Endothelin Receptor Antagonists Attenuate the Inflammatory Response of Human Pulmonary Vascular Smooth Muscle Cells to Bacterial Endotoxin

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ABSTRACT

Bacterial infections induce exacerbations in chronic lung diseases, e.g., chronic obstructive pulmonary disease (COPD), by enhancing airway inflammation. Exacerbations are frequently associated with right heart decompensation and accelerate disease progression. Endothelin receptor antagonists (ERAs) might have therapeutic potential as pulmonary vasodilators and anti-inflammatory agents, but utility in exacerbations of chronic lung diseases is unknown. We hypothesized that cytokine releases induced by lipopolysaccharide (LPS), the major bacterial trigger of inflammation, are reduced by ERAs in pulmonary vascular smooth muscle cells (PVSMCs). Ex vivo cultivated human PVSMCs were preincubated with the endothelin-A-receptor selective inhibitor ambrisentan, with the endothelin-B-receptor selective inhibitor BQ788 [sodium (2R)-2-[[2S)-2-[[2R,6S)-2,6-dimethyl-1-piperidinyl]carbonyl]amino]-4,4-dimethylpentanoyl][1-(methoxycarbonyl)-o-tryptophyl]amino)hexanoate], or with the dual blocker bosentan before stimulation with smooth LPS (S-LPS), rough LPS (Re-LPS), or a mixture of long and short forms (M-LPS). Expression of cytokines and LPS receptors (TLR4, CD14) were analyzed via enzyme-linked immunosorbent assay (ELISA) and/or quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). All LPS forms induced interleukin (IL)-6-, IL-8-, and granulocyte macrophage–colony stimulating factor (GM-CSF) release. Bosentan and BQ788 inhibited M-LPS-induced release of all cytokines and soluble CD14 (sCD14) but not TLR4 expression. Ambrisentan blocked M-LPS-induced IL-6 release but not IL-8, GM-CSF, or LPS receptors. IL-8 release induced by Re-LPS, which requires CD14 to activate TLR4, was blocked by bosentan and BQ788. IL-8 release induced by Re-LPS, which does not require CD14 to activate TLR4, was insensitive to both bosentan and BQ788. In conclusion, PVSMCs contribute to inflammation in bacterial-exacerbated chronic lung diseases. Inhibition of the endothelin-B receptor suppresses cytokine release induced by long/smooth LPS attributable to sCD14 downregulation. ERAs, particularly when targeting the endothelin-B receptor, might have therapeutic utility in exacerbations of chronic lung diseases.

Introduction

Airway inflammation drives progression of chronic inflammatory lung diseases, such as chronic obstructive pulmonary disease (COPD) (Barnes 2008a; Stockley, 2009). Airway infections with Gram-negative bacteria induce exacerbations in COPD and other chronic lung diseases (Jackson et al., 2011; Decramer et al., 2012). This aggravates inflammation and inflammation-induced airway remodeling, leading to accelerated disease progression including the development of comorbidities (Barnes 2008a; Stockley, 2009; Decramer et al., 2012).

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ABBREVIATIONS: ANOVA, analysis of variance; BQ788, sodium (2R)-2-[[2S)-2-[[2R,6S)-2,6-dimethyl-1-piperidinyl]carbonyl]amino]-4,4-dimethylpentanoyl][1-(methoxycarbonyl)-o-tryptophyl]amino)hexanoate; CLI-095 (TAK-242), ethyl (6R)-6-[(2-chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate; COPD, chronic obstructive pulmonary disease; DMEM, Dulbecco’s modified Eagle’s medium; ELISA, enzyme-linked immunosorbent assay; ETa, endothelin A receptor; ETb, endothelin B receptor; ERAs, endothelin receptor antagonists; GM-CSF, granulocyte macrophage–colony-stimulating factor; IL, interleukin; LPS, lipopolysaccharide; mCD14, membrane-bound CD14; M-LPS, LPS with an undefined mixture of long and short LPS forms; PBS, phosphate-buffered saline; PBT, PBS + 0.1% Triton X-100; PH, pulmonary hypertension; PVSMCs, pulmonary vascular smooth muscle cells; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; Re-LPS, shortest form of rough LPS; RT, room temperature; sCD14, soluble CD14; S-LPS, smooth LPS; SMA, α-smooth muscle actin; SMC, smooth muscle cell; SMMHC, smooth muscle myosin heavy chain; TLR, Toll-like receptor; TNFα, tumor necrosis factor α; VSMCs, vascular smooth muscle cells.
COPD patients with pulmonary hypertension (PH) have more frequent exacerbations, and inflammation contributes to PH (Price et al., 2012; Wells et al., 2012). Therefore, inflammation might contribute to the pathogenesis of right heart failure in acute exacerbations of COPD. As therapies for exacerbations in chronic lung diseases are insufficient, alternative therapeutic strategies are required (Adcock and Barnes, 2008).

