth and apoptosis are at present not entirely clear and may differ both between cells and species.

The receptor responsible for the effects of Ang II on cardiac contractility is less controversial. Both the positive and negative inotropic effects of Ang II are mediated via AT_1 receptors,[19-21] either directly or indirectly, via facilitation of norepinephrine release from noradrenergic nerve endings.[22-24] Furthermore, the Ang II-induced positive chronotropic effects are also mediated via AT_1 receptors.[25] AT_2 receptors may counteract the latter effect, since Ang II infusion in transgenic mice overexpressing AT_2 receptors on cardiomyocytes resulted in bradycardia.[26]

Finally, it is generally accepted that Ang II constricts coronary arteries exclusively via AT_1 receptors.[27,28] Although studies with coronary arteries[29] did not support a role for AT_2 receptors in Ang II-mediated vasoactive effects, recent *in-vivo* studies[30,31] have shown that AT_2 receptors antagonize AT_1 receptor-dependent vasoconstriction, thus suggesting that AT_2 receptors induce vasodilation in the coronary vascular bed.

Cardiac angiotensin II: its generation

Renin

In agreement with the virtual absence of renin mRNA in the heart under normal and pathological conditions,[32,33] renin could not be demonstrated in myocardial tissue of nephrectomized animals.[34,35] Cardiac renin is therefore most likely kidney-derived.[33-35] Renin diffuses into the interstitial space,[36-38] and may bind to renin receptors on the cell surface.[39] These receptors, one of which is the mannose 6-phosphate/insulin-like growth factor II (M6P/IGFII) receptor, do not appear to make a distinction between renin and its inactive precursor, prorenin. Remarkably, (pro)renin binding to M6P/IGFII receptors on cardiomyocytes is followed by rapid internalization and, in the case of prorenin, proteolytic activation to renin,[40] thus providing a basis for intracellular angiotensin generation.

The above *in-vivo* data contrast with studies demonstrating renin by Western blot in cultured, serum-deprived cardiomyocytes.[41,42] There are several explanations for this

discrepancy. Cultured cardiac cells may not be representative for cardiac cells *in vivo*, the cells may have sequestered (pro)renin from the serum-containing medium during culturing,[43] or the antibodies may have recognized renin-like enzymes like cathepsin. In support of the latter, using a specific rat renin inhibitor, we were unable to demonstrate renin-dependent Ang I-generating activity in neonatal rat cardiomyocytes,[43] while Katz et al.[44] recently showed that cathepsin D is fully responsible for Ang I generation in adult rat cardiomyocytes. Finally, the renin demonstrated by Western blot could represent a recently discovered second renin transcript that lacks the coding sequence for the prefragment[45] (i.e., that cannot be targeted to the secretory pathway and thus remains intracellular). This renin is expressed in rat hearts,[45] although it is currently not known whether it is catalytically active.

Angiotensinogen

The lack of angiotensinogen release from the isolated perfused rat heart[46,47] and the high correlation between myocardial and plasma angiotensinogen levels,[48] both under normal and pathological conditions, strongly suggest that myocardial angiotensinogen is largely blood- (and thus liver-) derived. Myocardial angiotensinogen levels, expressed per g tissue, are within the range expected based on the assumption that plasma angiotensinogen diffuses freely into the interstitial compartment.[34,47,49] The rise in renin in the infarcted and failing heart may explain why cardiac angiotensinogen under these conditions decreases rather than increases. *In vitro*, cardiomyocytes have been reported to release angiotensinogen,[41,42] although not all investigators confirmed this finding.[43,50] Moreover, cardiac angiotensinogen mRNA levels are low or undetectable.[51] Taken together therefore, local synthesis of angiotensinogen contributes marginally, if at all, to cardiac angiotensinogen levels.

Unlike renin, angiotensinogen does not bind to cardiac membranes,[34,39] nor has evidence been obtained to support angiotensinogen internalization.[52] These findings, combined with the fact that angiotensinogen-synthesizing cells normally release angiotensinogen into the medium, without storing it intracellularly,[53,54] suggest that angiotensinogen is restricted to the extracellular compartment. Without intracellular angiotensinogen, neither the alternative renin transcript mentioned above nor internalized activated prorenin may have a functional role. In support of this concept, evidence for intracellular Ang II generation in rat hepatoma cells was only obtained following transfection of the cells with a mutated angiotensinogen cDNA that produces a nonsecreted form of angiotensinogen.[55]

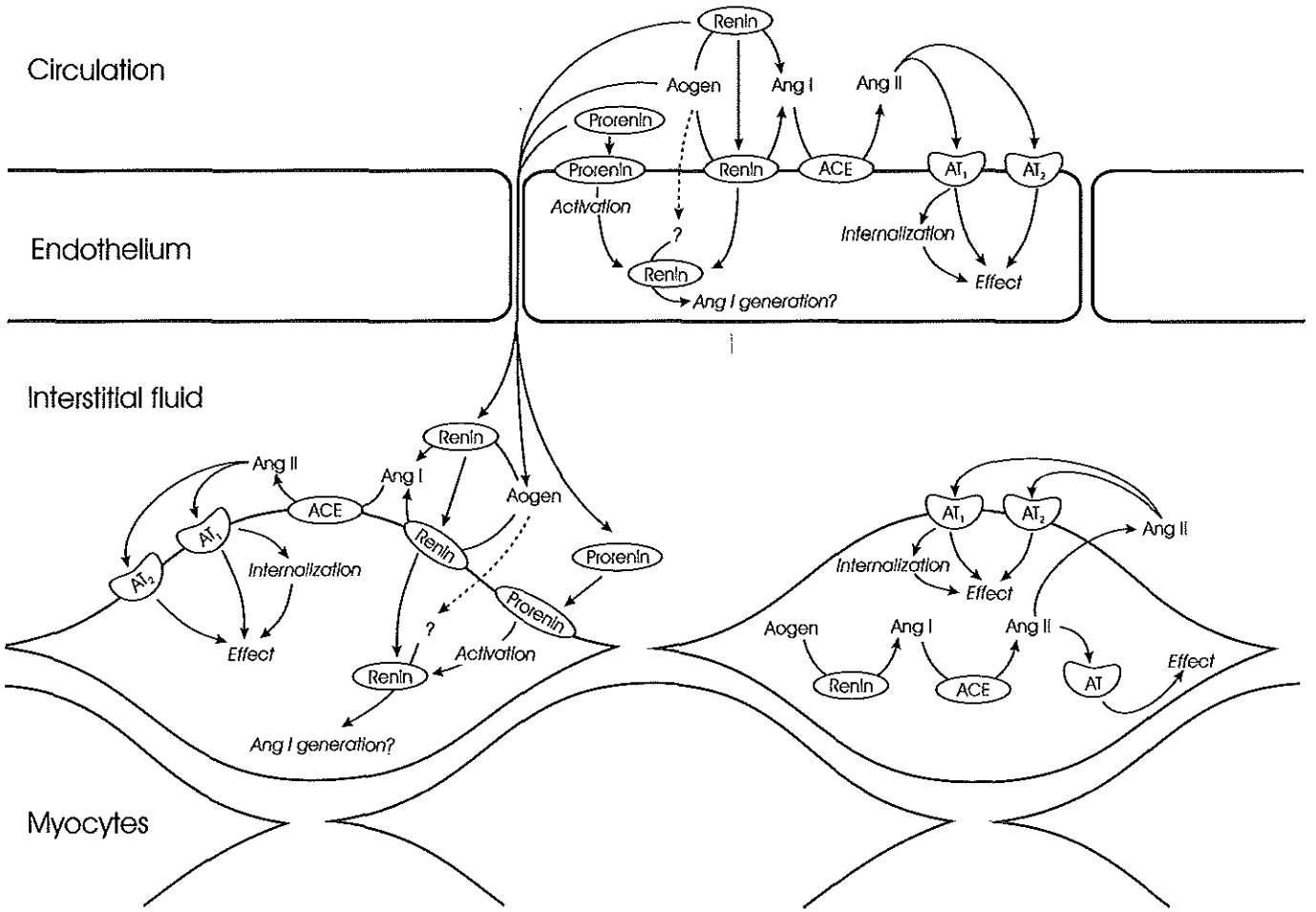

Figure 1. Possible angiotensin generation in the heart. Ang, angiotensin; Aogen, angiotensinogen: AT, angiotensin II receptor.

ACE and chymase

Cardiac ACE is not limited to endothelial cells. Particularly under pathological conditions ACE has also been shown on cardiomyocytes, macrophages and fibroblasts.[56] Although it is well accepted that ACE is a membrane-associated enzyme, the question arises whether ACE might also act intracellularly. Dostal et al.[57] reported intensive staining for ACE protein in the perinuclear region of rat cardiomyocytes. This finding, however, does not necessarily imply that ACE contributes to intracellular Ang II production; it may simply illustrate ACE synthesis in cardiomyocytes.[43] Normally, ACE is directed to the cell membrane,[58] where it is anchored with its active domain located extracellularly. Beldent et al.[58] were unable to measure ACE activity in the cytosol. Furthermore, ACE inhibition did not affect stretch-induced release of Ang II from intracellular storage sites in cardiomyocytes,[50] suggesting that these Ang II storage sites do not depend on intra- (or even extra-) cellular ACE. In contrast, enalaprilat blocked the decrease of junctional conductance induced by intracellularly-applied Ang I in rat cardiomyocytes.[59] However, the latter may have been due to a nonspecific effect of enalaprilat, since the ACE inhibitor also affected conductance in hamster cardiomyocytes in the absence of Ang I.[60]

Finally, *in-vitro* studies in cardiac homogenates have provided ample evidence for Ang I-II conversion by a serine protease, chymase.[61] Remarkably, however, chymase is stored in mast cells and it is therefore unlikely that chymase is a contributor to Ang II production by cardiomyocytes. Moreover, although its *in-vivo* importance still needs to be investigated, α_1-antitrypsin acts as an endogenous inhibitor of any chymase that is released into the interstitium.[62,63] Thus, it is not surprising that studies applying Ang I to the interstitium of intact dog and rat hearts observed ACE-dependent Ang I-II conversion only.[64,65]

Angiotensin I and II

The presence of both renin and angiotensinogen in cardiac tissue allows local angiotensin production in the heart. Indeed, studies using systemic infusions of ^{125}I-labeled Ang I to correct for uptake from plasma, have demonstrated that the majority of cardiac Ang I and II is not derived from the circulation, but is synthesized locally.[34,66,67] The most likely site for cardiac Ang I production is the interstitial space. First, as discussed above, there is little evidence for the intracellular presence of angiotensinogen and ACE. Second, both *in-vitro* and *in-vivo* studies have shown that cardiac Ang I, unlike cardiac Ang II, is present in extracellular fluid only.[47,67] Thus, renin and angiotensinogen may either react in the interstitial fluid or, in the case of membrane-bound renin, on the cell surface. Membrane-associated renin

displays increased catalytic activity as compared to soluble renin.[68] Ang I generated on the cell surface by renin may be rapidly converted to Ang II by membrane-bound ACE, and such efficient local Ang II generation might explain why similar Ang II-mediated effects are observed during application of Ang II, Ang I or renin combined with angiotensinogen to coronary arteries and cardiomyocytes, despite the fact that the extracellular Ang II levels required to induce effects during the latter two applications are 100-fold lower than during Ang II application.[29,69]

Support for intracellular Ang II synthesis is derived from studies reporting that Ang II is present in cardiomyocytes,[57,70,71] that intracellularly applied Ang II exerts effects,[59,60,72] and that cultured cardiomyocytes release Ang II into the medium.[42,50,73] How should this be reconciled with the above line of reasoning on extracellular Ang I generation? First, Ang II is internalized via AT_1 receptor-mediated endocytosis[74] and this may explain its intracellular presence. In fact, most or all tissue Ang II is cell-associated, suggesting that if Ang II is generated extracellularly, it is immediately internalized via AT_1 receptors,[36,67,75] for instance because ACE and AT_1 receptors are colocalized.[76] Second, at least part of the Ang II-mediated effects depend on internalization of the Ang II-AT_1 receptor complex[77] and thus it is not surprising that cytosolic application of Ang II, via binding to internalized AT receptors, may exert effects. The mere fact that intracellularly applied Ang II exerts effects, of course, does not prove that Ang II is synthesized in the cell. Third, the question arises whether internalized Ang II can be released again. This could explain the stretch-induced release of Ang II that has been demonstrated by

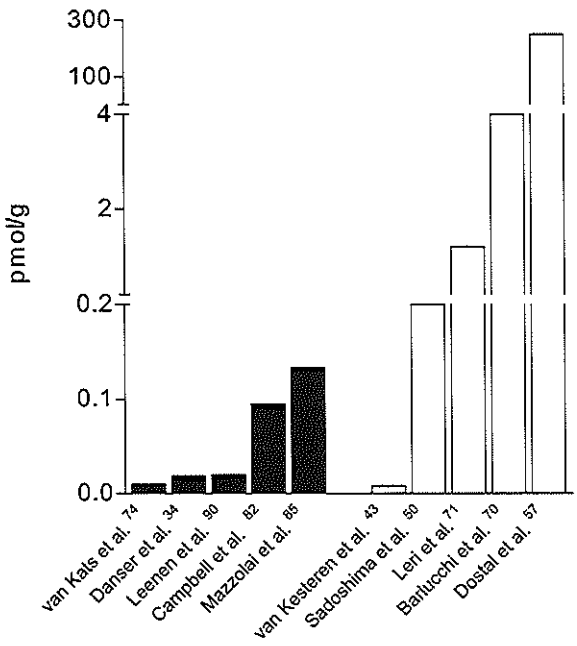

Figure 2. Angiotensin II levels in whole heart in vivo (black bars) and in cultured myocytes (open bars) under control conditions.

some,[50,78] but not all investigators.[43] *In vivo*, internalized Ang II disappears with a half-life of 10-15 min, in full agreement with the concept of lysosomal degradation of internalized Ang II.[74] In cultured cardiomyocytes the Ang II half-life is much longer[3] and thus, if the cells are loaded with Ang II during their exposure to serum-containing (i.e. renin- and angiotensinogen-containing) medium this Ang II might be released again in the absence of serum, in particular after stretch. However, Ang II levels in serum[43] are in the order of fmol/ml, and thus an argument against this concept are the exceptionally high Ang II levels that have been demonstrated by some investigators[71,78,79] in cardiomyocytes and cardiomyocyte-conditioned medium (Figure 2). Since the volume of a cardiomyocyte[79] is ~25.000 μm^3, it can be calculated, assuming that 1 ml volume corresponds with approximately 1 g weight,[34] that cultured cardiomyocytes contain up to 250 pmol Ang II per g[43,50,57,70,71] (Figure 2) and secrete 0.2-15 pmol Ang II/g per hour[78,79] during basal conditions. This is several orders of magnitude higher than the Ang II levels reported in myocardial tissue *in vivo*[34,66,80] (Figure 2). Not all studies, however, reported such high levels[43,81] and part of this discrepancy may be due to the fact that angiotensins were measured by direct radioimmunoassay (i.e., without prior purification and/or separation from material crossreacting with the Ang I and Ang II antibodies applied in these assays).

Transgenic experiments

Male transgenic rats with liver-specific overexpression of rat prorenin (resulting in a 500-fold rise in plasma prorenin) displayed severe cardiac hypertrophy.[83] This hypertrophy occurred in the absence of hypertension or a rise in plasma renin activity, in agreement with the concept that circulating prorenin, following its uptake in the heart, is capable of exerting toxic effects locally. Unfortunately, this study did not address cardiac prorenin-renin conversion or circulating (pro)renin-induced angiotensin generation in cardiac tissue. Prescott et al.[84] investigated the latter by mating transgenic mice that release human active renin from the liver with transgenic mice expressing human angiotensinogen in the heart. Double-transgenic mice displayed cardiac fibrosis and a substantial rise in the cardiac content of both Ang I and II, without a change in the circulating levels of these peptides. These data are therefore direct evidence that circulating renin contributes to the synthesis of functionally important Ang II in the heart.

Further support for Ang II generation at cardiac tissue sites comes from studies in transgenic mice overexpressing rat angiotensinogen in cardiomyocytes.[85] Plasma angiotensin levels were not altered in these mice, whereas cardiac Ang I and II were 4- and 2-fold

elevated, respectively, as compared to control mice. Importantly, both ACE inhibition and AT_1 receptor blockade prevented or normalized ventricular hypertrophy in the transgenic mice, confirming that the angiotensinogen-induced effects depend on AT_1 receptor stimulation by Ang II.

Finally, although transgenic rats overexpressing cardiac ACE 40-fold had normal Ang II levels,[86] such overexpression did result in cardiac hypertrophy and fibrosis.[87] These data therefore confirm that cardiac ACE is not rate-limiting for Ang II generation, and suggest that interference with other ACE substrates (e.g., bradykinin) may underlie the functional consequences of cardiac ACE overexpression.

Remarkably, in contrast with the studies supporting a growth-stimulatory role for Ang II released from cultured cardiomyocytes,[50,79] transgenic mice expressing an Ang II-producing fusion protein in cardiomyocytes did not develop cardiac hypertrophy.[88] Although intracellular cleavage of the fusion protein by furin led to Ang II secretion into the interstitial space, thereby raising cardiac Ang II 20- to 40-fold, the authors only observed a modest fibrosis in the hearts of these transgenic mice. Cardiac hypertrophy did occurr when replacing Ang II by a degradation-resistant analogue that resulted in much higher cardiac Ang II levels (>600-fold higher) and subsequent Ang II overflow into the circulation. These data suggest that extracellular production of Ang II, for instance on the cell surface in the immediate vicinity of AT_1 receptors by membrane-bound renin and ACE, is much more efficient than intracellular Ang II synthesis, since Ang II-dependent cardiac remodeling in non-transgenic animals occurs at cardiac Ang II levels that are maximally 2- to 3-fold elevated as compared to control.[89-91]

References are presented in the general reference list.

Aim of the thesis

Whether cardiac Ang II generation occurs in an auto-, para-, or intracrine manner as well as whether Ang II stimulation of the AT_2 receptor mediates vasodilation, has not yet been thoroughly investigated. Therefore, we performed *in-vivo* studies in rats and pigs and *in-vitro* studies in porcine isolated vessels with the following aims:

1. To study the vasoactive role of AT_2 receptors under normal and pathological conditions, as well as to investigate which vasodilator compounds counterbalance Ang II-mediated vasoconstriction.

2. To determine the site of local Ang II formation, and the enzyme(s) (ACE and/or chymase) involved in its generation.

To address issue 1, we made use of the radioactive microsphere method under various conditions, which allowed us to measure blood flow in all organs of one animal. In normal anesthetized rats, blood flow was measured during Ang II infusion in the absence or presence of antagonists at AT_1 or AT_2 receptors (chapter 2) and following inhibition of nitric oxide synthase or cyclooxygenase (chapter 3). Similar studies were performed in rats 4 weeks after coronary artery ligation, i.e, at the time when AT_2 receptors are upregulated (chapter 4). To address issue 2, a detailed analysis of cardiac angiotensin generation was made in myocardial infarcted pigs with and without RAS blockade (chapter 5). Interstitial Ang I and Ang II levels were measured in porcine hearts *in vivo*, using the microdialysis technique, (chapter 6) and in isolated porcine arteries (chapters 7). Finally, the enzyme responsible for interstitial Ang I-to-Ang II conversion was determined using a modified version of the rat Langendorff heart, which allows separate collection of coronary effluent and interstitial fluid (chapter 8).

Chapter 2

No vasoactive role of the angiotensin II type 2 receptor in normotensive Wistar rats

J Hypertens. 1999; 17: 1879-1884

Summary

To investigate the vasoactive consequences of angiotensin (Ang) II type 2 (AT_2) receptor stimulation *in vivo*, three consecutive 10-min intravenous infusion of Ang II (100, 300 and 1000 ng/kg/min) were given to 20 pentobarbital-anesthesized normotensive Wistar rats (weight 330±6 g, mean±SEM). The rats had been pretreated with saline (n=8), the Ang II type 1 (AT_1) receptor antagonist, irbesartan (100 μg/kg/min for 30 min, n=6), or the AT_2 receptor antagonist, PD123319 (20 μg/kg/min for 30 min followed by continuous infusion throughout the entire experiment, n=6). Regional hemodynamic effects of Ang II were studied using the radioactive microsphere method. Ang II increased mean arterial blood pressure (MAP) and heart rate by maximally 44±9 and 26±6%, respectively (P<0.05 compared with baseline), and decreased cardiac output and systemic vascular conductance (=cardiac output/MAP) by maximally 24±8 and 47±4%, respectively (P<0.05 compared with baseline). The Ang II-induced decrease in systemic vascular conductance was caused by decreases in vascular conductances (regional flow/MAP) of the gastrointestinal tract (52±4%), kidney (63±3%), skeletal muscle (39±8%), skin (63±4%), mesentery+pancreas (32±11%), adrenal (27±11%) and spleen (57±6%) (all P<0.05 compared with baseline). Irbesartan increased baseline vascular conductances in adrenal, brain and kidney, and inhibited all hemodynamic responses induced by Ang II. PD123319 affected neither baseline values, nor the Ang II-induced hemodynamic responses. In conlusion, Ang II-induced systemic and regional hemodynamic effects in normotensive Wistar rats are mediated exclusively via AT_1 receptors. No evidence for AT_2 receptor-mediated vasoactive responses was obtained.

Introduction

Angiotensin (Ang) II, the active end-product of the renin-angiotensin system, is a potent vasoconstrictor with growth-promoting properties. Its effects are mediated by specific receptors, of which two major subtypes, termed AT_1 and AT_2 have been characterized to date.[92] The AT_1 receptor subtype appears to mediate essentially all of the known effects of Ang II, including the mentioned above; a physiological role for the AT_2 receptor has not yet been clearly established. Initially it was believed that this receptor existed in fetal tissue only and that it disappeared after birth.[93-95] However, more recent studies support its existence in adult animals also.[96-101] On the basis of its abundant presence in fetal animals, it has been speculated that the AT_2 receptor is involved in the regulation of growth and development. Indeed, in isolated cells, AT_2 receptor stimulation results in growth inhibition and apoptosis,[3,15,102] thereby antagonizing the growth-stimulatory effects that are mediated via AT_1 receptors.

AT_2 receptor knockout mice display a significant increase in blood pressure or an increased sensitivity to the pressor action of Ang II, or both,[103,104] which suggests that AT_2 receptors may cause vasodilation. In support of this concept, Arima *et al.*[105] and Endo *et al.*[106] demonstrated AT_2 receptor-dependent vasodilation in isolated microperfused rabbit afferent and efferent glomerular arterioles. In the intact rat however, AT_2 receptor-mediated blood pressure decreasing effects have not been found by all investigators;[107-111] indeed, in some cases, AT_2 receptor-mediated increases in blood pressure were reported.[111-113] These discrepancies may be explained by the short half-life of PD123319,[114] (the AT_2 receptor antagonist that was applied in most studies), *in-vivo* biotransformation of PD123319 to a metabolite with AT_1 receptor antagonist properties,[113] or displacement of non-specifically bound AT_1 receptor antagonist by AT_2 receptor antagonists in studies where both types of receptors were combined.[112]

It was our aim in the present study to investigate the systemic and regional hemodynamic effects of AT_2 receptor stimulation in the rat, using the radiolabeled microsphere method.[115,116] Injection of radiolabeled microspheres, which are trapped in end-arterioles because of their size, allows one to obtain detailed information on the regional hemodynamic effects of Ang II, both in the presence and absence of AT receptor antagonists. By using microspheres with different isotope labels it is possible to perform several measurements in one animal. Our working hypothesis was that Ang II causes vasorelaxation via AT_2 receptors and vasoconstriction via AT_1 receptors. Consequently, we expected enhanced vasoconstrictor

responses to Ang II in rats that were pretreated with the AT_2 receptor antagonist, PD123319, and vasodilator responses to Ang II in animals that were pretreated with the AT_1 receptor antagonist, irbesartan.

Materials and Methods

General procedures

All experiments were performed in accordance with the 'Guiding Principles in the Care and Use of Animals' approved by the American Physiological Society and under the regulation of the Animal Care Committee of the Erasmus University Rotterdam, Rotterdam, The Netherlands. Experiments were carried out in 28 male Wistar rats (body weight: 331±5g, mean±SEM) obtained from Harlan, Zeist, The Netherlands. Animals were anesthetized with an intraperitonial injection of sodium pentobarbital (60 mg/kg; Apharma, Arnhem, The Netherlands). To maintain an adequate depth of anesthesia, intravenous bolus injections of sodium pentobarbital (5-10 mg/kg) were administered via the right external jugular vein every 15 min during the stabilization period. A catheter was placed in the trachea for intermittent positive pressure ventilation with a mixture of oxygen and air, using a ventilatory pump (Small Animal Ventilator, Harvard Apparatus, Natick, MA, USA). The ventilatory rate was adjusted to keep arterial blood gases within the physiological range. Blood pressure and heart rate were recorded with a pressure transducer (Combitrans Disposable Pressure Transducer, Braun, Melsungen, Germany) in the left femoral artery. Radioactive microspheres were injected into the left ventricle via a catheter in the right carotid artery. Drugs were administered via the right external jugular vein. The right femoral artery was cannulated to allow the withdrawal of reference blood samples.

Experimental protocol

After a 1-h stabilization period after completion of instrumentation, the animals were given a 30-min infusion of the AT_1 receptor antagonist, irbesartan (100 μg/kg/min; n=6), the AT_2 receptor antagonist, PD123319 (20 μg/kg/min; n=6) or vehicle (saline, 0.1 ml/min; n=8). Next, three consecutive 10 min infusions of Ang II (100, 300 and 1000 ng/kg/min) were given to each animal. At the end of each Ang II infusion, when a steady state had been reached, hemodynamic parameters were measured and the distribution of aortic blood flow was determined by injecting radioactive microspheres (see below). In view of the short half-life of PD123319,[114] the administration of this drug (20 μg/kg/min) was continued during the Ang II

infusions. Hemodynamic parameters and the distribution of aortic blood flow were also measured in rats that were pretreated with a 30-min infusion of vehicle and then given three consecutive 10-min infusions of vehicle. The results revealed and excellent stability of the preparation (n=8, data not shown), in agreement with previous studies.[115]

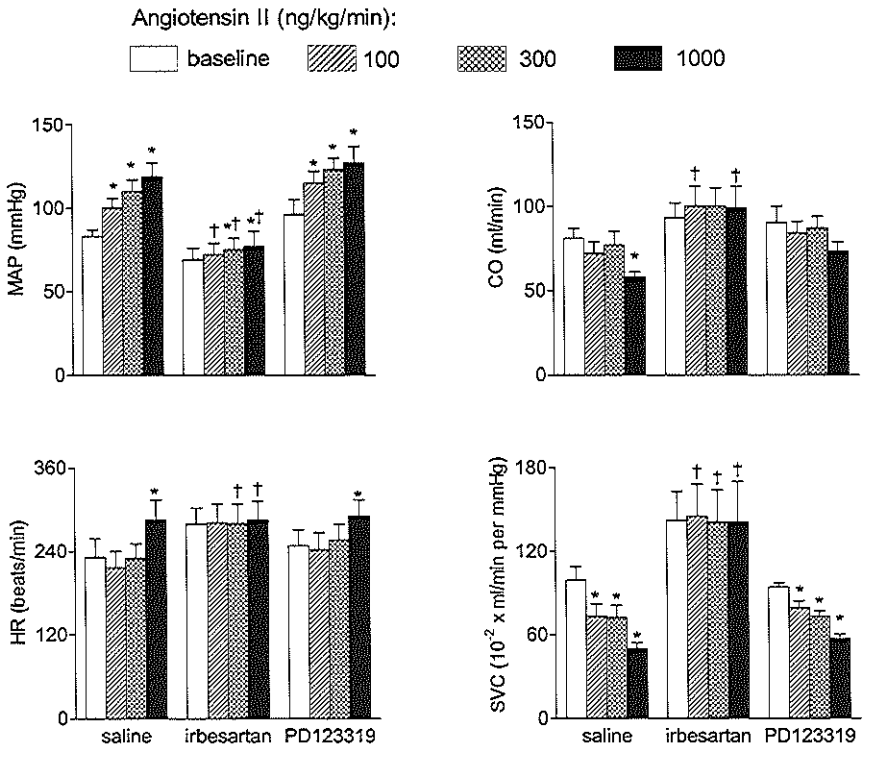

Figure 1. Effects of 10-min intravenous infusions of Ang II on mean arterial blood pressure (MAP), cardiac output (CO), heart rate (HR) and systemic vascular conductance (SVC) in anesthesized rats pretreated with saline (0.1 ml/min; n=8), irbesartan (100 µg/kg/min; n=6) or PD123319 (20 µg/kg/min; n=6). Values are mean±SEM. Significant differences (P<0.05): * compared with baseline, † compared with corresponding dose in saline-pretreated animals.

Distribution of cardiac output

The distribution of aortic blood flow was determined with 15.5±0.1 µm diameter (mean±SD) microspheres labeled with ^{141}Ce, ^{103}Ru, ^{95}Nb or ^{46}Sc (NEN Dupont, Boston, MA, USA). For each measurement about 200,000 microspheres, suspended in 0.2 ml saline and labeled with one of the isotopes, were mixed and injected into the left ventricle over a 15-s period. After

each injection, the catheter was flushed thoroughly with 0.5 ml saline. Starting 10 s before microsphere injection and lasting 70 s, an arterial reference blood sample was drawn from the left femoral artery at a constant rate of 0.5 ml/min, using a withdrawal pump (Model 55, Harvard Apparatus). At the end of the experiment, the animal was killed with an overdose of pentobarbital and all tissues and organs were removed, weighed and put into vials. The following tissues were dissected: gastrointestinal tract, kidney, skeletal muscle, skin, mesentery+pancreas (for practical reasons, these two tissues were not studied separately), adrenal, brain, liver, heart, spleen and lungs. Lungs were not evaluated further, because the amount of radioactivity in these organs represents microspheres that have bypassed the peripheral vascular beds (via arteriovenous anastomoses with a diameter larger than that of the microspheres), rather than pulmonary flow.[116] The radioactivity in the reference blood samples and the tissues was counted for 5 min in a γ-scintillation counter (Packard, Minaxi Auto-Gamma 5000 series, Downers Grove, IL, USA), using suitable windows discriminating the different isotopes (^{141}Ce: 120-167 KeV; ^{103}Ru: 450-548 KeV; ^{95}Nb: 706-829 KeV; ^{46}Sc: 830-965 KeV).

Drugs

Irbesartan was a kind gift of Bristol-Myers Squibb, Princeton, NJ, USA. PD123319 was a kind gift of Parke-Davis, Natick, MA, USA. Irbesartan (330 μg/ml) was dissolved in 1.2 mM KOH as described by Trippodo et al.[117] PD123319 (70 μg/ml) and Ang II (0.33, 0.99 and 3.30 μg/ml) were dissolved in saline.

Data presentation and statistical analysis

Data were processed as described previously.[118] Cardiac output and regional blood flow were calculated as follows:

$$\text{cardiac output} = \frac{\text{amount of radioactivity injected} \times \text{withdrawal rate of arterial blood sample}}{\text{radioactivity of arterial blood sample}}$$

$$\text{regional blood flow} = \frac{\text{tissue radioactivity} \times \text{cardiac output}}{\text{amount of radioactivity injected}}$$

Systemic and regional vascular conductances (i.e., cardiac output and regional blood flow corrected for mean arterial blood pressure) were calculated to quantify the vasoconstrictor effects of Ang II with or without its receptor antagonists.

All data are presented as mean±SEM. Duncan's new multiple range test was used to test differences from baseline when two-way ANOVA had revealed that differences existed between the consecutive infusions. Student's unpaired *t*-test was used to evaluate the effects of the AT receptor antagonists when two-way repeated measures ANOVA followed by Bonferroni's correction had revealed differences between the groups. Statistical significance was accepted at $P<0.05$ (two-tailed).

