Endotoxin Induces Differentiated Contractile Responses in Porcine Pulmonary Arteries and Veins

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Key Words

Pulmonary circulation • Endothelin-1 • Constriction • Relaxation • Western blot • Immunohistochemistry • Acute lung injury

Abstract

Background/Aims: Sepsis-induced lung injury is characterized by pulmonary hypertension, edema and deteriorated gas exchange. As in vivo studies have indicated that bacterial endotoxin predominantly induces a pulmonary venous constriction, we aimed to investigate effects of endotoxin on isolated porcine pulmonary vessels. **Methods:** Pulmonary arteries and veins were examined using in vitro isometric force recordings. Endothelin-receptor protein expression and distribution were analyzed by Western blot and immunohistochemistry. Freshly isolated preparations and vessels incubated (24 h) with/without endotoxin ($10 \mu\text{g}\cdot\text{ml}^{-1}$) were compared. The contractile responses to phenylephrine, UK14,304, U46619, $\text{PGF}_{2\alpha}$, endothelin-1 (ET-1) and sarafotoxin were recorded, as well as the relaxation in response to acetylcholine, isoproterenol and nitroprusside. **Results:** In freshly isolated vessels, phenylephrine-induced contractions had a 5-times larger amplitude in arteries than in veins. The amplitude of the contractions in response to sarafotoxin was

nearly 2 times larger in veins than in arteries, but there was no difference in responses to ET-1. Endotoxin markedly reduced phenylephrine-induced contractions in both arteries and veins, whereas the responses to ET-1 and sarafotoxin were augmented in veins only. No apparent changes in ET receptor expression or distribution were detected with Western blot or immunohistochemistry. **Conclusion:** Endotoxin differentially and selectively alters the contractile responses of porcine pulmonary vessels in vitro, towards a situation where the α -1 adrenergic responses of arteries are attenuated and the ET responses of veins are augmented. In situations with high adrenergic activity and high circulating ET levels, such as sepsis, these results may provide a mechanism contributing to pulmonary hypertension and edema formation.

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Introduction

Acute lung injury (ALI), characterized by deteriorated gas exchange and pulmonary hypertension, is a frequent and severe complication of sepsis in man and is associated with considerable mortality [1]. This condition can be reproduced and examined in large animal experimen-

tal models where intravenous endotoxin (lipopolysaccharide), isolated from the cell wall of Gram-negative bacteria, induces a powerful sepsis-like response as well as the manifestations of ALI [2–4]. The reaction to endotoxin includes major regional and systemic vasomotor disturbances, widely affecting both systemic and pulmonary circulations [5]. In the systemic circulation, the lowered resistance contributes to arterial hypotension and hypoperfusion of vital organs, leading to ischemia and organ failure. In the pulmonary circulation, endotoxin-induced hypertension contributes to formation of pulmonary edema, deteriorated gas exchange and to potential strain on the right ventricle of the heart. The hydrostatic pressure in the pulmonary capillaries is a crucial determinant of edema formation and, depending on the site where the constrictor effect dominates, pulmonary vascular constrictors may affect this pressure differentially. Thus, given an identical overall increase in pulmonary resistance, a constrictor with a predominantly venous site of action will generate a relatively higher capillary pressure with potential subsequent edema formation as compared with a constrictor with arterial predominance. Endotoxin has been shown to induce pulmonary hypertension largely characterized by increased venous resistance [6], potentially causing edema, impaired gas exchange and reduced pulmonary compliance, all hallmarks of ALI.

The endogenous, pro-inflammatory and highly vasoconstrictive peptide endothelin-1 (ET-1) is largely produced and released by the endothelium [7]. The levels of ET-1 are elevated in patients with sepsis [8] and ALI and correlate with the severity of illness [9–11]. We and others have shown that ET-1 is also involved in the formation of extravascular lung water and development of pulmonary hypertension during experimental endotoxemia. Furthermore, these detrimental pulmonary effects are counteracted by ET-receptor antagonism [3, 12, 13]. The mechanisms involved in the ET-1-mediated effects on edema formation during endotoxemia are not yet resolved. Possibly they include effects of ET-1 on pulmonary capillary pressure, capillary permeability and alveolar fluid clearance. Previous studies from our laboratory have shown that ET-receptor stimulation predominantly induces a venous constriction in the porcine pulmonary circulation, both in vivo and in isolated pulmonary vessels. Thus, ET-1 may play an important role in endotoxin-induced lung injury.

In the vasculature, ET-1 mediates its effects via two receptor subtypes: endothelin type A (ET_A) and endothelin type B (ET_B) receptors. The ET_A receptors are mainly

located on the vascular smooth muscle cells and mediate not only vasoconstriction but also smooth muscle cells proliferation [14]. The ET_B receptors are localized both on the vascular smooth muscle cells and on the endothelial cells, mediating contraction in the former and relaxation in the latter through release of nitric oxide (NO) and prostacyclin. In addition, the endothelial ET_B receptors clear ET-1 from the circulation via endocytosis of the receptor-ligand complex [14]. As the ET_A and ET_B receptors in part have different effects on vascular tone, changes in receptor distribution and density can have an impact on vascular resistances and pressures.