Pulmonary vascular smooth muscle cells (PVSMCs) are involved in airway remodeling in chronic inflammatory lung diseases and drive vascular remodeling in PH (Dupuis and Hoeper, 2008; Barbera and Blanco, 2009; Al-Muhsen et al., 2011). They contribute to inflammation during bacterial infection by releasing cytokines in response to lipopolysaccharide (LPS), which is the major inflammatory trigger synthesized by Gram-negative bacteria (Gatheral et al., 2012). Therefore, PVSMCs might contribute to the link between infection-induced inflammation and vascular remodeling associated with the development of PH as a comorbidity in chronic inflammatory lung diseases such as COPD. This renders PVSMCs interesting therapeutic targets in early disease stages regarding the reduction of inflammation-induced airway vascular remodeling in consequence of infection-caused exacerbations.

PVSMCs express both subtypes of endothelin receptors, ET₄R and ET₃R (Henno et al., 2009). Two types of ERAs are approved in therapy of PH: ET₁R-selective blockers, namely ambrisentan, and nonselective/dual blockers, such as bosentan. However, studies comparing the efficacy of ET₁R-selective versus dual blockers are missing. Endothelin-1 does not only trigger remodeling but also inflammation (Dupuis and Hoeper, 2008). ERAs have anti-inflammatory and antifibrotic potential in bronchial smooth muscle cells: they reduce TNFα-induced release of cytokines/chemokines and remodeling proteins (Knobloch et al., 2009, 2013). This was the first evidence for roles of ET₁R and ET₃R in inflammation-induced remodeling processes. From this, we hypothesized that the anti-inflammatory properties of ERAs could have a utility in early stages of chronic lung diseases with regard to attenuating inflammation-induced remodeling processes and the consequent development of comorbidities like PH.

Here, we aimed to support this hypothesis by means of clinical basic research. We focused on the question whether ERAs might suppress the inflammatory response of PVSMCs to bacterial infection, which is critical in disease exacerbations and promotes remodeling. Given the importance of LPS in infection-induced inflammation, LPS-stimulated PVSMCs are a suitable cell culture model for addressing this question. We further aimed to investigate the underlying mechanisms for putative anti-inflammatory effects of ERAs. This requires consideration of the specific characteristics of the response of PVSMCs to LPS and of the receptors involved.

LPS sensitivity is inconsistent among different human arterial vascular smooth muscle cell preparations (Yang et al., 2005): VSMCs from coronary arteries recognize LPS via the TLR4/CD14 receptor complex on their surface. However, PVSMCs do not express membrane-bound CD14 (mCD14), although CD14 mRNA is detectable. Nonetheless, LPS induces cytokine release in PVSMCs (Gatheral et al.; 2012), suggesting that soluble CD14 (sCD14) might compensate for the lack of mCD14, as it has been shown for other cell types including VSMCs of venous origin (Loppnow et al., 1995; Bäckhed et al., 2002).

Complete LPS (smooth LPS, S-LPS) consists of lipid A, a core oligosaccharide and an O-polysaccharide (King et al., 2009). Some bacteria that are relevant for exacerbations in chronic lung diseases synthesize truncated forms (rough LPS) with oligosaccharide structures in different degrees of completion. Re-LPS, the shortest form, lacks the O-polysaccharide and has the most incomplete core oligosaccharide (Huber et al., 2006). For example, Pseudomonas aeruginosa strains, which are frequently found in COPD patients, synthesize a broad spectrum from S-LPS to shortest rough LPS forms (Bantroch et al., 1994; King et al., 2009). This suggests that the whole LPS spectrum has clinical relevance. Whereas S-LPS requires mCD14 or sCD14 to activate TLR4, this CD14 dependence diminishes with increasing degrees of LPS truncation. Re-LPS activates TLR4 completely independent from CD14 (Huber et al., 2006).