Results

Systemic hemodynamic effects

Mean arterial blood pressure, cardiac output, heart rate and systemic vascular conductance were not affected by acute administration of irbesartan or PD123319 (Figure 1). Ang II increased mean arterial blood pressure and heart rate by maximally 44±9 and 26±6%, respectively ($P<0.05$ compared with baseline), and decreased cardiac output and systemic vascular conductance by maximally 24±8 and 47±4%, respectively ($P<0.05$ compared with baseline). Irbesartan reduced or abolished the effects of Ang II, whereas PD123319 did not affect any of the Ang II-induced systemic hemodynamic responses.

Regional hemodynamic effects

Acute administration of irbesartan increased the vascular conductance in adrenal, brain and kidney. Regional vascular conductances were not affected by acute administration of PD123319 (Figure 2). Ang II decreased vascular conductance in gastrointestinal tract, kidney, skeletal muscle, skin, mesentery+pancreas, adrenal and spleen (by maximally 52±4, 63±3, 39±8, 63±4, 32±11, 27±11 and 57±6%, respectively; all $P<0.05$ compared with baseline). No Ang II-induced effects on vascular conductance were observed in brain, liver and heart. This was most probably because of the effects of autoregulatory compensatory mechanisms in these organs. Irbesartan reduced or abolished the effects of Ang II, whereas PD123319 did not affect any of the Ang II-induced regional hemodynamic responses.

Discussion

The findings of the present study does not support a systemic or regional vasoactive role for AT_2 receptors; all hemodynamic responses observed were mediated exclusively via AT_1 receptors. We observed neither enhanced vasoconstriction in response to Ang II during AT_2 receptor blockade with PD123319, nor Ang II-induced vasorelaxation during AT_1 receptor blockade with irbesartan. Thus, our results are not in agreement with the concept of a vasodilatory AT_2 receptor, which has been proposed on the basis of AT_2 receptor knockout experiments in mice.[103,104]

We used the radiolabeled microsphere method, because it allows one to obtain detailed *in-vivo* information on the regional hemodynamic effects of a

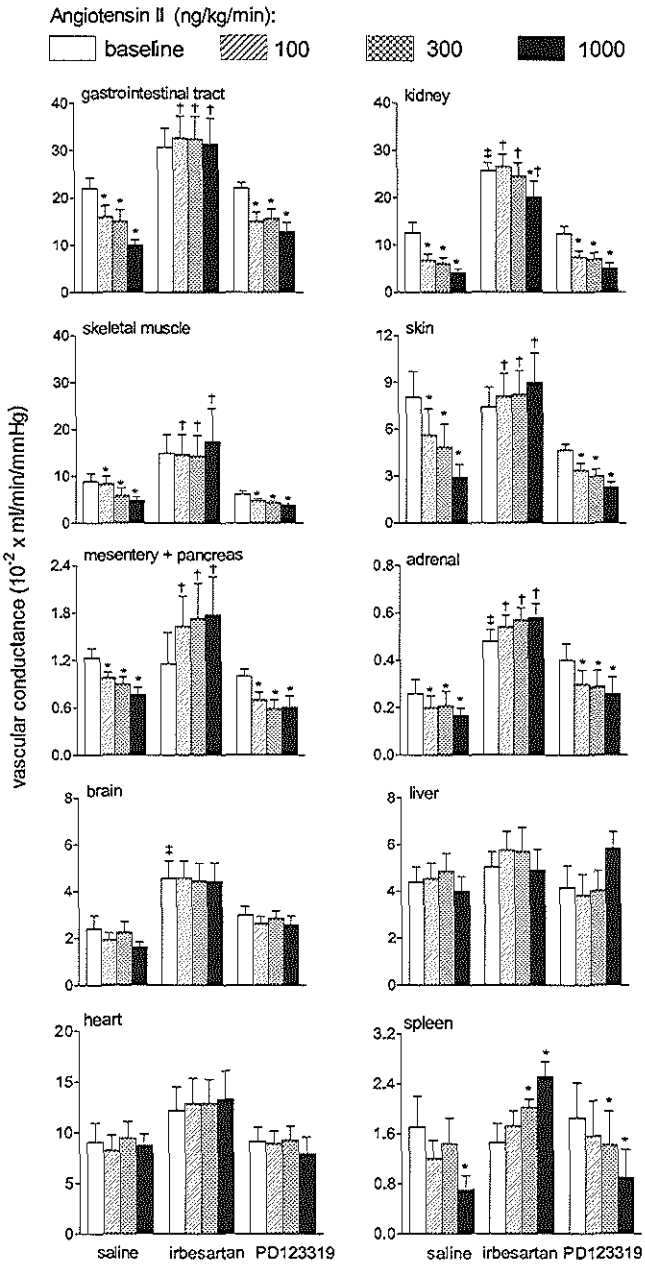

Figure 2. Effects of 10 min intravenous infusions of Ang II on regional vascular conductance in anesthesized rats pretreated with saline (0.1 ml/min; n=8), irbesartan (100 µg/kg/min; n=6) or PD123319 (20 µg/kg/min); n=6). Values are mean±SEM. Significant differences (P<0.05): * compared with baseline, † compared with corresponding dose in saline-pretreated animals, ‡ compared with baseline of saline-pretreated animals.

vasoactive peptide such as Ang II. Until now, this information was not available, because previous investigations were limited either to the systemic hemodynamic effects of Ang II,[107-110] or to its effects in one particular vascular bed.[105,106]

The dose of PD123319 that was applied in the present study was sufficient to result in micromolar blood plasma concentrations of the agent[114] - that is, concentrations that selectively block AT_2 receptors. Higher doses have been reported to lead to concentrations that also interfere with AT_1 receptors.[114] PD123319 was infused throughout the entire experiment in view of its short *in-vivo* half-life. Munzenmaier and Greene[109] and Ohkubo *et al.*[12] have demonstrated that PD123319, when given in doses similar to those applied in the present study, blocks the growth-inhibitory effects that are mediated by AT_2 receptors. Thus it is unlikely that insufficient AT_2 receptor blockade was the cause of our failure to observe an AT_2 receptor-mediated response.

Are AT_2 receptors present in adult rats? Originally, it was believed that AT_2 receptors disappeared after birth,[93-95] to return only under pathological conditions.[119,120] However, numerous studies have now established that these receptors are also normally present in the brain,[96] kidney,[105,121] heart[97,98] and vascular wall[101] of adult rats. Moreover, AT_2 receptors have been demonstrated in the heart and kidney of adult humans.[99,100,122,123] Stimulation of AT_2 receptors in the kidney and the vascular wall results in the release of bradykinin, nitric oxide and cGMP[101,121,124] - a mechanism that may well explain the vasorelaxation ascribed to this receptor. Remarkably, however, and in agreement with the present study, the AT_2 receptor-mediated increase in aortic cGMP levels found in stroke-prone spontaneously hypertensive rats was not accompanied by a reduction in blood pressure.

Pretreatment with the AT_1 receptor antagonist, irbesartan, in a dose that is selective for AT_1 receptors,[125-127] increased vascular conductance in the kidney, adrenal, and brain. This demonstrates the importance of endogenous Ang II as a regulator of vascular tone in anesthesized rats. Interestingly, in the spleen Ang II increased vascular conductance (i.e., it caused vasorelaxation) in the presence of irbesartan. This effect was not mediated via AT_2 receptors, as the Ang II-induced effects with PD123319 were indistinguishable from those without PD123319. Possibly, vasorelaxant Ang-(1-7) receptors, stimulated by either Ang II or its metabolite, Ang-(1-7), were involved in this effect.[128]

In agreement with previous findings,[129] Ang II increased heart rate via AT_1 receptors. Masaki *et al.*[26] reported that overexpression of AT_2 receptors in the mouse heart attenuated this AT_1 receptor-mediated chronotropic response. We were unable to confirm this finding in the present study.

In conclusion, the Ang II-induced systemic and regional hemodynamic effects in normotensive Wistar rats are mediated exclusively via AT_1 receptors. No evidence for AT_2 receptor-mediated vasoactive responses was obtained during Ang II infusion.

References are presented in the general reference list.

Chapter 3

Prostanoids, but not nitric oxide, counterregulate angiotensin II mediated vasoconstriction in vivo

Eur J Pharmacol. 2001; 428: 331-336

Summary

To evaluate the modulating effects of nitric oxide and prostanoids during angiotensin (Ang) II-mediated vasoconstriction, male Wistar rats (n=25) were infused with increasing doses of Ang II following pretreatment with the cyclooxygenase inhibitor indomethacin, the nitric oxide-synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) plus sodium nitroprusside to restore mean arterial blood pressure, or saline. Hemodynamics were studied with the radioactive microsphere method. Indomethacin did not alter systemic or regional hemodynamics. L-NAME + sodium nitroprusside reduced cardiac output, as well as systemic and renal vascular conductance. Ang II increased mean arterial blood pressure and heart rate, and decreased systemic vascular conductance as well as vascular conductance in gastrointestinal tract, kidney, skeletal muscle, skin, mesentery+pancreas, spleen and adrenal. Indomethacin enhanced the Ang II-mediated effects in all vascular beds, whereas L-NAME + sodium nitroprusside enhanced its effect in mesentery+pancreas only. In conclusion, vasodilator prostanoids, but not nitric oxide, counterregulate Ang II-mediated vasoconstriction *in vivo*.

Introduction

Angiotensin (Ang) II induces vasoconstriction via stimulation of Ang II type 1 (AT_1) receptors. Simultaneous vasodilation via stimulation of the Ang II type 2 (AT_2) receptor[130] may counteract this effect, although not all studies agree on this matter.[131,132] In addition, vasodilator factors, such as nitric oxide (NO) and vasodilator prostanoids, may oppose the vasoconstrictor effects of Ang II. These factors are either released in response to vasoconstriction or via stimulation of AT_1 and/or AT_2 receptors on endothelial and vascular smooth muscle cells.[133-137]

In support of this concept, NO-synthase (NOS) inhibition enhanced Ang II-induced vasoconstriction systemically as well as in the carotid artery and kidney.[107,138] However, studies with NOS inhibitors have to be interpreted with care, because of the persistent rise in blood pressure which occurs during NOS inhibition. Indeed, an enhanced effect of Ang II was not observed in the kidney when NO was normalized by application of a NO donor during renal NOS inhibition[139-141] Furthermore, Ang II infusion is accompanied by a rise in the levels of prostacyclin and/or prostaglandin E_2 in blood plasma[133] and interstitial fluid.[134,142] Receptor-mediated release of such vasodilator prostanoids may explain why Ang II induces vasodilation in cerebral[143] and mesenteric[144] arteries. However, Ang II also stimulates the release of vasoconstrictor prostanoids (e.g. thromboxane A_2).[145] Moreover, the cyclooxygenase inhibitor indomethacin attenuated the blood pressure lowering effects of both captopril and losartan, suggesting that blockade of Ang II synthesis or AT_1 receptors is also accompanied by release of vasodilator prostanoids.[146]

In view of these discrepancies, it was the aim of the present study to investigate the role of NO and cyclooxygenase products in the Ang II-induced systemic and regional hemodynamic effects *in vivo*, using the radiolabeled microsphere method.[132] By measuring radioactivity of microspheres trapped in end-arterioles detailed information on systemic hemodynamics as well as regional hemodynamics in all organs can be obtained, thereby extending previous studies on this isssue that were either limited to the systemic hemodynamic effects of Ang II, or its effect in one particular vascular bed.

Materials and Methods

Instrumentation and hemodynamic measurements

All experiments were performed in accordance with the 'Guiding principles in the care and use of animals' approved by the American Physiological Society and under the regulation of the Animal Care Committee of the Erasmus University Rotterdam, Rotterdam, The Netherlands. Experiments were carried out in male Wistar rats (body weight: 364±7 g, mean±SEM; n=25) obtained from Harlan, Zeist, The Netherlands.

Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg). To maintain an adequate depth of anesthesia, intravenous bolus injections of sodium pentobarbital (5-10 mg/kg) were administered via the right external jugular vein every 15 min during the stabilization period. A catheter was placed in the trachea for intermittent positive pressure ventilation with a mixture of oxygen and air, using a respiratory pump (Small Animal Ventilator, Harvard Apparatus, Natick, MA, USA). The ventilatory rate was adjusted to keep arterial blood gases within the physiological range. Blood pressure and heart rate were recorded with a pressure transducer (Combitrans Disposable Pressure Transducer, Braun, Melsungen, Germany) in the left femoral artery. Radioactive microspheres were injected into the left ventricle via a catheter in the right carotid artery. Drugs were administered via the right external jugular vein. The right femoral artery was cannulated to allow the withdrawal of reference blood samples.

After a 1-h stabilization period following completion of instrumentation, the animals were given a 30-min infusion of the cyclooxygenase inhibitor indomethacin (5 mg/kg; n=8), the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 10 mg/kg; n=8) followed by a continuous infusion of sodium nitroprusside at a rate required (approximately 4 µg/kg/min) to restore mean arterial blood pressure to baseline, or vehicle (saline, 0.1 ml/min; n=9).

Next, three consecutive 10-min infusions of Ang II (100, 300 and 1000 ng/kg/min) were given to each animal. Prior to the Ang II infusions (baseline) and at the end of each Ang II infusion, when a steady state had been reached, hemodynamic parameters were measured and the distribution of aortic blood flow was determined by injecting 15.5±0.1 (mean±SD) µm diameter microspheres labeled with ^{141}Ce, ^{103}Ru, ^{95}Nb or ^{113}Sn (NEN Dupont, Boston, MA, USA). For each measurement about 200,000 microspheres, suspended in 0.2 ml saline and labeled with one of the isotopes, were mixed and injected into the left ventricle over a 15-s period. Following each injection, the catheter was thoroughly flushed with 0.5 ml saline. Starting 10 s before microsphere injection and lasting 70 s, an arterial reference blood sample

was drawn from the right femoral artery at a constant rate of 0.5 ml/min, using a withdrawal pump (Model 55, Harvard Apparatus, Natick, MA, USA). At the end of the experiment the animal was sacrificed with an overdose of pentobarbital and all tissues were removed, weighed and put into vials. The radioactivity in the reference blood samples and the tissues was counted for 5 min in a γ-scintillation counter (Packard, Minaxi Auto-Gamma 5000 series, Downers Grove, IL, USA), using suitable windows discriminating the different isotopes. Lungs and heart were not evaluated, because the amount of radioactivity in the lungs mainly represents accumulation of microspheres that have bypassed the peripheral vascular beds,[118] whereas radioactivity in the heart represents both coronary flow and microspheres entrapped in the trabeculae during injection into the left ventricle. The latter may lead to falsely high values of coronary flow,[147] but will only marginally affect cardiac output (the sum of all tissue radioactivities) since it accounts for less than 2 % of total body radioactivity.

Drugs

Indomethacin, L-NAME and sodium nitroprusside were obtained from Sigma (St. Louis, MO, USA) and Ang II was from Bachem Feinchemikalien AG (Bubendorf, Switzerland). Indomethacin (4 mg/ml) was dissolved in phosphate buffered saline (140 mM NaCl, 2.6 mM KCl, 1.4 mM KH_2PO_4 and 8.2 mM Na_2HPO_4) containing 7 mM NaOH and titrated to pH 7.4 with 6 M HCl. L-NAME (27 mg/ml), sodium nitroprusside (40 μg/ml) and Ang II (0.33, 0.99 and 3.30 μg/ml) were dissolved in saline.

Data presentation and statistical analysis

Data were processed as described previously.[118] Cardiac output and regional blood flow were calculated as follows:

$$\text{cardiac output} = \frac{\text{amount of radioactivity injected} \times \text{withdrawal rate of arterial blood sample}}{\text{radioactivity of arterial blood sample}}$$

$$\text{regional blood flow} = \frac{\text{tissue radioactivity} \times \text{cardiac output}}{\text{amount of radioactivity injected}}$$

Systemic and regional vascular conductances (i.e., cardiac output and regional blood flow corrected for mean arterial blood pressure) were calculated to quantify the vasoconstrictor effects of Ang II with or without inhibition of NOS or cyclooxygenase.

All data are presented as mean±SEM. Duncan's new multiple range test was used to test differences from baseline, once a two-way analysis of variance (ANOVA) had revealed that differences existed between the consecutive infusions. Student's unpaired *t*-test was used to evaluate the effects of the inhibitors, once two-way repeated measures ANOVA followed by Bonferroni's correction had revealed differences between the groups. Statistical significance was accepted at $P<0.05$ (two-tailed).

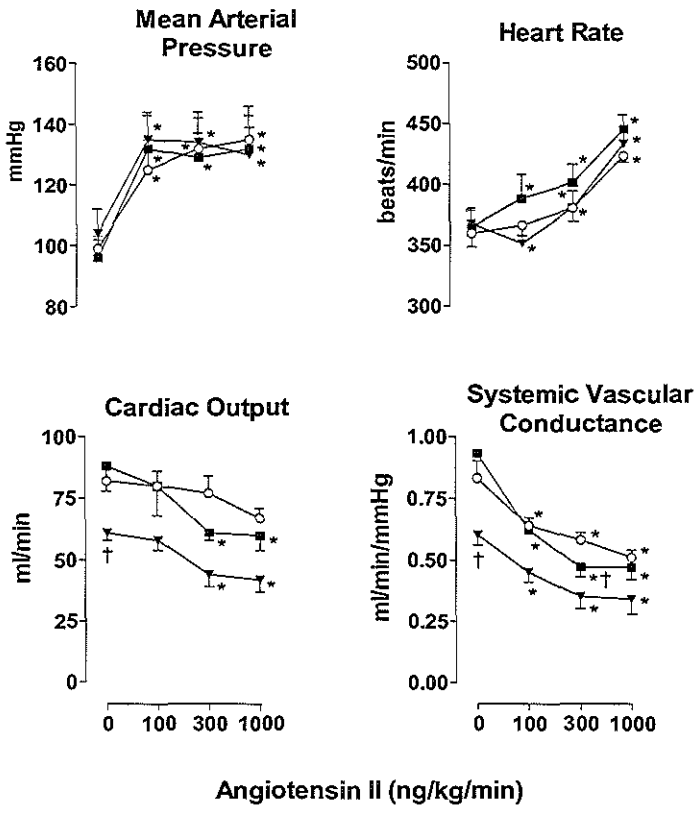

Figure 1. Effects of 10-min intravenous infusions of angiotensin II on systemic hemodynamic parameters in rats pretreated with vehicle (0.1 ml/min; *n*=9; open circles), indomethacin (5 mg/kg; *n*=8; closed squares) or L-NAME (10 mg/kg; followed by a continuous infusion of sodium nitroprusside to restore mean arterial blood pressure to pre-L-NAME level; *n*=8; closed triangles). Values are mean±SEM. * $P<0.05$ vs. baseline, † $P<0.05$ vs. vehicle.

Results

Systemic hemodynamic effects (Figure 1)

Mean arterial blood pressure, cardiac output, heart rate and systemic vascular conductance were not affected by indomethacin. L-NAME + sodium nitroprusside reduced cardiac output and systemic vascular conductance by 26% and 28%, respectively ($P<0.05$), but did not affect mean arterial blood pressure and heart rate. Ang II increased mean arterial blood pressure and heart rate by maximally 35±7% and 18±3%, respectively, and decreased systemic vascular conductance by maximally 36±7%. Cardiac output was not affected by Ang II. Neither indomethacin nor L-NAME + sodium nitroprusside affected any of the Ang II-induced systemic hemodynamic effects on mean arterial blood pressure, heart rate or cardiac output. Indomethacin, but not L-NAME + sodium nitroprusside, augmented the Ang II-induced decrease in systemic vascular conductance.

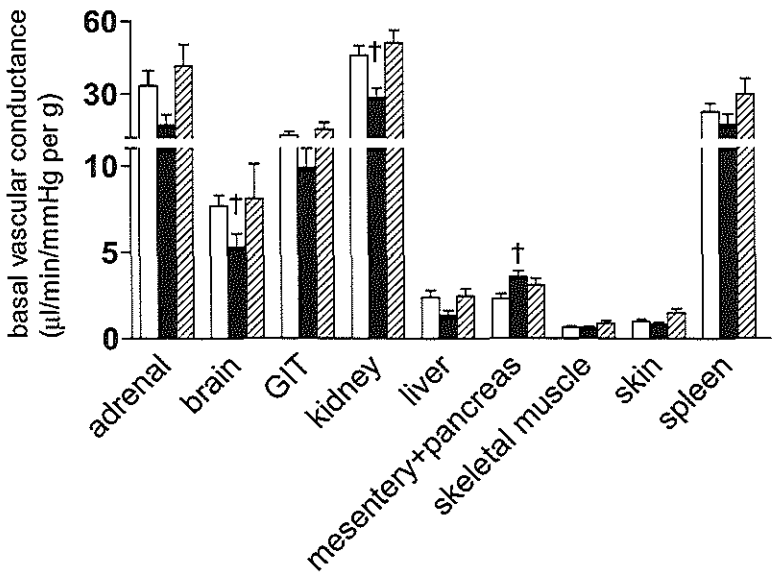

Figure 2. Effects of pretreatment with vehicle (0.1 ml/min; n=9; open bar), L-NAME (10 mg/kg; followed by a continuous infusion of sodium nitroprusside to restore mean arterial blood pressure to pre-blockade level; n=8; black bar), or indomethacin (5 mg/kg; n=8; hatched bar) on basal regional vascular conductance in rats. Values are mean±SEM. GIT: gastrointestinal tract. † $P<0.05$ vs. vehicle.

Regional hemodynamic effects

Basal blood flow values in adrenal, brain, gastrointestinal tract, kidney, liver, mesentery+pancreas, skeletal muscle, skin and spleen were 3.36 ±0.70, 0.76±0.05, 1.27±0.13, 4.52±0.36, 0.23±0.04, 0.23±0.03, 0.06±0.01, 0.10±0.01 and 2.3±0.37 ml/min per g,

respectively. L-NAME + sodium nitroprusside reduced renal and brain vascular conductance and increased vascular conductance in mesentery+pancreas. No significant changes were observed in the vascular conductance of other organs after L-NAME + sodium nitroprusside, nor did indomethacin alter the vascular conductances in any of the organs that were studied (Figure 2).

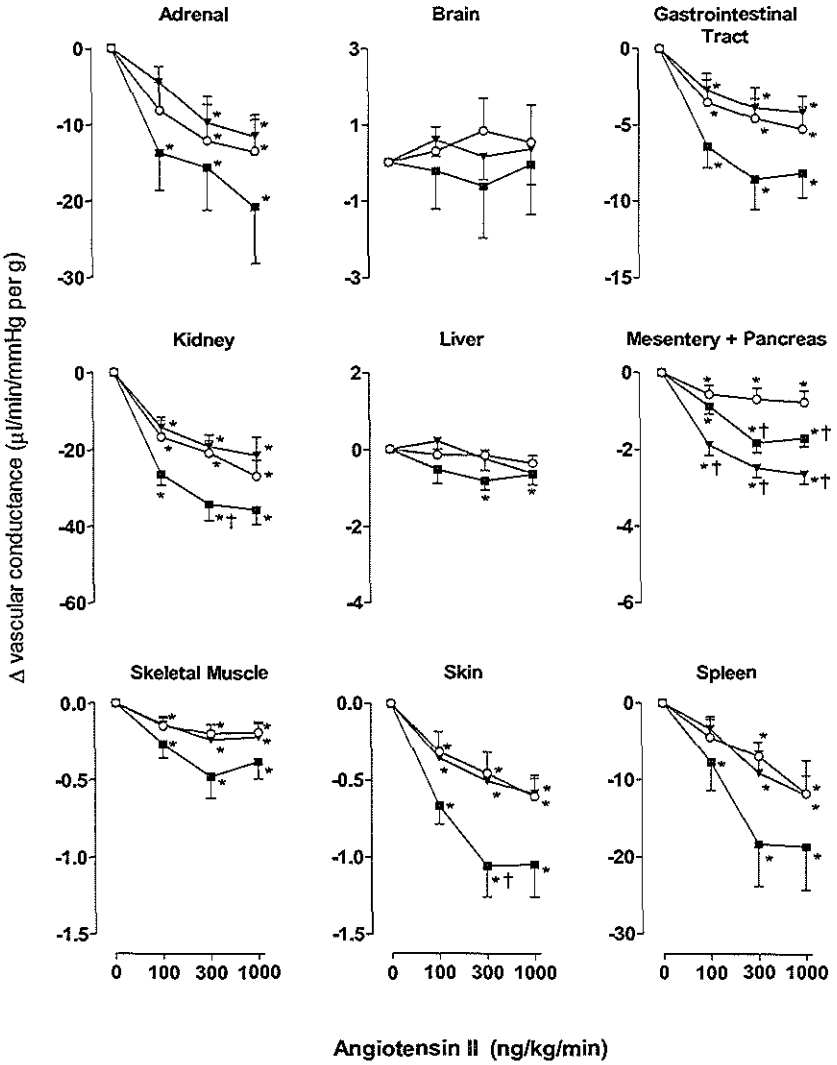

Figure 3. Changes in regional vascular conductance during 10-min intravenous infusions of angiotensin II in rats, pretreated with vehicle (0.1 ml/min; *n*=9; open circles), indomethacin (5 mg/kg; *n*=8; closed squares) or L-NAME (10 mg/kg; followed by a continuous infusion of sodium nitroprusside to restore mean arterial blood pressure to pre-blockade level; *n*=8; closed triangles). Values are mean±SEM. * $P<0.05$ vs. baseline, † $P<0.05$ vs. vehicle.

Ang II decreased vascular conductance in gastrointestinal tract, kidney, skeletal muscle, skin, mesentery+pancreas, spleen and adrenal by maximally 37±8%, 56±5%, 26±7%, 55±7%, 28±9%, 54±6% and 33±11%, respectively (Figure 3). No effects of Ang II were observed on vascular conductance in brain and liver, which is most likely due to autoregulatory compensatory mechanisms in these organs. The effects of Ang II were augmented by indomethacin in all organs, although the differences were significant in skin, kidney and mesentery+pancreas only. L-NAME + sodium nitroprusside did not augment the Ang II-induced effects in any of the organs studied, with the exception of the mesentery+pancreas.

Discussion

The present study demonstrates that vasodilator prostanoids, but not NO, counterregulate the acutely induced systemic and regional vasoconstrictor effects of Ang II *in vivo*. The absence of NO-mediated counterregulatory effects in virtually all organs is in full agreement with previous studies in the kidney, where endogenous NO also did not counteract the vasoconstrictor effects of Ang II.[139-141] In these renal studies, like in our *in-vivo* study, NO levels were normalized during NOS inhibition via application of sodium nitroprusside or *S*-nitroso-*N*-acetylpenicillamine. The dose of sodium nitroprusside administered during L-NAME application in our study was high enough to restore mean arterial blood pressure to preblockade level, although it did not restore cardiac output and vascular conductance in every vascular bed. The reduction in cardiac output might either be the consequence of the increase in systemic vascular resistance or it may reflect NO saturation in the heart and a subsequent decrease in cardiac inotropy.[148-150]

NO normalization was not applied by Champion *et al.*,[107] and this may explain why these authors did observe an enhanced effect of Ang II in the renal vascular bed with NOS inhibition. Furthermore, our data do not argue against the concept that the renal vasoconstriction observed during NO inhibition, particularly during high renin states, is the consequence of unopposed Ang II-mediated effects.[150,151] They merely demonstrate that Ang II infusion *per se* does not mediate NO release, neither directly (via angiotensin receptors) nor indirectly (i.e., as a consequence of shear stress).

The absence of NO-mediated effects during Ang II infusion opposes previous reports on Ang II-induced NO release via endothelial AT_1 or AT_2 receptors.[107,134,138] In an earlier study, using the same approach (Ang II infusions and regional blood flow measurements with microspheres), we were also unable to demonstrate AT_2 receptor mediated hypotensive

effects[132] and similar *in-vivo* data were obtained by others.[107,111] Moreover, the initial reports on AT_2 receptor-mediated vasorelaxation in AT_2 receptor knockout mice[103,104] were disputed by subsequent studies[131,152] demonstrating that the enhanced Ang II-mediated vasoconstriction in AT_2 receptor knockout mice is due to and upregulation of AT_1 receptors rather than the absence of vasodilator AT_2 receptors. Furthermore, removal of the endothelium as well as preincubation with L-NAME did not affect the AT_1 receptor-mediated contractions in human subcutaneous arteries,[153] thereby supporting our data on the absence of endothelial AT_1 receptor-mediated NO release.

Prostacyclin is known to counteract Ang II-induced vasoconstriction.[154] Moreover, stimulation of both AT_1 and AT_2 receptors on endothelial and vascular smooth muscle cells has been reported to result in the release of vasodilator and vasoconstrictor prostanoids, including prostacyclin, prostaglandin E_2[136] and thromboxane A_2.[145] A recent study in human isolated subcutaneous resistance arteries,[153] however, does not support the latter concept, suggesting that if prostanoids are released during Ang II infusion, this is due to the rise in blood pressure. Based on the present study, it cannot be concluded whether the release of prostanoids is a direct consequence of angiotensin receptor stimulation or occurs in response to the regional and systemic vasoconstrictor effects of Ang II (i.e., occurs as a consequence of shear stress). Although the Ang II-induced release of prostanoids differs depending on the vascular bed, resulting in both vasodilator[143,144] and vasoconstrictor[145] effects *in vitro*, our data indicate a predominance of vasodilator prostanoids during Ang II infusion in virtually all vascular beds. Our study, using the radiolabeled microsphere method, is the first to investigate the role of NO and prostanoids in all regional vascular beds at the same time.

The dose of indomethacin used in the present study has been reported to attenuate the responses mediated by the prostanoid precursor, arachidonic acid.[155,156] Indomethacin did not alter systemic and regional hemodynamics, thereby indicating that, normally, prostanoids do not play a major regulatory hemodynamic role. Furthermore, the rise in blood pressure occuring during L-NAME administration in this study confirms its NOS-inhibitory effect at the dose applied. In conclusion, vasodilator prostanoids are more important than NO in counterregulating the Ang II-mediated systemic and regional hemodynamic effects in Wistar rats.

References are presented in the general reference list.

Chapter 4

AT_2 receptor-mediated vasodilation in the heart: effect of myocardial infarction

Am J Physiol. 2001; 281: H2590-H2596

Summary

To investigate the functional consequences of postinfarct cardiac angiotensin (Ang) type 2 (AT_2) receptor upregulation, rats underwent coronary artery ligation or sham operation and were infused with Ang II 3-4 weeks later, when scar formation is complete. Ang II increased mean arterial pressure (MAP) more modestly in infarcted than in sham animals. The AT_1 receptor antagonist irbesartan, but not the AT_2 receptor antagonist PD123319, decreased MAP and antagonized the Ang II-mediated systemic hemodynamic effects. Myocardial (MVC), but not renal vascular conductance (RVC) was diminished in infarcted versus sham rats. Ang II did not affect MVC and reduced RVC in all rats. MVC was unaffected by irbesartan and PD123319 in all animals. However, with PD123319, Ang II reduced MVC in sham but not infarcted animals, and, with irbesartan, Ang II increased MVC in infarcted but not sham animals. Irbesartan increased RVC and antagonized the Ang II-mediated renal effects in all animals. RVC, at baseline or with Ang II, was not affected by PD123319 in infarcted and sham animals. In conclusion, coronary but not renal AT_2 receptor stimulation results in vasodilation, and this effect is enhanced in infarcted rats.

