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Prostacyclin therapy increases right ventricular capillarisation in a model for flow-associated pulmonary hypertension[☆]

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Abstract

Pulmonary hypertension, and consequently right ventricular failure, complicates several congenital heart defects. Although intervention in the prostacyclin–thromboxane ratio is known to improve outcome, the underlying mechanism is not clear. Therefore, effects of acetyl salicylic acid and iloprost are studied in an animal model for flow-associated pulmonary hypertension. Male Wistar rats with flow-associated pulmonary hypertension, an aortocaval shunt in addition to monocrotaline induced pulmonary hypertension, were treated with low-dose aspirin (25 mg/kg/day) or iloprost (72 µg/kg/day). Effects on pulmonary hemodynamics and pulmonary vascular remodeling as well as right ventricular hemodynamics and remodeling were evaluated. Ninety percent ($n=7/8$) of the untreated pulmonary hypertensive rats developed dyspnea and pleural fluid, whereas this was seen in 50% ($n=4/8$, ns) and 10% ($n=1/8$, $P<0.05$ vs. untreated animals) of the aspirin and iloprost-treated rats, respectively. This could not be attributed to changes in pulmonary artery pressure, wall–lumen ratio of the pulmonary vasculature or right ventricular hypertrophy. However, both therapies restored reduced right ventricular capillary to myocyte ratio in pulmonary hypertensive rats (0.95 ± 0.10 in untreated rats vs. 1.38 ± 0.18 in control animals; $P<0.05$, and 1.32 ± 0.11 in aspirin-treated and 1.29 ± 0.9 in iloprost-treated rats; both $P<0.05$ vs. non-treated animals), which was associated with improved right ventricular contractility (iloprost). Thus, interventions in the prostacyclin–thromboxane metabolism improve outcome in rats with flow-associated pulmonary hypertension. However, these effects may be attributed to effects on cardiac rather than on pulmonary vascular remodeling.

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Keywords: Pulmonary circulation; Heart failure; Histopathology; Cyclo-oxygenase; Angiogenesis

1. Introduction

Pulmonary arterial hypertension is a progressive and often fatal disease. It is characterized by a disturbed vasodilator–vasoconstrictor balance, associated with medial hypertrophy,

muscularization of normally non-muscularized arteries and endothelial and smooth muscle cell proliferation (Wagenvoort, 1980; Wagenvoort and Mooi, 1989; Pietra et al., 2004; Humbert et al., 2004). Consequently, pulmonary vascular resistance increases, leading to right ventricular hypertrophy and eventually right ventricular failure.

Patients with pulmonary arterial hypertension have an increased ratio of urinary thromboxane to prostacyclin-metabolites (Christman et al., 1992). Both thromboxane and prostacyclin are metabolites from the cyclo-oxygenase pathway of arachidonic acid degradation. Prostacyclin has vasodilator and antiproliferative actions and is a potent inhibitor of platelet

Abbreviations: bpm, beats per minute; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor.

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aggregation, whereas thromboxane has vasoconstrictive, smooth muscle mitogenic and pro-coagulant activities (Fletcher, 1993).

No curative therapy for pulmonary arterial hypertension is available. However, therapy with prostacyclins has beneficial effects on exercise tolerance and survival in patients (Barst et al., 1994, 1996; Rubin et al., 1990; Higenbottam et al., 1984), indicating that an altered balance of cyclo-oxygenase metabolites in favour of the vasodilating prostacyclin at the expense of the vasoconstrictor thromboxane is associated with an improved clinical outcome. The underlying mechanism is largely unknown.

We recently developed a rat model for flow-associated pulmonary hypertension, showing advanced pulmonary vascular disease (van Albada et al., 2005). The effects of inhibition of thromboxane production by low-dose aspirin and the administration of a prostacyclin analogue were investigated in a rat model for flow-associated pulmonary hypertension, with regard to hemodynamics as well as cardiac and pulmonary vascular remodeling.

2. Methods

2.1. Animals and design of the study

Thirty-two male Wistar rats, weighing 250–350 g, were housed under 12 h/12 h light/dark conditions and fed ad libitum. Animal care and experiments were conducted according to the Dutch Animal Experimental Act and the investigation therefore conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The Erasmus University and the University of Groningen Animal Care and Use Committees approved the experimental protocols.

Rats were randomly assigned to four experimental groups: 1) control ($n=8$), 2) untreated pulmonary hypertension ($n=8$), in which a model for flow-associated pulmonary hypertension was created as previously used in our laboratory, with a combination of monocrotaline injections (60 mg/kg, Sigma Chemical Co., St. Louis, MO) followed by the creation of an abdominal aortocaval shunt one week later (van Albada et al., 2005), 3) pulmonary hypertension treated with acetyl salicylic acid ($n=8$) and 4) pulmonary hypertension treated with the prostacyclin analogue iloprost ($n=8$).