We hypothesized that PVSMCs differentially respond to both S-LPS and Re-LPS with the release of IL-6, IL-8, and GM-CSF, all of which are important for inflammation-induced remodeling. We further hypothesized that selective and dual ERAs differentially suppress LPS-induced cytokine release from PVSMCs by interfering with TLR4 signaling and, therefore, could be considered candidate drugs for attenuating bacteria-induced inflammation in exacerbations of chronic lung diseases.

Materials and Methods

Human PVSMC Isolation and Cultivation. Human PVSMCs were dissected from main pulmonary artery explants from patients (4 women, 2 men) undergoing lung resection for carcinoma of the bronchus as described previously (Peng et al., 1996). For PVSMC isolation, healthy tumor-free tissue was used. In brief, the arteries were separated from their adventitia and endothelium and then cut into 1- to 3-mm² pieces with sterile scalpels. These tissue pieces were incubated for 30 minutes at 37°C and 5% CO₂ in Hanks’ balanced salt solution (H6648; Sigma-Aldrich, St. Louis, MO) containing 10 mg/ml bovine serum albumin (BSA; A0281; Sigma-Aldrich) and elastase (type I, 3.3 U/ml; E7885; Sigma-Aldrich). Tissue pieces were then incubated for a further 30–60 minutes in the enzyme solution outlined above with elastase content increased to 15 U/ml. To separate the dispersed cells from the enzyme solution they were centrifuged (10g, 5 minutes) at 4°C and then resuspended in Dulbecco’s modified Eagle’s medium (DMEM; 31885-023; Invitrogen), sodium pyruvate (1 mM; 11360-039; Invitrogen), L-glutamine (2 mM; G-7513; Sigma-Aldrich), nonessential amino acids (1%; A-5953; Sigma-Aldrich) and elastase (type I, 0.9 U/ml; E7885; Sigma-Aldrich). Tissue pieces were then incubated for a further 30–60 minutes in the enzyme solution outlined above with elastase content increased to 15 U/ml. To separate the dispersed cells from the enzyme solution they were centrifuged (10g, 5 minutes) at 4°C and then resuspended in Dulbecco’s modified Eagle’s medium (DMEM; 31885-023; Invitrogen), sodium pyruvate (1 mM; 11360-039; Invitrogen), L-glutamine (2 mM; G-7513; Sigma-Aldrich), nonessential amino acids (1%; 11140-035; Invitrogen), penicillin (100 U/ml), streptomycin (100 μg/ ml; P-4333; Sigma-Aldrich), and amphotericin B (1.5 μg/ml; A-2942; Sigma-Aldrich). The PVSMC cellular suspension was placed in a tissue culture flask (25 cm²; 156367; Nunc, Roskilde, Denmark) containing 10 mg/ml bovine serum albumin (BSA; A0281; Sigma-Aldrich), sodium pyruvate (1 mM; 11360-039; Invitrogen), L-glutamine (2 mM; G-7513; Sigma-Aldrich), nonessential amino acids (1%; 11140-035; Invitrogen), penicillin (100 U/ml), streptomycin (100 μg/ml; P-4333; Sigma-Aldrich), and amphotericin B (1.5 μg/ml; A-2942; Sigma-Aldrich). The PVSMC cellular suspension was placed in a tissue culture flask (25 cm²; 156367; Nunc, Roskilde, Denmark) containing 8 ml of supplemented DMEM and incubated in a humidified atmosphere at 37°C in 5% CO₂. The culture medium was replaced after 4–5 days and subsequently every 2–3 days. After 4–8 weeks the cells reached confluence. Subconfluent cells were passaged with trypsin (1% in Hanks’ balanced salt solution; T-4674; Sigma-Aldrich). This study was approved by the ethics committee of the University of Bochum (4257-12), Germany, and all patients gave their written consent.