Introduction

The effects of angiotensin (Ang) II are mediated by specific receptors, of which two major receptor subtypes, termed AT_1 and AT_2, have been characterized to date. AT_1 receptors mediate essentially all of the known effects of Ang II, including vasoconstriction and cell proliferation.[157] Much less is known about the physiological role of AT_2 receptors. On the basis of its high expression in fetal tissues, it has been speculated that AT_2 receptors are involved in cell growth and differentiation.[119] Indeed, AT_2 receptor stimulation in isolated cells results in growth inhibition and apoptosis,[3,15,102] thereby antagonizing the AT_1 receptor-mediated growth-stimulatory effects. AT_2 receptor knockout mice are more sensitive to the pressor action of Ang II than wildtype mice,[103,104] suggesting that AT_2 receptors antagonize the AT_1 receptor-mediated rise in blood pressure. However, this increase in sensitivity may also be explained on the basis of the increased vascular AT_1 receptor expression in AT_2 receptor knockout mice.[131] Moreover, AT_2 receptor-mediated blood pressure decreases have not been found consistently in normal animals.[107-109,132]

Although initially it was thought that cardiac AT_2 receptors disappear after birth, it is now widely accepted that both AT_1 and AT_2 receptors are expressed in the normal adult heart, either at equal levels or with AT_1 receptors predominating.[97,158-162] Pathophysiological conditions such as postinfarct remodeling and heart failure are accompanied by increased AT_2 receptor expression[120,161,163-165] and/or decreased AT_1 receptor expression,[99,166] resulting in a relative AT_2 receptor upregulation.[158,167] Stimulation of cardiac AT_2 receptors results in inhibition of cell growth and fibrillar collagen metabolism, thereby counteracting the AT_1 receptor-mediated effects on cardiac remodeling after myocardial infarction (MI).[10,89] The contribution of AT_2 receptors to coronary blood flow regulation is currently unknown. A recent study[168] in dogs that underwent a 15-min coronary artery occlusion followed by 4-h reperfusion, observed an increase in myocardial blood flow after pretreatment the the AT_1 receptor antagonist candesartan. This finding is in agreement with the concept that AT_1 and AT_2 receptors mediate vasoconstriction and vasodilation, respectively.

In the present study, we investigated Ang II-mediated effects in the rat coronary circulation *in vivo* using the radiolabeled microsphere method. Microspheres are trapped in end-arterioles, thus allowing one to obtain information on regional hemodynamics by measuring tissue radioactivity. In view of the relative upregulation of AT_2 receptors after MI, we also studied the effects of Ang II on coronary blood flow in MI rats. These studies were performed at 3-4 wk after coronary ligation, when scar formation is complete,[169] i.e., at the

compensated stage of cardiac remodeling.[170] Finally, for comparison we investigated the Ang II-mediated effects on renal hemodynamics in sham and MI rats in view of the AT_2 receptor-mediated vasodilation that has been reported in glomerular arterioles.[105] All studies were performed with and without the AT_1 receptor antagonist irbesartan or the AT_2 receptor antagonist PD123319. The effect of combined AT_1 receptor and AT_2 receptor blockade was not investigated, because PD123319 displaces AT_1 receptor antagonists from their plasma protein-binding sites.[112,113,171] Non-specific displacement of irbesartan would increase its free (effective) plasma concentration, thereby making the interpretation of such combination studies highly complex.

Methods

The investigation conforms with the National Institutes of Health 'Guide for the Care and Use of Laboratory Animals' (NIH publication No. 85-23, revised 1996). Experiments were carried out in male Wistar rats (260-280 g body wt) obtained from Harlan (Zeist, The Netherlands). Rats were housed with a 12:12-h light-dark cycle with standard rat chow and water available *ad libitum*.

Myocardial infarction

Rats were subjected to either coronary artery ligation (n=64) or sham operation (n=28). Under sodium pentobarbital (60 mg/kg ip, Apharma; Arnhem, The Netherlands) anesthesia, the left anterior descending coronary artery (LADCA) was ligated.[170] Briefly, after the trachea had been intubated, an incision was made in the skin and the muscles overlying the fourth intercostal space were placed aside. The animals were put on positive pressure ventilation (frequency, 65 breaths/min; tidal volume, 3 ml), and the thoracic cavity was opened by cutting the intercostal muscles. The heart was then carefully pushed to the left and a 6-0 silk suture was looped under the LADCA ~2 mm from its origin. After the heart was returned to its normal position, the suture was tied. The intercostal space was closed by pulling the ribs together with 2-0 silk. Subsequently, the muscles were returned to their normal position, and the skin was sutured. Sham-operated animals underwent the same surgical procedure, without the actual LADCA ligation. Proper occlusion of the LADCA resulted in an extensive transmural infarction comprising a major part of the left ventricular tissue, with small variations in size.[170]

Systemic and regional hemodynamics

At 3-4 wk after surgery, animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg). To maintain an adequate depth of anesthesia, intravenous bolus injections of sodium pentobarbital (5-10 mg/kg) were administered via the right external jugular vein every 15 min during the stabilization period. A catheter was placed in the trachea for intermittent positive pressure ventilation with a mixture of oxygen and air, using a respiratory pump (Small Animal Ventilator, Harvard Apparatus; Natick, MA). The ventilatory rate was adjusted to keep arterial blood gases within the physiological range. Blood pressure and heart rate (HR) were recorded with a pressure transducer (Combitrans Disposable Pressure Transducer, Braun; Melsungen, Germany) in the left femoral artery. Radioactive microspheres were injected into the left ventricle via a catheter in the right carotid artery. Drugs were administered via the right external jugular vein. The right femoral artery was cannulated to allow the withdrawal of reference blood samples.

After a 1-h stabilization period after completion of instrumentation, animals were given a 30-min infusion of the AT_1 receptor antagonist irbesartan (100 μg/kg/min), the AT_2 receptor antagonist PD123319 (20 μg/kg/min, continued throughout the experiment to ensure blockade)[132] or vehicle (saline, 0.1 ml/min). Two consecutive 10-min infusions of Ang II (100 and 300 ng/kg/min) were then given to each animal. At the end of each Ang II infusion, when a steady state had been reached, hemodynamic parameters were measured and the distribution of aortic blood flow was determined by injecting 15.5±0.1 (mean±SD) μm diameter microspheres labeled with ^{141}Ce, ^{103}Ru or ^{95}Nb (NEN Dupont; Boston, MA). For each measurement, ~200,000 microspheres, suspended in 0.2 ml of saline and labeled with one of the isotopes, were mixed, and injected into the left ventricle over a 15-s period. After each injection, the catheter was thoroughly flushed with 0.5 ml saline. Starting 10 s before microsphere injection and lasting 70 s, an arterial reference blood sample was drawn from the right femoral artery at a constant rate of 0.5 ml/min, using a withdrawal pump (Model 55, Harvard Apparatus). At the end of the experiment the animal was euthanized with an overdose of pentobarbital, and the heart and kidneys were removed. The ventricles were separated from atria and large vessels, and subsequently divided into right and left ventricular tissue and interventricular septum tissue. The left ventricular tissue of MI hearts were further divided into viable tissue and scar tissue based upon macroscopic appearance. Tissues were washed thoroughly, to remove radioactive microspheres not trapped in arterioles, weighed, and put into vials. The radioactivity in the reference blood samples and the tissues was counted for 5 min in a γ-scintillation counter (Minaxi Auto-Gamma 5000 series, Packard;

Downers Grove, IL), using suitable windows, discriminating the different isotopes. Tissues other than heart and kidney were also removed and counted, but since the findings in these tissues resembled those in the kidney, they will not be discussed here.

Drugs

Irbesartan was a kind gift of Bristol-Myers Squibb (Princeton, NJ). PD123319 was a kind gift of Parke-Davis (Natick, MA). Irbesartan (330 μg/ml) was dissolved in 1.2 mM KOH as described by Trippodo et al.[117] PD123319 (70 μg/ml) and ANG II (0.33 and 0.99 μg/ml, respectively) were dissolved in saline.

Data presentation and statistical analysis

Data were processed as described previously.[118] Cardiac output (CO) and regional blood flow were calculated as follows:

$$CO = \frac{\text{amount of radioactivity injected x withdrawal rate of arterial blood sample}}{\text{radioactivity of arterial blood sample}} \quad (1)$$

and

$$\text{regional blood flow} = \frac{\text{tissue radioactivity x CO}}{\text{amount of radioactivity injected}} \quad (2)$$

Systemic and regional vascular conductances [i.e., CO and regional blood flow corrected for mean arterial blood pressure (MAP)] were calculated to quantify the vasoconstrictor effects of Ang II with or without its receptor antagonists.

All data are presented as mean±SEM. Duncan's new multiple-range test was used to test differences from baseline once a two-way ANOVA had revealed that differences existed between the consecutive infusions. Student's unpaired *t*-test was used to evaluate the effects of the AT receptor antagonists once two-way repeated-measures ANOVA followed by Bonferroni's correction had revealed differences between the groups. To evaluate differences between sham and MI animals Student's unpaired *t*-test was used. Statistical significance was accepted at $P<0.05$ (two-tailed).

Results

Mortality

All 28 sham animals survived the follow-up period. Three sham animals were excluded from analysis due to technical failure during microsphere injection. Of the 64 MI animals, 32 animals died within 24 h after LADCA ligation and 8 animals died due to technical failure during microsphere injection. Three MI animals were excluded from analysis because the infarcted area comprised only a minor part (<20%) of the left ventricular free wall.

Systemic hemodynamic effects

Acute administration of irbesartan or PD123319 did not affect CO or HR in sham and MI animals (Figure 1). Irbesartan, but not PD123319, reduced MAP and tended to increase (*P*=not significant) systemic vascular conductance in sham and MI rats. Ang II reduced systemic vascular conductance similarly in sham and MI animals. Ang II did not affect HR, and

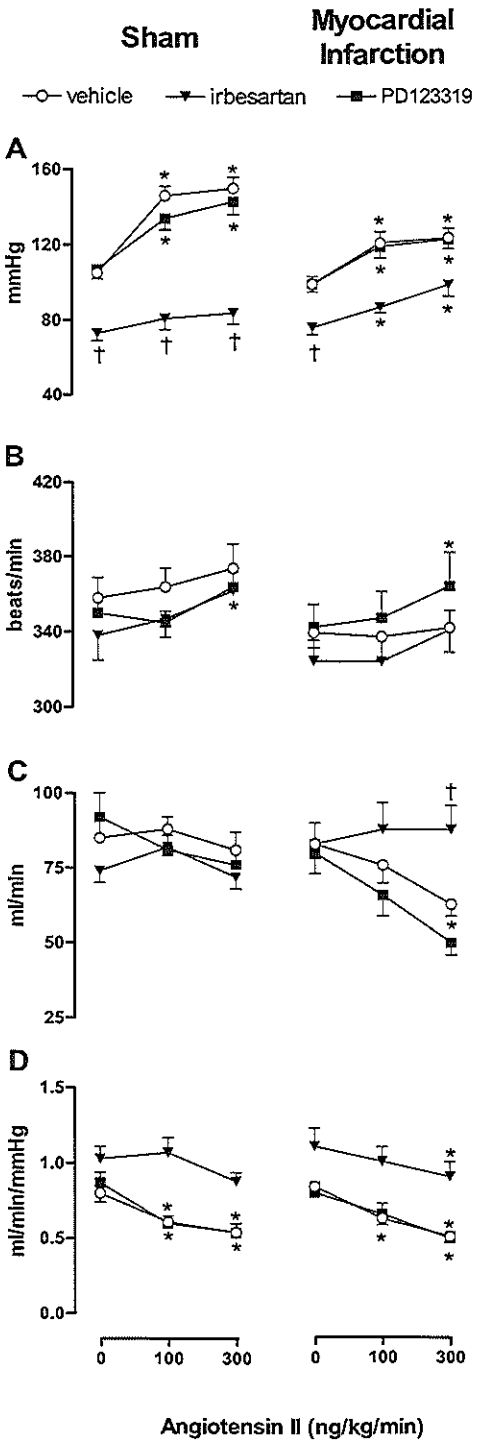

Figure 1. Effects of 10 min-intravenous infusions of angiotensin II on mean arterial blood pressure (*A*), heart rate (*B*), cardiac output (*C*) and systemic vascular conductance (*D*) in sham-operated and myocardial infarcted (MI) rats, pretreated with vehicle (0.1 ml/min, *n*=9 and *n*=7, respectively), irbesartan (100 µg/kg/min; *n*=8 and *n*=7, respectively) or PD123319 (20 µg/kg/min, *n*=8 and *n*=7, respectively). Values are mean±SEM. * *P*<0.05 vs. baseline, † *P*<0.05 vs. vehicle.

reduced CO in MI animals only. The Ang II-induced rise in MAP was larger in sham (44±6 mmHg) than in MI (26±5 mmHg) animals ($P<0.05$). In both sham and MI rats, irbesartan reduced or abolished the systemic hemodynamic effects of Ang II, while PD123319 did not affect these effects.

Cardiac hemodynamic effects

MI reduced left ventricular blood flow by 35%, but did not significantly affect right ventricular, interventricular septal, or atrial blood flow (Figure 2). As a consequence, myocardial blood flow (i.e., the sum of left and right ventricular, interventricular septal, and atrial blood flow) and myocardial vascular conductance were lower ($P<0.05$) in MI than in sham animals (Figures. 2 and 3). Irbesartan and PD123319 did not affect myocardial vascular conductance. In sham animals Ang II, with or without irbesartan, did not affect myocardial vascular conductance. Only in the presence of PD123319 did Ang II infusions decrease myocardial vascular conductance (i.e., caused coronary vasoconstriction) in sham animals. This effect was due to vasoconstriction in the left ventricle (Table 1). In MI animals, Ang II also did not affect myocardial vascular conductance, nor did PD123319 affect the myocardial vascular response to Ang II. In the presence of irbesartan, however, Ang II increased myocardial vascular conductance (i.e., caused vasodilation) in MI animals. These vasodilatory effects were limited to the right ventricle and the viable part of the left ventricle (Table 1).

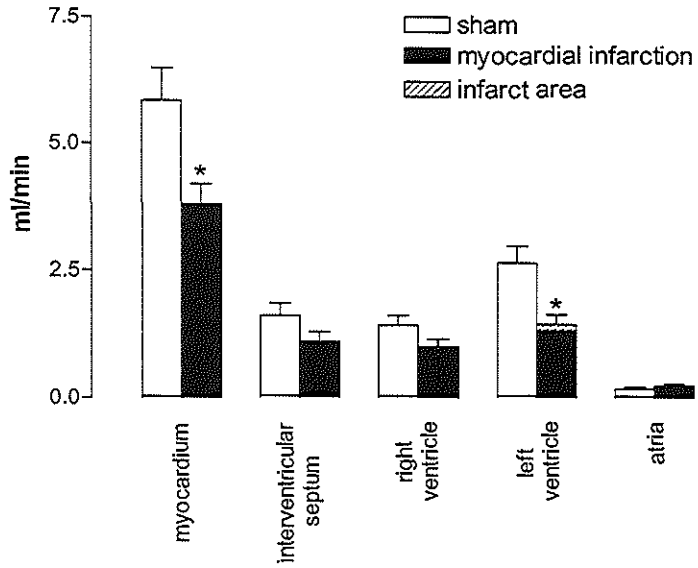

Fig. 2. Baseline regional myocardial blood flow values in sham-operated (n=9) and MI rats (n=7). Myocardium represents the sum of interventricular septum, left and right ventricles, and atria. Values are mean±SEM. * $P<0.05$ vs. sham.

Renal hemodynamic effects

MI did not affect renal vascular conductance (Figure 3). Irbesartan, but not PD123319, increased renal vascular conductance. Ang II decreased renal vascular conductance and this effect was reduced or blocked by irbesartan. PD123319 did not affect the Ang II-mediated responses in the kidney.

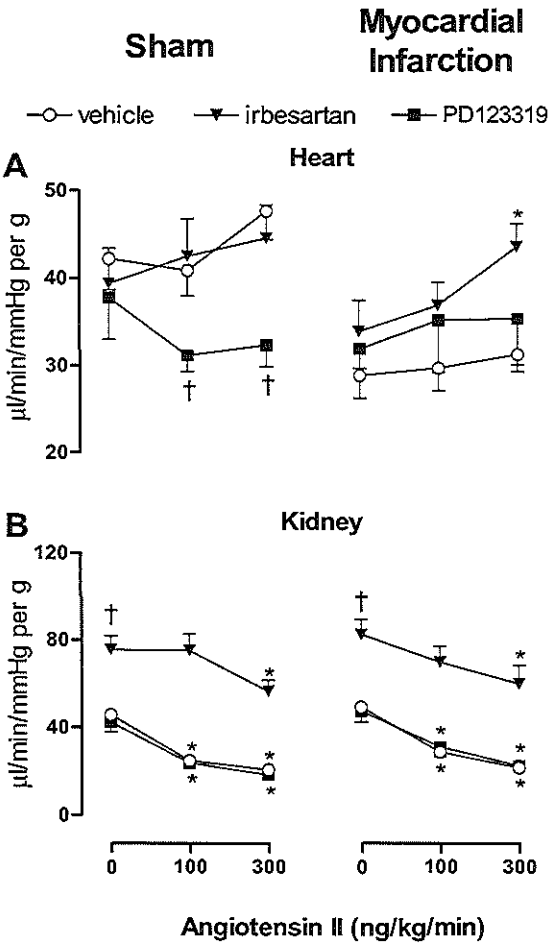

Figure 3. Effects of 10 min-intravenous infusions of angiotensin II on myocardial (*A*) and renal vascular conductance (*B*) in sham-operated and MI rats pretreated with vehicle (0.1 ml/min; n=9 and n=7, respectively), irbesartan (100 µg/kg/min; n=8 and n=7, respectively) or PD123319 (20 µg/kg/min; n=8 and n=7, respectively). Values are mean±SEM. * P<0.05 vs. baseline, † P<0.05 vs. vehicle.

Discussion

The present study supports the concept of AT_2 receptor-mediated vasodilation in the rat coronary vascular bed. No such vasodilation was observed in the renal vascular bed or the systemic circulation, in either normal animals or in MI animals.

Ang II infusion into sham-operated rats did not affect myocardial vascular conductance despite its significant pressor effects, thereby indicating either a balance between AT_1 and AT_2 receptors in the coronary circulation in the normal heart or autoregulatory mechanisms overruling any Ang II-mediated coronary effects. In support of the first concept, myocardial vascular conductance did decrease (i.e., vasoconstriction occurred) when Ang II was infused in the presence of the AT_2 receptor antagonist PD123319. These data are in agreement with studies providing evidence for the presence of both AT_1 and AT_2 receptors in coronary arteries of normal hearts.[123,162] Remarkably, however, coronary vasodilation did not occur

during Ang II infusion in the presence of the AT_1 receptor antagonist irbesartan, at a dose that fully prevented the systemic pressor effects of Ang II. This indicates that, normally, AT_2 receptor-mediated coronary vasodilation is of limited importance, and mainly serves to counteract AT_1 receptor-mediated effects. This may be different under pathological conditions, when AT_2 receptors are upregulated[164,167] relative to AT_1 receptors either because AT_2 receptor density increases and/or because AT_1 receptor density decreases.[99,120,158,161,163,165-167] Indeed, 3-4 wk after MI, we observed Ang II-mediated coronary vasodilation in the presence of irbesartan. In agreement with a reduced density of AT_1 receptors in the infarcted heart, Ang II did not cause coronary vasoconstriction in the presence of PD123319. An alternative, less likely, explanation for this lack of Ang II-mediated vasoconstriction in the presence of PD123319 is that, due to the upregulation of AT_2 receptors in the infarcted heart, the applied dose of PD123319 was insufficient to obtain full blockade of cardiac AT_2 receptors. Previous studies, however, have demonstrated that this dose of PD123319 is sufficient to result in micromolar blood plasma concentrations (i.e., concentrations that selectively block AT_2 receptors) and that higher doses will lead to concentrations that also interfere with AT_1 receptors.[114] Moreover, the previously described increases in AT_2 receptor density are relatively modest, i.e., less than three- to fourfold.[12,167]

We were unable to demonstrate a vasodilator role for AT_2 receptors in the systemic circulation and the kidney. The irbesartan-induced increases in systemic and renal vascular conductance in sham and MI rats are suggestive for AT_1 receptor-mediated vasoconstriction by endogenous Ang II in anesthetized animals. No PD123319-induced decreases in systemic or renal vascular conductance were observed in normal or MI rats, nor did the AT_2 receptor antagonist affect the Ang II-induced systemic and renal hemodynamic responses in these rats. Thus MI does not appear to result in AT_2 receptor upregulation in organs other than the heart, including the kidney, at least at 3-4 wk after coronary ligation, i.e., at the compensated stage of cardiac remodeling.[170]

Our *in-vivo* data showing no AT_2 receptor-mediated vasodilation in the rat kidney contrast with *in-vitro* data demonstrating AT_2 receptor-dependent vasodilation in microperfused rabbit glomerular afferent and efferent arterioles[105] One explanation for this discrepancy might be a difference in shunting in the kidney as compared to the heart, as a consequence of the use of microspheres of a single size (15.5 μm in the present study). However, we[172] demonstrated earlier that for the measurement of regional blood flow, for the vast majority of tissues

Table 1. Regional myocardial conductances during 10-min intravenous infusions of Ang II in sham-operated and MI rats pretreated with vehicle, irbesartan, or PD123319.

	Sham-operated (ng Ang II/kg/min)			MI (ng Ang II/kg/min)		
	0	100	300	0	100	300
Interventricular Septum						
Vehicle	45± 5	44± 3	48± 3	34± 4	33± 4	37± 3
Irbesartan	38± 5	43± 3	46± 4	44±10	47± 6	56± 7
PD123319	38± 6	33± 4	33± 3	33± 2	37± 5	40± 6
Right Ventricle						
Vehicle	47± 7	49± 6	52± 6	34± 3	39± 4	42± 6 *
Irbesartan	48±13	48±10 *	47± 8	42± 4	45± 4	54± 3
PD123319	52± 6	42± 5	44± 6	45± 5	49±10	49± 9
Left Ventricle						
Viable Tissue						
Vehicle	47± 5	43± 3	55± 3	32± 2	33± 3	34± 3 *
Irbesartan	44± 2	49± 5 †	53± 5 †	36± 4	40± 3	48± 4
PD123319	41± 6	34± 2	35± 2	34± 3	38± 6	38± 5
Infarct Area						
Vehicle				11± 3	11± 3	9± 2
Irbesartan				13± 4	13± 4	16± 3
PD123319				11± 3	14± 6	10± 3
Atria						
Vehicle	12± 1	10± 1	10± 1	16± 3	13± 2	13± 1
Irbesartan	15± 3	15± 3	14± 2	14± 3	14± 2	16± 3
PD123319	11± 3	9± 2	10± 3	15± 3	13± 4	11± 3

Values are mean±SEM; n=9 sham-operated and 7 myocardial infarcted (MI) rats pretreated with vehicle (0.1 ml/min), 8 sham-operated and 7 MI rats pretreated with irbesartan (100 µg/kg/min), and 8 sham-operated and 7 MI rats pretreated with PD123319 (20 µg/kg/min). Values of myocardial conductance are µl/min/mmHg per g. * P<0.05 vs. baseline, † P<0.05 vs. vehicle.

(including the kidney) it does not matter whether one uses microspheres of 10, 15, 25 or 35 µm diameter.[172] It is also unlikely that drawing the reference blood sample from a relatively distal vessel such as the femoral artery and/or non-appropriate admixture of microspheres with blood after their injection into the left ventricle underlie this phenomenon. First, although using femoral arterial blood as a reference may result in a modest overestimation of CO,[147] this will not affect regional blood flow or mask regional AT_2 receptor-mediated effects. Second, in this study, as in many previous studies,[115,173,174] we observed similar blood flow values in the left and right kidney, both in sham-operated and infarcted animals (data not shown), thereby supporting the concept of appropriate admixture. A more likely explanation for the lack of renal AT_2 receptor-mediated vasodilation is therefore that AT_1 receptors predominate in renal blood vessels other than the glomerular arterioles. Indeed, our results obtained in whole kidney do not rule out the possibility of regional hemodynamic changes with no change in total renal hemodynamic blood flow.

The MI model used in the present study is well-established,[170] and results in extensive transmural infarction comprising >20% of the left ventricle. As a consequence, and in full

agreement with previous studies,[175] baseline left ventricular blood flow was found to be reduced by 35% in MI animals as compared to sham-operated rats. No flow reductions were observed in other parts of the heart. The Ang II-induced effects on myocardial vascular conductance were limited to the left ventricle in sham-operated animals and to the right ventricle and viable left ventricle in the MI animals, indicating that the MI-induced changes in AT receptor density were most prominent in these areas of the heart. Such changes, which need to be confirmed in future studies, most likely relate to the vascular growth and remodeling process that occurs in the noninfarcted myocardium.[10,12,175,176]

Ang II reduced systemic vascular conductance similarly in sham-operated and MI rats. However, in MI rats, this increase in systemic vascular conductance was accompanied by a reduction in CO, thereby attenuating the rise in blood pressure in these rats.

In conclusion, this study is the first to demonstrate the counteracting effect of AT_2 receptors on AT_1 receptor-mediated coronary vasoconstriction. This effect appears to be enhanced after MI and parallels similar findings on AT_2 receptor-mediated growth inhibition opposing AT_1 receptor-mediated growth stimulation.[3,15,102]

References are presented in the general reference list.

Chapter 5

Angiotensin-converting enzyme inhibition and angiotensin II type 1 receptor blockade prevent cardiac remodeling in pigs after myocardial infarction

Role of tissue angiotensin II

Circulation. 2000; 102: 1556-1563

Summary

The mechanisms behind the beneficial effects of renin-angiotensin system blockade after myocardial infarction (MI) are not fully elucidated but may include interference with tissue angiotensin (Ang) II. Forty-nine pigs underwent coronary artery ligation or sham operation and were studied up to 6 weeks. To determine coronary Ang I to Ang II conversion and to distinguish plasma-derived Ang II from locally synthesized Ang II, ^{125}I-labeled and endogenous Ang I and II were measured in plasma and in infarcted and in noninfarcted left ventricle (LV) during ^{125}I-Ang I infusion. Ang II type 1 (AT_1) receptor-mediated uptake of circulating ^{125}I-Ang II was increased at 1 and 3 weeks in noninfarcted LV, and this uptake was the main cause of the transient elevation in Ang II levels in the noninfarcted LV at 1 week. Ang II levels and AT_1 receptor-mediated uptake of circulating Ang II were reduced in the infarct area at all time points. Coronary Ang I to Ang II conversion was unaffected by MI. Captopril and the AT_1 receptor antagonist eprosartan attenuated postinfarct remodeling, although both drugs increased cardiac Ang II production. Captopril blocked coronary conversion by >80% and normalized Ang II uptake in the noninfarcted LV. Eprosartan did not affect coronary conversion and blocked cardiac Ang II uptake by >90%. In conlusion, both circulating and locally generated Ang II contribute to remodeling after MI. The rise in tissue Ang II production during angiotensin-converting enzyme inhibition and AT_1 receptor blockade suggests that the antihypertrophic effects of these drugs do not only result from diminished AT_1 receptor stimulation, but also from increased stimulation of growth-inhibitory Ang II type 2 receptors.

Introduction

Angiotensin-converting enzyme (ACE) inhibitors prevent cardiac remodeling (i.e., ventricular hypertrophy and chamber dilatation) following myocardial infarction (MI). This occurs, in part, independent of their blood pressure-lowering effect,[177] suggesting interference with tissue angiotensin (Ang) II. However, ACE inhibitors do not always lower cardiac Ang II[66,80,90] because of (1) their pharmacokinetic properties (short half-life, lack of tissue penetration),[80,90] (2) ACE upregulation during prolonged ACE inhibition,[178] or (3) alternative converting enzymes such as chymase.[61] Furthermore, previous Ang II measurements in infarcted hearts[90,91,179] have not taken into account that cardiac Ang II is partly derived from the circulation. Plasma Ang II accumulates in the heart through Ang II type 1 (AT_1) receptor-mediated endocytosis,[66,74] and changes in AT_1 receptor density following MI[167,180] may influence this process.

AT_1 receptor antagonists have opened new avenues to investigate the importance of cardiac Ang II after MI. In general, these drugs increase rather than decrease Ang II levels,[181] and this may lead to activation of unoccupied, growth-inhibitory[3] Ang II type 2 (AT_2) receptors. Currently, the effect of AT_1 receptor antagonism on the Ang II content of the infarcted heart is unknown. Furthermore, it is still controversial whether AT_1 receptor antagonism prevents the development of cardiac hypertrophy and increases survival following MI.[182,183]

Therefore, the aim of the present study was to investigate changes in cardiac Ang II content and origin (local synthesis versus uptake from plasma) after MI in pigs, to compare the effects of ACE inhibition and AT_1 receptor antagonism on these changes, and to evaluate whether the effects of both renin-angiotensin system (RAS) blockers on cardiac angiotensin content are related to their effects on postinfarct remodeling.

Methods

Animals

Experiments were performed in accordance with the 'Guide for the Care and Use of Laboratory Animals' (NIH Publication 86-23, revised 1985). Forty-nine 2-3 month-old Yorkshire×Landrace pigs of either sex entered the study. Adaptation of animals to laboratory conditions started 1 week before surgery.

Surgical procedure

Animals were sedated with ketamine (30 mg/kg IM), anesthetized with thiopental (10 mg/kg, iv), intubated and ventilated with a mixture of O_2 and N_2O to which 0.2 to 1.0% (vol/vol) isoflurane was added.[184] Anesthesia was maintained with midazolam (2 mg/kg + 1 mg/kg per hour IV) and fentanyl (10 μg/kg per hour IV). The chest was opened via a left intercostal space and a fluid-filled polyvinylchloride catheter was inserted into the aortic arch for hemodynamic monitoring and blood sampling.[184] Subsequently, the pericardium was opened, the proximal left circumflex coronary artery (LCXCA) dissected out, and a suture placed around the LCXCA. In 35 animals, the LCXCA was permanently ligated (MI group), whereas in 14 animals the suture was removed (sham group). The pericardium was closed and the aortic catheter 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.[184]

Experimental groups

Animals were followed for 1, 3 or 6 weeks. Of the 3-week MI animals, 6 received captopril (25 mg PO BID), and 7 received the AT_1 receptor antagonist eprosartan (400 mg PO BID, a gift of Dr. P.K. Weck, SmithKlineBeecham, Collegeville, PA). This dose of eprosartan blocks Ang II-induced pressor responses by >95% (n=3). Treatment started 12 to 24 hours after LCXCA ligation and was continued for 3 weeks.

Echocardiography

At the end of the follow-up period, animals were sedated with ketamine, and 2D echocardiographic recordings of the left ventricular (LV) short axis at midpapillary level were obtained (Sonos 5500, Hewlett-Packard) and stored for off-line analysis. LV end-diastolic cross-sectional areas (EDA) and end-systolic cross-sectional areas (ESA) were determined and ejection fraction (EF) was calculated as (EDA-ESA)/EDA x 100%.

Infusion of ^{125}I-Angiotensin I

After echocardiography, pigs were anesthetized and prepared for hemodynamic monitoring, administration of ^{125}I-Ang I, and blood and tissue sampling.[74,129] After baseline measurements were collected, the animals were subjected to a 60-minute infusion of ^{125}I-Ang I ($\approx 4 \times 10^6$ c.p.m./min) into the LV cavity. ^{125}I-Ang I and II reach steady-state levels in plasma and cardiac tissue within 10 and 60 min, respectively.[74]

Blood and tissue sampling

During follow-up, arterial blood samples were collected in the morning from awake animals, for measurement of norepinephrine, epinephrine, atrial natriuretic peptide (ANP), N-terminal ANP, Ang I and Ang II.[66,185,186] During ^{125}I-Ang I infusion, arterial and coronary venous blood samples were collected from anesthetized animals for measurement of endogenous and ^{125}I-labeled Ang I and II.[66,74] With the ^{125}I-Ang I infusion still running, the heart was stopped by fibrillation, the LV and right ventricle (RV) were separated and weighed, and 0.5- to 1-g samples were rapidly obtained from noninfarcted anterior LV wall, lateral LV wall (containing the infarct area and borderzone after MI), interventricular septum, and RV wall. Samples were immediately frozen in liquid nitrogen and stored at -70°C.

