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

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ABSTRACT

Both phosphodiesterase 5 (PDE5) inhibition and endothelin (ET) receptor blockade have been shown to induce pulmonary vasodilation. However, little is known about the effect of combined blockade of these two vasoconstrictor pathways. Since nitric oxide (NO) exerts its pulmonary vasodilator influence via production of cyclic guanosine monophosphate (cGMP) as well as through inhibition of ET, we hypothesised that interaction between the respective signaling pathways precludes an additive vasodilator effect. We tested this hypothesis in chronically instrumented swine exercising on a treadmill by comparing the vasodilator effect of the PDE5 inhibitor EMD360527, the ET_A/ET_B antagonist tezosentan, and combined EMD360527 and tezosentan. In the systemic circulation, vasodilation by tezosentan and EMD360527 was additive, both at rest and during exercise, resulting in 17±2% drop in blood pressure. In the pulmonary circulation, both EMD360527 and tezosentan produced vasodilation. However, tezosentan produced no additional pulmonary vasodilation in the presence of EMD360527, either at rest or during exercise. Moreover, in isolated preconstricted porcine pulmonary small arteries (~300 μm) EMD360527 (1nM-10 μM) induced dose-dependent vasodilation, whereas tezosentan (1nM-10 μM) failed to elicit vasodilation irrespective of the presence of EMD360527. However, both PDE5 inhibition and 8Br-cGMP, but not 8Br-cAMP, blunted pulmonary small artery contraction to ET and its precursor Big ET in vitro. In conclusion, in healthy swine, either at rest or during exercise, PDE5 inhibition and the associated increase in cGMP produce pulmonary vasodilation that is mediated in part through inhibition of the ET pathway, thereby precluding an additional vasodilator effect of ET_A/ET_B receptor blockade in the presence of PDE5 inhibition.

INTRODUCTION

Under basal resting conditions, the pulmonary circulation is a low-pressure, low-resistance system.[1,2] During exercise, however, flow through the pulmonary vasculature increases, which is accompanied by an increase in pulmonary arterial pressure. This requires the right ventricle not only to pump more blood but to do so against a higher afterload.[3] Although vascular tone in the pulmonary vasculature is low as compared to the systemic vasculature, pulmonary vasodilation during exercise does occur and limits the increase in pulmonary artery pressure (PAP) and thereby the increase in right ventricular afterload.[1,2]

Pulmonary vascular tone is determined by an interplay between vasodilators and vasoconstrictors, such as nitric oxide (NO) and endothelin (ET). Exercise-induced pulmonary vasodilation is largely NO mediated[1,4], and can be enhanced by prolonging the half-life of its second messenger cGMP through inhibition of phosphodiesterase 5 (PDE5).[5] Since PDE5 is abundantly expressed in pulmonary vascular smooth muscle in particular[6,7], PDE5 inhibition has clinically been used to selectively evoke pulmonary vasodilation without inducing systemic hypotension in patients with pulmonary hypertension.[8-10] NO induces pulmonary vasodilation not only through a direct cGMP-mediated effect on vascular smooth muscle but also indirectly by blunting ET mediated pulmonary vasoconstriction in swine.[11,12] Nevertheless, ET exerts a vasoconstrictor influence on the pulmonary vasculature[13,14], that is relatively small under basal resting conditions, but, surprisingly, becomes more pronounced during exercise, thereby limiting the exercise-induced pulmonary vasodilation.[11,15] Thus both PDE5 inhibition and ET receptor blockade cause vasodilation in the pulmonary vasculature, particularly during exercise, and combined inhibition of both pathways may have an additive vasodilator effect and may therefore synergistically decrease right ventricular afterload. However, since NO blunts ET-mediated pulmonary vasoconstriction[11] and since ET can enhance NO production via ET_B receptor stimulation[1], it is also possible that interaction between the respective signaling pathways precludes such additive vasodilator effect. Therefore, the aim of the present study was to evaluate the vasodilator effect of ET receptor blockade on the pulmonary vasculature in vivo in the presence of PDE5 inhibition not only at rest but also during exercise.