Endotoxin has been shown to induce major vasomotor disturbances involving effects on responses to both vasoconstrictive and vasodilating substances in various animal models and vascular beds [15–19]. Many of these substances in the vascular wall are also highly involved in the pathophysiology of sepsis, but their integrated action in the pulmonary vasculature during endotoxaemia, and ALI, is not fully elucidated. In particular, an analysis of differentiated effects on pulmonary arterial and venous resistance would be of importance for understanding of the mechanisms involved in edema formation. Although the in vivo experiments on animal models are of key clinical relevance, a more mechanistic analysis of the effects of endotoxin on specific vessels and on vascular signaling requires an approach where the effects on vessel segments can be isolated. To address this issue, we have developed an in vitro organ culture/incubation model for porcine pulmonary arteries and veins and examined the direct effects of endotoxin on contractile function and vascular reactivity. The vessel preparations were examined using in vitro experiments after 24 h incubation with or without endotoxin. In addition, Western blot and immunohistochemistry was performed to investigate endotoxin-induced changes of ET receptor expression and distribution.

Parts of this study were presented in a preliminary form at the 10th conference of the World Federation of Societies of Intensive and Critical Care Medicine, Florence, Italy in 2009.

Material and Methods

Vascular Preparations

Freshly harvested lungs from domestic landrace pigs of both sexes, 60–90 days of age, were obtained at the regional abattoir. The basal third of the lower lobe was cut and rinsed with a cold transport buffer solution (content see below). The lung tissue was stored in the buffer solution and transported on ice to the laboratory within 1 h. Pulmonary arteries and veins were identified and

carefully dissected under sterile conditions. Three rings (internal circumference 8.9–15.2 mm corresponding to 2nd to 3rd branching, segment length 1.1–4.3 mm) were cut from each type of vessel. After dissection, two rings from each vessel type were incubated, one with endotoxin and one without, while the third vessel was used for immediate experimentation.

Vessel Incubation

After dissection, the vessel rings were placed in 5 ml sterile Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin 100 U·ml⁻¹, streptomycin 100 µg·ml⁻¹ and L-glutamine 2 mM. One vessel of each type was exposed to endotoxin (*Escherichia coli*, serotype O127:B8) at a concentration of 10 µg·ml⁻¹ during the incubation, whereas the corresponding control vessels were kept in endotoxin-free medium. The vessels were then kept for 24 h at 37°C in a humidified atmosphere with 5% CO₂ in air.

Experimental Protocol

In vitro Pharmacology

The arterial and venous vessel rings were mounted on two parallel stainless steel pins (diameter 0.2 mm) in 6-ml organ baths of a 610M myograph system (DMT Danish Myo Technology A/S, Aarhus, Denmark) filled with Krebs-Ringer physiological salt solution (PSS) continuously gassed with 95% O₂/5% CO₂ at a temperature of 37°C. One pin was connected to a force transducer and the other to a micrometer screw for length adjustment. Force data were collected with a computerized A/D converter (ADInstruments Ltd., Chalgrove, UK).

The vessels were stretched to the optimal circumference for maximal isometric force development [high K⁺ (125 mM)-induced contraction is maximal]. This length was defined as the length (that is, circumference) at which the high potassium-induced contraction was maximal. In previous studies [20, 21], this length was found to correlate with the initial slack circumference allowing an estimate of optimal length based on the slack circumference of the vessels. After stretching and equilibration for 60 min, the viability of the preparations was tested by measuring the contractile response to high K⁺. Preparations not contracting to K⁺ were excluded from the study. This initial procedure was used for all preparations studied. After the baseline had returned to pre-K⁺-challenge levels, all preparations were challenged with 10 nM U46619 (stable thromboxane A₂ analogue). After the contraction to this agonist had stabilized (approx. 5 min), bradykinin (1 µM, arteries) or acetylcholine (ACH; 10 µM, veins) were added. Failure to relax to these two agonists (in the freshly isolated vessels) excluded the preparations from further experimentation.

The responses to α -receptor agonists were recorded by adding 10 µM phenylephrine (PHE; α -1 receptor agonist) or 10 µM UK14.304 (brimonidine, α -2 agonist). The effects of the β -receptor agonist isoproterenol (ISO) were assessed by adding the drug to a final concentration of 10 µM in vessels precontracted with 10 nM U46619. The relaxation to ACH was measured in rings precontracted using 10–30 µM U46619 to a steady-state force of at least 50% of that induced by high K⁺. To measure responses to ET-1 (ET_A and ET_B receptor agonist) or sarafotoxin 6c (S6c; selective ET_B receptor agonist), the peptides were added cumulatively (0.01–100 nM, log unit steps) and concentration-response relationships were recorded. These recordings ended the experiment as the effects of the peptides could not be washed away.