Biochemical measurements

Norepinephrine, epinephrine, ANP, and N-terminal ANP were measured as described before.[185,186] Endogenous and ^{125}I-labeled Ang I and II were measured, after SepPak extraction and high-performance liquid chromatography separation, by γ-counting and radiommunoassay, respectively.[66,74]

Data analysis

Fractional conversion and degradation of ^{125}I-Ang I in the coronary vascular bed, i.e., the percentage of arterially delivered ^{125}I-Ang I that is converted to ^{125}I-Ang II or degraded to other angiotensin metabolites during coronary passage, were calculated as described previously.[187] To quantify cardiac Ang I and II synthesis, tissue levels of Ang I and II were corrected for uptake from plasma by using the steady-state plasma and tissue levels of ^{125}I-Ang I and II.[66] Cardiac tissue ^{125}I-Ang I was undetectable in nearly all animals. To prevent underestimation of the contribution of plasma Ang I to tissue Ang I, *in-situ* synthesized tissue Ang I was quantified under the assumption that tissue ^{125}I-Ang I equals 5% of the steady-state plasma levels of ^{125}I-Ang I.[66] Differences between sham and MI animals and differences between treated and untreated animals were tested by 2-way ANOVA or multivariate ANOVA, followed by Student *t* test. Statistical significance was accepted at $P<0.05$. Data are expressed as mean±SEM.

Table 1. Hemodynamics and morphology

	1 wk		3 wk				6 wk	
	Sham	MI	Sham	MI	MI+Captopril	MI+Eprosartan	Sham	MI
Parameter	(n=4)	(n=4)	(n=5)	(n=6)	(n=6)	(n=5)	(n=3)	(n=4)
MAP, mm Hg	95±4	90±3	93±4	96±6	85±4	89±2	94±6	100±3
Heart rate, bpm	115±5	124±11	101±6	103±8	103±5	113±8	118±9	130±8
Cardiac output, L/min	2.2±0.1	1.9±0.2	3.0±0.2	2.7±0.1	2.8±0.3	2.2±0.3	2.8±02	3.5±0.2
LV dP/dt_{max}, mm Hg/s	2343±214	2221±301	2053±138	1706±78	1872±162	1690±127	1940±104	2053±99
LVEDP, mm Hg	7±2	10±2	8±2	14±2*	11±1	11±3	7±1	9±2
PAP, mm Hg	21±4	31±1	16±3	28±2*	25±4	24±3	23±5	31±3
EDA, cm^2	8.1±0.1	13.6±0.3*	9.7±1.4	12.8±1.5	11.8±1.2	11.6±0.7	10.0±0.3	15.8±4.2
ESA, cm^2	4.1±0.5	10.4±0.6*	4.6±0.8	8.6±1.6*	6.9±1.0	7.0±1.3	5.7±0.8	9.7±2.8
EF, %	50±6	24±3*	53±4	33±9*	42±3*	42±8	43±6	40±4
BW, kg	23.7±0.5	23.5±1.2	30.0±1.4	28.7±1.1	28.3±1.4	28.4±0.6	36.5±1.5	40.1±1.5
LVW, g	75.1±1.4	90.6±1.1*	82.7±6.2	102.2±5.2*	88.2±3.2†	82.5±4.4†	102.1±1.0	131.3±12.2*
RVW, g	30.7±2.7	32.5±2.8	27.3±1.0	40.1±3.1*	31.9±1.5*†	30.3±2.7†	27.8±2.5	52.9±4.7*
LVW/BW, g/kg	3.17±0.11	3.90±0.22*	2.75±0.16	3.57±0.14*	3.17±0.25†	2.91±0.16†	2.80±0.09	3.32±0.42
RVW/BW, g/kg	1.30±0.10	1.39±0.12	0.91±0.04	1.39±0.06*	1.14±0.06*†	1.06±0.08†	0.77±0.10	1.34±0.16*

Values are mean±SEM. MAP indicates mean arterial pressure; LVEDP, LV end-diastolic pressure; PAP, pulmonary arterial pressure; EDA, end-diastolic cross-sectional area; ESA, end-systolic cross-sectional area; EF, ejection fraction; BW, body weight; LVW, LV weight; and RVW, RV weight. * $P<0.05$ vs sham; † $P<0.05$ vs untreated MI.

Results

Mortality

All sham animals survived the follow-up period, but 2 sham animals died during ^{125}I-Ang I infusion because of technical failure. Eight MI animals died within 12 hours after induction of MI, and 2 during eprosartan treatment on days 7 and 15.

Hemodynamic and neurohumoral characteristics

One day after surgery, mean arterial pressure was lower and heart rate was higher in MI pigs than in sham pigs (Figure 1). Mean arterial pressure partially recovered in the MI group during the first week but remained below sham levels during follow-up. Plasma norepinephrine and epinephrine levels were similar in MI and sham animals, wherease plasma ANP and N-terminal ANP were higher in MI animals during the entire follow-up period. Captopril and eprosartan did not affect any of the hemodynamic or neurohumoral parameters (data not shown).

Remodeling

LV EDA and ESA were increased at 1 week after MI, so that EF was lower in MI animals (Table 1). EF recovered during the follow-up period, although LV EDA and ESA remained increased compared to sham values. Captopril and eprosartan blunted the increases in LV dimension, thereby slightly increasing EF.

After MI, the surviving myocardium hypertrophied, as reflected by the increased LV and RV weight and the ratios of LV and RV weight to body weight (Table 1). Captopril and eprosartan attenuated LV and RV hypertrophy.

Hemodynamics during anesthesia

Hemodynamics did not differ between MI and sham animals, except for LV end-diastolic pressure and pulmonary arterial pressure, which were higher ($P<0.05$) in MI pigs at 3 weeks (Table 1). Neither captopril nor eprosartan affected hemodynamics.

Angiotensin levels in plasma

In animals in the awake state, plasma Ang I and II levels (Figure 1) were somewhat higher than those levels in anesthetized animals (Table 2). However, the changes produced by MI and RAS blockade in animals in the awake state parallelled those in anesthetized animals. The Ang I and II levels in arterial and coronary venous plasma in MI animals were marginally higher (P=NS) than those in corresponding sham animals at 1 week. At 3 and 6 weeks, the

levels were similar in both groups. Captopril increased plasma Ang I 5- to10-fold, but did not alter plasma Ang II in MI animals. The plasma Ang II/Ang I ratio decreased by ≈80% during captopril treatment, indicating effective ACE inhibition. Eprosartan increased plasma Ang I and II in MI animals 5- to 10-fold, without altering the plasma Ang II/Ang I ratio. Neither the steady-state ^{125}I-Ang I and II levels nor the ^{125}I-Ang II/Ang I ratios in arterial and coronary venous plasma differed between sham and MI animals at any time point. Captopril reduced the ^{125}I-Ang II levels and the ^{125}I-Ang II/Ang I ratio at both sampling sites, whereas eprosartan did not alter the steady-state plasma ^{125}I-Ang I and II levels.

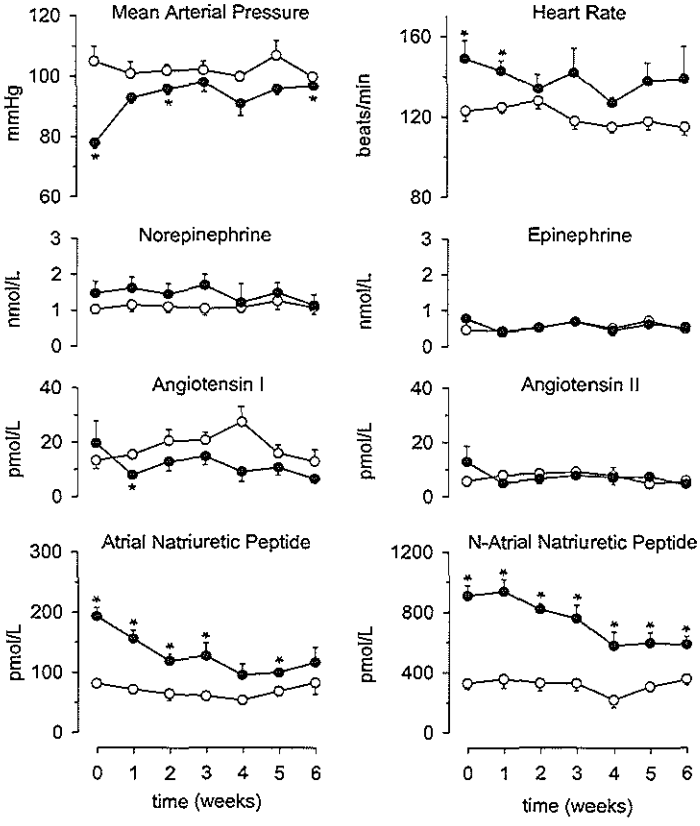

Figure 1. Mean arterial blood pressure, heart rate, and arterial plasma levels of neurohormones in sham (o) and MI (•) animals. N-artrial natriuretic peptide indicates N-terminal atrial natriuretic peptide. * P<0.05 vs sham.

Angiotensin I metabolism in the coronary vascular bed

Coronary ^{125}I-Ang I-Ang II conversion and ^{125}I-Ang I degradation in sham animals (Table 2) were not different from those reported in noninstrumented normal pigs.[187] MI did not alter Ang I conversion or degradation at any time point. Captopril inhibited conversion but did not affect degradation. Eprosartan affected neither conversion nor degradation.

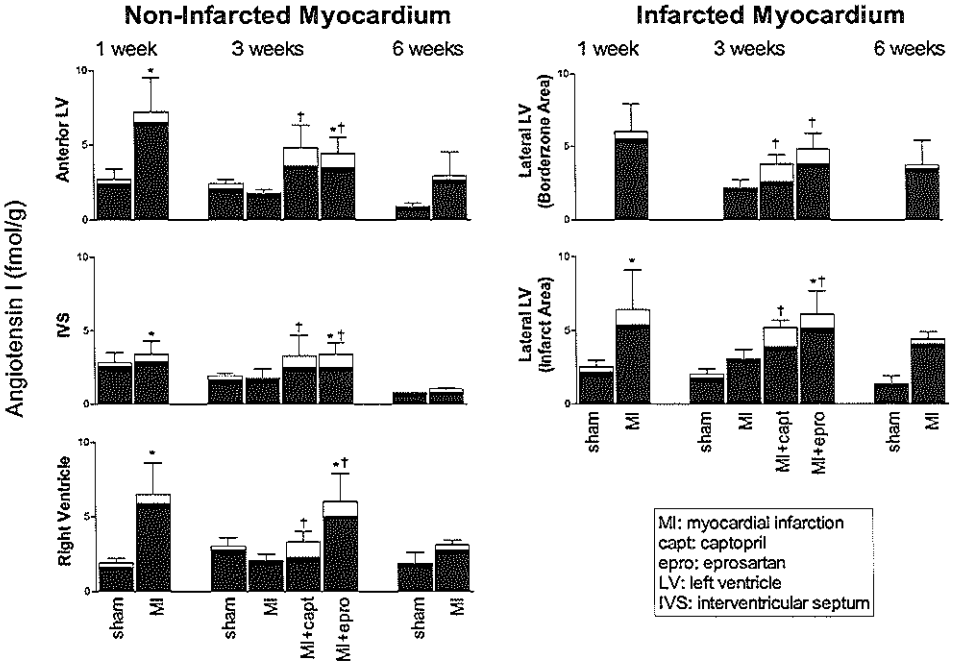

Figure 2. Cardiac Ang I levels. Black area in bar represents amount of locally synthesized Ang I. * $P<0.05$ vs sham; † $P<0.05$ vs untreated MI.

Angiotensin levels in tissue

Ang I and II levels in the noninfarcted myocardium were higher in MI animals than in sham animals at 1 week but not at 3 and 6 weeks (Figures 2 and 3). In sham animals, Ang II levels were similar at all myocardial sites, whereas in MI animals, Ang II levels were lowest in the center of the infarct region and intermediate in the borderzone. Both captopril and eprosartan increased Ang I and II 2- to 3-fold at all tissue sites in MI animals (Figures 2 and 3), without altering the tissue Ang II/Ang I ratios significantly (data not shown).

^{125}I-Ang I was undetectable in all regions, indicating that virtually all tissue Ang I had been produced *in situ* (Figure 2). ^{125}I-Ang II accumulated in cardiac tissue, reaching steady-state

levels in the noninfarcted myocardium that were comparable to or higher than those in arterial plasma (Figure 4). ^{125}I-Ang II levels in the infarcted and border-zone lateral LV wall were ≈20% to 30% and ≈60% to 70% of those in arterial plasma. Blockade of cardiac ^{125}I-Ang II accumulation by eprosartan (Figure 4) indicates that this process is AT_1 receptor-mediated. There were no changes in ^{125}I-Ang II accumulation in the RV wall or in the infarcted or border-zone lateral LV wall during the follow-up period. In sham animals compared with normal noninstrumented animals, ^{125}I-Ang II accumulation in the noninfarcted anterior LV wall and the interventricular septum was increased at 1 week but not at 3 and 6 weeks,[74] whereas it was elevated in MI animals at 1 week and 3 weeks but not at 6 weeks. Captopril abolished the increased ^{125}I-Ang II accumulation in the noninfarcted myocardium of MI animals at 3 weeks. After the tissue ^{125}I-Ang II levels were used to correct for uptake of circulating Ang II, it appeared that the increase in cardiac Ang II levels in MI animals at 1 week was mainly due to uptake from plasma, whereas the increases in cardiac Ang II levels in captopril- and eprosartan-treated animals were due to Ang II generated in situ from locally synthesized Ang I (Figure 3).

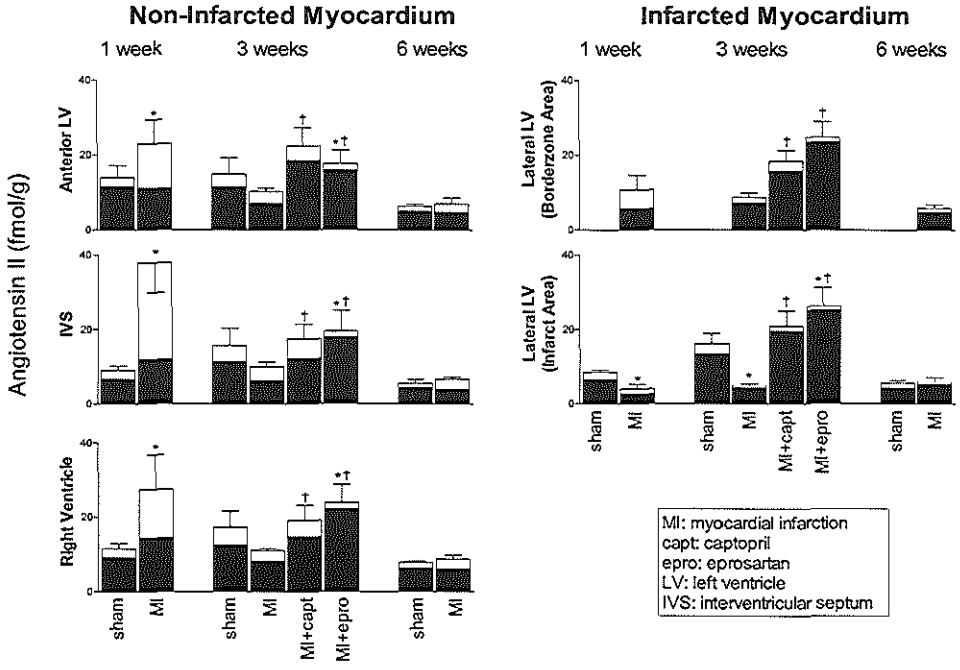

Figure 3. Cardiac Ang II levels. Black area in bar represents amount of locally synthesized Ang II. * $P<0.05$ vs sham; † $P<0.05$ vs untreated MI.

Discussion

The present study demonstrates that after MI, the Ang II levels in the noninfarcted LV are transiently increased because of enhanced AT_1 receptor-mediated uptake of circulating Ang II. Captopril and eprosartan normalized or blocked this uptake and attenuated postinfarct remodeling but increased local cardiac Ang II production. These results suggest that the antihypertrophic effects of RAS blockade results not only from diminished AT_1 receptor stimulation but also from increased stimulation of growth-inhibitory AT_2 receptors.

LCXCA ligation caused LV and RV hypertrophy within 1 to 3 weeks. MI was accompanied by a transient but severe decrease in mean arterial pressure, which, via stimulation of renal renin release, may have caused the modest (nonsignificant) rise in plasma angiotensin levels in the first week. The hemodynamic and neurohumoral profile of the MI animals indicates that permanent LCXCA occlusion, which results in 15% to 25% infarction of the porcine LV, was associated with mild-to-moderate LV dysfunction, requiring minimal sympathetic activation to maintain cardiovascular homeostasis. This contrasts with an atrial pacing model in pigs, which led to severe heart failure and significant neurohumoral activation within 3 weeks.[188] Interestingly, in the latter model, LV AT_1 receptor density was decreased, whereas after MI, LV AT_1 receptors are usually upregulated.[167,189] The present findings are in agreement with such upregulation, inasmuch as the amount of plasma-derived ^{125}I-Ang II sequestered by the noninfarcted LV myocardium via AT_1 receptor-mediated internalization at 1 week and 3 weeks after MI was twice as high as that in a previous study in normal pigs.[74] However, similar increases were observed in sham animals at 1 week after surgery, suggesting that this procedure, possibly through the induction of a fibrogenic response,[189] is partly responsible for the increase in AT_1 receptor density. It must be realized that ^{125}I-Ang II uptake in the heart is mediated exclusively via AT_1 receptors, because AT_2 receptors do not internalize Ang II.[74,119] Furthermore, ^{125}I-Ang II formation from ^{125}I-Ang I that has diffused into the interstitial space does not contribute significantly to the steady-state tissue levels of ^{125}I-Ang II.[36,74]

The increase in AT_1 receptor-mediated uptake of circulating Ang II is responsible for the transient rise in Ang II levels in the noninfarcted LV myocardium, suggesting that circulating Ang II is among the factors initiating the development of LV hypertrophy, even when plasma Ang II is only marginally increased. This observation extends previous studies demonstrating that elevation of circulating Ang II, either through infusion of Ang II or through renovascular hypertension, induces biventricular hypertrophy within 2 weeks.[190] Increased ^{125}I-Ang II

Table 2. Plasma angiotensin levels, Ang II/Ang I ratios, and coronary Ang I metabolism

	1 wk		3 wk				6 wk	
	Sham	MI	Sham	MI	MI+Captopril	MI+Eprosartan	Sham	MI
Parameter	(n=4)	(n=4)	(n=5)	(n=6)	(n=6)	(n=5)	(n=3)	(n=4)
Aorta								
Ang I, pmol/L	4.5±1.7	8.4±4.3	4.9±1.6	2.9±1.0	24.7±8.5*†	19.0±4.7*†	2.1±0.3	3.0±1.0
Ang II, pmol/L	1.4±0.1	6.6±2.9	3.6±1.2	1.9±0.5	4.2±1.6	16.5±3.4*†	1.1±0.1	2.1±0.7
Ang II/I ratio	0.54±0.22	0.82±0.08	0.62±0.08	0.73±0.04	0.16±0.05*†	0.92±0.09	0.50±0.12	0.49±0.20
^{125}I-Ang I, 10^3 cpm/L	546±76	501±52	420±28	545±106	620±63	563±73	369±28	459±50
^{125}I-Ang II, 10^3 cpm/L	263±19	255±33	232±46	286±62	100±15*†	385±15	186±12	352±81
^{125}I-Ang II/I ratio	0.50±0.05	0.51±0.05	0.56±0.12	0.57±0.14	0.17±0.03*†	0.73±0.09	0.51±0.01	0.74±0.13
Coronary vein								
Ang I, pmol/L	2.6±0.8	7.1±3.2	4.7±1.1	2.5±1.0	21.8±7.6*†	15.8±3.9*†	1.9±0.3	3.1±0.8
Ang II, pmol/L	1.1±0.2	4.8±2.0	2.8±0.8	1.4±0.3	4.5±1.4	18.0±3.2*†	1.3±0.2	1.9±0.6
Ang II/I ratio	0.58±0.22	0.63±0.0.8	0.49±0.08	0.66±0.10	0.18±0.06*†	0.96±0.16	0.65±0.25	0.59±0.13
^{125}I-Ang I, 10^3 cpm/L	257±35	323±35	203±31	267±74	455±50	225±28	193±25	250±26
^{125}I-Ang II, 10^3 cpm/L	277±23	268±38	223±48	289±52	97±11*†	346±16	223±33	349±58
^{125}I-Ang II/I ratio	1.12±0.08	0.82±0.05	1.14±0.16	1.22±0.20	0.21±0.04*†	1.67±0.26	1.17±0.17	1.21±0.17
Coronary ^{125}I-Ang I metabolism								
Fractional conversion, %	24±1	17±1	20±4	25±2	4±1*†	26±4	26±4	24±1
Fractional degradation, %	29±2	20±4	31±6	31±4	23±1	31±5	22±4	22±2

Values are mean±SEM. * $P<0.05$ vs sham; † $P<0.05$ vs untreated MI.

levels in the noninfarcted LV myocardium were no longer observed at 6 weeks, nor were they present in captopril-treated animals at 3 weeks. This, in combination with the notion that eprosartan fully blocked the uptake of circulating ^{125}I-Ang II at all myocardial locations, suggests that RAS blockade prevents the development of LV hypertrophy, at least in part, by attenuating or blocking the AT_1 receptor-mediated uptake of circulating Ang II.

In addition to their effects on cardiac ^{125}I-Ang II uptake, both captopril and eprosartan increased plasma and cardiac Ang I. Because cardiac renin is derived from the circulation, both under normal circumstances and after MI,[34,38,49] these increases most likely reflect the rise in renal renin release that normally accompanies RAS blockade. It is currently unknown whether enzymes other than renin (e.g., cathepsin D[191]) contribute to Ang I generation in infarcted hearts. Similarly, the origin of cardiac angiotensinogen after MI has not been fully elucidated, although recent data suggest that most angiotensinogen in infarcted hearts,[38] like in normal hearts,[34] is plasma-derived.

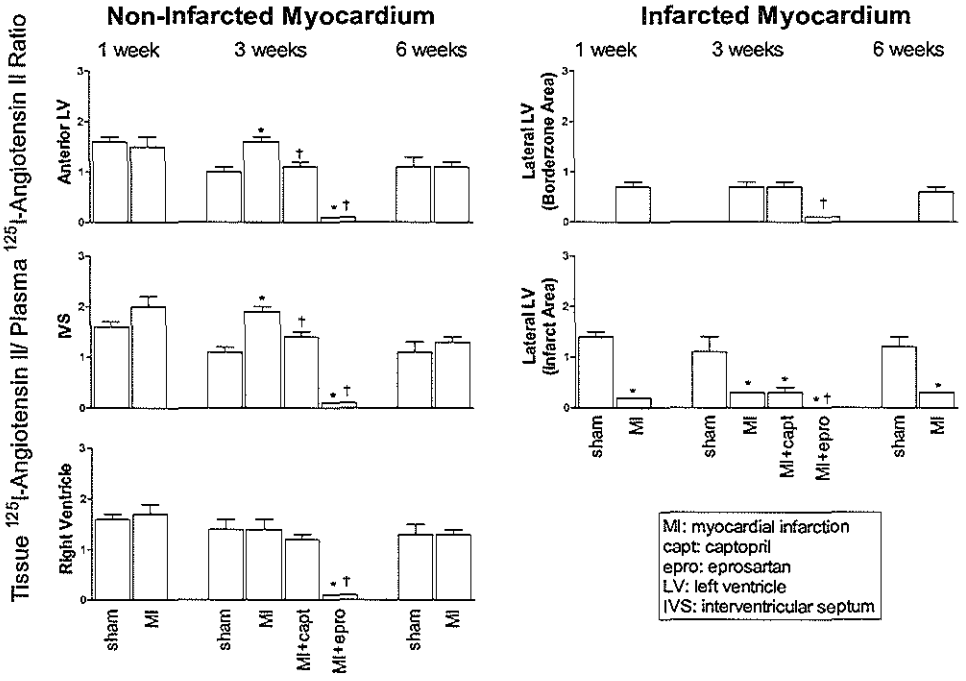

Figure 4. Tissue/plasma concentration ratios of ^{125}I-Ang II. * $P<0.05$ vs sham; † $P<0.05$ vs untreated MI.

In the eprosartan-treated pigs, plasma and cardiac Ang II rose in parallel with Ang I, whereas in the captopril-treated pigs, Ang II did not change in the circulation but increased in the heart. Consequently, the Ang II/Ang I ratio, a measure of ACE activity, was decreased in plasma but not in the heart after captopril treatment.

The rise in cardiac Ang II synthesis with captopril may have several causes. First, captopril may not have entered the heart in sufficient quantities. This seems unlikely, because in an earlier study, we observed a clear reduction in the cardiac Ang II/Ang I ratio after 3 days of treatment with the same dose of captopril.[66] It is also unlikely in view of the favorable cardiac effects of captopril in humans following MI.[177] Second, cardiac Ang II synthesis might occur at intracellular sites[36] that cannot be reached by ACE inhibitors. Third, the initial captopril-induced blockade of cardiac tissue Ang I-Ang II conversion[66] may have been compensated for during long-term treatment with this drug, either through an upregulation of ACE[178] or because alternative converting enzymes such as chymase[61] have come into play. Our findings do not provide evidence for changes in cardiac or coronary ACE activity, because neither the cardiac Ang II/Ang I ratio nor coronary Ang I-Ang II conversion were altered in MI animals. Although MI-induced ACE upregulation has been described,[56,189] transgenic rats overexpressing cardiac ACE 40-fold have normal cardiac Ang II levels.[86] Moreover, we failed to observe ACE gene insertion/deletion polymorphism-related differences in vascular Ang I-Ang II conversion despite profound effects of this polymorphism on plasma and tissue ACE levels.[192] Thus, elevated cardiac ACE levels, if present in MI pigs, do not necessarily result in elevated Ang II levels or Ang II/Ang I ratios but may explain why captopril decreases the cardiac Ang II/Ang I ratio less effectively. Furthermore, chymase is present in the porcine heart,[193] and its concentration increases following MI.[194]

Does the rise in cardiac Ang II production during RAS blockade have a physiological function? If caused by chymase, one must realize that chymase is present in the cytosol of mast cells and in the extracellular matrix,[61] whereas ACE is located on the cell membrane, in proximity to AT_1 receptors.[76,189] Consequently, Ang II generated by chymase may couple less efficiently to AT_1 receptors than Ang II generated by ACE.[29]

AT_2 receptor antagonism abolishes the beneficial effects of AT_1 receptor blockade in MI rats.[10] This raises the possibility that the rise in Ang II in the present study results in stimulation of growth-inhibitory AT_2 receptors.[3,119] The AT_2 receptor density is increased in infarcted and failing hearts,[167,180] and because the net effect of Ang II depends on the AT_1/AT_2 receptor ratio,[3] it is indeed conceivable that growth inhibition occurs, not only during AT_1 receptor antagonism, but also during ACE inhibition, because the latter prevents the rise in

AT_1 receptor density following MI (the present study), and is possibly accompanied by chymase-dependent Ang I-Ang II conversion at sites distant from AT_1 receptors.[29,76]

Enhanced AT_2 receptor stimulation, together with diminished AT_1 receptor stimulation, might also explain why captopril and eprosartan prevented RV hypertrophy in MI animals. Both drugs minimally affected the increase in pulmonary arterial pressure that, in combination with the elevated RV Ang II levels at 1 week after MI, may have contributed to this hypertrophy.

Interestingly, the lowest ^{125}I-Ang II accumulation and local Ang II production were observed in the infarct area. The latter finding could indicate that in this area, possibly secondary to the reduced blood flow, renin uptake was diminished in parallel with the diminished ^{125}I-Ang II uptake. Alternatively, and perhaps more likely in view of the unaltered local Ang I production in this area, the low ^{125}I-Ang II and Ang II levels might be the consequence of increased local Ang II degradation.

In summary, MI results in a transient upregulation of AT_1 receptors in spared noninfarcted myocardium, which will cause enhanced sequestration of plasma Ang II even in the absence of changes in the circulating RAS. RAS inhibitors prevent the rise in plasma Ang II sequestration, either by interfering with myocardial AT_1 receptor upregulation or by blocking these receptors. Furthermore, these inhibitors increase tissue Ang II production, which through stimulation of cardiac AT_2 receptors may minimize postinfarct remodeling.

Acknowledgements

Drs van Kats and Duncker were supported by the Dutch Kidney Foundation (grant NSN 96.1585) and the Royal Netherlands Academy of Arts and Sciences, respectively.

References are presented in the general reference list.

Chapter 6

Cardiac interstitial fluid levels of angiotensin I and II in the pig

J Hypertens. 1999; 17: 1185-1891

Summary

To study whether cardiac interstitial fluid levels of angiotensin (Ang) I and II can be monitored *in vivo*, using the microdialysis technique, and to assess the contribution of plasma-derived angiotensins to the interstitial fluid levels of these peptides, microdialysis probes were placed in the left ventricular (LV) myocardium of eight anesthesized pigs, three of which were untreated and five treated with the Ang II type 1 (AT_1) receptor antagonist L-158,809 (10 mg intracoronary). All pigs were given a 1-h intracoronary infusion of ^{125}I-Ang II. Aortic and coronary venous blood samples were taken under steady-state conditions, and interstitial dialysate was collected during the entire infusion period. Immediately after stopping the infusion, LV tissue pieces were obtained at various time points. L-158,809 did not affect the levels of endogenous Ang I and II or the levels of plasma ^{125}I-Ang II. Aortic Ang I and II (22 and 16 fmol/ml; geometric mean of eight pigs) were comparable to coronary venous Ang I and II levels, whereas the coronary venous ^{125}I-Ang II levels (6650 c.p.m./ml) were approximately 30 times higher than those in the aorta. Tissue Ang I and II were 5 and 17 fmol/g, respectively. In untreated animals, the ^{125}I-Ang II levels per g LV tissue were similar to the levels per ml coronary venous plasma, and the *ex-vivo* half-life of tissue ^{125}I-Ang II was >30 min. In treated animals, tissue ^{125}I-Ang II was <5% of coronary venous ^{125}I-Ang II and became undetectable within 15 min. ^{125}I-Ang II, Ang I and Ang II in interstitial fluid were close to or below the detection limit (200 c.p.m., 60 fmol and 20 fmol per ml, respectively) in all animals. In conclusion, plasma and myocardial interstitial fluid angiotensin levels are of the same order of magnitude. Plasma Ang II does not contribute to the interstitial fluid level of Ang II, most likely because of its rapid metabolism in the vascular wall. Binding to AT_1 receptors protects Ang II against metabolism.

Introduction

It is now generally accepted that angiotensin (Ang) I and II are not only produced in circulating blood, but also at tissue sites. Using systemic infusions of ^{125}I-labeled Ang I and II we have recently demonstrated that, in the heart, the majority of tissue Ang I and II is produced locally and not derived from the circulation.[66] Uptake of circulating Ang II, which occurs predominantly via AT_1 receptor-mediated endocytosis,[74,195] accounts for less than 20% of cardiac tissue Ang II.

It is not yet known where tissue angiotensin production occurs. Renin and angiotensinogen diffuse into the interstitial space,[35,37,47] and thus angiotensin generation might occur in cardiac interstitial fluid. In a modified version of the isolated perfused rat Langendorff heart, which allows separate collection of interstitial fluid and coronary effluent, Ang I and II are generated in interstitial fluid if renin and angiotensinogen are provided via the perfusion buffer.[36,47] The angiotensin levels in interstitial fluid during perfusion of the isolated rat heart with renin and angiotensinogen were only marginally higher than in coronary effluent. In contrast, using the microdialysis technique, Dell'Italia *et al.*[196] demonstrated that the angiotensin levels in interstitial fluid, obtained from the dog heart *in vivo*, are 100 times higher than in plasma. Such high angiotensin levels have also been found in interstitial fluid obtained from rat kidneys[197]. In general, the angiotensin levels in the cardiac atria and ventricles, unlike those in the kidney, are not several orders of magnitude higher than the angiotensin levels in plasma.[66,80] Consequently, high cardiac interstitial fluid angiotensin levels do not appear to be in accordance with the reported angiotensin levels in the heart.