Since we found no additive pulmonary vasodilator effect of ET receptor blockade following PDE5 inhibition, we further investigated whether this lack of effect was the result of a direct interaction between the NO-cGMP and the ET pathway or due to a lack of residual tone in the pulmonary vasculature following PDE5 inhibition, using isolated pulmonary small arteries. For this purpose, we investigated whether PDE5 inhibition and ET_A/ET_B receptor blockade could act synergistically when pulmonary tone was increased with the stable thromboxane A_2 analogue U46619, and we evaluated the responsiveness of the isolated pulmonary small arteries to ET and its precursor Big ET, in the absence and presence of PDE5 inhibition as well as increased cGMP levels. Finally, to test whether the

interactions were specific for cGMP signalling, we studied pulmonary artery ET and Big ET responsiveness in the absence and presence of cAMP.

METHODS

In vivo studies

Animals

Studies were performed in accordance with the Council of Europe Convention (ETS123)/ Directive) (86/609/EEC) for the protection of vertebrate animals used for experimental and other scientific purposes, and with approval of the Animal Care Committee of the Erasmus University Medical Center Rotterdam. Thirteen crossbred Yorkshire X Landrace swine (2-3 mo old, 22±1 kg at the time of surgery, 9 females and 4 neutered males) entered the study. Daily adaptation of animals to laboratory conditions started 1 wk before surgery and continued during the first week after surgery.

Surgical procedures

Swine were sedated with ketamine (20 mg/kg im), and midazolam (1 mg/kg im), anesthetised with thiopental (10 mg/kg iv), intubated, and ventilated with a mixture of O_2 and N_2 (1:2) to which 0.2-1% (vol/vol) isoflurane was added.[4,11,16] Anesthesia was maintained with midazolam (1 mg kg^{-1} per hour iv) and fentanyl (10 μg kg^{-1} per hour iv). Under sterile conditions, the chest was opened via the fourth left intercostal space and fluid-filled polyvinylchloride catheters were directly inserted into the aortic arch, left atrium, and pulmonary artery by puncture of these structures for blood sampling and blood pressure measurement (Combitrans pressure transducers, Braun, Melsungen, Germany). A Transonic flow probe (16 mm; Transonic Systems) was positioned around the ascending aorta for measurement of cardiac output. Catheters were tunnelled to the back and animals were allowed to recover, receiving analgesia (0.3 mg buprenorphine im) for 2 days and antibiotic prophylaxis (25 mg/kg amoxicillin and 5 mg/kg gentamycin iv) for 5 days.

Exercise protocols

Experimental design

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

EMD360527 (a gift from Merck, Darmstadt, Germany) and *3)* exercise in the presence of combined EMD360527 and tezosentan.

Effects of ET_A/ET_B receptor blockade during exercise

With swine resting (lying and standing) quietly on the treadmill, resting hemodynamic measurements consisting of left atrial, aortic, and pulmonary artery blood pressures; heart rate; and cardiac output were obtained; arterial and mixed venous blood samples were collected (lying); and rectal temperature measured. Subsequently, a five-stage exercise protocol (1, 2, 3, 4 and 5 km/h) was started, with each stage lasting 2-3 min. Hemodynamic variables were continuously recorded and blood samples collected during the last 30 s of each exercise stage, at a time when hemodynamics had reached a steady state. Following the exercise trial, animals were allowed to rest for 90 min, resulting in a complete return of hemodynamic variables to baseline values.[4] In the second exercise protocol, tezosentan was infused intravenously over 10 min in a dose of 3 mg/kg, followed by a continuous intravenous infusion of tezosentan in a dose of 6 mg kg^{-1} h^{-1}, and the five-stage exercise protocol was repeated. We have previously shown that this dose of tezosentan abolishes the increase in blood pressure in response to endothelin.[15] Tezosentan has a pA_2 of 9.5 for ET_A and a pA_2 of 7.7 for ET_B receptors, indicating only a 63-fold selectivity for ET_A receptors compared with ET_B receptors.[17,18]

Effects of ET_A/ET_B receptor blockade in the presence of PDE5 inhibition during exercise