Three weeks after the start of treatment, rats were placed in a metabolic cage for 24 h to collect urine for measurements of thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ levels.

Animals were weighed three times a week, daily watched for dyspnea, defined as the use of accessory respiratory muscles and/or gasping, and sacrificed when a 15% weight loss or debilitating dyspnea occurred. Matched rats in the other groups were sacrificed simultaneously.

2.2. Treatment protocols

Osmotic minipumps (model 2004, Alzet, Palo Alto, California) with iloprost (72 $\mu\text{g/kg/day}$; a generous gift of Schering, the Netherlands) were implanted subcutaneously in the same operative session as the creation of the abdominal aortocaval shunt.

Aspirin (acetyl salicylic acid) was given via intraperitoneal injections in a dosage of 25 mg/kg/day once daily. We previously validated this aspirin dosage to inhibit thromboxane production by approximately 60% without affecting prostacyclin production (Kalkman et al., 1995). Control animals underwent sham surgery and sham implantation of a minipump.

2.3. Echocardiography

In 4 rats of each group, echocardiographic studies were performed under pentobarbital anesthesia 4 weeks after the administration of monocrotaline using a 12 MHz phased array transducer (Sonos 5500, Hewlett-Packard Inc, Andover, Massachusetts, USA). Ventricular dimensions and the presence of tricuspid insufficiency were assessed in standard views (Kato et al., 2003; van Albada et al., 2005).

2.4. Hemodynamic measurements

At the time of sacrifice, animals were anesthetized with pentobarbital (60 mg/kg intraperitoneally) and ventilated with room air. Pulmonary artery pressures were measured with a technique described by Rabinovitch (Rabinovitch et al., 1979) routinely used in our laboratory (van Albada et al., 2005). If pulmonary artery pressures could not be obtained, right ventricular systolic pressure was recorded as being equal to systolic pulmonary pressure ($n=2$ control animals). By introducing a catheter via the left carotid artery into the aorta, systemic arterial pressures as well as heart rate were measured.

2.5. Pulmonary vascular remodeling

After completion of hemodynamic measurements, the thorax was opened and the presence of pleural fluid was noted. The lungs were weighed and fixed in 3.6% formalin. Pulmonary sections (5 μm thickness) were stained with resorcin-fuchsin elastic stain for morphometric analysis of vascular dimensions according to a previously described protocol (van Albada et al., 2005). In lung sections all transversally cut arteries with a diameter equal to or more than 50 μm (pre-acinar arteries) and 40 randomly chosen vessels (10 in each left lung quadrant) with a diameter less than 50 μm (intra-acinar vessels) were assessed at 200 and 400 times magnification using an image analysis system (CZ KS400, Imaging Associates, Bicester, UK) (van Albada et al., 2005).

2.6. Cardiac remodeling

The heart was divided into atria, ventricles and septum. Sections were weighed separately and fixed in 3.6% formalin. Deparaffinized 5 μm thick transverse cardiac sections at mid-ventricular level were stained with Gomori silver staining for analysis of myocyte size and with lectin GSL staining (Sigma-Aldrich) to stain endothelial cells for analysis of capillary density. In the right ventricle, myocyte size and capillary density were measured as described in detail before (Van Kerckhoven et al., 2004). Myocyte cross-sectional area was measured in

transversally cut myocytes showing a nucleus. Myocyte density was calculated as total myocyte area by average myocyte area, corrected for total tissue area in a field, and expressed as the number of myocytes per mm². In consecutive slices in the same tissue area, capillary density was obtained by counting the number of capillaries per total tissue area (#capillaries/mm²). The capillary to myocyte ratio was calculated by dividing capillary density by myocyte density.

Sirius Red staining was used to determine collagen content, as described previously (Van Kerckhoven et al., 2004). Percentage of collagen was corrected for hypertrophy of the right ventricle by multiplying collagen percentage by right ventricular weight, to obtain collagen content.

2.7. Arachidonic acid metabolites

After three weeks of treatment, 24 h urinary samples were collected. At sacrifice, 2 ml of blood was collected in an EDTA-tube. Urine and plasma were used to measure the stable metabolites of thromboxane and prostacyclin, thromboxane B₂ and 6-keto-prostaglandin F_{1α} respectively, using radioimmunoassay (antibodies: Advanced Magnetics, USA; standards: Sigma, USA) as described in detail by Zijlstra et al. (1992).