The present study aimed to compare the *in-vivo* angiotensin levels in blood plasma, cardiac interstitial fluid and cardiac tissue in the pig, in order to investigate whether tissue Ang I and II are restricted to extracellular fluid or cell-associated. Interstitial fluid was collected during intracoronary infusion of ^{125}I-Ang II to quantify the contribution of circulating Ang II to the interstitial fluid levels of this peptide. To address the possibility that binding to AT_1 receptors contributes to the interstitial clearance of Ang II, we infused both untreated pigs and pigs treated with the AT_1 receptor antagonist L-158,809.

Methods

In-vivo studies

All experiments were performed under the regulations of the Animal Care Committee of the Erasmus University Rotterdam, Rotterdam, The Netherlands, in accordance with the 'Guiding Principles in the Care and Use of Animals' approved by the American Physiological Society. Eight female pigs (crossbred Yorkshire × Dutch Landrace) with a body weight of 25-30 kg were included in the study. Two of these pigs had been used, immediately prior to the present experiments, in acute pharmacological studies investigating the effects of the calcium sensitizer EMD 57033. All animals were prepared for hemodynamic measurements, administration of anaesthetic and ^{125}I-labeled Ang II, and for blood and tissue sampling as described previously.[74,129] After a stabilization period of 30-45 minutes following completion of instrumentation, baseline hemodynamic measurements were made. Subsequently, five animals were given a 10-min infusion of the AT_1 receptor antagonist L-158,809 into the left anterior descending coronary artery (LADCA), at a dose (1 mg/min) previously shown to block the Ang II-mediated effects in the area perfused by the LADCA[129]. The other three animals were not pretreated. Thirty minutes later, a 1-h infusion of ^{125}I-Ang II (approximately 7×10^5 c.p.m./min) into the LADCA was started in all eight animals.

Blood, interstitial fluid and tissue sampling

Blood samples (10 ml) were taken from the aorta and great cardiac vein 20 and 55 min after the start of the infusion of ^{125}I-Ang II, i.e., under steady-state conditions.[74,187] The blood was rapidly drawn with a plastic syringe containing the following inhibitors (0.5 ml inhibitor solution in 10 ml blood): 0.2 mM of the renin inhibitor remikiren, 125 mM disodium EDTA and 25 mM 1,10-ortho-phenanthroline. The blood was immediately transferred into prechilled polystyrene tubes and centrifuged at 3000 *g* for 10 min at 4°C. Plasma was stored at -70°C and assayed within 3 days.

To collect interstitial fluid, microdialysis probes (CMA/20, Carnegie Medicine AB, Sweden; cut-off value 20 kDa) were inserted into the left ventricular myocardium in the region perfused by the LADCA (i.e., the area exposed to ^{125}I-Ang II) and in the region perfused by the left circumflex coronary artery (LCXCA; control area), as described before.[196,197] The probes were perfused by a microinjection pump (CMA/100, Carnegie Medicine AB) at a flow rate of 2 µl/min with isotonic Ringer's solution. The dialysate was

collected over the entire 60-min infusion period in chilled polystyrene tubes containing 10 µl of the above inhibitor solution and 10 µl 0.1% bovine serum albumin in saline.

The heart was removed immediately after the infusion of ^{125}I-Ang II had been stopped. Prior to its removal from the body, the heart was stopped by fibrillation, while the infusion was still running. Immediately after the heart had been removed from the body, pieces of left ventricular free wall tissue (1-2 g) from the areas perfused by the LADCA and the LCXCA were excised and transferred into liquid nitrogen. The tissues were frozen within 15 s after the heart had been stopped. To study the *ex-vivo* degradation of ^{125}I-Ang II in tissue, further parts of left ventricular wall tissue from the LADCA area were cut off and rapidly frozen in liquid nitrogen at various time points after the heart had been stopped. The frozen tissues were stored at -70°C and assayed within 3 days.

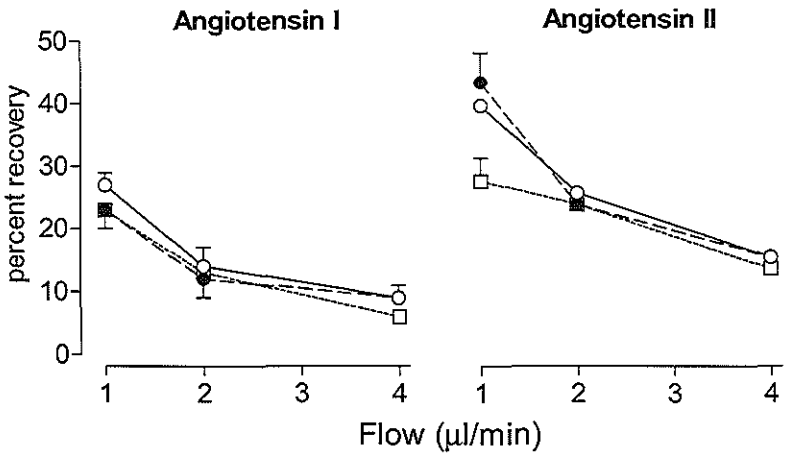

Figure 1. Recovery of angiotensin I and II in dialysate collected with microdialysis probes perfused at varying flow rates in Eppendorf cups containing 2 (o), 5 (•) and 10 (□) pmol angiotensin/ml. Data are means±SEM of three experiments.

In-vitro studies

Microdialysis is based on the principle that, as the dialysate solution passes through the microdialysis fibre, diffusion occurs between the fluid within the fibre and the interstitial fluid surrounding the fibre. Since it is unlikely that complete equilibration occurs between the solution within the fibre and the interstitial fluid in the vicinity of the fibre, we estimated the recovery from our microdialysis probes by perfusing microdialysis probes at 1, 2 and 4 µl/min with Ringer's solution in an Eppendorf cup containing Ang I and II in Ringer's solution

(maintained at 37°C) at concentrations ranging from 2-10 pmol/ml. Recovery was determined by comparing the concentration in the dialysis probe effluent to that of the solution in the Eppendorf cup. Figure 1 shows the recovery of Ang I and Ang II at different concentrations and flow rates. Recovery was unrelated to angiotensin concentration, but diminished rapidly at high flow rates. At 2 µl/min, the rate that was used for the present *in-vivo* experiments, the recovery was 13±2% (mean±SEM of all experiments, n=9) for Ang I and 23±1% for Ang II. Recent studies on noradrenaline kinetics in the porcine heart have shown that *in-vitro* probe recoveries are in good agreement with those *in vivo*.[198] The *in-vitro* recovery percentages for Ang I and Ang II were therefore used to correct the angiotensin concentrations measured in the dialysate collected during the ^{125}I-Ang II infusions. We assumed the recovery of ^{125}I-Ang II to be similar to that of Ang II, although in reality it may have been somewhat lower because the presence of the ^{125}I-label will increase the molecular weight of Ang II by about 10%.

Measurement of angiotensins

Frozen tissue samples were homogenized in 20 ml ice-cold 0.1 M HCL/80% ethanol as previously described[34,74]. The homogenate was centrifuged at 20,000 *g* for 25 minutes at 4°C. Ethanol in the supernatant was evaporated under constant air flow and the remainder of the supernatant was diluted in 20 ml 1% ortho-phosphoric acid and centrifuged again at 20,000 *g*. The supernatant was diluted with an equal volume of 1% ortho-phosphoric acid and then concentrated on SepPak cartridges. Plasma was directly applied to the SepPak cartridges. The preparation of the SepPak extracts for high-performance liquid chromatographic (HPLC) separation of the angiotensins and the HPLC procedure have been described elsewhere[34,74,187]. Interstitial fluid dialysates were applied directly to the HPLC. The concentration of intact ^{125}I-Ang II and the concentrations of intact Ang I and II in the HPLC eluate fractions were measured by gamma counting and radioimmunoassay, respectively. Data were not corrected for losses occurring during extraction and separation. These losses were less than 10% in plasma, and maximally 20-30% in tissue extracts. The lowest Ang I levels that could be measured were 1 fmol/ml in plasma, 60 fmol/ml in interstitial fluid and 2 fmol/g in tissue. The lowest Ang II levels that could be measured were 0.5 fmol/ml in plasma, 20 fmol/ml in interstitial fluid and 1 fmol/g in tissue. The lowest ^{125}I-Ang II levels that could be measured were 5 c.p.m./ml in plasma, 200 c.p.m/ml in interstitial fluid and 10 c.p.m./g tissue.

Statistics

Hemodynamic data and data from the *in-vitro* experiments are expressed as mean±SEM. All other data are expressed as geometric mean and range, because of non-normal distribution. Angiotensin levels that were below the detection limit were taken to be equal to the lower limit of detection in order to allow statistical calculations. Differences in angiotensin levels between untreated and L-158,809-treated animals were tested by Mann-Whitney U test. A P value of 0.05 was taken to indicate statistical significance.

We calculated the predicted cardiac tissue levels of Ang I, Ang II or ^{125}I-Ang II in the area perfused by the LADCA, assuming that angiotensins are restricted to extracellular fluid (i.e., blood plasma and interstitial fluid, accounting for 5 and 15% of tissue weight, respectively),[35,37,47,199,200] as follows:

$$\text{predicted } [\text{Ang}]_{\text{tissue}} = 0.05 \times [\text{Ang}]_{\text{coronary vein}} + 0.15 \times [\text{Ang}]_{\text{interstitial fluid}}$$

in which [Ang] represents the level of Ang I, Ang II or ^{125}I-Ang II per g tissue or per ml fluid.

Predicted tissue levels were compared with measured tissue levels. Higher measured tissue levels were taken as evidence for the presence of angiotensins outside the extracellular fluid compartment (i.e., as evidence for their localization on or in cells).

Table 1. Endogenous angiotensin I and II levels and angiotensin II/I ratios in aortic blood plasma, coronary venous blood plasma, cardiac interstitial fluid and cardiac tissue obtained from eight pigs. Interstitial fluid and tissue were obtained from the left ventricular free wall regions perfused by the LADCA and the LCXCA. Predicted cardiac tissue angiotensin levels in the LADCA area were calculated based upon the assumption that angiotensins are restricted to extracellular fluid

	Blood		Interstitial Fluid		Cardiac Tissue		
Parameter	Aorta	Great Cardiac Vein	LADCA area	LCXCA area	LADCA area (measured)	LADCA area (predicted)	LCXCA area (measured)
Ang I (fmol/ml or fmol/g)	22 (1.0-281)	24 (1.0-251)	<129 (ND-1236)	<120 (ND-1785)	4.9 (2.0-21)	22** (9.0-189)	3.4 (2.0-19)
Ang II (fmol/ml or fmol/g)	16 (1.0-283)	22 (3.0-158)	<30 (ND-262)	<37 (ND-819)	17 (7.0-195)	6.6** (3.2-45)	27 (9.0-210)
Ang II/I ratio	0.72 (0.09-6.8)	0.95 (0.39-3.0)	-	-	3.5* (1.0-14)	-	8.0* (4.5-17)

Data are geometric mean and range in parentheses; ND=not detectable; * P<0.05 versus aorta and coronary vein; ** P<0.05 versus measured tissue level in the LADCA area.

Results

The baseline mean arterial pressure, heart rate and cardiac output were 74±5 mmHg, 128±11 beats/min and 2.4±0.2 l/min. These hemodynamic parameters were not affected by acute intracoronary administration of L-158,809 or by the infusion of ^{125}I-Ang II (data not shown). In agreement with previous findings,[129] the levels of endogenous Ang I and II did not differ between untreated and L-158,809-treated animals. Table 1 summarizes the findings in all animals. Aortic Ang I and II levels were comparable to coronary venous Ang I and II levels. Tissue Ang I and II levels in the area perfused by the LADCA and the LCXCA, measured in tissue that was frozen within 15 s of the heart being stopped, did not differ. Measurements in tissue pieces that had been frozen at later time points did not differ from those in tissue pieces that were frozen within 15 s (data not shown). The Ang II/I ratio in cardiac tissue was five- to ten-fold higher than in plasma. The angiotensin levels in interstitial fluid were close to or below the detection limit of our assay. This is not due to rapid metabolism in interstitial dialysate prior to its mixture with angiotensinase inhibitors, since the addition of Ang I and II (2 pmol/ml) to freshly obtained dialysate at 37°C revealed no detectable breakdown over a 1-h period in the absence of inhibitors (n=3, data not shown).

Table 2. Levels of ^{125}I-Ang II in aortic blood plasma, cardiac interstitial fluid and cardiac tissue obtained from three untreated pigs and five pigs pretreated with the AT_1 receptor antagonist L-158,809. Interstitial fluid and tissue were obtained from the left ventricular free wall regions perfused by the LADCA and the LCXCA. Predicted cardiac tissue ^{125}I-Ang II levels in the LADCA area were calculated based upon the assumption that angiotensins are restricted to extracellular fluid.

	Blood		Interstitial Fluid		Cardiac Tissue		
Parameter	Aorta	Great Cardiac Vein	LADCA area	LCXCA area	LADCA area (measured)	LADCA area (predicted)	LCXCA area (measured)
^{125}I-Ang II (c.p.m./ml or c.p.m./g) without AT_1 receptor blockade	263 (94-1174)	7094* (4870-14685)	<200 (ND)	<200 (ND)	3303 (603-12602)	388** (274-764)	122 (27-580)
^{125}I-Ang II (c.p.m./ml or c.p.m./g) with AT_1 receptor blockade	201 (138-256)	6330* (3299-13842)	<279 (ND-642)	<247 (ND-575)	240*** (86-693)	251 (49-722)	19*** (ND-85)

Data are geometric mean and range in parentheses; ND=not detectable; * P<0.01 versus aorta and coronary vein; ** P<0.01 versus measured tissue level in the LADCA area; *** P<0.01 without versus AT_1 receptor blockade

In untreated animals, the steady-state ^{125}I-Ang II levels in the coronary vein were approximately 30 times higher than in the aorta (Table 2). The tissue ^{125}I-Ang II levels in the area exposed to ^{125}I-Ang II (LADCA area) were comparable to or somewhat lower than the levels in coronary venous plasma, whereas the tissue ^{125}I-Ang II levels in the control area

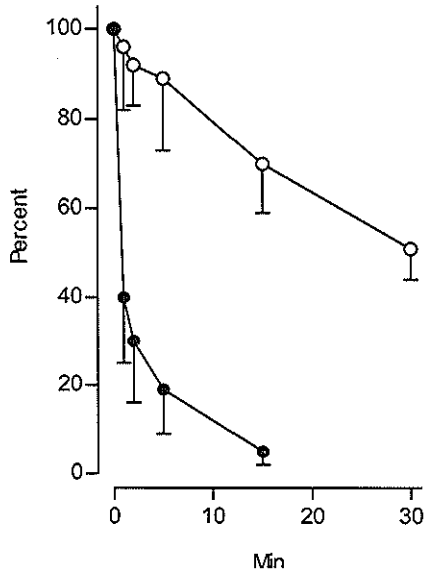

Figure 2. *Ex-vivo* decrease in ^{125}I-Ang II in cardiac tissue obtained from the LADCA area in three untreated pigs (o) and five pigs pretreated with the AT_1 antagonist L-158,809 (10 mg intracoronary; •). Values (mean±SEM) are expressed as a percentage of the levels measured immediately after removal of the heart from the body

(LCXCA area) were in the same range as the aortic ^{125}I-Ang II levels. L-158,809 did not affect the plasma levels of ^{125}I-Ang II, but greatly reduced the tissue levels of the radiolabeled peptide. The *ex vivo* half-life of tissue ^{125}I-Ang II in the LADCA area was >30 min in untreated animals (Figure 2). In L-158,809-treated animals, tissue ^{125}I-Ang II in the LADCA area decreased rapidly, and became undetectable within 15 min.

Interstitial dialysate collected during the course of the experiment contained radioactivity (2,570 c.p.m./ml; range 1,137-8,524 c.p.m./ml). After correction for recovery, on the basis of the recoveries for Ang I and II, this concentration is comparable to the concentration of radioactivity in coronary venous plasma (19,413 c.p.m./ml; range 1,137-60,259 c.p.m./ml). Thus, radiolabeled angiotensins had reached a steady state in the interstitium after 1 h of ^{125}I-Ang II infusion. HPLC separation showed that the majority of the radioactivity in interstitial dialysate eluted in a peak corresponding with ^{125}I-tyrosine (Figure 3). Intact ^{125}I-Ang II could be demonstrated in two samples from the LADCA area and in one sample from the LCXCA area. It was below the detection limit in all other dialysates.

The predicted tissue Ang I levels in the LADCA area were comparable to or higher than the measured Ang I levels, whereas the predicted tissue Ang II levels were at least three-fold lower than the measured Ang II levels. This suggests that tissue Ang I is predominantly extracellular, whereas tissue Ang II is largely cell-associated. In untreated animals, the predicted tissue ^{125}I-Ang II levels were also much lower than the measured tissue levels, while in L-158,809-treated animals the predicted and measured tissue ^{125}I-Ang II levels were similar. Thus, in pigs treated with AT_1 receptor blocker, but not in untreated pigs, tissue ^{125}I-Ang II is located extracellularly.

Discussion

The use of the microdialysis technique in the present study, which allows the collection of interstitial dialysate under *in-vivo* circumstances, shows that the interstitial fluid levels of Ang I and II in the pig heart are of the same order of magnitude as the plasma levels of these peptides. These data are in accordance with previous *in-vitro* studies in a modified version of the rat Langendorff heart, where, during perfusion of the heart with renin and angiotensinogen, the angiotensin levels in interstitial transudate were two- to three-fold higher than those in coronary effluent.[36,47]

Comparison of the tissue, interstitial fluid and plasma levels of Ang I reveals that tissue Ang I is largely extracellular. In fact, the level of Ang I expected per g of tissue based upon the assumption that Ang I is restricted to trapped blood plasma and interstitial fluid (the 'predicted tissue level'),[35-37,47] was higher than the tissue level that was actually measured. The reason for this discrepancy is either an underestimation of tissue Ang I because of *ex vivo* metabolism of extracellular angiotensin, or the fact that in some cases the actual Ang I level in interstitial fluid was below the detection limit of our assay. In such cases we assumed interstitial Ang I to be equal to the detection limit in order to predict the tissue level. Since tissue Ang I levels remain constant for at least 1 h after removal of the heart from the body,[66] the second possibility is the most likely.

Interestingly, the measured tissue levels of Ang II were three-fold higher than the predicted tissue levels of this peptide. Thus, Ang II, in contrast with Ang I, is largely cell-associated, either due to AT_1 receptor-dependent internalization[36,74] or intracellular generation. Our findings with ^{125}I-Ang II support the first possibility. Experimental evidence demonstrating binding and internalization of renin and prorenin and intracellular activation of prorenin by cardiac and endothelial cells[40,201] supports the second possibility. Additional studies involving long-term treatment with AT_1 receptor antagonists are needed to solve this issue.

The low levels of ^{125}I-Ang II in interstitial fluid suggest that circulating Ang II only marginally contributes to the interstitial fluid levels of Ang II. This is most likely due to rapid metabolism in the vascular wall[36,47] by angiotensinases such as aminopeptidases A and M,[202] prolyl endopeptidase[203] and carboxypeptidases.[204] Consequently, and in agreement with our *in-vitro* observations in the rat Langendorff heart,[36] the majority of Ang II present in interstitial fluid must have been synthesized locally. These results therefore fully support the concept of tissue Ang II generation as opposed to Ang II generation in the circulation. They are also in agreement with recent studies showing that tissue angiotensin-converting enzyme (ACE)

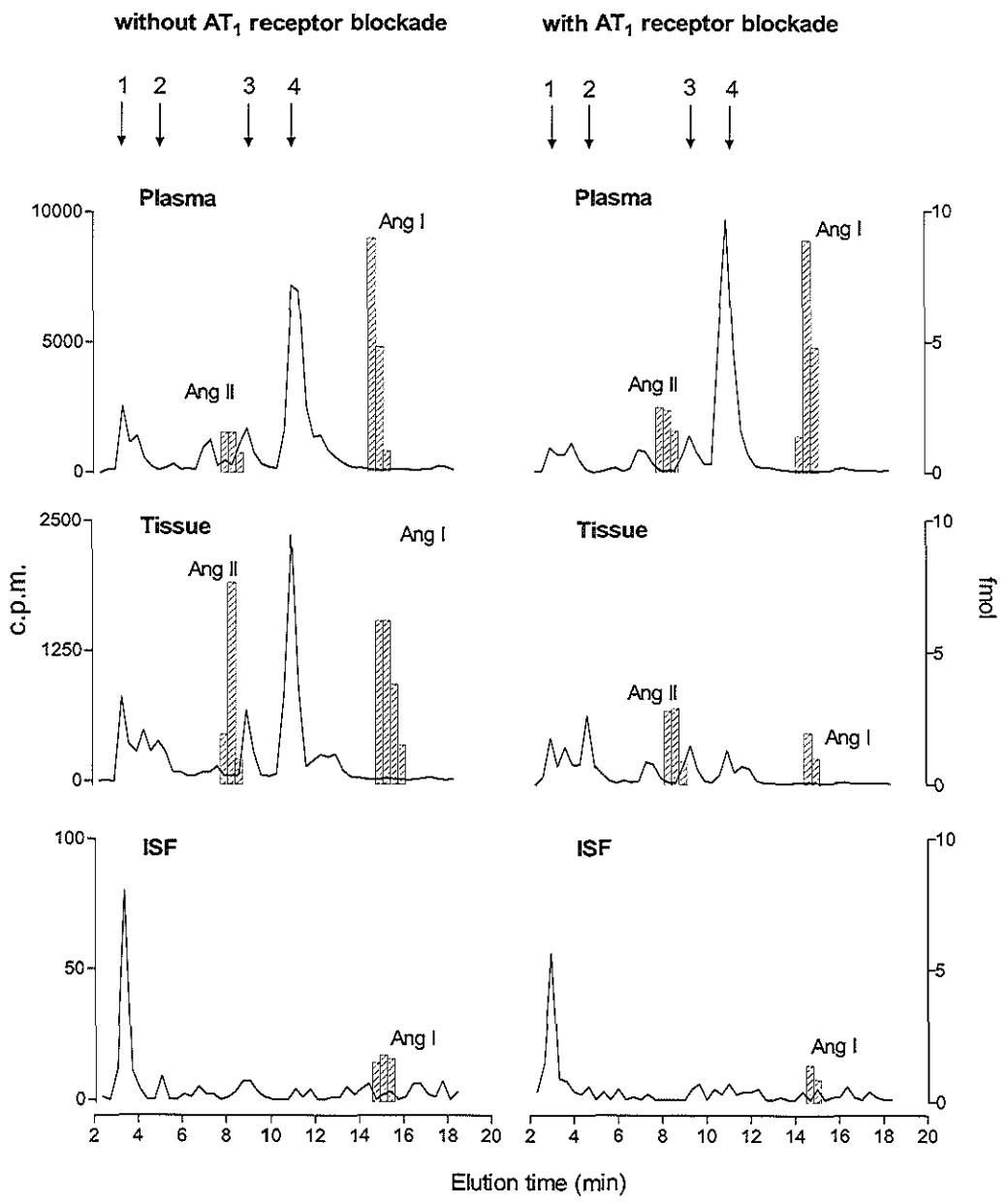

Figure 3. HPLC elution profile of endogenous (hatched bars) and ^{125}I-labelled (lines) angiotensins in coronary venous blood plasma, cardiac tissue and interstitial fluid (ISF) from the area perfused by the LADCA in an untreated pig (left panels) and a pig pretreated with the AT_1 receptor antagonist L-158,809 (right panels). The retention times of ^{125}I-labeled standards are indicated by arrows at the top of the figure: 1, ^{125}I-tyrosine; 2, ^{125}I-angiotensin-(1-7); 3, ^{125}I-angiotensin-(4-8); 4, ^{125}I-angiotensin II.

knockout mice, despite the fact that ACE is present in the plasma of these animals, display the same hemodynamic and vascular abnormalities as mice that are completely ACE deficient.[205,206]

Treatment with the AT_1 receptor antagonist L-158,809 did not result in elevated interstitial fluid levels of ^{125}I-Ang II. Moreover, the *ex vivo* half-life of tissue ^{125}I-Ang II in the presence of this drug was in the order of 1 min or less, which is comparable to the half-life of angiotensins in the circulation.[187] Since ^{125}I-Ang II is restricted to the extracellular fluid compartment during AT_1 receptor blockade, these data suggest that the clearance of Ang II in the interstitial space is comparable to its clearance in the circulation, and that receptor binding does not make an important contribution importantly to the disappearance of Ang II from extracellular fluid.

Why do the *in-vivo* interstitial fluid levels of Ang I and II resemble the plasma levels of these peptides? Clearly, this is not due to the fact that angiotensins diffuse freely from the circulation into the interstitial space and vice versa, because such diffusion is accompanied by rapid metabolism.[36,47] The most likely reason for these similar angiotensin levels is the fact that circulating renin and angiotensinogen also diffuse into the interstitial space, and that, under steady-state conditions, their levels in cardiac interstitial fluid are similar to those in plasma.[35,37,47] Thus, in tissues where local angiotensin production synthesis depends on uptake of renin and angiotensinogen from the circulation (such as the heart and the vascular wal),[198,207] the Ang I-generating capacity will be similar in plasma and interstitial fluid. High interstitial fluid angiotensin levels might only be expected under conditions where interstitial angiotensin clearance is reduced as compared to clearance in the vascular compartment (e.g., *in vitro*, in the isolated perfused heart),[36,47] or when renin and/or angiotensinogen are produced locally (e.g., in the kidney).[197] The high interstitial fluid levels reported in the dog heart[196] suggest that in this animal, in contrast with humans, pigs and rats,[47,49,187] renin is synthesized at cardiac tissue sites.

References are presented in the general reference list.

Chapter 7

Vasoconstriction is determined by interstitial rather than circulating angiotensin II

Br J Pharmacol. 2002; 135: 275-283

Summary

We investigate why angiotensin (Ang) I and II induce vasoconstriction with similar potencies, although Ang I-II conversion is limited. Construction of concentration-response curves to Ang I and II in porcine femoral arteries, in the absence or presence of the AT_1 or AT_2 receptor antagonists irbesartan and PD123319, revealed that the ≈2-fold difference in potency between Ang I and II was not due to stimulation of different AT receptor populations by exogenous and locally generated Ang II. Measurement of Ang I and II and their metabolites at the time of vasoconstriction confirmed that, at equimolar application of Ang I and II, bath fluid Ang II during Ang I was ≈18 times lower than during Ang II and that Ang II was by far the most important metabolite of Ang I. Tissue Ang II was 2.9±1.5% and 12.2±2.4% of the corresponding Ang I and II bath fluid levels, and was not affected by irbesartan or PD123319, suggesting that it was located extracellularly. Since ≈15% of tissue weight consists of interstitial fluid, it can be calculated that interstitial Ang II levels during Ang II resemble bath fluid Ang II levels, whereas during Ang I, they are 8.8-27 fold higher. Consequently at equimolar application of Ang I and II, the interstitial Ang II levels differ only 2-4 fold. In conclusion, interstitial, rather than circulating Ang II determines vasoconstriction. Arterial Ang I, resulting in high interstitial Ang II levels via its local conversion by ACE, may be of greater physiological importance than arterial Ang II.

Introduction

Angiotensin (Ang) I and II both induce vasoconstriction *via* stimulation of AT_1 receptors, the former following its conversion to Ang II by ACE and/or chymase.[64,208] In healthy volunteers, during intrabrachial Ang I infusion, one third of arterially delivered Ang I is converted to Ang II in the forearm vascular bed,[209] whereas in isolated human coronary arteries mounted in organ baths, the Ang II levels reached in the organ bath at the time of contraction are <1% of the levels of Ang I.[29] Yet, despite the relatively low Ang II levels during Ang I administration, the potencies of Ang I and Ang II *in vivo* as well as *in vitro* are similar.[29,209-211] Likewise, in isolated perfused rat hindquarters, renin infusion causes equal increases in perfusion pressure as Ang II infusion, although the Ang II levels during renin perfusion are five times lower than during Ang II perfusion.[212]

Several explanations may solve these discrepancies. Firstly, in view of the vasodilator effects of AT_2 receptors,[213,214] it is possible that locally generated Ang II reaches different AT receptors than exogenously applied Ang II. For instance, exogenous Ang II may stimulate both endothelial (vasodilatory) AT_2 receptors and AT_1 receptors on smooth muscle cells, whereas locally generated Ang II might stimulate the latter predominantly. Secondly, Ang I and Ang II are degraded to vasoconstrictor (Ang-(2-8) and Ang-(3-8), also known as Ang III and Ang IV) and vasodilator (Ang-(1-7)) metabolites,[128,215,216] and this may affect their vasoconstrictor potency. Thirdly, the Ang II levels in the circulation and organ bath may not be representative for the tissue (interstitial fluid) levels in the immediate vicinity of the AT receptors that actually cause vasoconstriction. This theory has also been put forward to explain the beneficial effects of ACE inhibitors on blood pressure and cardiac remodelling in the absence of clear reductions in circulating Ang II.[89,217] Fourthly, results obtained with Ang I and Ang II in organ baths, as well as during systemic infusion *in vivo*, may be of little physiological relevance, since normally vascular Ang II (i.e., the Ang II that is responsible for vasoconstriction) is largely derived from local synthesis by renin and converting enzymes from angiotensinogen.[218-220] In this respect, it is of interest to note that the majority of vascular Ang I-II conversion, at least *in vitro*, is mediated by chymase, a converting enzyme that is located in the adventitia.[29,61,210]

To investigate these issues, we constructed Ang I and Ang II concentration-response curves (CRCs) in porcine femoral and carotid arteries mounted in organ baths. Ang I and Ang II-mediated effects were also studied following preconstriction, to facilitate the detection of vasodilator effects, and in the presence of AT_1 and AT_2 receptor antagonists. Additional

studies with Ang I were performed in the presence of the ACE inhibitor quinaprilat, to investigate the importance of ACE versus chymase. Subsequently, we compared the tissue and bath fluid levels of Ang I and Ang II and their metabolites at the time of maximal vasoconstriction. The amount of vascular Ang II present in interstitial fluid was estimated after correction of the tissue levels for Ang II bound to AT receptors.[74] Finally, we studied the vasoconstrictor effects of Ang I and Ang II applied to the adventitial side of perfused carotid arteries, thereby mimicking the vascular origin of Ang II mediating vasoconstriction.

Methods

Chemicals

Angiotensin I and II, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619) and substance P (acetate salt) were purchased from Sigma (Zwijndrecht, The Netherlands). Irbesartan was a kind gift of Bristol-Myers Squibb (Princeton, NJ, U.S.A.). PD123319 and quinaprilat were a kind gift of Parke Davis (Hoofddorp, The Netherlands). Irbesartan was dissolved in ethanol and quinaprilat in dimethylsulphoxide. All other chemicals were dissolved in distilled water.

Tissue collection

Porcine femoral and carotid arteries were obtained from 33 2-3 month-old pigs (Yorkshire x Landrace, weight 10-15 kg). The pigs had been used in *in-vivo* experiments studying the effects of α-adrenoceptor and serotonin receptor (ant)agonists under pentobarbital (600 mg, i.v.) anesthesia.[221] The Ethics Committee of the Erasmus University Rotterdam dealing with the use of animals for scientific experiments approved the protocol for this investigation. After sacrificing the animal with an overdosis of pentobarbital, the right femoral artery and both carotid arteries were removed and stored overnight in a 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.