Animals from the second group underwent the same exercise protocol as those in the first group, but consisting of three consecutive exercise trials. First, a control exercise trial was performed as described above. Following the exercise trial, animals were allowed to rest for 90 min, resulting in a complete return of hemodynamic variables to baseline values. Subsequently, the EMD360527 was infused continuously in a dose of 300 μg min kg^{-1} min^{-1} intravenously, and 10 min after starting the infusion the five-stage exercise protocol was repeated while the infusion was continued.[19] EMD360527 demonstrates at least 45-fold selectivity for PDE5 (IC_{50}=0.007 μM) compared to PDE6 (IC_{50}=0.32 μM), 94-fold selectivity for PDE1 (IC_{50}=0.66 μM), 137-fold selectivity for PDE10 (IC_{50}=0.96 μM), and > 1400-fold selectivity for PDE2, PDE3, PDE4 and PDE7 (all IC_{50}>10 μM). In these assays PDE1-4 was from guinea pig heart muscle, PDE5 from human thrombocytes, PDE6 from bovine retina, whereas PDE7 and PDE10 were obtained by expression of cDNA in COS-7 cells and Escherichia coli, respectively.

Subsequently, the EMD360527 infusion was stopped and the animals were allowed to rest for another 90 min. Then, animals received an intravenous infusion of EMD360527 together with the ET_A/ET_B antagonist tezosentan, in dosages identical to those described above for the individual infusions, and the five-stage exercise protocol was repeated. We have previously observed excellent reproducibility of consecutive exercise protocols.[16]

In vitro studies

Tissues

Pig lungs (n=23) were collected at a local slaughterhouse. Pulmonary small arteries (diameter ~300 μm) were removed and stored overnight in cold, oxygenated Krebs bicarbonate solution of the following composition (mM): 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, and glucose 8.3; pH 7.4.

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

Effects of PDE5-inhibition and ET_A/ET_B blockade

Pulmonary small arteries were preconstricted with 100 nM U46619, and concentration-response curves were constructed to EMD360527 (1nM-10 μM; n=7), tezosentan (1nM-10 μM[21]; n=7), and combined EMD360527 and tezosentan (n=7).

Effects of PDE inhibition on ET receptor sensitivity and ET production

The responses to cumulative concentrations of ET (1-100 nM[22]) and Big ET (10 nM-1μM) were measured in control vessels and vessels pretreated with EMD360527 (3 μM, n= 7 for ET and Big ET), 8Br-cGMP (100 μM n= 4 for ET and Big ET), and 8Br-cAMP (300 μM n= 4 for ET and Big ET). Big ET has no direct vasomotor effect; therefore Big ET-induced vasoconstriction is used as an index of Big ET conversion to vasoactive ET.

Data analysis and statistics

Digital recording and offline analysis of hemodynamic data have been described in detail elsewhere.[16,23] Pulmonary vascular resistance (PVR) and systemic vascular resistance (SVR) were calculated as PAP minus left atrial pressure divided by cardiac output and mean aortic pressure divided by cardiac output, respectively. Pulmonary vascular conductance (PVC) and systemic vascular conductance (SVC) were calculated as 1/PVR and 1/SVR.[24] Total pulmonary resistance (TPR) was calculated as PAP divided by cardiac output.[25] Body oxygen consumption (BVO_2) was calculated as the product of cardiac output and the difference between arterial and mixed venous oxygen content of the blood. To accommodate for the

varying weights between animals and groups, cardiac output, PVC, SVC, TPR, and BVO_2 were indexed to body weight. Pulmonary distensibility (α) was estimated using the formula described by Linehan and Reeves[26,27], minimizing the difference between predicted and PAP measured with Solver in Excel using six data points (rest and 5 levels of exercise) per animal.

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

In this formula R_0 is assumed to be PVR measured at rest. Values for α could be obtained in all animals except one, which showed a paradoxical increase in PVR during exercise. Correlations between measured PAP and predicted PAP were calculated in Excel.

Vasodilator responses in vitro to EMD360527, tezosentan and combined EMD360527 and tezosentan were expressed as percentage of contraction to U46619. Vasoconstrictor responses to ET and Big ET were measured when contraction had reached a steady state, and normalized to maximal constriction to 100 mM KCl.

The effects of EMD360527 and tezosentan during exercise were analyzed by using linear regression analysis with BVO_2 as independent variable and assigning a dummy variable to each animal. The effect of EMD360527 and tezosentan on relation between the transpulmonary pressure gradient and cardiac index was analyzed by linear regression. Since we found no statistically significant differences between the hemodynamic response to exercise between male and female swine, in either the pulmonary or the systemic vasculature, data from both sexes were pooled.