PVSMC Characterization. PVSMCs were characterized by positive immunostaining for smooth muscle α-actin (SMA) and myosin heavy chain (MHC). Fluorescence-based immunostaining was done as described before (Knobloch et al., 2007). In brief, cells were seeded on coverslips in six-well plates (83.1839; Sarstedt, Bochum (4257-12), Germany, and all patients gave their written consent.

This study was approved by the ethics committee of the University of Bochum (4257-12), Germany, and all patients gave their written consent.
According to previous studies (Knobloch et al., 2009, 2013), PVSMCs detectable protein or DNA contaminants with agonistic TLR activity. LPS from *Escherichia coli* (EnzoLifeSciences GmbH, Lörrach, Germany) was done in serum-free and low glucose (1 g/l) DMEM supplemented with 100 U/ml penicillin, 100 

\[ \text{m} \] g/ml streptomycin, 1.5 \[ \text{m} \] g/ml; D9542; Sigma-Aldrich) was done for 15 minutes at RT in the dark. Cells were washed in Dako fluorescent mounting medium (S3023; Dako, Hamburg, Germany) and analyzed by fluorescence microscopy. Cells were (∼95% positive for SMA and MHC. Cell viability was assessed by counting cells after staining with trypan blue (15250; Invitrogen) in a Neubauer hemocytometer. Cell viability was found to be greater than 95%.

**PVSMC Stimulation.** Before stimulation, subconfluent cell monolayers (∼80% confluence) in six-well cell culture plates at passages 3 to 6 were deprived of serum for 24 hours in serum-free and low glucose (1 g/l) DMEM (41966-029; Invitrogen) supplemented with 1 mM sodium pyruvate, 2 mM l-glutamine, 1% nonessential amino acids, 100 U/ml penicillin, 100 \[ \mu \text{g} \]/ml streptomycin, 1.5 \[ \mu \text{g} \]/ml amphotericin B, 1 mM insulin (I882; Sigma-Aldrich), 5 \[ \mu \text{g} \]/ml apo-transferrin (T1147; Sigma-Aldrich), and 100 \[ \mu \text{g} \]/ml ascorbic acid (A4403; Sigma-Aldrich). Subsequent stimulation with different LPS forms and ERAs (as below) was done in serum-free and low glucose (1 g/l) DMEM supplemented with 1 mM sodium pyruvate, 2 mM l-glutamine, 1% nonessential amino acids, 100 U/ml penicillin, 100 \[ \mu \text{g} \]/ml streptomycin, and 1.5 \[ \mu \text{g} \]/ml amphotericin B for 4, 16 (for RNA analyses), or 72 hours (for cytokine measurements in cell culture supernatants).