2.8. Right ventricular function and gene expression

In an additional experiment, including control ($n=12$), untreated ($n=14$) and iloprost-treated ($n=13$) groups, effects of iloprost administration on right ventricular hemodynamics and gene expression of angiogenic factors were investigated in more detail. Iloprost-treated, but not aspirin-treated rats were used for this experiment, since beneficial effects of these treatments appeared qualitatively similar, but most pronounced in the iloprost group. In contrast to the previous experiment, rats were sacrificed at exactly four weeks after monocrotaline administration. Pulmonary arterial pressure measurements were performed as described, while a Microtip pressure transducer (Millar Instr. Inc., Houston, TX, USA) was inserted into the right ventricular cavity to determine right ventricular systolic pressure and right ventricular end-diastolic pressure. As index of contractility, the maximal rate of increase in right ventricular pressure ($dP/dt_{\max}$) was taken and corrected for right ventricular systolic pressure ($dP/dt_{\max}$ ind).

In order to look at angiogenic activity in the myocardium, real-time PCR was performed on right ventricular material for the expression of known angiogenic factors as vascular endothelial growth factor (VEGF), its two receptor subtypes VEGF receptor type 1 (VEGF R1 or flt-1) and VEGF receptor type 2 (VEGF R2 or flk-1), basic fibroblast growth factor (bFGF), angiopoietin-1 and angiopoietin-2. RNA was extracted from right ventricular tissue using the Qiagen RNEasy Mini Kit (Qiagen, Frankfurt, Germany). Real-time PCR experiments were performed on a Gene Amp 5700 Sequence detector (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands) as described previously (Sandovici et al., 2004) (for primer sequences see Table 1). RTQ-PCR results were obtained from a dilution standard curve.

Table 1

Primer sequences used for experiments on gene expression levels of angiogenic factors in the right ventricular myocardium

Gene	Primer sequence
VEGF	F 5'-GTACCTCCACCATGCCAAGT-3' R 5'-AATAGCTGCGCTGGTAGACG-3'
VEGF R1 (flt-1)	F 5'-GACCTGCGAAGCCACAGTTA-3' R 5'-GTCAATCCGCTGCCTGATAG-3'
VEGF R2 (flk-1)	F 5'-GCCTTATGATGCCAGCAAGT-3' R 5'-GCCAATGTGGATGAGGATCT-3'
bFGF (1)	F 5'-AAGGATCCCAAGCGGCTCTA-3' R 5'-TGCCCAGTTCGTTTCAGTGC-3'
Angiop. 1	F 5'-CCTTCAAGGCTTGGTTACTC-3' R 5'-ATCTAGGCTTCCATCCTCAC-3'
Angiop. 2 (2)	F 5'-GCTGGGCAACGAGTTGTCT-3' R 5'-CAGTCCTTCAGCTGGATCTTCA-3'

(1) Primer designed by Jensen et al., Angiogenesis. 2004; 7 (3): 255–67.

(2) Primer designed by Sarlos et al., Am J Pathol. 2003 Sep; 163 (3): 879–87.

2.9. Statistical analysis

Data are represented as mean $\pm$ S.E.M. Morbidity data (the presence of pleural fluid) were analyzed using a Fisher's exact test with Bonferroni correction. Mortality data were analyzed with a log-rank test with Bonferroni correction to compare between the untreated and the treated animals. Other differences between healthy control animals and untreated pulmonary hypertensive animals were analyzed using Student's *t*-test or a Mann–Whitney test when data were not normally distributed. Thereafter, the effect of therapeutic intervention was analyzed using a one-way ANOVA on the three groups with pulmonary hypertension with 2-sided Dunnett's post-hoc testing using the untreated group as reference, or a Kruskal–Wallis test when data were not normally distributed. Alfa was chosen as 0.05.

3. Results

3.1. Morbidity and mortality

All pulmonary hypertensive animals displayed weight loss (Table 2), lethargy and severe dyspnea in the 5th and 6th week after monocrotaline administration, leading to preliminary death in 4 rats of the untreated group. Consequently, hemodynamic data could not be obtained in these animals. Seven out of eight untreated animals determined the moment of sacrifice of the other matched animals, where this was 1 aspirin-treated animal (log-rank test on survival curves $P=0.054$ vs. untreated animals; Table 2). None of the iloprost-treated animals determined the moment of sacrifice of the other animals (log-rank test on survival curves $P<0.05$). At sacrifice, seven out of eight untreated animals displayed an excessive volume of intra-thoracic fluid (>10 ml), vs. 4 out of 8 aspirin-treated and 1 out of 8 iloprost-treated animals ($P=0.01$ vs. EXP, Table 2).