The effects of EMD360527, tezosentan, and combined EMD360527 and tezosentan on the preconstricted isolated pulmonary arteries were assessed by two-way ANOVA for repeated measures. The effects of EMD360527, cGMP, and cAMP on the vasoconstrictor response to ET and Big ET were also assessed by two-way ANOVA for repeated measures. In all ANOVAs post hoc testing was performed by Bonferroni's method. Statistical significance was accepted when $P<0.05$ (two-tailed). Data are presented as means± SE.

RESULTS

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

Graded treadmill exercise up to 5 km/h resulted in an increase in heart rate up to 90% of estimated maximal heart rate[28], and a doubling of cardiac output (Table 1), which in combination with an increase in body oxygen extraction from 49±2% at rest to 72±2% during maximal exercise, resulted in a threefold increase in BVO_2 (Figure 1) up to 65% of estimated

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

		Rest	Exercise (km/h)				
		Standing	1	2	3	4	5
HR	Control	144 ± 4	168 ± 4*	177 ± 6*	194 ± 8*	223 ± 10*	256 ± 8*
(beats/min)	Tezo	158 ± 6†	177 ± 6*†	188 ± 10*	207 ± 9*	233 ± 10*†	257 ± 8*
	Control	135 ± 14	152 ± 14*	167 ± 13*	181 ± 12*	216 ± 12*	249 ± 9*
	EMD	159 ± 7	195 ± 12*†	199 ± 11*†	223 ± 9*†	243 ± 8*	259 ± 8*
	EMD+Tezo	177 ± 9†‡	207 ± 11*†‡	214 ± 9*†	236 ± 8*†	257 ± 7*†	270 ± 6*†
MAP	Control	86 ± 6	81 ± 4	83 ± 5	85 ± 5	87 ± 5	91 ± 7*
(mmHg)	Tezo	71 ± 6†	73 ± 4†	74 ± 4†	76 ± 6†	78 ± 4†	82 ± 5†
	Control	83 ± 3	83 ± 4	83 ± 3	84 ± 3	86 ± 4	89 ± 4
	EMD	75 ± 4	73 ± 5†	73 ± 4†	74 ± 3†	73 ± 4†	76 ± 5†
	EMD+Tezo	68 ± 4†	68 ± 5†	65 ± 6†	67 ± 3†‡	67 ± 4†	72 ± 4†
PAP	Control	12 ± 2	15 ± 1	18 ± 1	20 ± 1*	24 ± 2*	28 ± 2*
(mmHg)	Tezo	8 ± 1†	12 ± 1	15 ± 1†	17 ± 1†	21 ± 1*	26 ± 1*
	Control	12 ± 1	16 ± 2	17 ± 2	19 ± 2*	24 ± 3*	27 ± 3*
	EMD	11 ± 1	13 ± 2†	15 ± 2†	17 ± 2*†	19 ± 2*†	22 ± 3*†
	EMD+Tezo	12 ± 1	14 ± 1	15 ± 1	17 ± 2†	20 ± 2*	24 ± 3*
LAP	Control	−0.4 ± 1.2	1.0 ± 0.8	3.9 ± 0.4*	4.8 ± 0.8*	6.4 ± 1.3*	8.7 ± 1.4*
(mmHg)	Tezo	−2.2 ± 0.4†	1.3 ± 0.7	3.3 ± 0.9*	4.1 ± 1.3*	7.0 ± 1.5*	10.0 ± 1.6*
	Control	−2.3 ± 1.8	1.2 ± 0.9	1.8 ± 1.1	3.5 ± 1.1*	5.6 ± 2.0*	5.9 ± 2.2*
	EMD	1.1 ± 2.1	0.8 ± 1.4	2.8 ± 1.2	3.9 ± 1.2	5.4 ± 2.1*	6.6 ± 2.5*
	EMD+Tezo	0.2 ± 1.4	1.6 ± 1.0	3.2 ± 1.3	4.1 ± 1.5*	6.6 ± 2.1*	9.1 ± 2.6*

Table 1. Hemodynamic effects of ET antagonism, PDE5 inhibition, and the combination (*continued*)