**Mixed LPS (M-LPS) preparations** (containing undefined amounts of S-LPS and short-chain LPS variants) from *Salmonella enteritidis* (LT770; Sigma-Aldrich) contained <1% protein and 10–20% nucleic acids. Preparations of S-LPS from *Salmonella minnesota* (ALEXIS/EnzoLifeSciences GmbH, Lörrach, Germany) cat no. 581-020) and Re-LPS from *S. minnesota* R595 (581-008; ALEXIS) did not contain detectable protein or DNA contaminants with agonistic TLR activity. According to previous studies (Knobloch et al., 2009, 2013), PVSMCs were preincubated for 2 hours with ambrisentan ([2S]-2-[4-(6,6-dimethylpyrimidin-2-yl)-3-methoxy-3,3-diphenylpropionic acid; kindly provided by GSK, Munich, Germany), bosentan [4-tert-butyln-N-[2-(2-hydroxyethyl)-5-[2-methoxyphenyl)-2-(pyrimidin-2-yl)pyrimidin-4-yl]benzene-1-sulfonamide; Actelion Pharmaceuticals, Freiburg, Germany], or BQ788 [sodium (2R)-2-[[2S]-2-[[2S]-2-[[2R,6S]-2,6-dimethyl-1-piperidinyl][carboxyl]amino]-4,4-dipentanoyl][4-(1-methoxyacyrnyl)-d-thryptophyl][amino]-hexanoate; B157; Sigma-Aldrich] (each at 0.1–10 \[ \mu \text{M} \]) before stimulation with LPS. According to previous studies (Knobloch et al., 2011a), PVSMCs were preincubated with polymyxin B [N-[4-amino-1-[[4-amino-1-oxo-1-[[6,9,18-triazole-2-aminoethy]-15-benzyl-1(1-hydroxyethyl)-12-(2-methoxypropyl)-2,5,8,11,14,17,20-heptacosano-1,4,7,10,13,16,19-heptadecacarboxylic acid][2-aminobutane-2-ylamine]-3-hydroxy-1-oxobutan-2-ylamine]-1-oxobutan-2-yl-6-methylcarbamoylamine; 1-10 \[ \mu \text{M} \]; cat no. tlr-pml; Invivogen; with blocking antibodies for TLR2 (10 \[ \mu \text{g} \]/ml; cat no. pab-hstlr2; Invivogen) or CD14 (1-10 \[ \mu \text{g} \]/ml; clone bgl14; cat no. 021-3c-2; Biometeq, Greifswald, Germany), with an isotype control (MAB003; R&D Systems, Wiesbaden, Germany) or with the TLR9-specific inhibitory nucleotide ODN TTAGGG (5’ttt agg gtt agg gtt agg gtt g3; 2.5 \[ \mu \text{M} \]; Invivogen) 30–60 minutes before stimulation with LPS.

**RNA Isolation and Quantitative RT-PCR.** DNA-free total RNA was extracted from 80% confluent PVSMCs with the chromatography-based RNeasy technique (74106; QIAENJ GmbH, Hilden, Germany). Quantitative RT-PCR (qRT-PCR) was done as described previously (Knobloch et al., 2011a). In brief, after cDNA synthesis (205115; QIAGEN) with random primers (C1181; Promega, Mannheim, Germany), quantitative PCRs (PCR kit; 203205; QIAGEN) were done with gene-specific and intron-spanning primers. The housekeeping gene EF1a was used as a reference. PCR conditions were established for each primer pair in the exponential range, allowing quantification of signal intensities after ethidium bromide staining and standard agarose gel electrophoresis by densitometry using Alpha Innotech (San Leandro, CA) software, version 1.3.0.7. As a prerequisite for reliable quantification of signal intensities, Alpha Innotech software recognizes oversaturation (e.g., attributable to excess loading of the agarose gel) and nonlinear gamma or contrast correction of signals and disables densitometry tools in those cases. Values for TLR4 and CD14 under the different stimulation conditions were normalized to EF1a reference signals to correct for putative differences in RNA/DNA load. Primer sequences have been published previously (Knobloch et al., 2011a,b).

**ELISA.** IL-6, IL-8, GM-CSF, and sCD14 concentrations in cell culture supernatants were measured via ELISA (Duo Sets or Quantikine, R&D systems, catalog nos. DY206, DY208, DY215, DC140) according to standard protocols (Koch et al., 2004; Knobloch et al., 2011a, 2011b).

**Statistical Analysis of qRT-PCR and ELISA Data.** Drug effects on cytokine expression in LPS-stimulated PVSMCs were expressed as percent inhibition versus stimulation with LPS alone. According to previous studies (Knobloch et al., 2010, 2011b), Gaussian distributions of the data describing LPS and drug effects on cytokine expression in primary cells were expected and were confirmed for all data sets by Kolmogorov-Smirnov tests. Therefore, the data were expressed as mean ± S.E.M., and parametric tests (with 95% confidence intervals) were used for analyses. \( \alpha < 0.05 \) was considered as statistically significant. All calculations were done with GraphPad Prism.

The effects of LPS on cytokine release investigated in concentration-response experiments were analyzed by one-way repeated-measures ANOVA tests. If \( \alpha < 0.05 \) was reached, post hoc Bonferroni-Holm tests were used to analyze for differences between the data for individual LPS concentrations versus unstimulated controls.