To assess endothelium-independent vasorelaxation, a separate set of precontracted incubated venous rings was exposed to cumulative concentrations (1 nM to 1 mM, log unit steps) of sodium nitroprusside (SNP; NO donor) and concentration-response relationships were recorded. The segment lengths of the preparations were recorded at the end of the experiments.

Solutions and Chemicals

Serum-free DMEM was purchased from Invitrogen (Paisley, UK). ET-1 and S6c were purchased from American Peptide Inc. (Sunnyvale, Calif., USA). The other drugs used, including endotoxin, were obtained from Sigma-Aldrich (St. Louis, Mo., USA). All drugs were dissolved according to the instructions of the manufacturer and stored in aliquots at –80°C until use. The transport buffer solution was composed of (in mM): NaCl 145, KCl 5, CaCl₂ 2, MgSO₄ 1, NaH₂PO₄ 1, EDTA 0.02, MOPS 3, pyruvate 2, glucose 5 and titrated to pH 7.4 with NaOH. The Krebs-Ringer PSS solution contained (in mM): NaCl 118.5, KCl 4.7, KH₂PO₄ 1.2, MgCl₂ 1.2, CaCl₂ 2.5, EDTA 0.026, glucose 11.1 and NaHCO₃ 25. The high-K⁺ PSS was made by replacing NaCl with KCl in the solution. The PSS solutions were gassed with 95% O₂/5% CO₂.

Immunohistochemistry

Approximately 10-mm-long intact pieces of pulmonary artery and vein (freshly isolated, incubated 24 h with or without endotoxin 10 µg·ml⁻¹) were transferred to 4% paraformaldehyde for fixation for 24 h, followed by 30% sucrose (24 h), washed in phosphate-buffered saline (PBS; 1 h) and embedded for cryosectioning (8-µm sections). Sections were rehydrated and permeabilized in PBS + 0.1% Triton X-100 and unspecific binding was blocked with 5.5% horse serum (1 h), followed by primary antibody incubation overnight at 4°C. Antibodies for the ET_A or ET_B receptors (sc-21193 and sc-21196, respectively; Santa Cruz Biotechnology, Santa Cruz, Calif., USA) and Cy3-conjugated SM- α -actin (Sigma-Aldrich) were diluted 1:100. After washing, the sections were incubated with fluorescent secondary antibodies (Alexa Fluor 555 donkey-anti goat to detect ET_A- and ET_B-receptors; Molecular Probes, Carlsbad, Calif., USA). After washing, the sections were finally incubated 2 min with 20 µM nuclear stain DRAQ5 (Bioss Ltd., Shephed, UK), rinsed briefly and mounted with fluorescent mounting medium (Dako, Carpinteria, Calif., USA). The mounted sections were refrigerated dark until confocal microscopy analysis using a Zeiss META LSM 510 microscope (Carl Zeiss GmbH, Jena, Germany).

Western Blots

Samples from pulmonary vessels were extracted in a 50 mM Tris, pH 7.5, 1% Triton X-100, 0.1% mercaptoethanol, 1% protease inhibitor cocktail (all reagents from Sigma-Aldrich), using a glass mortar and pestle for homogenization. Extracts were left on ice for 30 min, further homogenized until completely dissolved, centrifuged at 12,000 g for 10 min and supernatant collected for protein determination in a NanoDrop (Thermo Scientific, Rockford, Ill., USA).

Immunoblotting

Seventy-five micrograms protein of tissue extracts was mixed with 5× SDS-Laemmli buffer and denatured at 100°C for 5 min, separated by 4–12% polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes (Hybond ECL; Amers-

Table 1. Inner circumference, active force responses and ET receptor protein levels (Western blot) of freshly isolated pulmonary arteries and veins, and of vessels incubated in organ culture for 24 h without and with 10 $\mu\text{g}\cdot\text{ml}^{-1}$ endotoxin

	Artery (freshly isolated)	Vein (freshly isolated)	Artery		Vein	
			incubated	+ endotoxin	incubated	+ endotoxin
Number of vessels	10	9	9	15	10	15
Circumference, mm	11.6 ± 1.0	9.5 ± 0.7	8.5 ± 0.8	9.0 ± 1.1	8.5 ± 0.7	9.6 ± 1.1
Max. high K^+ tension, mN mm^{-1}	22.2 ± 1.8	24.7 ± 2.2	22.2 ± 2.5	16.9 ± 1.7	29.4 ± 3.8	$19 \pm 2^*$
ET-1 responses						
EC_{50} , log M	-8.6 ± 0.3	-8.6 ± 0.3	-8.2 ± 0.1	-8.1 ± 0.1	-8.3 ± 0.1	-8.3 ± 0.1
E_{max} , %	129 ± 7	138 ± 6	134 ± 9	149 ± 7	126 ± 7	182 ± 24
S6c responses						
EC_{50} , log M	-8.6 ± 0.3	-8.6 ± 0.3	-8.2 ± 0.1	-8.1 ± 0.1	-8.3 ± 0.1	-8.3 ± 0.1
E_{max} , %	74 ± 20	116 ± 13	130 ± 16	130 ± 17	124 ± 7	$153 \pm 6^{**}$
ET_A receptor protein levels, % of fresh	100	100	99 ± 1	107 ± 3	106 ± 14	90 ± 8
ET_B receptor protein levels, % of fresh	100	100	92 ± 4	88 ± 3	110 ± 12	101 ± 10