		Rest	Exercise (km/h)				
		Standing	1	2	3	4	5
CO	Control	0.20 ± 0.01	0.24 ± 0.01*	0.25 ± 0.01*	0.28 ± 0.01*	0.31 ± 0.01*	0.33 ± 0.02*
(L min^{-1} kg^{-1})	Tezo	0.20 ± 0.01	0.24 ± 0.01*	0.26 ± 0.01*	0.29 ± 0.01*	0.32 ± 0.01*†	0.35 ± 0.02*†
	Control	0.19 ± 0.02	0.22 ± 0.02*	0.24 ± 0.02*	0.26 ± 0.02*	0.30 ± 0.02*	0.34 ± 0.02*
	EMD	0.21 ± 0.02	0.26 ± 0.03*†	0.28 ± 0.03*†	0.31 ± 0.02*†	0.34 ± 0.02*†	0.36 ± 0.02*†
	EMD+Tezo	0.24 ± 0.02‡	0.28 ± 0.03*†	0.30 ± 0.03*†	0.32 ± 0.03*†	0.36 ± 0.03*†	0.37 ± 0.02*†
SV	Control	1.40 ± 0.11	1.44 ± 0.07	1.45 ± 0.06	1.46 ± 0.07	1.43 ± 0.09	1.41 ± 0.11
(ml kg^{-1})	Tezo	1.31 ± 0.07	1.41 ± 0.07	1.45 ± 0.06*	1.47 ± 0.07*	1.45 ± 0.10*	1.41 ± 0.10
	Control	1.42 ± 0.12	1.46 ± 0.10	1.47 ± 0.09	1.45 ± 0.08	1.39 ± 0.05	1.36 ± 0.07
	EMD	1.31 ± 0.08	1.34 ± 0.07	1.40 ± 0.07	1.38 ± 0.06	1.39 ± 0.08	1.39 ± 0.09
	EMD+Tezo	1.34 ± 0.10	1.33 ± 0.10	1.39 ± 0.10	1.35 ± 0.08	1.38 ± 0.09	1.38 ± 0.08

Values are mean±SE. ET, endothelin, PDE5, phosphodiesterase 5; HR, heart rate; MAP, mean aortic pressure; LAP, left atrial pressure; CO, cardiac output; SV, stroke volume; Tezo, tezosentan; EMD, EMD360527. * $P < 0.05$ vs. Standing; † $P < 0.05$ effect of Tezo; ‡ $P < 0.05$ effect of Tezo in the presence of EMD360527.

Figure 1. Effects of phosphodiesterase 5 (PDE5) inhibition and endothelin $(ET)_A/ET_B$ receptor blockade on the systemic vasculature in vivo. Shown are the effects of the ET_A/ET_B receptor blocker tezosentan (Tezo), the PDE5 inhibitor EMD360527, and Tezo in the presence of EMD360527 on mean aortic pressure (MAP), systemic vascular conductance (SVC), and systemic vascular resistance (SVR) at rest and during exercise. Values are means±SE. * $P < 0.05$, drug effects vs. corresponding control; † $P < 0.05$, Tezo in the presence of EMD360527 vs. EMD360527 alone.

maximal BVO_2.[29] Mean aortic pressure was maintained constant (Fig. 1, A and D), as the increase in cardiac output was balanced by a 66±9% increase in SVC (Fig. 1, B and E).

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

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

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

Exercise resulted in a significant increase in pulmonary arterial pressure (Fig. 2, A and D), which was principally the result of increase in cardiac output and to a lesser extent of increase in left atrial pressure (that is transmitted backward into the pulmonary vasculature) (Table 1). TPR increased (Fig. 2, C and F), suggesting an increase in right ventricular afterload. The transpulmonary pressure gradient (PAP minus left atrial pressure) increased slightly less than cardiac output (Table 1) indicating that exercise-induced pulmonary vasodilation occurred as evidenced by an increase in PVC (Fig. 2, B and E). Distensibility of the pulmonary vasculature under control conditions was on average 0.5±0.1%/mmHg and ranged from 0.1 to 1.1 %/mmHg (r^2= 0.91±0.03).