The percent inhibition data for Polymyxin B, CLI-095, αTLR2AB, IgG control antibody, and ODN TTAGGG regarding IL-8 release in M-LPS-treated cells were analyzed for differences to a hypothetical value 0, which stands for no inhibition or stimulation with M-LPS in the absence of inhibitors, respectively, with one-sample \( t \) tests and Bonferroni correction for multiple testing.

Concentration response curves showing the reducing effects of ERAs on cytokine release in M-LPS-treated cells were calculated by nonlinear (sigmoidal) regression with variable parameters. The percent inhibition data for each ERA at 10 \[ \mu \text{M} \] were analyzed for differences for a hypothetical value 0, which stands for no reduction or stimulation with M-LPS in the absence of ERAs, respectively, with one-sample \( t \) tests and Bonferroni correction for multiple testing. If \( \alpha < 0.05 \) was reached for an ERA, the same analysis was done with the percent inhibition data for this ERA at 1 \[ \mu \text{M} \]. In addition, the percent inhibition data were analyzed for differences between the three ERAs at the same concentration. This was done by one-way repeated-measures ANOVA tests. If \( \alpha < 0.05 \) was reached, post hoc Bonferroni-Holm tests were performed.

The effects of ERAs on sCD14 release as well as on TLR4 and CD14 mRNA levels in the presence and absence of LPS were analyzed by one-way repeated-measures ANOVA. If \( \alpha < 0.05 \) was reached, post
The effects of different concentrations of the CD14 blocking antibody on cytokine release in S-LPS-exposed cells were analyzed by one-way repeated-measures ANOVA tests and, if χ < 0.05 was reached, post hoc Bonferroni-Holm tests were performed to analyze for drug effects versus unstimulated and LPS-stimulated cells.

The results of these experiments are presented in Table 1. M-LPS, S-LPS, and Re-LPS Induce Cytokine Release from PVSMCs. There is considerable similarity between LPS forms and spectra of Salmonella sp. and P. aeruginosa, which is frequently isolated in exacerbations of chronic lung diseases (King et al., 2009). As it is easily available, LPS from Salmonella has been established as a model LPS in experimental basic research regarding exacerbations of chronic inflammatory lung diseases (Knobloch et al., 2011a). Thus, we have used LPS from Salmonella sp. to experimentally investigate the responses of PVSMCs to different LPS forms and the effects of ERAs. M-LPS is an undefined mixture of smooth LPS and multiple short-chain LPS forms of S. enteritidis. S-LPS and Re-LPS are pure and defined preparations of smooth LPS or short-chain LPS, respectively, both from S. minnesota. All LPS forms induce a robust release of IL-8, IL-6, and GM-CSF from PVSMCs in a concentration-dependent manner (Fig. 1). There were no statistically significant differences between the inductive effects of S-LPS and Re-LPS on all cytokines analyzed (Fig. 1, data not shown), suggesting that PVSMCs almost equally respond to LPS forms of different length. In contrast to S-LPS and Re-LPS, which are highly purified, M-LPS preparations might contain small amounts of bacterial lipopeptides and nucleic acids, which are putative TLR2 or TLR9 agonists (see Materials and Methods). M-LPS-induced IL-8 release from PVSMCs was completely blocked by polymyxin B, which neutralizes LPS (Morrison and Jacobs, 1976), and by CL1-095 (TAK-242), a specific inhibitor for TLR4 that binds to its intracellular TIR domain (Kawamoto et al., 2008), but was not affected by a TLR2 blocking antibody and by ODN TTAGGG (Fig. 2), a TLR9-specific inhibitory nucleotide (Gursel et al., 2003). All blocking reagents did not modulate baseline IL-8 release (data not shown). These data confirmed that true M-LPS effects and demonstrate that LPS signals via TLR4 but not TLR2.