The active tension in response to depolarizing high K^+ solution is related to segment length and given as wall tension. The maximal responses to ET-1 and sarafotoxin 6c are given in percentage of the high K^+ responses. The E_{max} and EC_{50} values were obtained by fitting a sigmoidal function to the force-concentration

data. ET receptor protein levels are expressed as percentage of values for freshly isolated vessels ($n = 4$ in each group).

* $p < 0.05$; ** $p < 0.01$ for difference between incubated veins with and without endotoxin analyzed by Student's t test.

ham Biosciences, Piscataway, N.J., USA). Ponceau staining was used to evaluate protein loading. Nonspecific binding sites were blocked by incubation in 3% BSA. Membranes were incubated with specific antibodies (goat polyclonal) anti- ET_A and ET_B receptor (sc-21193 and sc-21196, respectively; Santa Cruz), or mouse monoclonal antibody specific for smooth muscle-specific α -actin (Sigma-Aldrich) diluted 1:1,000 at $+4^\circ\text{C}$ overnight. After primary antibody incubation, membranes were washed 3 times 10 min in PBS containing 0.1% Tween. Bands were visualized after 1 h incubation with an HRP-conjugated secondary antibody (1:1,000 donkey-anti goat for ET_A and ET_B receptors; Santa Cruz; goat-anti mouse 1:10,000 for SM α -actin; Pierce Biotech Inc.) using ECL technique (Pierce Biotech Inc.) according to the manufacturer's instructions. Equal amount of protein of the different samples were loaded on each gel and the band ECL intensities (using GelDoc and image software; Bio-Rad, Sundbyberg, Sweden) were expressed as percentage of the intensities of bands from freshly isolated vessels.

Data Analysis and Statistics

Data are expressed as means $\pm$ SEM. Numbers indicate the number of vessels obtained from different animals. Relaxation was calculated as percent of reduction of the active force at the stable plateau level.

Contractile responses to high K^+ (125 mM) are expressed as the ratio of force and segment length (active tension, mN/mm) and the other responses are expressed as percentage of the maximal contraction induced by high K^+ . The force (Y) and concentration (X) data were analyzed by fitting a sigmoidal function using nonlinear regression (implemented in Prism 4.0; GraphPad Software Inc., La Jolla, Calif., USA) using the formula: $Y = E_{\text{max}} \cdot X^h / (X^h + \text{EC}_{50}^h)$, where E_{max} denotes the estimated maximal amplitude of

the response, h the steepness of the relationship (Hill coefficient) and EC_{50} the concentration giving half maximal response.

Differences in contractions and relaxations between the in vitro preparations and between band intensities from Western blot were analyzed using Student's t test. Concentration-response data were assessed using repeated measures analysis of variance (ANOVA). The statistical calculations were made using Statistica (version 7.0; StatSoft Inc., Tulsa, Okla., USA). A p value of less than 0.05 was considered statistically significant.

Results

Vascular Reactivity of Freshly Isolated, Nonincubated, Vessels

The active wall tension (force per segment length) in response to depolarization with high K^+ solution was similar in the freshly isolated nonincubated arteries and veins (table 1). In contrast, the α -1 adrenergic agonist PHE induced a contraction that was more than 5-fold stronger in arteries than in veins (fig. 1a). No significant difference between arteries and veins was noted in response to the α -2 adrenergic agonist UK14.304, which caused only small contractions in both preparations (fig. 1a). The response to the thromboxane A_2 analogue U46619 or to $\text{PGF}_{2\alpha}$ did not differ between freshly isolated arteries and veins (fig. 1a). The ET_B receptor agonist S6c induced a nearly 2-fold stronger contraction and was