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

Similar to previous observations from our laboratory[19], infusion of PDE5 inhibitor EMD360527 resulted in a decreased PAP (Fig.2D), with minimal effect on left atrial pressure, whereas cardiac output increased significantly at all levels of exercise (Table 1). The decrease in TPR in response to EMD360527 was larger at higher levels of exercise (Fig. 2F), suggesting a larger effect of PDE5 inhibition on right ventricular afterload with incremental levels of exercise. EMD360527 caused a downward rotation of the relation between cardiac

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

Figure 3. Effects of PDE5 inhibition and ET_A/ET_B receptor blockade on the pulmonary pressure-flow relationships. Shown are the effects of the ET_A/ET_B receptor blocker Tezo, the PDE5 inhibitor EMD360527, and Tezo in the presence of EMD360527 on the pressure-flow relationship at rest and during exercise. CO, cardiac output; LAP, left atrial pressure. Values are means±SE. * $P < 0.05$, drug effects vs. corresponding control.

output and transpulmonary pressure-gradient (Fig. 3C), reflecting a significant increase in PVC (Fig. 2E). Distensibility of the pulmonary vasculature was not significantly altered by EMD360527.

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

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

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

To further investigate whether the lack of vasodilator effect of tezosentan in the presence of EMD360527 was the result of a direct suppression of the ET pathway by the NO-cGMP pathway, we measured constriction to ET and Big ET in the absence and presence of EMD360527 and 8Br-cGMP in isolated pulmonary small arteries. Both ET and Big ET

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

produced dose-dependent vessel segment contraction. EMD360527 blunted the response to ET and Big ET (Fig. 5, A and D), whereas 8Br-cGMP blunted the response to ET slightly more than the response to Big ET (P<0.05, Fig. 5, B and E). These data indicate inhibition of PDE5 decreases the sensitivity of the pulmonary vasculature to ET by increasing cGMP but has no effect on the conversion of Big ET to ET in the pulmonary vasculature. 8Br-cAMP had no effect on the response to either ET or Big ET (Fig 5, C and F).

DISCUSSION

The main findings of the present study were as follows: 1) both ET_A/ET_B receptor blockade with tezosentan and PDE5 inhibition with EMD360527 resulted in systemic and pulmonary vasodilation. 2) ET_A/ET_B receptor blockade resulted in further vasodilation in the presence of PDE5 inhibition in the systemic circulation. 3) However, in the presence of PDE5 inhibition, ET_A/ET_B receptor blockade failed to produce additional vasodilation in the pulmonary circulation in vivo or in isolated preconstricted pulmonary small arteries in vitro. 4) Both PDE5 inhibition and 8Br-cGMP blunted ET and Big ET-induced pulmonary small artery contraction in vitro and to a similar extent. The implication of these findings will be discussed below.

Methodological Considerations

Conductance versus resistance

In general, changes in vasomotor tone of a given vascular bed are extrapolated from changes in either the conductance or the resistance of the vascular bed. Although these measures

Figure 5. Effects of elevations in cGMP and cAMP on ET sensitivity and production from its precursor Big ET in pulmonary small arteries in vitro. Elevation of cGMP by either EMD360527 (3 μM, n=7, A and D) or the stable cGMP analog 8Br-cGMP (100 μM, n=4, B and E) attenuated constriction to ET and Big ET in isolated pulmonary small arteries, whereas elevation of cAMP with 8Br-cAMP (300 μM, n=4, C and F) had no effect. Values are means±SE. * $P < 0.05$, control vs. EMD360527, 8Br-cGMP, or 8Br-cAMP; † $P < 0.05$ effect of 8Br-cGMP different for ET and Big ET.

are mathematically related, interpretation can differ depending on whether one considers resistance or conductance.[24] Vascular conductance is calculated as flow corrected for pressure (flow/pressure) whereas vascular resistance is calculated as pressure divided by flow. These variables are interchangeable if one investigates the effect of only a single stimulus (e.g., exercise); however, interpretation of our results here are more complicated because we studied the effects of vasoconstrictor mechanisms at rest and during various levels of treadmill exercise in the systemic and pulmonary circulations.