Inhibition of ETBR Reduces S-LPS-Induced IL-8 Release but Not Re-LPS-Induced IL-8 Release. In contrast to S-LPS, Re-LPS activates TLR4 independent from CD14 (Huber et al., 2006). M-LPS contains long and short LPS forms, suggesting that the cytokine response to M-LPS is partially but not completely dependent on CD14 (Knobloch et al., 2011a). Thus, if CD14 expression by ETBR block is a major mechanism explaining the reduction of LPS-induced cytokine expression by bosentan and BQ788, S-LPS-induced but not Re-LPS-induced cytokine expression should be sensitive to these ERAs. We investigated this hypothesis as exemplary for IL-8; indeed, bosentan and BQ788 but not ambrisentan suppressed S-LPS-induced IL-8 release, whereas all three ERAs did not significantly modulate Re-LPS-induced IL-8 release (Fig. 6). These data provide evidence that sCD14 suppression caused by ETBR blockade explains the reductive effects of bosentan and BQ788 on LPS-induced cytokine expression in PVSMCs.

Discussion

IL-6 and IL-8 become upregulated in the airways in response to bacterial infections in chronic lung diseases, particularly in COPD, and are believed to have key roles in infection-induced exacerbations (Wedzicha and Donaldson,
GM-CSF is involved in the pathogenesis of chronic inflammatory lung diseases and might link inflammation with remodeling processes (Barnes 2008a; Knobloch et al., 2009). Our data suggest that PVSMCs contribute to the enhancement of inflammation during exacerbations induced by bacteria, as they respond to LPS by releasing these cytokines. The PVSMC response to bacterial infections might contribute to the development of PH in chronic inflammatory lung diseases, which is associated with IL-6 excess in the lung (Mathew, 2010, Pullamsetti et al., 2011). Therefore, PVSMCs might represent suitable targets in alternative therapies for infection-induced exacerbations of chronic inflammatory lung diseases, for example, to reduce the risk of developing PH.

Although PVSMCs do not express mCD14 (Yang et al., 2005) and were stimulated in sCD14-free medium, they responded almost equally to S-LPS and Re-LPS. As CD14 mRNA is detectable in PVSMCs (Yang et al.; 2005; this study) we hypothesized that endogenously released sCD14 might functionally replace mCD14. This was confirmed by data showing that sCD14 is constitutively released by PVSMCs and that sCD14 neutralization abolishes S-LPS-induced cytokine release. There are two ways how cells can produce sCD14. First, they can produce sCD14 by shedding from mCD14, which is attached via a glycosylphosphatidylinositol-anchor to the outside of the plasma membrane (Bufler at al., 1995). This protease-dependent mechanism is primarily found on activated cells and probably does not apply to PVSMCs because they do not carry mCD14. Second, cells can produce sCD14 by protease-independent mechanisms—these are based on intracellular post-translational mechanisms, allowing CD14 to escape from the glycosylphosphatidylinositol-anchoring mechanism and to become directly secreted (Bufler at al., 1995). This mechanism is suggested to primarily trigger constitutive sCD14 release and, therefore, might apply to the constitutive and LPS-independent sCD14 release from PVSMCs.

ERAs have been approved for therapy in PH but are also being considered for chronic inflammatory lung diseases. As endothelin has potent inflammatory potential, ERAs are believed to have anti-inflammatory properties. Experimental research supported this hypothesis, suggesting that ERAs could block the link between inflammation and remodeling (Teder and Noble, 2000; Knobloch et al., 2009, 2013). Recent clinical trials, however, failed to provide evidence for a utility...
of ERAs in inflammation-associated chronic lung diseases, such as idiopathic pulmonary fibrosis, severe asthma, or severe COPD, when administered at late disease stages with extensive tissue remodeling (Stolz et al., 2008; King et al., 2011). However, we believe that ERAs would have to be administered at early disease stages, characterized by airway inflammation but without extensive fibrosis or emphysema, to use a putative interference of ERAs with inflammation-induced remodeling. Here, we investigated by the use of ex vivo cultivated primary PVSMCs, whether ERAs have potential to counteract the amplification of inflammation induced by bacterial infections during exacerbations of chronic lung diseases. We showed that ERAs reduce cytokine release from PVSMCs activated with the bacterial endotoxin LPS. We used ambrisentan and bosentan at concentrations of 0.1–10 μM in PVSMC culture. This resembles peak plasma concentrations of bosentan (~1–2 μM) in vivo when taken twice daily at dosages of 62.5 or 125 mg per tablet (Treiber et al., 2007).