3.2. Echocardiography

Tricuspid insufficiency was mostly present in the untreated group, and less present in both treatment arms (Table 2). Right

Table 2
Animal characteristics at time of sacrifice

	Control	Untreated	Aspirin-treated	Iloprost-treated
<i>General</i>				
Animals with thoraxfluid (>12 ml)	0/8 (0%)	7/8 (87.5%)	4/8 (50%)	1/8 (12.5%) ^b
Animals determining the time of sacrifice	0/8 (0%)	7/8 (87.5%)	1/8 (12.5%)	0/8 (0%) ^b
Body weight at sacrifice (g)	403±8	338±13 ^a	352±13	350±7
<i>Echocardiography</i>				
Tricuspid insufficiency at echocardiography	0/4	3/4	1/4	2/4
RV wall thickness (mm)	0.75±0.03	1.25±0.10 ^a	0.90±0.08 ^b	0.95±0.06
RV internal diastolic diameter (mm)	4.15±0.33	5.73±0.46 ^a	4.80±0.41	4.60±0.68
RV wall–lumen ratio	0.019±0.002	0.022±0.002	0.019±0.002	0.022±0.004
<i>Systemic hemodynamics</i>				
Heart rate (bpm)	379±11	277±44 ^a	284±20	288±17
Systolic systemic arterial pressure (mmHg)	117±7	93±16	86±5	105±9

Number of animals displaying a symptom vs. the total number of animals in that group. Percentages are given between brackets. Body weight, heart rate and systolic systemic arterial pressure are shown as mean±SEM. Bpm = beats per minute. ^a*P*<0.05 vs. control, ^b*P*<0.05 vs. untreated group.

ventricular wall thickness and internal right ventricular diameter during diastole increased in the untreated animals, although wall to lumen ratios were preserved. Because of low numbers of rats, statistical significance was not reached, but both interventions tended to reverse these effects (Table 2).

3.3. Hemodynamic measurements

Systolic as well as diastolic pulmonary artery pressure was significantly increased in the pulmonary hypertensive rats, whereas heart rate was significantly reduced. Systemic arterial

Fig. 1. Pulmonary arterial pressures (mean±SEM). dPAP = diastolic pulmonary arterial pressure, mPAP = mean pulmonary arterial pressure and sPAP = systolic pulmonary arterial pressure. CON = control animals (*n*=6), PAH = untreated experimental group (*n*=3), PAH+ASA=experimental animals treated with acetyl salicylic acid (*n*=7) and PAH+ILO=experimental animals treated with the prostacyclin analogue iloprost (*n*=7). **P*<0.05 vs. control, †*P*<0.05 vs. PAH.

pressure was slightly lower in pulmonary hypertensive rats. No major effects of therapy were observed (Fig. 1, Table 2).

3.4. Arachidonic acid metabolism

Whereas urinary levels of prostaglandin $F_{1\alpha}$ were not changed in the untreated animals, thromboxane B_2 displayed a non-significant increase (40%) in this group compared to the control animals. The ratio between prostacyclin and thromboxane metabolites was decreased in untreated animals without any obvious effect of therapy (Fig. 2A).

In contrast, plasma prostaglandin $F_{1\alpha}$ increased more than tenfold in untreated animals and was reduced by both therapeutic interventions. Plasma levels of thromboxane B_2 increased in untreated animals, while therapy with aspirin or iloprost decreased plasma thromboxane B_2 to near normal levels (Fig. 2B).

3.5. Cardiac and pulmonary weights

Untreated pulmonary hypertensive rats showed increased heart weights (heart to body weight ratio 4.41 ± 0.22 g/kg in untreated vs. 2.66 ± 0.07 g/kg in control animals, *P*<0.001), which could be attributed to the right part of the heart (Fig. 3). Therapy did not influence this (heart to body weight ratio 4.18 ± 0.13 g/kg in aspirin-treated animals, ns vs. untreated animals, and 4.08 ± 0.22 g/kg in iloprost-treated animals, ns vs. untreated

Fig. 2. A: Urinary levels of 6-keto-prostaglandin $F_{1\alpha}$ and thromboxane B_2 in pg/24 h. B: Plasma levels of 6-keto-prostaglandin $F_{1\alpha}$ and thromboxane B_2 in pg/ml. The inserts are showing the ratio of 6-keto-prostaglandin $F_{1\alpha}$ to thromboxane B_2 . CON = control animals, PAH = untreated experimental group, PAH+ASA = experimental animals treated with acetyl salicylic acid and PAH+ILO = experimental animals treated with the prostacyclin analogue iloprost. **P*<0.05 vs. control, †*P*<0.05 vs. PAH.

Fig. 3. Cardiac weight. RV = right ventricle, IVS = inter ventricular septum, LV = left ventricle, RA = right atrium, LA = left atrium, CON = control animals, PAH = untreated experimental group, PAH+ASA = experimental animals treated with acetyl salicylic acid and PAH+ILO = experimental animals treated with the prostacyclin analogue iloprost. * $P < 0.05$ vs. control. No differences were observed between treated and untreated animals.

animals). Right ventricular to left ventricular plus septal weight ratio increased in the untreated animals (0.617 ± 0.040 in untreated animals compared to 0.273 ± 0.004 in control animals, $P < 0.001$ vs. CON), while no effects of therapy could be demonstrated (0.584 ± 0.016 in aspirin-treated and 0.561 ± 0.043 in iloprost-treated rats, both ns vs. the untreated animals).