The systemic circulation is a system with a low-flow state (high resistance, low conductance) at rest that transforms into a high-flow state (low resistance, high conductance) during exercise. Consequently, under low-flow conditions at rest, vasodilation causes a large decrease in resistance while the increase in conductance is small. In contrast, the same vasodilation under high-flow conditions during exercise causes a large increase in conductance, with only a small decrease in resistance. When quantifying the magnitude of the vasodilator responses, it appears in terms of conductance that a greater vasodilation occurs during exercise, whereas in terms of resistance it appears that vasodilation is larger at rest. This is illustrated by Fig. 1, which shows that the increase in SVC produced, for example, by the combination of PDE5 inhibition and ET_A/ET_B blockade is similar at rest and during exercise, whereas the decrease in vascular resistance wanes with incremental levels of exercise. Interpretation of vasomotor control thus critically depends on the variable examined. It has been forwarded that the variable (flow or pressure) that undergoes the primary change should be in the numerator of the index for vascular responses.[30] Since aortic blood pressure remains relatively constant while cardiac output markedly increases during exercise, the most appropriate measure for systemic vascular responses is SVC (cardiac output / aortic blood pressure). An additional argument in support of using conductance to determine systemic vascular responses is that the systemic circulation is comprised of vascular beds of various organs that are perfused principally in parallel. Parallel resistors add reciprocally, whereas parallel conductors add linearly, so that a change in conductance of one regional vascular bed results in an equal change of the total SVC.

The choice for either PVR or PVC as a measure of pulmonary vasomotor tone is less obvious as exercise increased both cardiac output and PAP (Table 1). However, the choice for either PVR or PVC also appears less critical, because exercise-induced changes in PVR and PVC are relatively minor compared to the vasodilation caused by PDE5 inhibition and ET_A/ET_B blockade. Consequently, the use of either resistance or conductance to assess the pulmonary vascular effects of a vasodilator will yield similar interpretations in the pulmonary bed.

Active vasodilation vs. passive distension

The increase in PVC during exercise in the present study could represent passive distension to the increase in pressure as well as vasodilation due to a decrease in pulmonary vascular tone. It is difficult to distinguish between passive distension and a decrease in pulmonary

Figure 6. Effects of PDE5 inhibition and ET_A/ET_B receptor blockade on the relation between PAP and PVC and pulmonary vascular resistance (PVR). Shown are the effects of the ET_A/ET_B receptor blocker Tezo, the PDE5 inhibitor EMD360527, and Tezo in the presence of EMD360527. Values are means±SE. $^*P < 0.05$, drug effects vs. corresponding control.

vascular tone. The pulmonary distensibility coefficient α of 0.5±0.1% is in accordance with the values found in literature.[27] To further address the question whether the increase in PVC is due to passive distension or active vasodilation, the relation between PAP and PVC in the different experiments was plotted in Fig 6. At low pressure, PVC increases with increasing PAP, whereas the relations show a plateau above ~20 mmHg, suggesting that the increase in PVC is not solely due to passive distension. Moreover, PDE5 inhibition or ET_A/ET_B receptor blockade resulted in an upward shift of the relation between PAP and PVC, which must be the result of a reduction in pulmonary vascular tone, since the increase in PVC occurred at any given level PAP.

Integrated control of pulmonary vascular tone by PDE5 and ET

The magnitude of these individual vasodilator effects of PDE5 inhibition and ET_A/ET_B receptor blockade in the systemic and pulmonary vascular beds are in good agreement with

their respective effects in previous studies from our laboratory.[11,15,19,24] Thus PDE5 inhibition resulted in pulmonary and systemic vasodilation that was similar in magnitude at rest and during exercise. PAP decreased in response to ET_A/ET_B receptor blockade at rest. The increase in PVC in response to ET_A/ET_B receptor blockade under resting conditions was nonsignificant (P=0.1), whereas the increase in PVC was significant at all levels of exercise. This smaller effect of ET_A/ET_B receptor blockade on PVC under resting conditions in vivo is consistent with previous studies from our laboratory[11,15] as well as with the observation that tezosentan failed to induce vasodilation in isolated pulmonary small arteries preconstricted with U46619. In the present study, we did not examine the effect of tezosentan on pulmonary veins, which are known to contribute to PVC as well and have been shown to be even more sensitive to ET than pulmonary arteries.[31]

Nevertheless, our in vivo and in vitro data, taken together, indicate that there is little ET released into the pulmonary vasculature under basal resting conditions. During exercise, however, pulmonary vasodilation occurred in response to ET_A/ET_B receptor blockade. It remains to be determined whether this vasodilator effect of ET_A/ET_B receptor blockade is due to its action on pulmonary arteries and/or pulmonary veins.