Fig. 1. a Effect of PHE (10 μ M, α -1 agonist, $n = 3$ and 3), UK14.304 (10 μ M, α -2 agonist, $n = 6$ and 6), U46619 (10 nM, TxA2-analogue, $n = 5$ and 5), PGF_{2 α} (1 μ M, $n = 3$ and 3), ET-1 (100 nM, $n = 3$ and 3) and sarafotoxin 6c (100 nM, S6c, $n = 6$ and 6) on freshly isolated rings of porcine pulmonary artery and vein. * $p < 0.05$; ** $p < 0.01$ for artery versus vein, analyzed by unpaired Student's t test. **b** Relaxing effects of ACH (10 μ M, α -1 agonist, $n = 3$ and 3) and ISO (10 μ M, β -agonist, $n = 5$ and 5) on freshly isolated rings of porcine pulmonary artery and vein precontracted with 10 nM U46619. ** $p < 0.01$ artery versus vein analyzed by unpaired Student's t test. **c, d** Concentration-response relationships for ET-1 ($n = 3$ and 3) and S6c ($n = 6$ and 6) in freshly isolated rings of porcine pulmonary artery and vein. Force values are given relative to the responses to high K⁺. *** $p < 0.001$ analyzed by repeated measures ANOVA. Values are presented as means $\pm$ SEM.

Fig. 2. a Effect of PHE (10 μ M, α -1 agonist, $n = 3$ and 3), UK14.304 (10 μ M, α -2 agonist, $n = 6$ and 6), U46619 (10 nM, TxA2-analogue

$n = 5$ and 5), PGF_{2 α} (1 μ M, $n = 3$ and 3), ET-1 (100 nM, $n = 3$ and 3) and S6c (100 nM, $n = 6$ and 6) on pulmonary veins incubated with or without endotoxin 10 μ g·ml⁻¹ during 24 h. * $p < 0.05$ without versus with endotoxin analyzed by unpaired Student's t test. **b** Effect of PHE (10 μ M, α -1 agonist, $n = 3$ and 3), UK14.304 (10 μ M, α -2 agonist, $n = 6$ and 6), U46619 (10 nM, TxA2-analogue, $n = 5$ and 5), PGF_{2 α} (1 μ M, $n = 3$ and 3), ET-1 (100 nM, $n = 3$ and 3) and S6c (100 nM, $n = 6$ and 6) on pulmonary veins incubated with or without endotoxin 10 μ g/ml during 24 h. * $p < 0.05$; ** $p < 0.01$ analyzed by unpaired Student's t test. **c, d** Concentration-response relationships for ET-1 on pulmonary artery and vein preparations incubated with or without endotoxin 10 μ g·ml⁻¹ during 24 h ($n = 3$ and 3). * $p < 0.05$ analyzed by repeated measures ANOVA. **e, f** Concentration-response relationships for S6c on pulmonary artery and vein preparations incubated with or without endotoxin 10 μ g·ml⁻¹ during 24 h ($n = 6$ and 6). * $p < 0.05$ analyzed by repeated measures ANOVA.

Fig. 3. a Effect of ACH (10 μ M, n = 6 and 6) and ISO (10 μ M, β -agonist, n = 5 and 5) on incubated and endotoxin-incubated pulmonary arteries preconstricted with 10 nM U46619. **b** Effect of ACH (10 μ M, n = 5 and 6), ISO (10 μ M, β -agonist, n = 5 and 5) and SNP (NO donor, n = 8 and 8) on incubated and endotoxin-incubated pulmonary veins preconstricted with 10 nM U46619. Values are means $\pm$ SEM.

active at lower concentrations in veins than in arteries (fig. 1a, d; table 1), whereas the response to the ET_A and ET_B receptor agonist ET-1 was equal (fig. 1a, c; table 1). In vessels preconstricted with U46619, the β -adrenergic agonist ISO induced a greater relaxation in veins compared to arteries. The responses to ACH were similar in the freshly isolated arteries and veins (fig. 1b).

Effects of Incubation on Vascular Reactivity

Incubation during 24 h induced only modest changes in active force generation of the vessels in response to membrane depolarization; the contraction induced by high K^+ (table 1) was not significantly altered compared to the freshly isolated vessels. In addition, no difference was found in response to the other constrictive substances apart from slightly augmented response to PHE (compare fig. 1a with 2b, 14.4 ± 0.2 vs. $16.9 \pm 0.7\%$ of high K^+ response, $p < 0.05$) in veins and a tendency towards an increase in response to S6c (compare fig. 1a with 2a, 61 ± 15 vs. $108 \pm 17\%$ of high K^+ response, $p = 0.06$) in arteries after incubation. In veins, incubation moderately attenuated the endothelium-dependent relaxation to ACH (compare fig. 1b with 3b, 79.3 ± 7.9 vs. $46.8 \pm 7.4\%$ relaxation of maximal U46619-induced contraction), while the artery was not significantly affected. Moreover, the effect of ISO, studied in arteries and veins and SNP, studied in vein only, was not altered by incubation (fig. 3a, b).

Effects of Incubation with Endotoxin on Vascular Reactivity

Incubation with endotoxin $10 \mu\text{g}\cdot\text{ml}^{-1}$ during 24 h induced a significant decrease in the high K^+ induced active tension of veins but not in arteries (table 1). Moreover, endotoxin also induced markedly lowered responses in both arteries and veins in response to PHE (fig. 2a, b). In veins selectively, the contraction induced by S6c and ET-1 was relatively augmented by endotoxin (fig. 2b, d, f), while the response to the other contractile agonists, except PHE, was not altered by endotoxin in arteries nor veins (fig. 2a, d).