Organ bath studies with femoral arteries

To study Ang I- and II-induced vasoconstriction and/or vasodilatation, femoral arteries were cut into segments of approximately 3-4 mm length, suspended on stainless steel hooks in 15-ml organ baths containing Krebs bicarbonate solution, aerated with 95% O_2/5% CO_2, and maintained at 37°C. All vessel rings were allowed to equilibrate for at least 30 min and the

organ bath fluid was refreshed every 15 min during this period. Changes in tissue contractile force were recorded with a Harvard isometric transducer (South Natick, MA, U.S.A.). The vessel rings, stretched to a stable force of about 50 mN, were exposed to 30 mM K^+ twice. Subsequently, the tissue was exposed to 100 mM K^+ to determine the maximal contractile response to K^+. The rings were then allowed to equilibrate in fresh organ bath fluid for 30 min. Next, the vessel rings were pre-incubated for 30 min with or without the AT_1 antagonist irbesartan (1 μM), the AT_2 antagonist PD123319 (1 μM) or the ACE inhibitor quinaprilat (10 μM). Thereafter, Ang I or Ang II (0.3 nM-10 μM) CRCs were constructed. To facilitate detection of vasodilation due to AT_2 receptor stimulation, Ang I or Ang II CRCs were also constructed following preconstriction with 1 μM $PGF_{2\alpha}$ or 10 nM U46619. Preconstrictions amounted to approximately 40-50% of the maximal contraction induced by 100 mM K^+. In the presence of irbesartan, the amount of $PGF_{2\alpha}$ or U46619 required to obtain such preconstrictions was approximately 5-6 times higher, because, at a concentration of 1 μM, irbesartan acts as an antagonist of the thromboxane A_2/prostaglandin endoperoxide receptor.[222] After the addition of Ang I or II, it took 10-15 min to reach a stable contraction. Subsequent angiotensin concentrations were applied as soon as a stable contraction had been reached. Construction of the CRC was discontinued when desensitisation occurred, i.e., when subsequent angiotensin doses elicited no response or decreased contraction. At the end of the experiment the functional integrity of the endothelium was verified by observing relaxation to 1 nM substance P or 100 nM bradykinin after pre-constriction with $PGF_{2\alpha}$ or U46619. To measure the release of newly formed Ang II into the organ bath fluid during Ang I CRCs, bath fluid samples (150 μl) for Ang II measurements were taken as soon as a stable contraction had been reached. All samples were collected in chilled tubes containing 15 μl inhibitor solution (125 mM disodium EDTA and 25 mM 1,10-ortho-phenanthroline) and 15 μl 0.1% bovine serum albumin (BSA) in distilled water. The samples were stored at -80°C until analysis.

Metabolism studies with femoral arteries

To study vascular Ang I-II conversion and Ang I and II metabolism in further detail, femoral arteries were cut into segments of approximately 3-4 mm length (weight 10-45 mg; mean 22 mg), put into test tubes containing 1.0 ml Krebs bicarbonate solution ('incubation fluid'), aerated with 95% O_2/5% CO_2, and maintained at 37°C. After a 10-min equilibration period, vessel rings were pre-incubated for 30 min with or without irbesartan (1 μM), PD123319 (1 μM) or quinaprilat (10 μM). Next, Ang I (10 or 100 pmol) or Ang II (10 pmol), randomly

combined with ^{125}I-Ang I (100,000 c.p.m.) or ^{125}I-Ang II (100,000 c.p.m.), were added to the incubation fluid. The use of radiolabeled angiotensins facilitates the detection of the various angiotenin metabolites, by gamma-counting after their high-performance liquid chromatographic (HPLC) separation (see below). After either 15 or 60 min the vessel segments were removed, washed in fresh Krebs solution and dried on tissue paper. The remaining incubation fluid (≈1 ml) as well as the dried segment were rapidly frozen in liquid nitrogen and stored at -80°C until analysis.

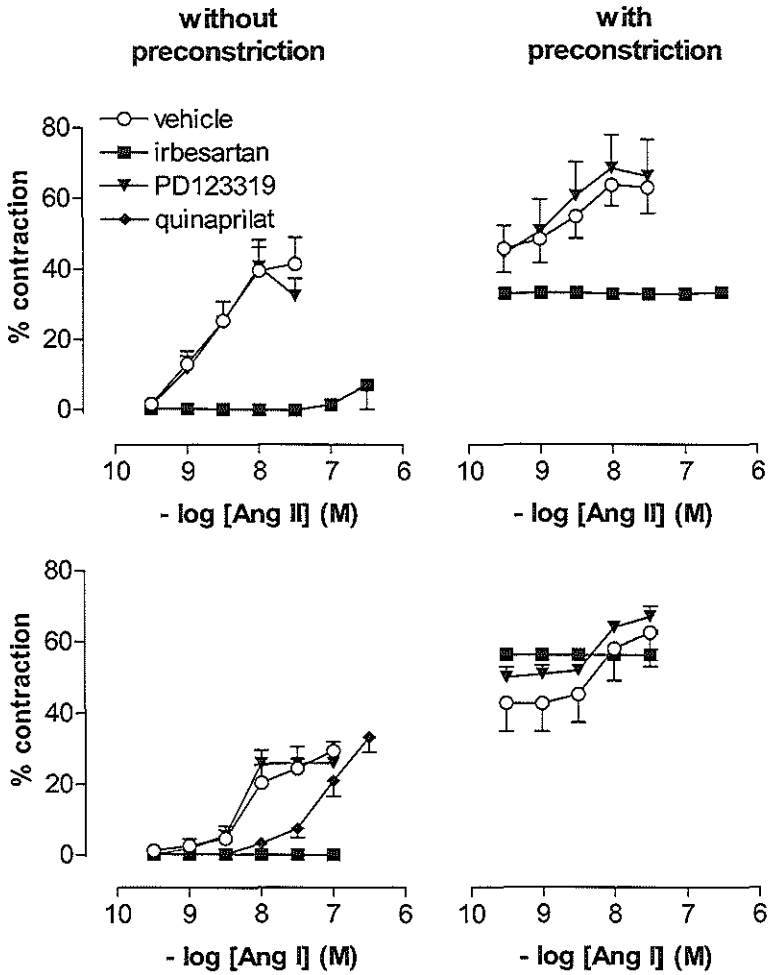

Figure 1. Contractions of femoral arteries, at baseline and after preconstriction with 1 µM $PGF_{2\alpha}$ or 10 nM U46619, to Ang II (upper panels; *n*=8) or Ang I (lower panels; *n*=5) in the absence or presence of 1 µM irbesartan, 1 µM PD123319 or 10 µM quinaprilat. Data are expressed as a percentage (mean±SEM) of the response to 100 mM K^+.

Perfusion and organ bath studies with carotid arteries

To study whether adventitial application of Ang I and II elicits a response that is different from the combined luminal and adventitial application obtained after addition of Ang I or II to the organ bath in the above set-up, carotid arteries (diameter 2-3 mm) were divided in 3-4 mm rings as well as 1-1.5 cm sections. The rings were mounted in organ baths as described above. The vessels sections, which did not contain side branches, were mounted horizontally in a double-jacketed 4-ml bath containing a carbogenated Krebs bicarbonate solution and maintained at 37°C, as described by Hulsmann et al.[223] Krebs solution was perfused through the vessels using a roller pump (Ismatec IPS, Zürich, Switzerland). This approach allows the adventitial application (i.e., into the bath) of drugs. Fluids were refreshed every 15 min. Changes in pressure were recorded with a Viggo-spectramed disposable pressure transducer (Bilthoven, The Netherlands). The vessel sections and rings, at a stable force of 44 mm Hg and 20 mN, respectively, were exposed to 30 mM K^+ twice. Subsequently the tissues were exposed to 100 mM K^+ to determine the maximal contractile response to K^+. The vessels were then allowed to equilibrate in fresh organ bath fluid for 30 min. Thereafter, Ang I or Ang II (1 nM-1 μM) CRCs were constructed as described above. At the end of the experiment the functional integrity of the endothelium of the vessel rings was verified by observing relaxation to 100 nM bradykinin after pre-constriction with 10 nM U46619.

Measurements of angiotensins

In the metabolism studies, Ang I and Ang II and radiolabeled Ang I and Ang II in vascular tissue and incubation fluid were measured as described previously,[74,187] using SepPak extraction and HPLC separation. In short, frozen vessel segments were homogenised in 4 ml ice-cold 0.1 M HCl/80% ethanol. The homogenate was centrifuged at 20,000×*g* for 10 min at 4°C. Ethanol in the supernatant was evaporated under constant air flow. The remainder of the supernatant was diluted in 8 ml 1% ortho-phosphoric acid and concentrated on SepPak cartridges. SepPak extracts were dissolved in 100 μl HPLC elution buffer and injected into the HPLC column. Incubation fluid was directly applied to the column without prior SepPak extraction. The concentrations of Ang I and Ang II and of radiolabeled Ang I and Ang II in the HPLC eluate fractions were measured by radioimmunoassays and gamma counting, respectively. Measurements were not corrected for losses occurring during extraction. These losses were maximally 20-30 %.[74] The lowest Ang I levels that could be measured were 50 fmol/g tissue and 10 fmol/ml incubation fluid. The lowest Ang II levels that could be measured were 25 fmol/g tissue and 5 fmol/ml incubation fluid. The lowest ^{125}I-Ang I and

^{125}I-Ang II levels that could be measured were 500 c.p.m./g tissue and 100 c.p.m./ml incubation fluid.

In the organ bath studies, Ang I and Ang II in organ bath fluid were measured by radioimmunoassay without prior SepPak extraction or HPLC separation.[29] The lowest Ang I and Ang II levels that could be measured in these experiments were 20 and 10 fmol/ml bath fluid, respectively.

Data presentation and statistical analysis

Data are given as mean±SEM. Data of the functional studies are expressed as a percentage of the maximal contraction to 100 mM K^+ (88±5 mN in the organ bath studies with femoral arteries (n=15); 220±20 mmHg in the perfusion studies with carotid arteries (n=7) and 111±8 mN in the organ bath studies with the carotid arteries (n=7)). Data of metabolism experiments are expressed as percentage of the angiotensin concentration in the bath fluid at the start (t=0) of the experiment, assuming that 1 g of tissue equals 1 ml of fluid.[36] To allow statistical evaluation, angiotensin levels that were below the detection limit were taken to be equal to this limit.

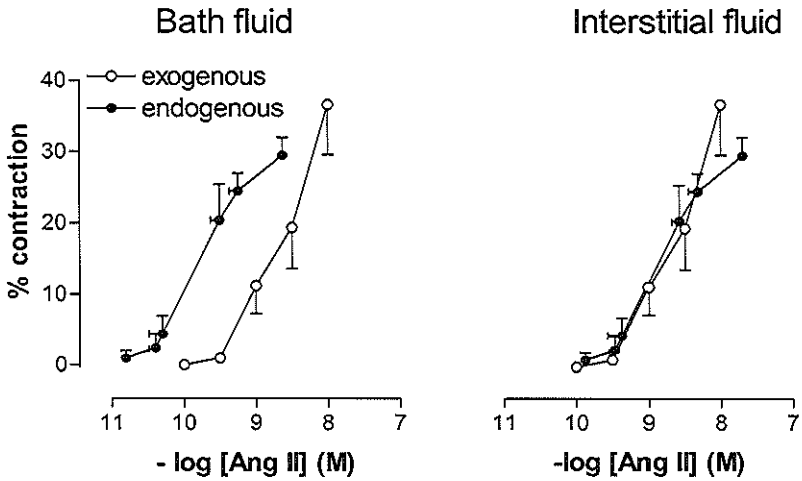

Figure 2. Contractile responses of femoral arteries versus organ bath fluid Ang II levels (left panel) or interstitial fluid Ang II levels (right panel), during Ang I ('endogenous') or Ang II ('exogenous') application. Contractions are expressed as a percentage (mean±SEM; n=5) of the response to 100 mM K^+. Interstitial fluid levels of endogenous and exogenous Ang II were calculated by multiplying the Ang II levels in the organ bath with the interstitial fluid/incubation fluid Ang II concentration ratios obtained 15 min following Ang I (8.5±4.4) or Ang II (1.1±0.2) administration in the metabolism studies.

CRCs were analysed using the logistic function described by de Lean et al.[224] to obtain pEC_{50} ($-^{10}logEC_{50}$) values. Statistical analysis was by ANOVA, followed by *post hoc* evaluation according to Dunnett. *P* values <0.05 were considered significant. Statistics were performed using the software package SigmaStat.

Results

Organ bath studies with femoral arteries

In non-preconstricted femoral arteries (Figure 1), Ang I and Ang II displayed similar maximal effects (E_{max} 31±2% and 37±7%, respectively, n=5). Ang I was 2 fold less potent than Ang II (pEC_{50} 8.21±0.13 and 8.59±0.09, respectively; P<0.05). In femoral arteries that had been preconstricted with $PGF_{2\alpha}$ or U46619 to 34±4 mN (or ≈45% of the maximal contraction induced by 100 mM K^+; n=13), Ang I and Ang II increased contraction further to 63±9% and 62±6% (Figure 1). The potencies of Ang I and Ang II in the preconstricted vessels (pEC_{50} 8.08±0.11 and 8.51±0.05, respectively) were not different from those in the non-preconstricted vessels.

The AT_1 receptor antagonist irbesartan abolished all contractile responses to both Ang I and Ang II, whereas the AT_2 receptor antagonist PD123319 was ineffective under all circumstances. In non-preconstricted vessels, the ACE inhibitor quinaprilat shifted the Ang I CRC approximately 10 fold to the right (pEC_{50} 7.12±0.10, n=5; P<0.001 vs control).

Despite the small difference in potency between Ang I and Ang II, the Ang II levels measured in the organ bath fluid during the Ang I CRC, at the time a stable contraction had been reached, were <3% of the levels of Ang I. Figure 2 (left panel) relates the organ bath fluid levels of Ang II measured during the Ang I CRC ('endogenous Ang II') to the contractile response. For comparison the CRC obtained with exogenous Ang II is also shown in this Figure. The pEC_{50} value of endogenous Ang II (9.84±0.14) illustrates that, for a given contractile response, the organ bath fluid levels of endogenous Ang II are ≈18-fold lower than for exogenous Ang II.

Metabolism studies with femoral arteries

Incubation fluid. Femoral artery segments slowly metabolized ^{125}I-Ang I and Ang I (Figure 3). After 1 h of incubation at 37°C, 56±6% (n=4) and 64±9% (n=6) of the incubation fluid levels at t=0 was still present as intact ^{125}I-Ang I and Ang I, respectively. Metabolism

was partly due to Ang I-II conversion, as evidenced by the rise in the ^{125}I-Ang II and Ang II levels over time (Figure 3). After 1 h, the incubation fluid levels of ^{125}I-Ang II and Ang II were 4.5±2.6 and 5.4±2.0% of the respective ^{125}I-Ang I and Ang I levels at t=0. HPLC separation of the incubation fluid samples revealed that ^{125}I-Ang II was by far the most important metabolite of ^{125}I-Ang I (Figure 4). The metabolism of ^{125}I-Ang II and Ang II occurred at a similarly slow rate as the metabolism of ^{125}I-Ang I and Ang I, resulting in incubation fluid ^{125}I-Ang II and Ang II levels after 1 h that were >60% of the levels at t=0 (data not shown). Metabolites that could be detected in the incubation fluid during incubation with ^{125}I-Ang II were ^{125}I-Ang-(4-8) and ^{125}I-tyrosine. Irbesartan and PD123319 did not affect the metabolism of ^{125}I-Ang I, ^{125}I-Ang II, Ang I or Ang II (Figure 3). Quinaprilat virtually completely prevented the appearance of ^{125}I-Ang II and Ang II in the incubation fluid during incubation of the vessel segments with ^{125}I-Ang I and Ang I, but did not significantly affect the decrease in ^{125}I-Ang I or Ang I over time (Figure 3), thereby indicating that Ang I-II conversion is not the only metabolic pathway which results in destruction of Ang I.

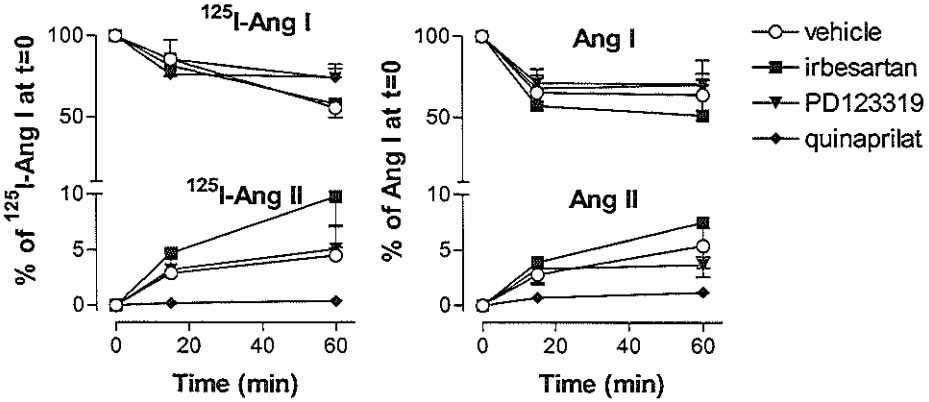

Figure 3. Metabolism of ^{125}I-Ang I and generation of ^{125}I-Ang II (left panel) and metabolism of Ang I and generation of Ang II (right panel) by femoral artery rings incubated in 1 ml incubation fluid containing ^{125}I-Ang I (100,000 c.p.m.) or Ang I (10 or 100 pmol) in the absence or presence of 1 μM irbesartan, 1 μM PD123319 or 10 μM quinaprilat. Incubation fluid levels are expressed as a percentage (mean±SEM, n=4-6) of the ^{125}I-Ang I and Ang I levels at t=0.

Vessel segment. Under all conditions that were investigated, the angiotensin levels measured in the vessel segment at t=15 min were not different from those at t=60 min. Therefore, the data in Figure 5 represent the average of the measurements at these two time points ('steady-state' levels). The ^{125}I-Ang I and Ang I levels in the vessel segments (expressed per g tissue) were 3-6 times lower than the concomitant levels in the incubation

fluid (expressed per ml fluid). In contrast, the tissue ^{125}I-Ang II and Ang II levels, measured after the addition of ^{125}I-Ang I and Ang I to the incubation fluid, were as high as (or slightly higher than) the incubation fluid ^{125}I-Ang II and Ang II levels. Quinaprilat reduced these levels by >75% (data not shown).

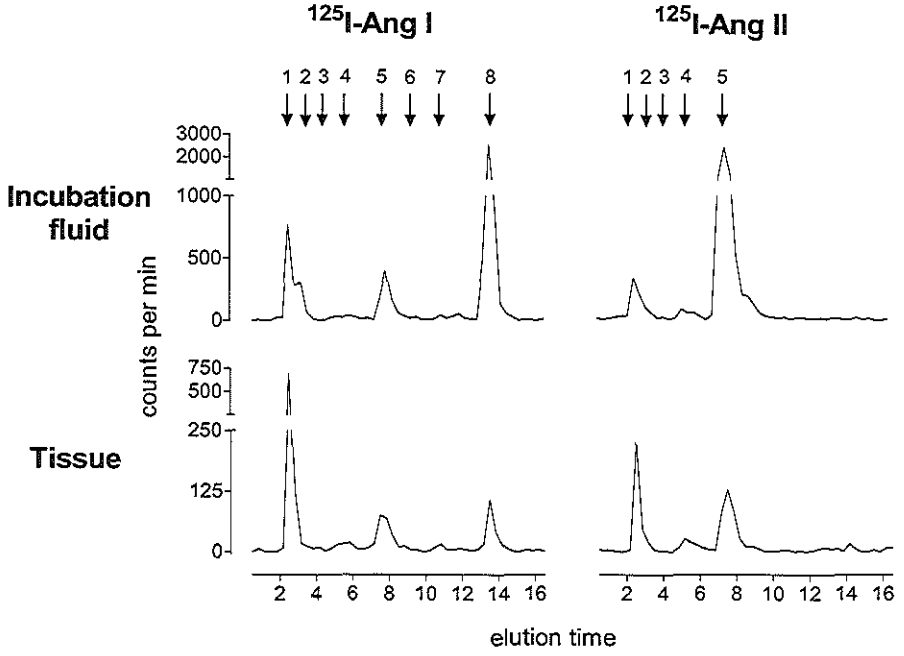

Figure 4. HPLC elution profile of ^{125}I-labeled angiotensins in femoral artery tissue (22 mg) and incubation fluid (100 µl) obtained after incubation of femoral artery rings at 37°C for 60 min in 1 ml incubation medium containing 100,000 c.p.m. ^{125}I-Ang I or ^{125}I-Ang II. The retention times of ^{125}I-labeled standards are indicated by arrows at the top (1, ^{125}I-tyrosine; 2, ^{125}I-Ang-(1-7); 3, ^{125}I-Ang III; 4, ^{125}I-Ang-(4-8); 5, ^{125}I-Ang II; 6, ^{125}I-Ang IV; 7, ^{125}I-Ang-(2-10); 8, ^{125}I-Ang I).

The tissue ^{125}I-Ang II and Ang II levels, measured after the addition of ^{125}I-Ang II and Ang II to the incubation fluid were 5-6 times lower than the concomitant levels in the incubation fluid. Other metabolites that could be detected in the vessel segments during ^{125}I-Ang I or ^{125}I-Ang II application were ^{125}I-Ang-(2-10), ^{125}I-Ang-(4-8) and ^{125}I-tyrosine (Figure 4).

Neither irbesartan nor PD123319 affected the tissue ^{125}I-Ang II and Ang II levels under any of the conditions studied, thereby indicating that the majority of tissue ^{125}I-Ang II and Ang II was not bound to cell surface - or internalized AT receptors. Assuming therefore that all tissue ^{125}I-Ang II and Ang II is located extracellularly (i.e., present in vascular interstitial

fluid, which accounts for ≈15% of tissue weight,[35,199,225,226] it can be calculated that during ^{125}I-Ang I and Ang I application, the interstitial ^{125}I-Ang II and Ang II levels are 49±17 and 19±10%, respectively, of the initial ^{125}I-Ang I and Ang I levels in the incubation fluid. Moreover, during ^{125}I-Ang II and Ang II application, the interstitial ^{125}I-Ang II and Ang II levels are 103±23 and 81±16%, respectively, of the initial ^{125}I-Ang II and Ang II levels in the incubation fluid. In other words, 15 min after the application of ^{125}I-Ang I and Ang I, the interstitial fluid ^{125}I-Ang II and Ang II levels are, respectively, 27±13 and 8.8±4.4 times higher than the ^{125}I-Ang II and Ang II levels in the incubation fluid (the latter levels are shown in figure 3), whereas 15 min after the application of ^{125}I-Ang II and Ang II, the interstitial fluid ^{125}I-Ang II and Ang II levels equal the levels in the incubation fluid.

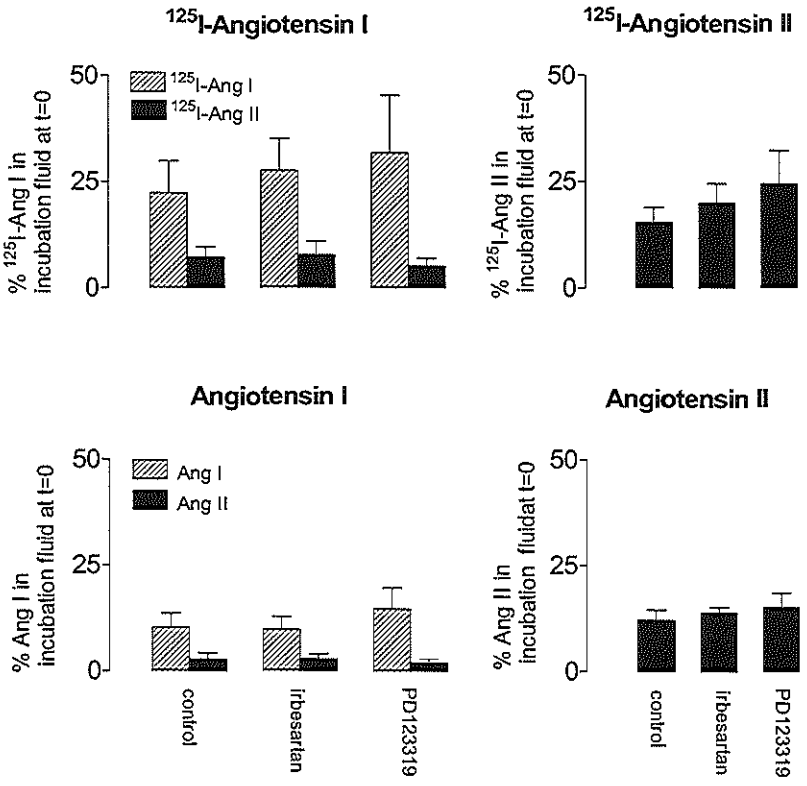

Figure 5. Steady-state tissue ^{125}I-Ang I, ^{125}I-Ang II, Ang I and Ang II levels in femoral artery rings incubated with ^{125}I-Ang I (100,000 c.p.m.) or Ang I (10 or 100 pmol) (left panels) or with ^{125}I-Ang II (100,000 c.p.m.) or Ang II (10 pmol) (right panels) in the absence or presence of 1 μM irbesartan or 1 μM PD123319. Data are expressed as a percentage of the radiolabeled and endogenous Ang I or II levels in incubation fluid at *t*=0 (mean±SEM, *n*=4-6).

Perfusion and organ bath studies with carotid arteries

Ang I and Ang II displayed similar maximal effects, both in the organ bath studies and the perfusion experiments (Figure 6). Under both conditions, Ang I tended to be less potent than Ang II (pEC_{50} 7.53±0.11 vs 7.76±0.08 in the organ bath studies and 7.73±0.07 vs 8.00±0.11 in the perfusion experiments), but the difference did not reach statistical significance. Ang II, but not Ang I, was more potent after adventitial application (perfusion experiment) than after application to the organ bath ($P<0.05$).

Discussion

In the present *in-vitro* study, in agreement with previous studies measuring Ang II following Ang I administration,[29,209,227] the levels of Ang II in the organ bath during the construction of the Ang I CRC in femoral artery rings were <3% of the Ang I levels at the time of maximal vasoconstriction. Yet, in femoral, as well as in carotid arteries, the potency of Ang I was only 2 fold lower than that of Ang II.

The results obtained with the AT_1 receptor antagonist irbesartan and the AT_2 receptor antagonist PD123319 do not support the possibility that exogenous Ang II and locally generated Ang II stimulate different AT receptors. Neither at baseline, nor following preconstriction, we were able to demonstrate Ang I- or Ang II-induced vasodilation *via* AT_2 receptors in femoral arteries. Thus, either such receptors do not exist in porcine femoral arteries, or AT_2 receptors in these vessels mediate other, non-blood pressure-related effects in this vessel (e.g., effects on vascular growth and remodeling.[3,15,102] Measurement of radiolabeled angiotensin metabolites during incubation of femoral artery rings with ^{125}I-Ang I and ^{125}I-Ang II, revealed that the metabolism of Ang I and Ang II by these rings is relatively slow (half-life > 1 h), and that Ang II is by far the most important metabolite of Ang I. The enzyme responsible for Ang I-II conversion is ACE, since the ACE inhibitor quinaprilat shifted the Ang I CRC 10 fold to the right and prevented the appearance of Ang II in the organ bath. The latter contrasts with previous findings in human vessels,[29,210] where chymase was by far the most important converting enzyme. Neither the vasoconstrictor metabolites Ang III and Ang IV,[216] nor the vasodilator metabolite Ang-(1-7)[128,215] were present in detectable amounts. Thus, the similar potencies of Ang I and Ang II are not related to the generation of different vasoconstrictor and/or vasodilator metabolites from Ang I and Ang II, nor to differences in the rapidity of their metabolism. Moreover, the rightward shift of Ang I CRC caused by quinaprilat, and the similar potencies of Ang I and Ang II despite the >100

fold lower affinity of Ang I for the AT_1 receptor,[228,229] exclude the possibility that Ang I mediated its vasoconstrictor effects directly, independent of its conversion to Ang II.

During adventitial application to perfused carotid arteries, Ang I and Ang II were equipotent, thereby showing that their similar potency in organ bath studies[29,210,211] or following arterial infusion *in vivo*[209] is not a consequence of the experimental set-up. The perfusion studies in the present investigation were performed with carotid arteries, since the many sidebranches of femoral arteries did not allow such studies with these vessels. The slightly higher potency of Ang II following adventitial application as compared to combined adventitial + luminal application (i.e., into the organ bath) is in agreement with earlier studies showing a higher metabolic clearance rate of angiotensins after luminal application than after adventitial application.[227] Apparently, the majority of the vascular angiotensin metabolizing enzymes is located on endothelial cells.

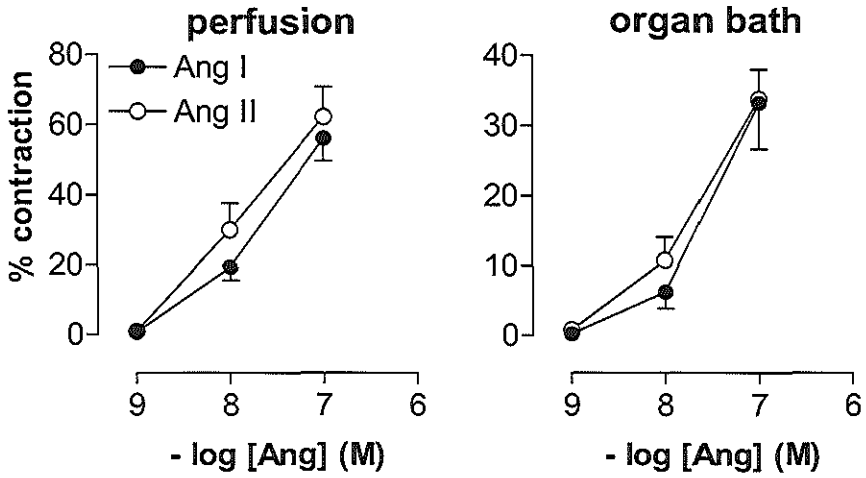

Figure 5. Contractions of carotid arteries to Ang I or Ang II added adventitially to perfused vessel sections (left panel) or into the bath fluid of vessel rings mounted in organ baths (right panel). Data are expressed as a percentage (mean±SEM; n=7) of the response to 100 mM K^+.

Based on the above findings, we reasoned that Ang I administration results in high tissue Ang II levels with limited Ang II release into the surrounding bath fluid, and that these high tissue Ang II levels might explain why the potency of Ang I is similar to that of Ang II. We therefore expected the vascular Ang II levels during Ang I administration to be much higher than the organ bath fluid Ang II levels. As shown in Figures 3 and 5, this was not the case. Fifteen minutes after Ang I administration (i.e., at the time maximal vasoconstriction had

occurred), the vascular Ang II levels were approximately as high as the bath fluid levels. The tissue concentrations at 15 min represented steady-state levels, since similar concentrations were measured after 60 min. Tissue Ang II, however, is localized in one or more different compartments, and a low tissue level therefore does not argue against the possibility that Ang II is present at high concentrations in a specific tissue compartment. For instance, previous *in-vivo*[196,197] and *in-vitro*[36,47] studies have shown that interstitial fluid Ang II may be higher than circulating Ang II. In addition, tissue Ang II is, at least partly, localized intracellularly, because of its internalization *via* AT_1 receptors.[74,75] Such internalization does not appear to occur after binding to AT_2 receptors,[74,119] nor *via* non-AT_1, non-AT_2 receptor-mediated mechanisms.[75] Finally, Ang II may be bound to AT receptors on the cell surface. Receptor binding protects Ang II against rapid metabolism by degrading enzymes.[67] Since in pigs, like in humans, there is currently no evidence for the existence of AT receptors other than the AT_1 and AT_2 receptor, we distinguished cellular (membrane bound or internalized) and extracellular Ang II by measuring tissue Ang II in the presence of irbesartan and PD123319. No significant differences were found in comparison with the control situation. This suggests that only a small fraction of tissue Ang II in femoral artery rings is bound to cell surface - and/or internalized AT_1 receptors, and, thus, that the majority of vascular Ang II is localized in interstitial fluid. Assuming therefore that all vascular Ang II is present in the interstitial space, and taking into account that this compartment represents ≈15% of tissue weight,[35,199,225,226] it follows that interstitial Ang II during Ang I administration is 8.8-27 times higher than bath fluid Ang II.