Similarly, lung weight to body weight ratio increased significantly in the untreated animals, and was not significantly affected by therapy (7.4 ± 0.5 g/kg in untreated animals compared to 3.3 ± 0.2 in control animals, $P < 0.001$, and 5.8 ± 0.5 in aspirin-treated and 6.7 ± 0.5 in iloprost-treated animals, both ns vs. the untreated animals).

3.6. Pulmonary vascular remodeling

3.6.1. Pre-acinar pulmonary arteries ($> 50 \mu\text{m}$)

Larger pulmonary arteries increased in number and size in the untreated group. Luminal diameter, wall thickness and wall to lumen ratio increased in these animals, suggesting outward remodeling of the larger pulmonary arteries. No intimal proliferation could be demonstrated in the pre-acinar arteries of the untreated animals. Treatment did not change either of these parameters (Table 3).

3.6.2. Intra-acinar pulmonary vessels ($< 50 \mu\text{m}$)

The smaller pulmonary vessels in the untreated group showed a decreased luminal diameter, while outer diameter remained similar to control animals, indicating inward remodeling. Increased wall thicknesses, wall to lumen ratios, and increased occlusion with increased muscularization support this observation. Therapy did not have an effect on any of these parameters (Table 3).

3.7. Cardiac remodeling

Fig. 4 shows typical examples of right ventricular tissue stained to measure myocyte size (Fig. 4A) and capillary density (Fig. 4B). Photographs show increased right ventricular myocyte size in the untreated pulmonary hypertensive animals. Therapy did not affect myocyte size. Capillary density decreased in the untreated animals. Treatment with both aspirin

and iloprost increased capillary density, while myocyte size did not alter. These observations are substantiated by the actual measurements (Fig. 5). In the untreated animals, right ventricular myocyte cross-sectional area increased by 45%, while capillary density decreased by 50% (Fig. 5A). In addition to dilution of capillaries per tissue by increasing myocyte size, the actual number of capillaries decreased, as is indicated by a reduced capillary-to-myocyte ratio (Fig. 5B). Both therapeutic interventions normalized this reduced capillary-to-myocyte ratio, by preserving capillaries rather than by reducing myocyte size (Fig. 5A and B).

Relative collagen percentages were $3.01 \pm 0.15\%$ in control animals, $2.72 \pm 0.39\%$ in untreated animals, $1.97 \pm 0.34\%$ in aspirin-treated ($P = 0.20$ vs. untreated animals) and $1.66 \pm 0.18\%$ in iloprost-treated animals ($P = 0.056$ vs. untreated animals). Collagen content, percentages adjusted for right ventricular weight, increased in the untreated animals. Therapy with acetyl salicylic acid decreased collagen content by 35% (ns) while iloprost treatment reduced collagen content by 45% ($P < 0.05$; Fig. 5C).

3.8. Right ventricular hemodynamics

Pulmonary arterial pressures were comparable to the pressures in the previous experiment; increased pulmonary arterial pressures in the untreated group, without a significant effect of

Table 3
Pulmonary vascular morphometry

	Control	Untreated	Aspirin-treated	Iloprost-treated
<i>Pre-acinar pulmonary arteries ($> 50 \mu\text{m}$)</i>				
Number	21 $\pm$ 3	43 $\pm$ 4 ^a	45 $\pm$ 5	33 $\pm$ 2
Outer diameter (μm)	101.1 $\pm$ 4.3	139.8 $\pm$ 6.0 ^a	125.9 $\pm$ 10.0	125.7 $\pm$ 7.5
Luminal diameter (μm)	82.7 $\pm$ 4.3	108.8 $\pm$ 5.1 ^a	99.0 $\pm$ 8.6	99.8 $\pm$ 7.2
Wall thickness (μm)	9.2 $\pm$ 0.4	15.5 $\pm$ 0.8 ^a	13.5 $\pm$ 1.6	12.9 $\pm$ 1.0
Wall–lumen ratio	0.13 $\pm$ 0.01	0.16 $\pm$ 0.01 ^a	0.15 $\pm$ 0.02	0.15 $\pm$ 0.02
<i>Intra-acinar pulmonary arteries ($< 50 \mu\text{m}$)</i>				
Outer diameter (μm)	33.3 $\pm$ 0.5	32.0 $\pm$ 0.8	32.2 $\pm$ 0.7	32.1 $\pm$ 0.8
Luminal diameter (μm)	32.4 $\pm$ 0.6	25.7 $\pm$ 0.8 ^a	26.4 $\pm$ 0.7	26.8 $\pm$ 0.9
Wall thickness (μm)	0.4 $\pm$ 0.1	3.1 $\pm$ 0.3 ^a	2.9 $\pm$ 0.3	2.7 $\pm$ 0.2
Wall–lumen ratio	0.015 $\pm$ 0.007	0.180 $\pm$ 0.022 ^a	0.158 $\pm$ 0.022	0.150 $\pm$ 0.019
Occlusion (%)	3.9 $\pm$ 1.2	32.6 $\pm$ 2.0 ^a	29.5 $\pm$ 2.7	27.2 $\pm$ 2.4
<i>Muscularization (% of vessels)</i>				
– % of vessels that is totally muscularized	6.9 $\pm$ 2.4	28.1 $\pm$ 2.6 ^a	33.3 $\pm$ 2.8	30.0 $\pm$ 4.2
– % of vessels that is partially muscularized	0.9 $\pm$ 0.7	5.0 $\pm$ 1.3 ^a	6.2 $\pm$ 1.5	7.5 $\pm$ 2.6
– % of vessels that is non-muscularized	92.2 $\pm$ 3.0	66.9 $\pm$ 1.9 ^a	60.4 $\pm$ 3.3	62.5 $\pm$ 3.1