The effects of the vasodilating compounds ACH and ISO were not significantly changed by endotoxin, although there was a weak tendency ($p = 0.16$) suggesting a modest impairment of the endothelium-dependent vasodilation of ACH in veins only. The effect of the NO-donor SNP in veins was unchanged by endotoxin incubation (fig. 3a, b).

Immunohistochemistry and Western Blotting

Immunohistochemistry was performed to localize the ET_A and ET_B receptors within the vascular tissue and to study the gross morphology of freshly isolated, incubated and endotoxin-incubated preparations. The vascular smooth muscle and the endothelial cell layer showed no signs of disruption or swelling after incubation or endotoxin exposure (data not shown). Staining against α -actin

Fig. 4. Confocal microscopy images displaying representative examples of immunohistochemistry staining for the ET_A receptor, ET_B receptor, secondary antibody only and smooth muscle α -actin. Freshly isolated preparations of pulmonary artery (top) and pulmonary vein (bottom). The vessel lumen is given on top of the images.

showed equally intense staining in the smooth muscle cell layer of all preparations (fig. 4). In fresh as well as in incubated vessels, the ET_A receptors were preferentially located in the vascular smooth muscle cells, whereas the ET_B receptors were located in the endothelial as well as the vascular smooth muscle cell layer (fig. 4, 5a, b). No difference in ET receptor staining intensity or distribution could be detected between freshly isolated arteries and veins or between incubated and endotoxin-exposed vessels (fig. 4, 5a, b).

The expression of ET_A and ET_B receptor protein was investigated using Western blot and compared to α -actin protein levels. In arteries, the levels of both ET_A and ET_B receptor protein expressions was higher compared to the levels in veins, using the same amount of protein loaded on the gel (data not shown). This difference was not affected by incubation per se or by endotoxin (table 1). Moreover, no difference in expression of ET receptor protein (table 1) or α -actin (data not shown) was detected between freshly isolated, incubated or endotoxin-exposed vessels.

Discussion

In the present study, we developed an organ culture system for porcine pulmonary vessels and investigated the effects of 24 h exposure to endotoxin. We examined effects on responses to contractile and relaxant substances that are considered important either for the pathogenesis or for the treatment of sepsis and ALI in man. The major findings are that endotoxin induces changes that differentially affect the reactivity of pulmonary arteries and veins. The sum of these endotoxin-induced disturbances in vascular reactivity might favor an increase in the pulmonary venous resistance compared to the arterial resistance, changes that in vivo may augment the filtration pressure in the pulmonary capillaries.

In freshly isolated vessels, the α -1 receptor agonist PHE induced a more than 5-fold stronger contraction in pulmonary arteries than veins. This finding is consistent with previous studies [22, 23] demonstrating a negligible constriction in pulmonary veins compared to arteries in-

duced by noradrenaline. Bäck et al. [22] suggested that this difference was the result of formation of endogenous NO in the vein, as inhibition of NO synthase markedly enhanced noradrenaline-induced contractions. In contrast to the prominent and preferentially arterial constriction induced by PHE, the α -2 receptor agonist UK14,304 elicited rather small and equal contractions in both preparations. Postjunctional α -2 adrenergic receptors mediating vasoconstriction have previously been described in the pulmonary circulation of various species [24–26] and the canine pulmonary vein has been reported to be more sensitive than the artery to α -2 receptor agonists [25]. This discrepancy to our findings could possibly be attributed to differences in used experimental model and species. Moreover, the clinical significance of the small contractions is unclear and α -2 receptor agonists have been used uneventfully in patients with pulmonary hypertension [27].

Thromboxane A_2 is regarded as a potent and important mediator of pulmonary vascular constriction [28]. Interestingly, *in vitro* and *ex vivo* studies have indicated that thromboxane A_2 not only induces a venous constriction [29, 30], but also promotes the formation of pulmonary edema [31]. Moreover, Walch et al. [29] studied human pulmonary veins reporting that U46619 induces a constriction 50% higher than that of noradrenaline. Results from arteries were not reported. Similar findings were seen in the present study where U46619 induced a contraction in pulmonary veins that was almost 5-fold stronger than that of PHE, a notable difference, compared with the relative response to these agonists in arteries. However, the contractions were equally powerful in arterial and venous preparations when related to the high K^+ response suggesting a limited difference in arterial and venous responses to thromboxane. In addition, the reaction to $PGF_{2\alpha}$, another highly vasoactive metabolite of arachidonic acid that is produced in inflammatory states like endotoxemia [32], was equal in arteries and veins.