The latter was not the case during Ang II administration. Tissue Ang II following Ang II administration was also restricted to the interstitial fluid compartment (i.e., not affected by irbesartan or PD123319), and the interstitial Ang II fluid levels during Ang II administration were not different from the bath fluid Ang II levels. The latter contrasts with earlier findings in isolated perfused rat Langendorff hearts, where interstitial Ang II during Ang II infusion was <35% of arterial Ang II,[36] and with *in-vivo* studies in pigs[67] and dogs,[196] where interstitial fluid Ang II during Ang II infusion was <5% of arterial Ang II. These differences are most likely related to the amount of metabolizing enzymes present under the various experimental conditions. In addition, chronic exposure to Ang II is known to affect the level of angiotensin-metabolizing enzymes, including ACE.[178]

Why are the interstitial Ang II levels during Ang I administration higher than the bath fluid Ang II levels? In porcine arteries, Ang I-II conversion occurs both after luminal and after adventitial Ang I administration,[227] suggesting that ACE is present both in the endothelium

and the adventitia. In addition, ACE has been demonstrated on rat and human vascular smooth muscle cells.[230] Most likely therefore, Ang I-II conversion occurs by ACE within the interstitial space. This does not exclude Ang I-II conversion on either the endothelial or adventitial surface. In fact, in view of the much larger volume of the organ bath (15 ml) as compared to the interstitial fluid volume of a 22 mg vessel segment (≈3 µl), it can be calculated that the majority (>95%) of Ang II is present in the organ bath, and thus is probably generated on the vascular surface. In the artery segment, the interstitial space is continuously supplied, *via* diffusion,[47] with Ang I from the bath fluid, and this Ang I will be converted by ACE into Ang II in close proximity of the AT_1 receptors. The small volume of the interstitial space allows a rapid rise in the interstitial Ang II levels, resulting in a steady state within 15 minutes. The rate-limiting factor in this process is most likely the diffusion of Ang I into the interstitial space.[65] Although the bath fluid Ang II levels continue to rise over time, previous studies[29,227] have shown that it is unlikely that these levels will become as high as the interstitial Ang II levels. This is due to the small volume of the interstitial space, thereby allowing interstitial Ang II to contribute only marginally to the organ bath levels of Ang II. Indeed, in previous perfusion studies with porcine carotid arteries, we found no Ang II in the perfusion fluid upon adventitial Ang I administration, nor was Ang II detectable in bath fluid upon luminal Ang I administration.[227] Moreover, we were also unable to demonstrate significant release of Ang II from tissue sites into the circulation.[219,220]

Taken together, the similar potencies of Ang I and Ang II in the present and previous studies,[29,209,227] can be fully explained on the basis of the interstitial Ang II levels that are reached in the vessel wall during Ang I and Ang II application. During Ang I application, at the time of vasoconstriction, these levels are almost one order of magnitude higher than the levels in the organ bath, whereas during Ang II application the interstitial and bath fluid Ang II levels are virtually equal. The right panel of Figure 2 illustrates the consequences of this concept. Our results not only explain why circulating Ang II does not always represent tissue (interstitial) Ang II,[89,217,218] but they also raise the possibility that circulating Ang I, through its local conversion into Ang II, is of greater physiological importance than circulating Ang II. When considering the latter, it should be kept in mind that the circulating Ang I levels are higher than those of Ang II.[219,220]

References are presented in the general reference list.

Chapter 8

Angiotensin-converting enzyme is the main contributor to angiotensin I-II conversion in the interstitium of the isolated perfused rat heart

J Hypertens. 2001; 19: 959-965

Summary

Recent studies in homogenized hearts suggest that chymase rather than angiotensin-converting enzyme (ACE) is responsible for cardiac angiotensin (Ang) I to Ang II conversion. We investigated in intact rat hearts whether (i) enzymes other than ACE contribute to Ang I to Ang II conversion, and (ii) the localization (endothelial/extra-endothelial) of converting enzymes. We used a modified version of the rat Langendorff heart, allowing separate collection of coronary effluent and interstitial fluid. Hearts were perfused with Ang I (arterial concentration 5-10 pmol/ml) under control conditions, in the presence of captopril (1 μM) or after endothelium removal with 0.2% triton X-100. Endothelium removal was verified as the absence of a coronary vasodilator response to 10 nmol bradykinin. Ang I and Ang II were measured in coronary effluent and interstitial fluid with sensitive radioimmunoassays. In control hearts, 45% of arterial Ang I was metabolized during coronary passage, partly through conversion to Ang II. At steady-state, the Ang I concentration in interstitial fluid was three to four-fold lower than in coronary effluent, while the Ang II concentrations in both fluids were similar. Captopril and endothelium removal did not affect coronary Ang I extraction, but increased the interstitial fluid levels of Ang I two- and three-fold, respectively, thereby demonstrating that metabolism (by ACE) as well as the physical presence of the endothelium normally prevent arterial Ang I from reaching similar levels in coronary effluent and interstitial fluid. Captopril, but not endothelium removal, greatly reduced the Ang II levels in coronary effluent and interstitial fluid. With the ACE inhibitor, the Ang II/I ratios in coronary effluent and interstitial fluid were 83 and 93% lower, while after endothelium removal, the ratios were 33 and 71% lower. In conclusion, in the intact rat heart, ACE is the main contributor to Ang I to Ang II conversion, both in the coronary vascular bed and the interstitium. Cardiac ACE is not limited to the coronary vascular endothelium.

Introduction

Recent evidence suggests that angiotensin-converting enzyme (ACE) is not the only enzyme capable of converting angiotensin (Ang) I into Ang II. An important alternative candidate is a serine protease, chymase.[208,231] Chymase is located in the adventitia, in the cytosol of mast cells, and in the extracellular matrix.[61] *In-vitro* studies in human blood vessels and tissue homogenates clearly support its functional importance.[29,210,211] In contrast, the results of *in-vivo* studies, consisting of systemic or regional infusions of Ang I or ^{125}I-Ang I with simultaneous administration of an ACE inhibitor, are less convincing.[187,209,232] It has been suggested that this is due to the fact that chymase converts Ang I predominantly at tissue sites, e.g., in the interstitial space, rather than in the circulation.[231] Indeed, a study by Wei *et al.*[64] in dogs elegantly provided evidence for chymase-dependent Ang I to Ang II conversion in the cardiac interstitial space.

Furthermore, mammalian chymases are biochemically heterogenous, in that primate, dog and hamster chymase highly efficiently convert Ang I into Ang II, whereas rat chymase also degrades Ang II.[233,234] Despite the latter observation, Akasu *et al.*[193] demonstrated that virtually all Ang I to Ang II conversion in rat heart homogenates is due to chymase. Similarly, Müller *et al.*[28] were unable to block Ang I to Ang II conversion in rat left ventricular membranes with an ACE inhibitor. In view of the widespread use of the rat in cardiovascular studies, it is important to know (i) whether chymase contributes to Ang I to Ang II conversion in intact rat tissues, and (ii) whether this occurs in the circulation, in the interstitium or elsewhere. A unique model to address these issues is a modified version of the Langendorff heart, which enables the investigator to separately collect the coronary effluent and the transudate derived from the interstitial fluid compartment.[36,47] We report here on the use of this model to study the generation of Ang II during infusion of Ang I in the presence or absence of the ACE inhibitor captopril. In addition, we investigated the importance of extra-endothelial converting enzymes by infusing Ang I into de-endothelialized hearts.

Materials and methods

Chemicals

[Ile^5]-Ang-(1-10) decapeptide (Ang I) and [Ile^5]-Ang-(1-8) octapeptide (Ang II) were obtained from Bachem (Bubendorf, Switzerland). Bovine serum albumin (BSA), captopril and bradykinin were from Sigma (St. Louis, MO, USA). 1,10-Phenanthroline was from

Merck (Darmstadt, Germany). Sodium pentobarbital was obtained from Apharma (Arnhem, The Netherlands). Endothelin-1 was obtained from Neosystem Laboratoire (Strassbourg, France).

Preparation of the modified Langendorff heart

All experiments were performed under the regulation of the Animal Care Committee of the Erasmus University Rotterdam, Rotterdam, The Netherlands, in accordance with the 'Guiding principles in the care and use of animals' as approved by the American Physiological Society.

Male Wistar rats (Harlan, Zeist, The Netherlands; 280-400 g) were anesthesized with pentobarbital (60 mg/kg, intraperitoneally), and heparinized (5,000 U/kg, intravenously). The hearts were rapidly excised, cooled in ice-cold Tyrode's buffer (125 mM NaCl, 4.7 mM KCl, 1.4 mM $CaCl_2$, 20 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 1.0 mM $MgCl_2$, 10 mM D-glucose, pH 7.4) until contractions stopped, and prepared for Langendorff perfusion. Carbogen-gassed (95% O_2/5% CO_2) Tyrode's buffer at 37°C was continuously perfused immediately after cannulation of the aorta, at a constant perfusion pressure of 80 mmHg. Subsequently the pulmonary artery was cannulated and the caval and pulmonary veins were carefully ligated. After the ligation procedure, which took 30-45 minutes, the hearts were allowed to stabilize for 30 min.

With this modified Langendorff heart preparation it is possible to collect separately coronary effluent and interstitial fluid.[36,47] Coronary effluent, ejected by the right ventricle, was collected via the cannulated pulmonary artery. Interstitial fluid, which keeps dripping from the heart, was collected at the apex. An interstitial fluid flow >2% of the coronary flow was considered to be an indication of leakage, e.g., from veins that were not properly ligated.[47] Hearts with such high interstitial fluid flow were therefore not used.

Removal of the coronary endothelium

In some Langendorff hearts, the coronary endothelium was disrupted with a single bolus injection (0.1 ml) of triton X-100 (0.2% in Tyrode's buffer, v/v) into the aortic cannula. This approach has been reported previously to selectively destroy the endothelium of the isolated heart, without affecting the smooth muscle layer.[235] Endothelium disruption was verified in each heart by comparing the vasodilatory, endothelium-dependent effects of 10 nmol bradykinin[236] before triton X-100 with its effects after treatment. The viability of the underlying smooth muscle cells was tested by comparing the vasoconstrictor effects of 30 pmol endothelin-1 before and after treatment.

Perfusion with angiotensin I

After the 30-min stabilization period, Ang I was infused, via a T-connection, into the cannulated aorta at a speed of 40 pmol/min for a period of 15 min, either with or without captopril (1 μM in the perfusion buffer). In previous studies we have shown that the Ang II-mediated reduction in coronary flow at this infusion rate is transient, and that coronary flow returns to baseline within 2-3 min after the start of the Ang I infusion.[36,47] At the end of the infusion period, the hearts were rapidly frozen in liquid nitrogen for the measurement of tissue Ang I and Ang II. Coronary effluent and interstitial fluid were collected during the infusion over two time periods (from 7-10 and from 11-14 min) in order to measure the steady-state levels of Ang I and Ang II in coronary effluent and interstitial fluid. Coronary flow at the time of coronary effluent and interstitial fluid sampling was similar in control hearts (6±2 ml/min; mean±SD, n=15), de-endothelialized hearts (6±2 ml/min; n=5), and hearts that were exposed to captopril (8±2 ml/min; n=7). The coronary effluent samples were collected into BSA-coated 50-ml polystyrene tubes. Samples of interstitial fluid were collected into BSA-coated 1.5-mL Eppendorf cups. The Eppendorf cups and polystyrene tubes contained a mixture of inhibitors, 10 μl in the Eppendorf cups and 2,500 μl in the polystyrene tubes, in order to prevent the conversion of Ang I to Ang II and the degradation of Ang I and Ang II. The mixture consisted of 125 mM disodium EDTA and 25 mM 1,10-phenanthroline. The Eppendorf cups and polystyrene tubes were kept on ice during the perfusions, so that the samples were rapidly cooled during their collection and remained cold (0-4 °C) during the experiment. When the experiment was completed, the samples for Ang I and Ang II measurements were frozen at -80 °C.

Measurements of angiotensin I and angiotensin II

The Ang I and Ang II concentrations in coronary effluent and interstitial fluid samples were measured directly with sensitive radioimmunoassays.[29,36,187] Measurements were made in 50 μl of undiluted coronary effluent and in 50 μl interstitial fluid diluted with 25 μl of 0.25 mol/l phosphate buffer, pH 7.4, containing 0.15 mol/l NaCl. The lowest measurable Ang I concentration was 15 fmol/ml in coronary effluent and 40 fmol/ml in interstitial fluid. The lowest measurable Ang II concentration was 10 fmol/ml in coronary effluent and 25 fmol/ml in interstitial fluid.

The cardiac tissue concentrations of Ang I and Ang II were measured by radioimmunoassay, after SepPak extraction and reversed phase-high performance liquid chromatography (HPLC) separation.[36,187] The lowest levels of Ang I and Ang II that could be

measured with the radioimmunoassays after HPLC separation were 2.0 and 1.0 fmol/g cardiac tissue, respectively.

Data analysis

All data are given as mean±SD. To correct for differences in arterial Ang I delivery, the Ang I and Ang II levels in coronary effluent, interstitial fluid and cardiac tissue (Table 1) were expressed as a percentage of the arterial levels of Ang I, based upon the assumption that 1 g of tissue equals a volume of 1 ml.[36] The mean of the coronary effluent and interstitial fluid levels in the samples collected from 7-10 min and from 11-14 min after the start of the Ang I perfusion was taken as the steady-state concentration. Differences between experimental groups were evaluated for statistical significance by Student's unpaired *t*-test. Differences within groups were evaluated for statistical significance by Student's paired *t*-test. $P<0.05$ was considered statistically significant.

Table 1. Angiotensin I and angiotensin II levels (mean±SD) in coronary effluent interstitial fluid and tissue of rat Langendorff hearts perfused with angiotensin I in the absence (control; $n=15$) or presence of captopril ($n=7$), or following endothelium removal ($n=5$). The arterial angiotensin I levels in the three groups were 7.1±3.0, 5.1±1.9 and 6.7±1.5 pmol/ml, respectively.

	Control	Captopril	De-endothelialized
Angiotensin I (pmol/ml or pmol/g)			
Coronary effluent	3.7±1.5	2.8±0.8	3.6±0.6
Interstitial fluid	1.1±0.8	1.7±0.6	3.0±0.7
Tissue	0.27±0.08	0.20±0.06	0.44±0.15
Angiotensin II (pmol/ml or pmol/g)			
Coronary effluent	0.36±0.20	0.03±0.02	0.15±0.02
Interstitial fluid	0.50±0.30	0.03±0.01	0.24±0.02
Tissue	0.14±0.06	ND	0.08±0.01

ND, not detectable

Results

Removal of the coronary endothelium

Triton X-100 treatment did not affect baseline coronary flow but increased interstitial fluid flow to approximately 10% of the coronary flow. Prior to the addition of triton X-100, 10 nmol bradykinin increased coronary flow from 7.2±1.9 to 18.3±6.1 ml/min ($n=7$; $P<0.01$), whereas 30 pmol endothelin-1 decreased coronary flow from 11.7±7.1 to 5.9±4.8 ml/min ($n=4$; $P<0.05$). After triton X-100 treatment, bradykinin could no longer induce vasodilation

(increase in flow 0.4±0.6 ml/min; P=NS), whereas the effect of endothelin-1 was unchanged (decrease in flow 5.7±1.9 ml/min; P<0.05). These data, obtained in separate hearts that were not exposed to Ang I, show that endothelium removal was successful and did not affect the viability of the smooth muscle cells.

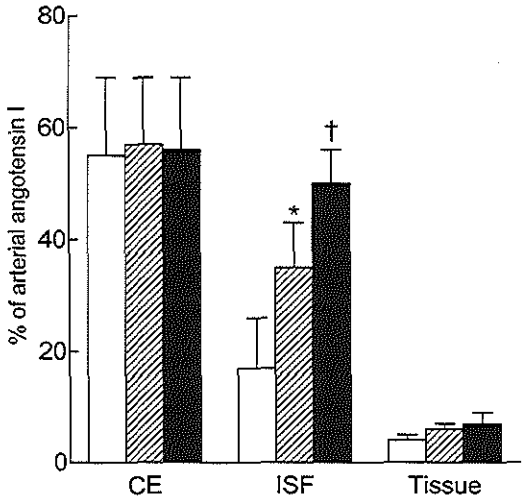

Figure 1. Angiotensin I concentrations in coronary effluent (CE), interstitial fluid (ISF) and tissue of rat Langendorff hearts perfused with angiotensin I in the absence (control, open bars; n=15) or presence of 1 μM captopril (hatched bars; n=7), or following endothelium removal (black bars; n=5). Data (mean±SD) are expressed as a percentage of the arterial angiotensin I levels (7.1±3.0, 5.1±1.9 and 6.7±1.5 pmol/ml, respectively). * P<0.05 or † P<0.01 versus control.

Angiotensin I and angiotensin II levels during perfusion with angiotensin I (Table 1)

Coronary effluent. During perfusion with Ang I, the steady-state Ang I level in coronary effluent was 55% of the arterial level (Figure 1). Thus, 45% of arterially delivered Ang I was removed from the perfusate by the heart. Part of the removal was caused by Ang I to Ang II conversion, since the perfusate contained Ang II levels corresponding to 3% of the arterial Ang I levels (Figure 2). Neither captopril, nor endothelium removal affected coronary Ang I extraction. In contrast, captopril greatly reduced the Ang II levels in coronary effluent, resulting in a 83% reduction of the Ang II/I ratio in coronary effluent (Figure 3). Endothelium removal marginally reduced the Ang II levels in coronary effluent, leading to 33% reduction (P<0.05) of the Ang II/I ratio in coronary effluent.

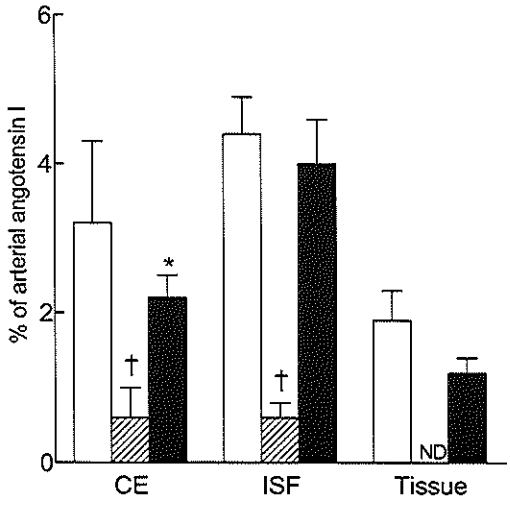

Figure 2. Angiotensin II concentrations in coronary effluent (CE), interstitial fluid (ISF) and tissue of rat Langendorff hearts perfused with angiotensin I in the absence (control, open bars; *n*=15) or presence of 1 µM captopril (hatched bars; *n*=7), or following endothelium removal (black bars; *n*=5). Data (mean±SD) are expressed as a percentage of the arterial angiotensin I levels (7.1±3.0, 5.1±1.9 and 6.7±1.5 pmol/ml, respectively). * $P<0.05$ or † $P<0.01$ versus control. ND, not detectable.

Interstitial fluid. Under control conditions, the steady-state Ang I levels in interstitial fluid were lower than in coronary effluent (Figure 1), whereas the steady-state Ang II levels in this compartment were comparable to or higher than in coronary effluent (Figure 2). Consequently, the Ang II/I ratio was approximately four- to five-fold higher in interstitial fluid than in coronary effluent (Figure 3). In the presence of captopril, the Ang I levels in interstitial fluid doubled, and the Ang II levels in interstitial fluid were reduced to levels close to the detection limit. The interstitial Ang II/I ratio was 93% lower after captopril. Endothelium removal allowed the Ang I levels in interstitial to equal the levels in coronary effluent; the interstitial Ang II levels were unchanged following de-endothelialization. However, due to the increased Ang I levels, the Ang II/I ratio in interstitial fluid was reduced by 71% after endothelium removal.

Tissue. The tissue level of Ang I in control hearts (per g tissue), immediately after the perfusion had been switched off, was 4% of the arterial level (per ml perfusate) (Figure 1). This is much lower than expected on the basis of the Ang I levels in coronary effluent and interstitial fluid, suggesting that Ang I was rapidly degraded in the short period between the moment the Ang I perfusion was stopped and the moment the tissue was transferred into

liquid nitrogen. In contrast, the tissue level of Ang II more closely resembled its level in coronary effluent and interstitial fluid (Figure 2). Consequently, the Ang II/I ratio in cardiac tissue was >10-fold higher than in coronary effluent (Figure 3). Captopril and endothelium removal did not significantly alter the tissue Ang I levels. Tissue Ang II was undetectable in the presence of captopril, and slightly reduced (*P*=NS) after endothelium removal. Both procedures significantly reduced the tissue Ang II/I ratio.

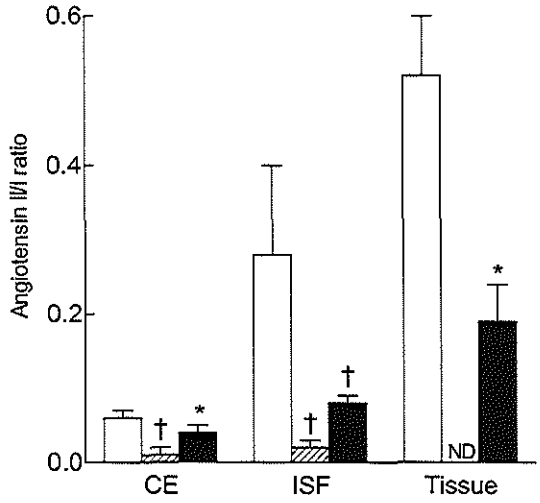

Figure 3. Angiotensin II/I ratios in coronary effluent (CE), interstitial fluid (ISF) and tissue of rat Langendorff hearts perfused with angiotensin I in the absence (control, open bars; *n*=15) or presence of 1 μM captopril (hatched bars; *n*=7), or following endothelium removal (black bars; *n*=5). Data are mean±SD. * *P*<0.05 or † *P*<0.01 versus control. ND, not detectable.

Discussion

The results of this study do not support the concept of Ang I to Ang II conversion by enzymes other than ACE in the intact rat heart, either in the coronary circulation, or in the interstitial space, or elsewhere in the heart. Thus, our findings do not confirm studies in heart homogenates that suggested that cardiac Ang I to Ang II conversion is mainly due to chymase.[28,193,208,237] There may be two reasons for this discrepancy. First, rat chymase, as opposed to human and dog chymase, also cleaves Ang II. It is unlikely that this property prevented us from detecting chymase-dependent Ang I to Ang II conversion in intact hearts, since such conversion was readily detectable in rat heart homogenates.[28,193]

Second, chymase might be located at a site that cannot be reached by arterially delivered Ang I. Indeed, the majority of chymase is present in the cytosol of mast cells, from where it will only be released upon cell disruption (e.g., after tissue homogenization). However, chymase is also present in the extracellular matrix.[61] Functional evidence for this contention was obtained in the dog heart by infusing micromolar concentrations of Ang I with a microdialysis probe directly into the interstitial space.[64] The Ang II levels measured in the interstitial dialysate under the latter circumstances were <5% of the dialysate Ang I levels. In the present study, the interstitial Ang II levels, relative to Ang I, were five to 10-fold higher, and yet none of this Ang II appeared to have been generated by chymase, since captopril reduced the Ang II/I ratio in interstitial fluid by >90%. Thus, the most likely explanation for the discrepancy between the results obtained in intact and homogenized rat hearts is indeed that, under normal circumstances, chymase is located at a site that is not accessible to either intravascular or interstitial Ang I.

Unfortunately, we were unable to further substantiate this conclusion by performing Ang I perfusion experiments in the presence of a selective chymase inhibitor. Such drugs are currently not available, and studies investigating chymase therefore make use of non-selective serine protease inhibitors such as soybean trypsin inhibitor and chymostatin.[28,29,64,193,208,210,211,237] In our experimental setup, the latter drug, at a concentration required to block chymase completely (100 μM),[29] as well as at a ten-fold lower concentration, immediately reduced coronary flow to zero (n=3, Danser *et al.*, unpublished observations).

The accessibility of chymase may be different under pathological conditions, e.g. after myocardial infarction. It might also be the case that tissue damage caused by the microdialysis technique is the underlying cause of the chymase-dependent Ang II generation in the normal dog heart.[64] Chymase-dependent Ang II generation in the intact dog heart would offer an explanation for the lack of effect of ACE inhibitor treatment on volume overload-induced cardiac hypertrophy.[238] Interestingly, and in support of the present study, ACE inhibitors do reduce cardiac hypertrophy in rats exposed to volume overload.[239]

The second aim of this study was to investigate Ang I to Ang II conversion by extra-endothelial converting enzymes. Removal of the endothelium, verified as the absence of a vasodilator response to bradykinin,[236] reduced, but not abolished Ang II generation. Based on the effects of captopril in intact hearts, it appears that such extra-endothelial conversion is due to ACE. Thus, our data clearly provide evidence for the presence and functional importance of ACE outside the endothelium, although no conclusions can be drawn on the exact location of the enzyme. Several cells in the heart and the vessel wall, including myocytes, fibroblasts,

valvular interstitial cells and vascular smooth muscle cells have been reported to synthesize ACE.[43,240-242] The modest reduction in the coronary effluent levels of Ang II following endothelium removal suggests that ACE located on vascular smooth muscle cells is an important contributor to circulating Ang II. Pipili *et al.*[243] demonstrated earlier that the majority of vascular ACE in the rat, unlike vascular ACE in humans[244] and pigs,[227] is present in the media. The low degree of Ang I to Ang II conversion in the coronary vascular bed in the present study, combined with our earlier observation that ACE-mediated conversion is flow-independent[209], suggests that ACE is rate-limiting with regard to coronary Ang II generation.

Removal of the endothelium allowed arterial Ang I to reach the same levels in the interstitial space as in the coronary circulation. This was not the case in intact hearts, in agreement with *in-vivo* studies demonstrating that circulating Ang I contributes little to the levels of interstitial and tissue Ang I.[74,196] Thus, either endothelial peptidases prevent circulating Ang I from entering the interstitium, or the endothelium is the most important physical barrier between the interstitium and the coronary circulation. The former explanation is supported by the increased interstitial Ang I levels in the presence of captopril. The latter explanation would imply that, in the absence of the endothelium, interstitial fluid and coronary effluent are virtually identical. Indeed, the angiotensin content of both fluids after triton X-100 treatment was similar. Therefore, although this concept does not alter our conclusions on the importance of extra-endothelial ACE, these experiments do not allow us to draw definite conclusions on the origin of interstitial Ang II in hearts lacking endothelium.

Tissue homogenization resulted in the immediate loss of virtually all cardiac Ang I. This was not the case for Ang II, indicating that Ang II, unlike Ang I, is located in a protected compartment with less exposure to degrading enzymes. In view of the long half-life of internalized Ang II[74] and the reduction in cardiac Ang II after pretreatment of the heart with the AT_1 receptor antagonist losartan,[36] this compartment is most likely the AT_1 receptor, either located on the cell surface or internalized.

In conclusion, in the intact rat heart, ACE is the main, if not the only, contributor to Ang I to Ang II conversion, both in the coronary vascular bed and the interstitium. Cardiac ACE is not limited to the coronary vascular endothelium.

References are presented in the general reference list.

Chapter 9

Summary and general discussion

Summary

Introduction

The beneficial effects of renin-angiotensin system blockers on cardiac structure and function are usually explained based on the capacity of these drugs to interfere with angiotensin (Ang) II synthesis at cardiac tissue sites. Such local synthesis may occur in the interstitial space, on the surface of, or within cardiac cells. Based on the studies reporting high intracellular Ang II levels, it seems likely that Ang II is an intracrine hormone. However, it is currently uncertain whether all other components required to synthesize Ang II are present in the cell. Furthermore, although most effects of Ang II are mediated via angiotensin II type 1 (AT_1) receptors, the effects occurring after angiotensin II type 2 (AT_2) receptor stimulation are not entirely clear. Therefore, it was the aim of the present thesis to investigate the consequences of AT_2 receptor stimulation and to unravel whether cardiac Ang II is an para-, auto- and/or intracrine hormone.

Chapters 2 and 3

To investigate the vasoactive role of the AT_2 receptor upon Ang II stimulation and the counterbalancing effects of vasodilator prostanoids and nitric oxide on AT_1 receptor-mediated vasoconstriction, normotensive Wistar rats were infused with increasing doses of Ang II after pretreatment with saline, antagonists of AT_1 or AT_2 receptors or inhibitors of cyclooxygenase or nitric oxide synthase, respectively. Systemic and regional hemodynamics were investigated using the radioactive microsphere method. Ang II increased mean arterial blood pressure and heart rate and decreased systemic vascular conductance. The latter was caused by decreases in the conductance of gastrointestinal tract, kidney, skeletal muscle, skin, mesentery+pancreas, adrenal and spleen. The AT_1 receptor antagonist, irbesartan, inhibited all hemodynamic responses induced by Ang II, whereas blockade of the AT_2 receptor with PD123319 was without effect, demonstrating that under normal conditions the Ang II-induced systemic and regional hemodynamic effects are mediated via the AT_1 receptor only. Cyclooxygenase inhibition by indomethacin, but not nitric oxide synthase inhibition by N^G-nitro-L-arginine methyl ester (L-NAME), enhanced the Ang II-induced vasoconstriction systemically as well as regionally, showing that vasodilator prostanoids, but not nitric oxide, are involved in counteracting the Ang II-induced vasoconstrictor effects.

Chapters 4 and 5

To study the functional consequences of AT_2 receptor upregulation[123,163] and the importance of local Ang II generation after myocardial infarction (MI), coronary artery-ligated rats and pigs were infused with Ang II and ^{125}I-labeled Ang I, respectively. In sham-operated and MI rats, consecutive Ang II infusions did not affect myocardial vascular conductance, whereas the conductance during Ang II infusion was decreased in sham-operated rats by PD123319 and increased in MI rats by irbesartan, demonstrating first, that stimulation of the AT_2 receptor in the heart causes coronary vasodilation and second, that this effect is enhanced after MI. The Ang II-induced systemic effects in the presence or absence of irbesartan or PD123319 were similar in sham-operated and MI rats, suggesting that AT_2 receptors do not contribute importantly to systemic hemodynamics after MI.

The AT_1 receptor antagonist, eprosartan, as well as the angiotensin-converting enzyme (ACE) inhibitor, captopril, prevented the development of left ventricular hypertrophy in pigs following MI. Remarkably, however, both drugs increased the Ang II generation at cardiac tissue sites, suggesting that not only diminished AT_1 receptor stimulation but also increased stimulation of the growth-inhibitory AT_2 receptor in the myocardium[123,163] might play a role in the beneficial effects of these drugs after MI. AT_1 receptor-mediated uptake of circulating ^{125}I-Ang II was increased at 1 and 3 weeks after MI in the noninfarcted LV, and this uptake was the main cause of the transient elevation in Ang II levels in the noninfarcted LV at 1 week. These data show that both circulating and locally generated Ang II contribute to the remodeling process after MI.

Chapter 6, 7 and 8

To determine the site of cardiac Ang II generation, as well as the enzyme(s) (ACE and/or chymase) contributing to this generation, the circulating and interstitial levels of Ang I and Ang II were compared *in vivo* and *in vitro* with and without application of exogenous (^{125}I-)Ang I and Ang II.

During intracoronary ^{125}I-Ang II infusion in the pig, the aortic Ang I and Ang II levels were comparable to their levels in coronary venous plasma, while the Ang I and Ang II levels in the myocardial interstitial fluid were of the same order of magnitude as those in the circulation. In untreated pigs, at steady-state, the ^{125}I-Ang II levels in left ventricular tissue were similar to the ^{125}I-Ang II level in coronary venous plasma. Under these circumstances the *ex-vivo* half-life of tissue ^{125}I-Ang II was >30 min. In contrast, after AT_1 receptor blockade with L-158,809, the ^{125}I-Ang II levels in the left ventricular tissue decreased to <5%

of the coronary venous ^{125}I-Ang II levels and tissue ^{125}I-Ang II disappeared within 15 min. These data demonstrate that binding to AT_1 receptors protects Ang II against metabolism. Since interstitial levels of ^{125}I-Ang II were <3 % of the coronary venous ^{125}I-Ang II levels, it appears that circulating Ang II does not contribute importantly to the interstitial levels of Ang II.