Data are shown as mean $\pm$ SEM. ^a $P < 0.05$ vs. control.

Fig. 4. A: Representative Gomori stained tissue sections of the right ventricular wall to determine myocyte cross-sectional area. B: Lectin stained tissue sections of the right ventricular wall to determine capillary density. CON = control animals, PAH = untreated experimental group, PAH+ASA = experimental animals treated with acetyl salicylic acid and PAH+ILO = experimental animals treated with the prostacyclin analogue iloprost.

Fig. 5. Cardiac histopathology. A: Capillary density and myocyte area, B: Capillary to myocyte ratio and C: Right ventricular collagen content. CON = control animals, PAH = untreated experimental group, PAH+ASA = experimental animals treated with acetyl salicylic acid and PAH+ILO = experimental animals treated with the prostacyclin analogue iloprost. * $P < 0.05$ vs. control, † $P < 0.05$ vs. PAH.

therapy. Accordingly, right ventricular systolic pressure was increased in the model (64 ± 4 mmHg in the untreated animals vs. 31 ± 1 in control animals, $P < 0.001$ vs. control animals), and was not significantly affected by therapy (58 ± 4 in iloprost-treated animals, ns vs. untreated group). Right ventricular end-diastolic pressure was increased in the untreated group (6.2 ± 1.0 mmHg in untreated rats vs. 3.2 ± 0.7 in control animals, $P = 0.03$), without a significant effect of prostacyclin therapy (6.0 ± 0.9 mmHg in iloprost-treated animals).

Absolute $dP/dt_{\max}$ was increased in the untreated group and in the iloprost-treated group compared to controls (4510 ± 230 mmHg/s in the untreated group and 4494 ± 179 in iloprost-treated animals vs. 2753 ± 194 mmHg/s in controls, both

Fig. 6. Gene expression patterns of angiogenic factors in right ventricular myocardium. CON = control animals, PAH = untreated experimental group, and PAH+ILO = experimental animals treated with the prostacyclin analogue iloprost. VEGF = vascular endothelial growth factor, VEGF R1 = vascular endothelial growth factor receptor type 1, VEGF R2 = vascular endothelial growth factor receptor type 2, bFGF = basic fibroblast growth factor, Angiopoietin 1 = Angiopoietin 1 and Angiopoietin 2 = Angiopoietin 2.

$P < 0.05$ vs. controls). $dP/dt_{\max}$ corrected for right ventricular pressure ($dP/dt_{\max}$ ind.) was decreased in the untreated group ($71 \pm 2 \text{ s}^{-1}$ in untreated rats vs. 91 ± 5 in controls, $P < 0.001$). Prostacyclin treatment increased $dP/dt_{\max}$ ind. ($80 \pm 3 \text{ s}^{-1}$ in iloprost-treated animals, $P = 0.03$ vs. untreated rats). Heart rate decreased significantly in the untreated group (310 ± 13 bpm in untreated animals vs. 384 ± 10 in controls, $P < 0.001$ vs. controls) and was partly restored by iloprost (334 ± 11 bpm, ns vs. untreated animals).

3.9. Right ventricular gene expression

Right ventricular angiotensin-1 expression decreased, while right ventricular basic fibroblast growth factor (bFGF) and angiotensin-2 expression increased in the experimental model. However, no changes in the expression of vascular endothelial growth factor (VEGF), VEGF receptors, bFGF or the angiotensins could be demonstrated after prostacyclin treatment (Fig. 6).

4. Discussion

In the present study, the effects of interventions in the prostacyclin and thromboxane metabolism in a rat model for flow-associated pulmonary hypertension were determined. Beneficial effects of both low-dose aspirin and iloprost treatment are presented by decreased mortality and morbidity. Interestingly, the improved outcome is associated with altered right ventricular remodeling and function, rather than with altered pulmonary vascular remodeling or pulmonary hemodynamics.