The differential effects of the ET system on the pulmonary vascular tone have previously been investigated in

various species, partly with conflicting results. Brink et al. [33], using human pulmonary vessel preparations, found the pulmonary vein to be more sensitive than the artery to ET-1, and similar results have been reported by others in different species [34, 35]. In contrast to those results, Kemp et al. [30] and Zellers et al. [36] found no arterial to venous difference in sensitivity to ET-1 using pulmonary vessels of ovine and porcine origin. The latter findings are in line with the present study, where freshly isolated vessels, in contrast to endotoxin exposed, showed no arterial to venous difference in response to ET-1.

The finding that the selective ET_B receptor agonist S6c causes a predominant venous constriction is consistent with previous studies using both *in vitro* [6, 36, 37] and *in vivo* [6] preparations, suggesting that the ET_B receptor expression/function is a major factor determining the differential responses to ET in arteries and veins.

The β -adrenoceptor agonist ISO was more potent in relaxing veins than arteries, and its actions were not affected by endotoxin. Even though clinical use of β -agonists in ALI primarily is aimed at treating bronchoconstriction, there is growing evidence that β -agonists can reduce pulmonary edema [38, 39]. These effects of β -agonists have mainly been attributed to enhanced clearance of alveolar fluid by stimulation of sodium and water reabsorption through the alveolar epithelium. The findings of the present study suggest that a venous dilatation by β -agonists can provide an additional effect leading to a reduction of the pulmonary capillary and filtration pressure. This potentially beneficial effect needs further investigations using *in vivo* experiments.

The vessel incubation procedure induced only minor changes in the contractile patterns of the porcine pulmonary vessels. This finding is partly in contrast to previous reports on human, rodent and porcine nonpulmonary systemic vessels which have indicated that organ culture may induce an upregulation of contractile ET_B receptors located on vascular smooth muscle cells [40, 41] or a downregulation of dilatory endothelial ET_B receptors [42]. In the present study, incubation did not induce any statistically significant changes in response to ET receptor stimulation even though there was a trend towards increased S6c sensitivity in the pulmonary arteries after incubation. However, the analysis of protein expression with immunohistochemistry and Western blot did not reveal any apparent changes of ET_B receptor distribution or expression after incubation. It is possible that pulmonary vessels are less affected by the short-term culture conditions as compared with nonpulmonary vessels used in the above-mentioned studies.

Fig. 5. Confocal images displaying representative examples of immunohistochemistry staining for the ET_A receptor, ET_B receptor and secondary antibody. Isolated preparations of pulmonary artery (**a**) and pulmonary vein (**b**), incubated for 24 h with (bottom) or without (top) endotoxin $10 \mu\text{g}\cdot\text{ml}^{-1}$. The vessel lumen is given on top of the images.

Endotoxin induced a substantial vascular hyporeactivity in response to PHE, which was evident both in arteries and veins. These findings from the pulmonary vasculature are consistent with reports demonstrating endotoxin-induced hyporeactivity to α -1 adrenergic agonists in other vessel beds and species [5, 15, 43], including humans [44]. These reactions have been reported both after in vitro and in vivo application of endotoxin and have been suggested to involve the actions of NO and an increased action of soluble guanylate cyclase. That NO is highly involved especially in early sepsis-induced vascular hyporeactivity has been suggested by several reports [45]. Increased levels of NO breakdown products in sepsis patients have been reported and inhibition of the actions of NO has successfully been used to increase blood pressure in sepsis patients [46]. The results of the present study indicate that vessels in the pulmonary circulation are also prone to develop hyporeactivity to α -adrenergic stimulation after exposure to endotoxin. It is, to our knowledge, unknown if these disturbances in adrenergic signaling are present in the pulmonary circulation during septic shock and whether these changes are important for development of sepsis-induced pulmonary edema remains to be investigated. In contrast to the diminished reactions to the α -1 agonist, the contractions induced by the α -2 adrenergic agonist UK14,304 and the arachidonic acid metabolites U46619 and PGF_{2 α} , were left unaffected in arteries and veins after endotoxin exposure. This indicates that endotoxin interacts with specific receptors or cellular signaling pathways in the pulmonary vessels.

Endotoxin reduced the responses to membrane high K⁺ depolarization in pulmonary veins without affecting the arteries significantly. This might relate to a general downregulation of contractile components or to a change in Ca²⁺ influx. The link between endotoxin exposure and these changes is not clarified. However, endotoxin did not attenuate the responses to ET-1 and S6c. Instead, the responses to ET receptor agonism in pulmonary veins were increased in relation to other contractile agonists. Interestingly, the ET receptor response in arteries was not increased. If these differential effects of endotoxin on the arterial and venous vessels shown in our in vitro study can be extrapolated to a change in the ET receptor-mediated reactivity in vivo remains to be clarified. The importance of pulmonary venous tone has gained increased attention during the last decades, since it may have an impact on pulmonary fluid filtration and ventilation/perfusion matching [28]. The results of the present study suggest that ET receptor-mediated venoconstriction