Measurement of Ang I and Ang II in organ bath fluid during equimolar Ang I and Ang II application to isolated porcine arteries, demonstrated that during Ang I application the Ang II levels in the bath were ≈18 times lower than during Ang II application. Remarkably, however, the potencies of both angiotensins differed only ≈2-fold. Ang II measured in vascular tissue was 2.9±1.5% and 12.2±2.4% of the corresponding Ang I and II bath fluid levels. Tissue Ang II was not affected by AT_1 or AT_2 receptor antagonists, suggesting that it was mainly located in the interstitial fluid. Since ≈15% of tissue weight consists of interstitial fluid, it can be calculated that the interstitial Ang II levels during Ang II application are similar to the Ang II bath fluid levels, whereas during Ang I application, they are 8.8-27 fold higher. Consequently, at equimolar application of Ang I and II, the interstitial Ang II levels differ only 2-4 fold. Thus, interstitial, rather than circulating, Ang II determines vasoconstriction. Furthermore arterial Ang I, which through regional conversion results in high interstitial Ang II levels, may be of greater physiological importance than arterial Ang II. In these *in-vitro* studies, the ACE inhibitor, quinaprilat, fully prevented Ang II generation during Ang I administration, suggesting that in porcine vessels ACE is the only converting enzyme. Similar observations were made in a modified version of the rat Langendorff heart, which allows separate collection of coronary effluent and interstitial fluid. Hearts were perfused with Ang I under control conditions, in the presence of captopril or after endothelium removal with 0.2% triton X-100. In control hearts, at steady-state, the Ang I concentration in interstitial fluid was three- to four-fold lower than in the coronary effluent, whereas the Ang II concentrations were similar. Captopril or endothelium removal did not alter coronary Ang I extraction, but increased the interstitial fluid levels of Ang I two- and three-fold, respectively. This demonstrates that ACE as well as the physical presence of the endothelium normally prevent the passage of intact arterial Ang I into the interstitial fluid. In the coronary effluent as well as the interstitial fluid, captopril, but not endothelium removal, greatly reduced the Ang II levels. Thus, ACE is the main contributor to Ang I-to-Ang II conversion in the intact rat heart, and its presence is not limited to the coronary vascular endothelium.

General discussion

Function of the AT_2 receptor

AT_2 receptors are assumed to decrease blood pressure and cardiac chronotropy and to inhibit growth. Inhibition of growth during pathological conditions is in agreement with our observations in pigs following MI. ACE inhibition as well as AT_1 receptor blockade increased Ang II levels in the infarcted myocardium, whereas both treatments prevented the development of left ventricular hypertrophy. The vasodilator effects mediated via AT_2 receptors are still highly controversial. For instance, the enhanced blood pressure responses to Ang II that were initially reported in AT_2 receptor knockout mice[103,104] are now attributed to the upregulation of AT_1 receptors in these animals.[131] We were unable to observe systemic vasodilator responses following AT_2 receptor stimulation in normotensive Wistar rats. All systemic hemodynamic effects during Ang II infusion could be attributed to AT_1 receptor stimulation. We also did not find evidence for Ang II-induced release of nitric oxide, the mediator that is assumed to be responsible for the AT_2 receptor-mediated vasorelaxation.[124,245] Remarkably, although not observed in other regional vascular beds, stimulation of the AT_2 receptor resulted in vasorelaxation in the myocardial vascular bed. Interestingly, this vasodilatory effect was enhanced after MI, in full agreement with the many studies reporting an upregulation of AT_2 receptor under pathological conditions.[123,163]

Tissue Ang II generation

Cardiac Ang II is largely formed at cardiac tissue sites. Circulating Ang II, which is sequestered by the heart via AT_1 receptor-mediated endocytosis, contributes little to the cardiac Ang II levels, except perhaps under pathological conditions (e.g. acutely after MI) when AT_1 receptors are upregulated. Rapid metabolism, most likely in the vascular wall, also prevents circulating Ang II from contributing importantly to interstitial Ang II.

Although several studies have suggested that myocardial Ang II acts as an intracrine hormone, our data do not support this concept. We therefore conclude that intracellular Ang II generation at cardiac tissue sites is unlikely to occur, except perhaps in transgenic animals expressing mutated angiotensinogen variants that cannot be secreted.[55] The impossibility of intracellular Ang II generation is mainly related to the fact that normally neither angiotensinogen nor ACE are available intracellularly. This conclusion implies that the internalization of renin and prorenin and the intracellular activation of prorenin via

M6P/IGFII receptors[40] represent (pro)renin clearance rather than the initial step leading to intracellular angiotensin generation. From our studies in isolated porcine vessels[246] and in the rat Langendorff heart,[36] it appears that tissue Ang II generation occurs in the extracellular fluid compartment and that Ang I-II conversion depends on ACE rather than chymase.

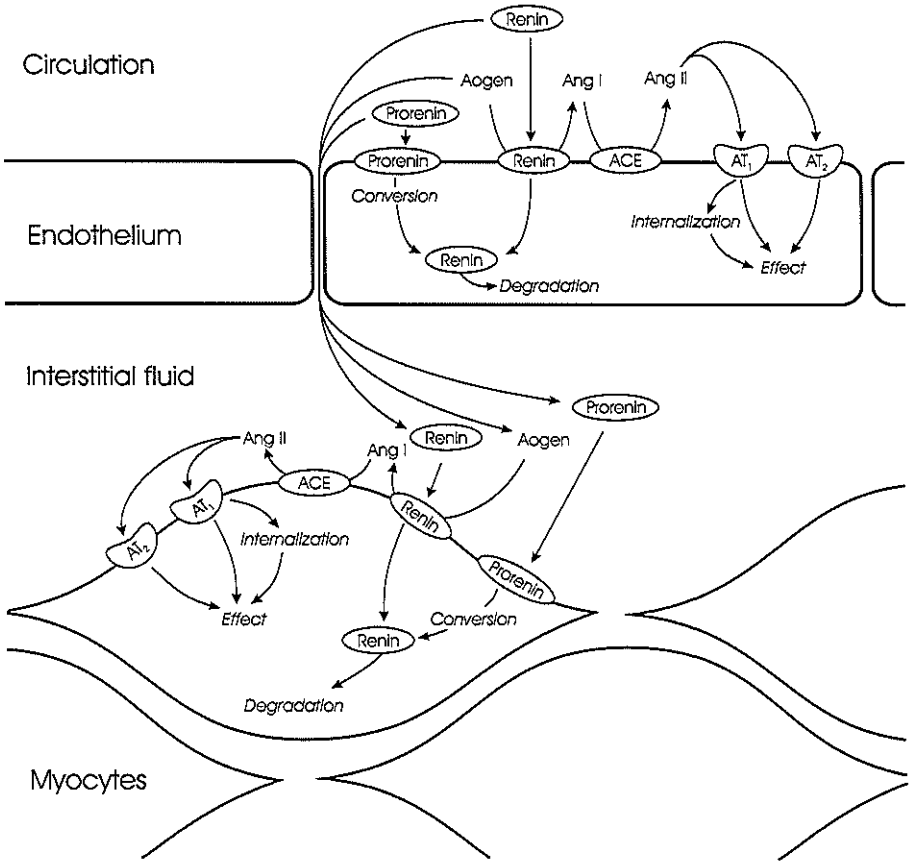

Figure 1. Model of cardiac angiotensin II generation. Ang, angiotensin; Aogen, angiotensinogen; AT, angiotensin II receptors.

Extracellular angiotensin generation in the heart may occur on the cell surface of cardiac cells and/or in the interstitial space. Cell surface angiotensin generation is particularly supported by studies revealing that membrane-bound renin cleaves angiotensinogen much more efficiently than soluble renin[68] and that Ang II-mediated effects occur at 100-fold or 20-fold lower extracellular Ang II levels during renin+angiotensinogen[29,69] or Ang I application (chapter 7), respectively, than during Ang II application. Apparently, angiotensin

generation on the cell surface results in high Ang II levels in the microenvironment of AT receptors, allowing immediate binding of Ang II to its receptors rather than leakage into the interstitium. This concept would explain why the modest changes in Ang II generation and/or AT receptor density that occur under pathological conditions[85,89,91] have functional consequences.

Cardiac Ang II levels, despite the dependency of the heart on circulating (pro)renin, do not always run in parallel with circulating Ang II. For instance, in our study in the infarcted pig,[89] cardiac, but not plasma, Ang II levels rose during ACE inhibition, and yet ACE inhibitors prevented cardiac remodeling following MI. Possibly, this rise in cardiac Ang II represents non-functional Ang II, generated by chymase at a site distant from AT receptors. Similarly, the hearts of transgenic mice expressing an Ang II-producing fusion protein in cardiomyocytes were virtually normal,[88] despite a 20- to 40-fold rise in their Ang II content. Taken together the data in this thesis as well as of other investigators stress the importance of Ang II generation at the right place (i.e., on the cell surface in the immediate vicinity of AT receptors; figure 1) in order to have functional consequences.

Finally, the lack of intracellular generation of Ang II does not mean that Ang II has no intracellular effects. It merely means that Ang II is not an intracrine hormone, i.e. a hormone that is synthesized and acts intracellularly. Since most tissue Ang II is cell-associated,[67,74] it is in fact very well possible that AT_1 receptor-mediated internalization of Ang II underlies its effects as an autocrine or paracrine hormone.

Clinical relevance

AT_2 receptor agonists might exert benificial effects in the heart. However, since AT_1 receptor blockade also results in stimulation of AT_2 receptors, it is questionable whether selective AT_2 receptor agonists would add to the treatment of heart failure. Moreover, if tissue Ang II is formed on the cell surface and not intracellularly, membrane-permeable ACE inhibitors and AT_1 receptor antagonists will not act differently from their nonmembrane-permeable counterparts. On the other hand, to not only treat hypertension, but also to improve heart structure and function following MI in subjects with heart failure, these RAS blockers do need to pass the endothelial layer in order to interfere with the close cascade of reactions of angiotensinogen to Ang II and the subsequent binding of Ang II to AT receptors on the surface of cardiac cells.

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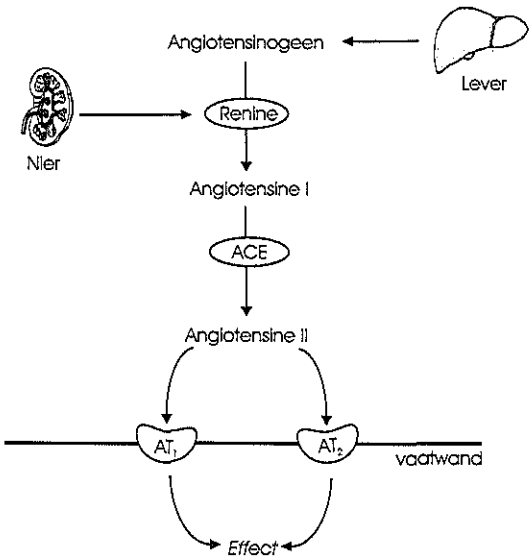
Nederlandse samenvatting

Angiotensine II, het eindprodukt van het renine-angiotensine systeem, is in het lichaam een belangrijk hormoon voor de regulatie van de bloeddruk en de herstrukturering van het hartweefsel na een hartinfarct. In de bloedbaan wordt angiotensine II gevormd door het angiotensine-converterend enzym (ACE) uit angiotensine I, wat op zijn beurt door renine gevormd wordt uit angiotensinogeen (Figuur 1). Activatie van de angiotensine II type 1 (AT_1) receptor door angiotensine II leidt in de bloedvaten tot vaatvernauwing, en in het hart tot groei van de hartspiercellen en tot een krachtiger pompfunctie. De effecten veroorzaakt door activatie van de angiotensine II type 2 (AT_2) receptor zijn momenteel nog niet helemaal duidelijk. Resultaten van studies naar de werking van de AT_2 receptor, wijzen voorlopig in de richting van effecten tegengesteld aan die van de AT_1 receptor.



Figuur 1. Schematisch weergave van het renine-angiotensine systeem in de bloedbaan.

Klinische en experimentele studies met medicijnen die het renine-angiotensine systeem blokkeren, laten zien dat de gunstige effekten van deze middelen op de struktuur en funktie van het hart niet volledig toegeschreven kunnen worden aan hun bloeddrukverlagende effekt. Daarom is het algemeen geaccepteerd dat angiotensin II niet alleen in de bloedbaan, maar ook lokaal in het hart gevormd wordt. Deze lokale synthese kan plaatsvinden in de vloeistof tussen de cellen (interstitiële vloeistof), op het oppervlak van, of in de hartcellen. Gezien studies die hoge concentraties van angiotensine II in de hartspiercellen melden, lijkt het erop dat angiotensine II in de cel gemaakt wordt. Het is echter onzeker of alle andere componenten betrokken bij de angiotensine II produktie aanwezig zijn in de cel.

Aangezien nog niet alle effecten veroorzaakt door angiotensine II, alsmede de plaats van synthese van dit hormoon bekend zijn, is het doel van dit proefschrift om de functie van de AT_2 receptor te onderzoeken en te bepalen op welke plek in het hartweefsel angiotensine II gevormd wordt.

De functie van de AT_2 receptor

In overeenstemming met een effect tegengesteld aan AT_1 receptoren, worden AT_2 receptoren verondersteld de bloeddruk en de hartslag te verlagen evenals de groei van cellen te remmen. Een remming van groei door activatie van de AT_2 receptor wordt ondersteund door onze studie in varkens met een hartinfarct; tijdens ACE remming of blokkade van de AT_1 receptor waren namelijk de angiotensine II spiegels in het geïnfarceerde hartweefsel verhoogd. Beide behandelingen voorkwamen echter de hypertrofie (vergroting van de hartspiercellen; een proces was tijdens hartinfarct optreedt) van de linker hartkamer, terwijl tijdens ACE remming de AT_1 receptor, die juist celgroei stimuleert, gewoon geactiveerd kon worden. Mogelijk werd de hypertrofie geremd door activatie van de AT_2 receptor als gevolg van de verhoogde Ang II spiegels.

Eventuele vaatverwijdende effecten veroorzaakt via AT_2 receptoren zijn nog steeds zeer controversieel. Bijvoorbeeld, de verhoogde bloeddruk tijdens angiotensine II toediening in muizen waarbij de AT_2 receptoren genetisch uitgeschakeld waren, werd eerst toegeschreven aan de afwezigheid van AT_2 receptoren, terwijl tegenwoordig meer wordt gedacht aan een vermeerdering van de AT_1 receptoren. Tijdens activatie van de AT_2 receptoren door angiotensine II toediening in ratten, konden wij gemiddeld in het gehele dier gezien geen vaatverwijdende effecten waarnemen. Alle gemiddelde effekten tijdens angiotensine II toediening, konden worden verklaard door activatie van de AT_1 receptoren. Hiermee in overeenstemming, hebben wij ook geen bewijs gevonden voor het vrijkomen van stikstofoxide tijdens de angiotensine II toediening. Stikstofoxide zou verantwoordelijk zijn voor de vaatverwijding tijdens activatie van de AT_2 receptor.

Alhoewel we geen gemiddelde vaatverwijding in de totale rat gevonden hebben en ook niet in de meeste afzonderlijke organen, bleek dat activatie van de AT_2 receptor in de bloedvaten van het hart wel vaatverwijding veroorzaakte en dat dit nog duidelijker aanwezig was na een hartinfarct. Dit laatste komt goed overeen met studies die een verhoging van AT_2 receptoren in het hart melden tijdens zulke omstandigheden.

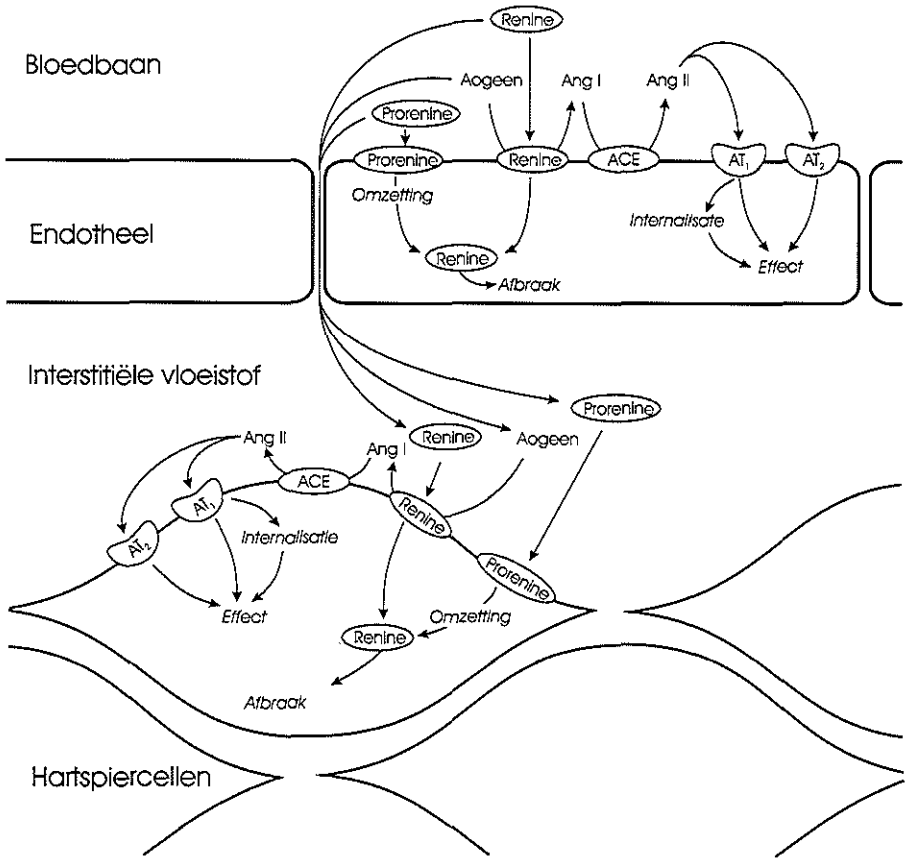
Vorming van angiotensin II in weefsel

Angiotensine II in weefsel van het hart wordt voornamelijk lokaal gevormd. Opname van angiotensine II vanuit de bloedbaan door hechting aan AT_1 receptoren, blijkt weinig bij te dragen aan angiotensine II in het weefsel. Waarschijnlijk wordt de opname verhinderd door afbraak van angiotensine II in de vaatwand. In tegenstelling tot de verschillende studies die suggereren dat angiotensine II in de cel gevormd zou worden, bleek in het varkenshart dat angiotensine I alleen aanwezig was in de interstitiële vloeistof en niet in de hartspiercellen. Dit, in combinatie met studies die aantonen dat angiotensinogeen en ACE niet aanwezig zijn in de hartspiercellen, maakt het onwaarschijnlijk dat angiotensine II in de hartspiercel gevormd wordt. Een logischer plek voor de vorming van angiotensine II is synthese buiten de cel. Dit wordt onderbouwd door het feit dat angiotensine II in geïsoleerde varkensbloedvaten gevormd werd in de interstitiële vloeistof en dat in de interstitiële vloeistof van geïsoleerde rattenharten angiotensine I door ACE omgezet werd in angiotensine II. Een ander enzym, chymase, waarvan studies met vermalen hartweefsel melden dat het angiotensine I kan omzetten in angiotensine II, bleek in intacte rattenharten nauwelijks bij te dragen aan deze omzetting. Voor de volledigheid moet vermeld worden, dat alhoewel angiotensine II niet in de cel gemaakt wordt, dit niet zonder meer betekent dat angiotensine II daar geen effect zou kunnen hebben. Door internalisatie via de AT_1 receptor kan angiotensine II zeker leiden tot effecten in de hartspiercel.

Vorming van angiotensine II buiten de hartcellen kan plaatsvinden op het oppervlak van deze cellen of in de interstitiële vloeistof. Synthese op het celoppervlak lijkt het meest aannemelijk. Ten eerste zet celgebonden renine angiotensinogeen veel effectiever om in angiotensine I dan ongebonden renine. Ten tweede zijn de concentraties van angiotensine II, bij een gelijk effect, op een afstand van de cel vele keren (20-100 x) lager tijdens angiotensine I of renine+angiotensinogeen toediening dan tijdens angiotensin II toediening. Blijkbaar leidt alleen synthese van angiotensin II op het celoppervlak tot hoge angiotensine II concentraties in de directe omgeving van de AT_1 en AT_2 receptoren, waardoor deze receptoren worden geactiveerd voordat angiotensine II weglekt. Dit concept zou eveneens verklaren waarom de geringe veranderingen in angiotensine II vorming en/of het aantal AT receptoren tijdens pathologische omstandigheden al functionele gevolgen hebben.

Conclusie en klinische relevantie

In dit proefschrift wordt aangetoond dat de AT_2 receptor voor vaatverwijding zorgt in het hart, maar niet in andere organen, en dat de rol van de AT_2 receptor belangrijker wordt na een hartinfarct. Verder blijkt dat lokaal angiotensine II niet in, maar juist buiten de hartspiercel gevormd wordt en dat dit zeer waarschijnlijk op het celoppervlak plaatsvindt (Figuur 2). Eveneens benadrukt dit proefschrift het belang van vorming van angiotensine II op de juiste plek (op het celoppervlak, dichtbij de AT receptoren) om effecten te hebben.



Figuur 1. Model van angiotensine II vorming in hartweefsel. Ang, angiotensine; Aogeen, angiotensinogeen; AT, angiotensine II receptoren.

Gezien de rol van de AT_2 receptor in het hart, zou activatie van de AT_2 receptoren door middel van medicijnen gunstig kunnen zijn voor het hart. Echter, medicijnen die AT_1 receptoren blokkeren leiden tot lokale verhoging van angiotensine II spiegels, waardoor deze AT_1 receptor blokkers automatisch zorgen voor een activatie van AT_2 receptoren. Daarom is

het twijfelachtig of medicijnen die selectief de AT_2 receptoren activeren iets zouden toevoegen aan de behandeling van hartfalen.

Tevens kan gesteld worden, als vorming van angiotensine II in het weefsel niet in de cel maar op het celoppervlak plaats vindt, dat ACE remmers en AT_1 receptor blokkers die door de celwand kunnen dringen niet verschillend zullen zijn van medicijnen die deze eigenschap missen. Aan de andere kant, om niet alleen hoge bloeddruk maar ook de struktuur en funktie van het hart na een hartinfarct te verbeteren, zullen deze remmers van het renine-angiotensine systeem wel de endotheellaag van de vaatwand moeten passeren om in te grijpen in de cascade van reacties van angiotensinogeen tot angiotensine II en de daaropvolgende binding van angiotensine II aan de receptoren op het celoppervlak van hartspiercellen.

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Letter

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Dankwoord

Als laatste dan het dankwoord. Aan de ene kant een erg leuke taak, omdat je nu eindelijk eens goed in de gelegenheid wordt gesteld om iedereen te bedanken die (in het klein en het groot) bijgedragen heeft aan de tot standkoming van dit proefschrift. Aan de andere kant toch ook een moeilijke taak, omdat ik me nu langzamerhand pas realizeer hoeveel mensen er eigenlijk aan meehelpen om te promoveren. En natuurlijk wil ik niemand te kort doen, maar omdat ik mijzelf wel een beetje ken, alvast bij deze mijn excuses. De tekortkoming ligt geheel bij mij.

In de tijd dat de universiteit de afdelingen steeds verder laat inkrimpen, was het wel erg luxe dat ik één van de weinige AIO was die werkelijk twee bureaus had. Hiervoor moest ik wel met mijn rugzakje op en neer pendelen tussen de afdelingen Inwendige Geneeskunde (I) in het Dijkzigt en Farmacologie in de EUR, maar dit heb ik er graag voor over gehad. Vooral in het begin was mijn thuisbasis de afdeling Inwendige Geneeskunde waar ik grondig ingewerkt werd in de bepaling van de angiotensines. Bedankt Sjors van Kats voor het optreden als persoonlijke coach, alle uitleg en geduld! Ook René de Bruin en Jeanette van Gool bedankt voor alle keren dat jullie klaar stonden als ik weer eens iets nodig had. Verder Gooitzen en Angelique voor alle HPLC ondersteuning en Marjolein, Bianca en Evelien voor het 'uitlenen' van materiaal van de onderzoekskamer. Frans Derkx, Ton, Frans Boomsma, Jaap, Carla, Annemiek, Thomas, Marinel (nog eventjes en dan ben jij aan de beurt), Richard Zuiderent, Usha, Nicole en Edith bedankt voor alle gezelligheid en gesprekken/discussies voor, na en tijdens de pauzes en tijdens de weekendjes weg.
Aangezien er al een goede samenwerking was met de Experimentele Cardiologie, was de introductie van mij op deze afdeling zo gebeurd en heb ik gebruik mogen maken van hun expertise. Dankzij het kundige team, te weten Sandra de Zeeuw, Jan van Meegen, René Stubeninsky en Rob van Bremen heb ik experimenten in varkens kunnen uitvoeren. In mijn dank hiervoor moet ik ook Dirk-Jan Duncker en prof. Pieter Verdouw betrekken, die het met een prettig kritische blik begeleid hebben.

Na de bepalingen van de angiotensines in varkensweefsel, verschoven mijn activiteiten meer naar studies in ratten en geïsoleerde bloedvaten waardoor ik de thuisbasis Inwendige Geneeskunde verruilde voor die van de Farmacologie. Hier heb ik mij gewaagd in het domein

van Jan Heiligers (het kratje bier moet toch nog een keer komen) waar ik samen met René de Vries en 'mijn' allereerste (en tot nu toe allerlaatste) student Munesh onder het mom van wetenschap vele ratten vervroegd aan hun einde heb laten komen.
Op deze afdeling waren ook de andere RAS-AIO's/postdocs aanwezig. Jasper, Beril, Mark en Wendy, ik heb het altijd erg prettig gevonden om samen met jullie onderzoek te doen aan hetzelfde onderwerp. In ieder geval begreep iemand mijn ideeën (of deed alsof) en daarnaast was het reizen naar congressen een stuk aangenamer.

Na(ast) het werk moet er natuurlijk ook enige afleiding zijn. Dit werd zeker gevonden op de verschillende kamers waar ik gezeten heb. Eerst op Interne en daarna vooral in de 'mannen'kamer, met Richard van Veghel, Roeland en Wiro/Mark op Farmacologie. Er was altijd een goede mix tussen gezelligheid en werk, alhoewel, vooral door de komst van de waterkoker en tosti-ijzer, soms de rest van de Farma's onze kamer verwarde met de koffiekamer. Verder was er natuurlijk de feestcommissie, waarbij de lol vooraf soms groter was dan de georganiseerde jaarlijkse labuitjes. Vooral het uitzetten van de fietstocht op de heetste dag van ongeveer 30 jaar zal me altijd heugen (of niet Mechteld?). Alle andere (ex-)Farma's wil ik niet ongenoemd laten: Antoinette, Peter, Ingrid, Sue, Erik, Pankaj, Edwin, Ria, Magda, Regien, Remon, Uday, Kapil, Wenxia, Andor, Inge, Hari, Anne, Marion, Larissa, Toos, Jeanette, Freek, Mariska, Emine, Saskia en Luc, met z'n allen maken jullie de afdeling Farmacologie.

Zonder bezielende begeleiding was dit proefschrift er echter nooit gekomen. Daarom wil ik mijn beide promotoren, prof. Schalekamp en prof. Saxena, hartelijk danken voor alle input die ze de laatste jaren hebben geleverd. Maarten, ik was altijd weer verbaasd dat je, terwijl je mij niet direct begeleidde, altijd precies wist wat voor experimenten ik had gedaan en altijd de beste ideeën had om de resultaten te verklaren. Pramod, ik vond het altijd erg prettig dat ik zo bij je binnen kon lopen om iets te vragen, vooral bij de microsfeermethode was dit erg handig. En natuurlijk Jan Danser, als mijn directe begeleider, ben ik je erg dankbaar voor alle tijd die je in mij gestoken hebt en het feit dat je het altijd weer positief inzag, zelfs als ik af en toe dacht dat een experiment totaal mislukt was. In de afgelopen jaren heb ik veel van je geleerd, maar helaas is het rood in mijn manuscripts nog steeds niet verdwenen. Verder moet ik je altijd erkentelijk blijven dat je me als AIO aangenomen hebt, ondanks dat ik aanvankelijk de baan afgeslagen had, maar na 5 minuten van gedachte veranderde.

Ook wil ik bij deze mijn ouders bedanken. Pap en mam, ik waardeer het heel erg dat jullie mij altijd gestimuleerd hebben en de vrijheid hebben gegeven om te studeren wat ik wilde. Natuurlijk wil ik ook de rest van mijn (schoon)familie en mijn vrienden niet vergeten. Chris, in het bijzonder bedankt voor je stimulerende interesse in mijn publicaties. Vaak hebben jullie je afgevraagd wat ik nu eigenlijk precies voor werk deed. Ik hoop dat jullie na het lezen van dit boekje iets wijzer geworden zijn. Arnoud en Richard, alvast bedankt dat jullie mij als paranimfen moreel willen steunen tijdens mijn verdediging.

Lieve Marinke, de ruimte is eigenlijk veel te klein om jou te bedanken. Zonder jouw steun en liefde op tijden dat ik het promoveren zat was, zou het een stuk moeilijker geweest zijn. Daarom hier alvast een kus en de rest houd je tegoed. Een promotie wordt weleens vergeleken met een bevalling. Alhoewel ik denk dat het laatste een stuk zwaarder is, zou ik niet weten wanneer ik zenuwachtiger ben. We zullen het de volgende maand wel zien, wanneer we met z'n drietjes zijn.

Curriculum vitae

De auteur van dit proefschrift werd geboren op 2 februari 1972 te Amersfoort, waar hij eveneens na enige jaren het VWO diploma haalde aan het Farel College. Alvorens te beginnen met studeren, volgde hij in 1990/1991 het examenjaar aan de Northwestern Lehigh Highschool te New Tripoli, PA, USA. Na terugkomst ving hij aan met de studie scheikunde aan de Universiteit van Utrecht en sloot deze in januari 1997 met succes af. Tijdens deze periode volgde hij het bijvak anorganische chemie aan het Anorganisches Institut van de Ruprecht-Karls-Universität, Heidelberg, Duitsland. Als extra bijvak klinische chemie bestudeerde hij bij het laboratorium Erfelijke Metabole Stoornissen van het Wilhelmina kinderziekenhuis te Utrecht, onder het toeziend oog van dr. R. Duran, of rhodanese deficiëntie een verhoogde thiosulfaat excretie veroorzaakte. Zijn hoofdvak voerde hij uit bij de vakgroep Biochemie van de Lipiden van de Universiteit van Utrecht onder begeleiding van dr. Y.C.M. de Hingh en prof. dr. J.A.F. Op den Kamp. In deze periode was hij betrokken bij het onderzoek naar de invloed van zuurstofradicalen op de peroxidatie van lipiden, een mogelijke oorzaak van mitochondriële encephalomyopathie. In zijn doctoraalscriptie behandelde hij de mogelijke mechanismen waarmee P-glycoproteins multidrug resistentie veroorzaken.

Vanaf juli 1997 is hij werkzaam geweest als assistent in opleiding op de afdelingen Farmacologie en Inwendige Geneeskunde (I) van het Erasmus MC te Rotterdam onder supervisie van zijn promotoren prof. dr. M.A.D.H Schalekamp en prof. dr. P.R. Saxena en onder directe begleiding van zijn co-promotor dr. A.H.J. Danser. De jaren onderzoek op deze afdelingen aan het renine angiotensine systeem hebben geresulteerd tot dit proefschrift. Sinds juli 2001 continueert hij zijn werk op de afdeling Farmacologie als wetenschappelijk medewerker.