4.1. Arachidonic acid metabolism

To monitor the effects of our intervention on circulating prostacyclin and thromboxane levels, plasma and urine samples were collected. Plasma thromboxane B_2 levels increased in the untreated pulmonary hypertensive animals. After treatment, plasma thromboxane levels decreased as expected with aspirin, but also after treatment with iloprost. This latter effect could be due to a direct inhibitory effect of prostacyclin on platelet activation (Czer et al., 1986; Flynn and Demling, 1982), or due to feedback suppression of cyclo-oxygenase activity.

Additionally, plasma prostacyclin metabolites tended to increase in the untreated rats. In the monocrotaline model for pulmonary hypertension, Stenmark showed increased 6-keto-prostaglandin $F_{1\alpha}$ concentrations in lung lavages from rats (Stenmark et al., 1985). Similarly, Czer et al. demonstrated an acute increase in plasma prostaglandin $F_{1\alpha}$ concentrations in mongrel dogs after monocrotaline administration (Czer et al., 1986). Urinary data from pigs with pulmonary emboli also showed both increased prostacyclin and thromboxane metabolites (Rautanen et al., 1997). Together, these results may indicate increased cox-2 activity in these animal models (Pidgeon et al., 2004).

However, other data do not demonstrate an increase in cox-2 activity, but rather a disturbed vasoconstrictor–vasodilator balance. Badesch et al. (1989) found decreased prostacyclin production in isolated pulmonary vascular rings from hypoxic

calves. Congruently, human studies show a decreased expression of pulmonary prostacyclin synthase (Tuder et al., 1999) and decreased urinary prostacyclin metabolites together with increased urinary thromboxane metabolites (Christman et al., 1992).

Surprisingly, in urine samples of our rats we found a trend to similar results, with a significant decreased prostacyclin to thromboxane ratio. Thus, although our plasma data agree with data from identical animal models, our urine data agree with urinary patient data. This discrepancy between plasma and urinary levels can not be explained from this study. In addition to a different time point in the progression of the disease – urine was collected three weeks after induction of the model and plasma at the end-stage of the disease – urinary 6-keto-prostaglandin $F_{1\alpha}$ -level might be a reflection of renal synthesis, more than reflecting circulating prostacyclin (Patrignani et al., 1982; Fitzgerald et al., 1983). It should be noted that iloprost is a synthetic prostacyclin analogue, and pilot studies in our lab showed that its metabolites are not detected in the 6-keto-prostaglandin $F_{1\alpha}$ assay.

4.2. Therapeutic effects on the lungs

Prostacyclin causes an intracellular increase in cyclic AMP, leading to pulmonary vascular dilatation, a decrease in pulmonary vascular resistance (McLaughlin et al., 1998) and a decrease in vascular smooth muscle cell proliferation (Tuder and Zaiman, 2002; Clapp et al., 2002; Li et al., 2004). Patients that show no acute hemodynamic reaction to prostacyclin often improve after several weeks of therapy, suggesting structural effects rather than direct effects on vascular tone (Clapp et al., 2002; Higenbottam et al., 1984; Barst et al., 1996).

Inhibition of thromboxane is thought to be beneficial because of limitation of its vasoconstrictive, mitogenic and pro-coagulant properties. A randomized placebo-controlled study regarding the effects of an orally active thromboxane inhibitor was performed in patients with idiopathic pulmonary hypertension (Langleben et al., 2002). However, this study had to be halted because of the occurrence of severe leg pain. Moreover, the patients that did reach the end of the trial did not show significant improvement.

In the present study, pulmonary arterial pressure did not significantly change with therapy. Similar results can be obtained from human studies, where survival did improve (Barst et al., 1994), but was associated with a very modest reduction in mean pulmonary pressure of maximal 10% (Rubin et al., 1990; Barst et al., 1996).

A detailed quantitative morphometric analysis of the complete pulmonary vasculature was performed in our study. However, we could not demonstrate significant effects of either therapy on pulmonary vascular remodeling. This is in contrast to a study of Schermuly et al. (2004) showing effects of iloprost administration on intra-acinar pulmonary vessel muscularization in the monocrotaline rat model. The difference may be explained by the fact that in their animal model, no additional increased pulmonary blood flow was applied. Furthermore, Sprague–Dawley rats were used, which may display different

sensitivity to monocrotaline (Mathew et al., 1989). Finally, as we aimed at a dose of iloprost that did not cause systemic hemodynamic effects, the chosen dose may be too low to interact with pulmonary arterial pressure and pulmonary vascular remodeling. Remains the observation that despite this lack of effect on pulmonary vascular remodeling, mortality and morbidity seemed improved.

Thus, the improved outcome observed after treatment in this study could not be attributed to effects on hemodynamics or morphology of the pulmonary vessels, indicating other responsible mechanisms as well.