could be enhanced in endotoxemia and have a significant impact on postcapillary resistances especially in conditions such as endotoxaemia or sepsis when circulating ET-1 levels are markedly elevated [8]. This is consistent with previous reports from our laboratory indicating that endotoxin in vivo predominantly induces an increase in the pulmonary venous resistance and enhances the formation of pulmonary edema [3, 6, 12]. Interestingly, these changes could be counteracted by ET receptor antagonists [3]. These changes in reactivity to ET receptor stimulation induced by endotoxin in pulmonary veins can involve alterations in expression or distribution of ET receptors, for example increased expression of contractile ET_A or ET_B receptors on the vascular smooth muscle cells or by a downregulation of relaxing ET_B receptors on the endothelium. We could, however, with immunohistochemistry, detect ET_B receptors in the endothelium and did not observe an upregulation of the ET receptors in the smooth muscle layer after endotoxin treatment. Furthermore, the Western blot analyses showed that the relative amounts of ET receptors was not changed by endotoxin incubation. It is possible that the sensitivity of these analyses is insufficient to resolve small but important changes of ET receptor expression or distribution, but other explanations are also possible. Since endotoxin is known to interact with several cellular processes, the observed changes in ET receptor responses after endotoxin incubation might involve changes in intracellular signaling pathways affecting the coupling between ET receptors and contractile activation/deactivation in combination with, or instead of, effects on the ET receptor expression per se.

The incubation attenuated the endothelium-dependent vasodilatation by ACH somewhat in veins, making analysis of endotoxin effects on endothelium-induced relaxation difficult in this model system. However, the ACH responses tended to be further diminished by endotoxin in the veins while the endothelium-independent responses to ISO and SNP remained unaffected, suggesting that the endothelial function was disturbed by incubation and endotoxin in the vein. In this context it should be noted that ET-1 and S6c also induce relaxation of vessels via endothelial ET_B receptors and release of NO and prostacyclin. It is therefore possible that the augmented responses to ET-1 and S6c in veins after endotoxin exposure may also include a component of endothelial impairment affecting the relaxing effects of endothelial ET_B receptor unmasking the constrictive effects of the ET_A and ET_B receptors on the smooth muscle cells. In the present study we could not detect any morphologic changes after

incubation or endotoxin exposure, suggesting that the endothelial impairment might be related to intracellular changes and not to endothelial disruption or shedding.

The concentration of endotoxin ($10 \mu\text{g}\cdot\text{ml}^{-1}$) was chosen after a pilot study and is similar to those used in other studies [15, 47]. Interestingly, O'Brien et al. [15] reported equal hyporeactivity of rat mesenteric artery induced by endotoxin (*Salmonella typhosa*) $1\text{--}100 \mu\text{g}\cdot\text{ml}^{-1}$ when assessed with PHE. Moreover, in their report the duration of incubation was more important than the concentration of endotoxin. Little is known about the time course of endotoxemia during sepsis and the level varies considerable between patients. However, the concentrations used in in vitro or in vivo studies [48] widely exceed those reported on plasma levels of septic patients in the intensive care setting (range $0.06\text{--}0.5 \text{ ng}\cdot\text{ml}^{-1}$) [49–51]. In vivo, an endotoxin insult will result in a generalized inflammatory response involving the actions of numerous cells and the release of several mediators, whereas in vitro the response is limited to the exposed experimental cell population, making comparisons between these setting complicated. Furthermore, the present study investigates pharmacological pathways which may affect the pulmonary capillary hydrostatic pressure in vivo. Even though this pressure is highly important for the formation of pulmonary edema, other forces of the Starling equilibrium (such as capillary permeability and oncotic pressures) as well as clearance of alveolar fluid are affected during sepsis and might also be of major importance for the formation of extravascular lung water. Moreover, although studies using isolated tissues exposed to separate pharmacologic stimuli may facilitate investigations of basic

mechanisms, additional studies in vivo are needed before in vitro results can be translated to whole animal settings, not to mention to the clinical situation.

In summary, this study shows that endotoxin induces differentiated effects on isolated porcine pulmonary arteries and veins. Endotoxin leads to lowered responses to α -1 adrenergic stimulation with a predominant effect on the arteries. Concurrently, endotoxin induces a relative augmentation of the contractile response to both ET-1 and S6c in the pulmonary veins, without altering any apparent expressions or distributions of ET-receptors. Taken together, these endotoxin-induced changes in the arterial and venous contractile responses to the adrenergic and ET pathways can, especially during increased sympathetic activity and high ET levels, influence the balance in tone between pulmonary arteries and veins. If these results are extrapolated to the clinical situation, this will increase the pulmonary capillary filtration pressure and have an effect on the formation of pulmonary edema, especially during conditions (for example sepsis) when other edema-promoting factors, such as increased capillary permeability or decreased oncotic pressure, are present.

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