The vasodilator effect of ET_A/ET_B receptor blockade on the pulmonary vasculature in vivo was lost in the presence of PDE5 inhibition. These data seem in contrast with a recent study in isolated pulmonary arteries preconstricted with ET, in which PDE5 inhibition and ET receptor blockade produced additive vasodilation.[32] This additive effect is likely due to the preconstriction with ET, since we failed to observe an additive vasodilator effect of PDE5 inhibition and ET receptor blockade on isolated pulmonary arteries preconstricted with the stable thromboxane analog U46619 in the present study.

In contrast to the observations in the pulmonary vasculature, we found that the systemic vasodilation produced by ET_A/ET_B receptor blockade was not influenced by prior PDE5 inhibition. In fact, the systemic vasodilation induced by combined treatment resulted in an average reduction in blood pressure of 17±2 mmHg, with average systolic blood pressure being 90±6 mmHg and average diastolic blood pressure being 49±3 mmHg following treatment, indicating marked hypotension. One pig, which was excluded from the analyses, was even unable to perform treadmill exercise following the combination treatment. In contrast to the findings in the present study, a recent study showed that the combination of ET_A/ET_B blockade by bosentan and PDE5 inhibition by sildenafil, at dosages that were ineffective in single treatments, did result in pulmonary vasodilation in rats without the occurrence of systemic hypotension.[33] The lack of systemic hypotension observed in that study is likely to be due to the smaller increase in SVC (~47%) in combination with the larger increase in cardiac output (~60%) that was observed in the rats[33] compared with the larger increase in SVC (55±14%) and the smaller increase in cardiac output (23±9%) in the present study. This difference in increase of cardiac output was potentially due to the decreased cardiac output at baseline in the rats with pulmonary hypertension, which was normalized following

the reduction in afterload of the right ventricle. Alternatively, the prolonged duration of the treatment[33] may have resulted in recruitment of compensatory long-term blood pressure regulation mechanisms, i.e., activation of the renin-angiotensin-aldosterone system, that resulted in peripheral vasoconstriction (and hence limited the increase in SVC), thereby contributing to restoration of systemic pressure.

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

We have previously shown that endogenous NO acts to suppress the pulmonary vasoconstrictor influence of endogenous ET[11], particularly during exercise, which together with our results in isolated pulmonary small arteries points toward a direct interaction between the NO-cGMP system and the ET system. NO has been shown to directly modulate binding of ET to the ET_A receptor.[34] In addition to such direct effect of NO that would not be enhanced by PDE5 inhibition, experiments in porcine aorta, rat hearts, and cultured pulmonary arterial endothelial cells show that an increase in cGMP induced by either NO or the nonhydrolyzable cGMP analog 8Br-cGMP suppresses ET production and release.[35-37] Moreover, plasma ET levels were lower in rats with pulmonary hypertension treated with the PDE5 inhibitor sildenafil.[38]

Although plasma ET levels do not always adequately reflect tissue ET levels, these data are consistent with a reduced ET release following PDE5 inhibition. In contrast, our experiments in isolated vessels, showing that elevation of cGMP levels either through administration of the cGMP analog 8Br-cGMP or through PDE5 inhibition attenuated the vasoconstrictor response to Big ET as well as to ET, indicating that the interaction between

the NO-cGMP system and the ET system in the porcine pulmonary vasculature occurs mainly at the level of the ET receptor, not ET production. The observation that 8Br-cAMP did not affect the response to either ET or Big ET suggests that this interaction in the pulmonary vasculature is specific for the NO-cGMP system.

Conclusions and implications

The present study shows that the interactions between the NO-cGMP system and the ET system in the pulmonary vasculature occurred at the level of ET receptor(s) and prevented an additive vasodilator effect of PDE5 inhibition and ET_A/ET_B receptor blockade in the healthy pulmonary vasculature. This inhibition of the ET_A/ET_B receptor-mediated vasoconstrictor influence by NO-cGMP signaling is already present at rest and is not further modulated during exercise. Future studies should investigate whether in pulmonary disease states the observed increased vasoconstrictor influences of PDE5[19] and ET[12] will unmask an additive vasodilator effect of combined PDE5 inhibition and ET receptor blockade.

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