4.3. Therapeutic effects on the heart

At end-stage pulmonary hypertension, no effect of treatment on cardiac hemodynamics was observed. However, when rats are sacrificed before overt signs of the disease, impaired right ventricular contractility was significantly improved by iloprost. The heart rate reduction observed in pulmonary hypertensive rats could be regarded as a sign of the severity of the disease as reduction was more pronounced at end-stage pulmonary hypertension (–26%) compared to the decrease measured after 3 weeks (–19%). Moreover, the general improvement with therapy coincided with a partly restored heart rate.

Right ventricular interstitial fibrosis decreased after treatment. This effect of therapy is in accordance with previous studies on the effect of aspirin treatment in the left ventricle (Kalkman et al., 1995). Collagen percentage itself did not increase in the untreated animals suggesting fibrosis in balance with myocyte growth. This is prevented by therapy. The functional consequence of interference in right ventricular collagen deposition is not clear yet.

Myocyte size in the pulmonary hypertensive animals increased, indicating concentric hypertrophy due to pressure overload. In addition to the decreased capillary number per tissue area because of increased myocyte size, the actual number of capillaries decreased as well, as indicated by reduced capillary to myocyte ratio. This phenomenon is also observed in the pressure overloaded left ventricle and called rarefaction. Decreased myocardial capillarisation is regarded as a general feature of pathological hypertrophy as it has been demonstrated in heart failure and hypertension (Rakusan et al., 1980; Rakusan and Wicker, 1990). Both therapy with prostacyclin and aspirin restored capillary to myocyte ratio in the right ventricle and hence prevented the indicated net loss of capillaries. Moreover, an improved capillary-to-myocyte ratio is associated with improved left ventricular contractility in heart failure in rats (van der Meer et al., 2005). Therefore, right ventricular angiogenesis could well be responsible for the improved right ventricular contractility and outcome in the present study. Research in end-stage ischemic heart failure patients showed similar results for treatment with prostaglandin E-1 (PGE1); induction of therapeutic angiogenesis was associated with a favourable clinical outcome (Mehrabi et al., 2002), attributed to upregulation of VEGF expression by PGE1. Current research addresses the role of prostacyclin as important downstream mediator of VEGF signalling (Zachary and Gliki, 2001).

In addition, prostacyclin could stimulate nuclear peroxisome proliferator-activated receptors (PPARs) (Kubis and Levy, 2004). This receptor exerts its angiogenic effects (Pola et al., 2004; Lim et al., 1999) also via VEGF upregulation. However, we could not demonstrate effects of prostacyclin treatment on the expression of VEGF/VEGF receptor, nor on several other angiogenetic growth factors in the right ventricular myocardium, although angiopoietin-expression altered in a pattern comparable to that after myocardial infarction (Sandhu et al., 2004). It should be noted that in this study, only mRNA expression levels were determined three weeks after initiation of the treatment. It could well be that initial changes in expression already had weaned off at sacrifice, so that we were unable to demonstrate them.

Furthermore, we can not exclude that in addition to indirectly improving cardiac contractility by improving cardiac perfusion, other mechanisms are involved, including direct effects of prostacyclin therapy on myocardial contractility (Sebbag et al., 2001; Oguchi et al., 1992; Rich and McLaughlin, 1999; Kisch-Wedel et al., 2003), mediated by the IP-receptor (Kisch-Wedel et al., 2003; Rich and McLaughlin, 1999).

Plasma thromboxane was decreased after both treatment forms. Whereas prostacyclin may stimulate angiogenesis, thromboxane is supposed to hamper it. Thromboxane A₂ mimetics prevent migration and tube formation of human epithelial cells (Ashton et al., 1999) and reduce angiogenesis (Beauchamp et al., 2001). Accordingly, in our study, inhibition of thromboxane was associated with improved capillarisation, and these properties could provide an explanation for the effects observed. Previous studies support this angiogenetic role of aspirin in left ventricular remodeling (Van Kerckhoven et al., 2004).

Finally, no ideal model for pulmonary arterial hypertension has been developed yet. The present rat model mimics the human disease with respect to pulmonary hemodynamics: increased flow and pressure in the pulmonary circulation, resulting in similar pulmonary vascular lesions, and clinical symptoms, rather than the model of monocrotaline alone. Moreover, clinically beneficial therapy with the prostacyclin analogue iloprost also appeared to improve outcome in our model, supporting that the present model provides a clinically relevant model for pathophysiology and therapy of pulmonary arterial hypertension.

In conclusion, in the present study, we investigated the effects of iloprost and low-dose aspirin in a rat model for flow-associated pulmonary arterial hypertension as is seen in children with large congenital left to right shunts. The study demonstrates that both iloprost and aspirin lead to an improved outcome in this model. However, the improved outcome could be attributed to restored right ventricular capillarisation, rather than to changes in pulmonary hemodynamics or pulmonary vascular remodeling.

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