The ETα-selective inhibitor ambrisentan only suppressed IL-6, whereas specific ETβR as well as dual blockade additionally suppressed IL-8 and GM-CSF. This indicates divergent roles for ETαR and ETβR in the upregulation of cytokines by LPS and might render ETαR antagonism interesting for therapy requiring selective IL-6 downregulation. IL-6 produced by airway cells is currently discussed as a major inflammatory mediator triggering the development of comorbidities in COPD (Barnes and Celli, 2009). Our cell culture data provide first indication that ETαR antagonism might be useful in this context. However, at high concentrations, ambrisentan non-significantly upregulated IL-8 and GM-CSF by trend. Translated to putative therapy, this indicates an increase in associated inflammatory processes at high ambrisentan dosages, which might promote chronic inflammation. This would have to be balanced with a possible benefit of IL-6 downregulation.

To therapeutically reduce chronic inflammation, e.g., in COPD, the development of approaches targeting a broad...
spectrum of cytokines might be more auspicious, because therapeutic neutralization of single mediators has been proven to be ineffective because of functional redundancy of inflammatory proteins (Barnes, 2008b). In this context, selective ETBR or a combined ETAR/ETBR blockade might have more putative therapeutic potential, because BQ788 as well as bosentan were both able to reduce not only IL-6 but also IL-8 and GM-CSF. IL-6, IL-8, and GM-CSF are believed to be important for the link between airway inflammation and remodeling (Knobloch et al., 2009; Mathew, 2010, Pullamsetti et al., 2011). Therefore, targeting the ETBR might not only have broader anti-inflammatory properties but also more

TABLE 1

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<th>EC_{50} \times 10^{-6}</th>
<th>logEC_{50} ± S.E.</th>
<th>E_{max} ± S.E.M.</th>
<th>% Reduction</th>
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<td>BQ788 4.30</td>
<td>-6.33 ± 0.59</td>
<td>55.25 ± 11.42</td>
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Fig. 4. Bosentan and BQ788 reduce sCD14 in PVSMCs. Cultured PVSMCs were preincubated for 2 hours with ambrisentan, bosentan, or BQ788 each at 1 μM before stimulation with M-LPS at 1 μg/ml. A-C, after 4 or 16 hours of incubation RNA was isolated and subjected to quantitative RT-PCR (see Materials and Methods) with specific primers for TLR4, CD14, and EF1α. A, representative set of RT-PCRs after agarose gel electrophoresis. B and C, densitometric analysis of PCR signals after gel electrophoresis. Values for TLR4 and CD14 were first normalized to the housekeeping gene and reference EF1α and, second, to the unstimulated control, which was set to 1. D, after 72 hours of incubation, sCD14 concentrations in supernatants were measured by ELISA. Data from n = 4 (B and C) or n = 5 (D) donors are presented as mean ± S.E.M. One-way repeated-measures ANOVA (B, P > 0.05; C, P < 0.001; D, P = 0.0042) with post hoc Bonferroni-Holm test: *P < 0.05; ***P < 0.001 versus unstimulated controls; +P < 0.05; ++P < 0.01; +++P < 0.001 versus stimulations with M-LPS alone.
potential to attenuate remodeling than selective ETAR blockade. Remodeling processes in part are mediated by PVSMCs, for example, when chronic inflammatory lung diseases become associated with PH (Barbera and Blanco, 2009; Al-Muhsen et al., 2011). ETBR-selective inhibitors have not yet been successfully developed for therapy, but our data indicate that dual blockers might have similar anti-inflammatory properties.

A direct molecular link between endothelin receptors and LPS/TLR4 signaling has not yet been reported. This raises the question of mechanisms, by which ERAs could interfere with LPS-induced cytokine release in PVSMCs. In bronchial smooth muscle cells, ERAs suppress TNFα-induced GM-CSF release by an indirect mechanism: ERAs suppress an endothelin-1 autoregulatory feedback mechanism, which is activated by TNFα and is crucial for maintenance but not for initiation of TNFα-induced GM-CSF transcription. Thus, ERAs reduce TNFα-induced long-time but not short-time GM-CSF expression (Knobloch et al., 2009). We expected a similar kind of indirect mechanism to be responsible for cytokine suppression by ERAs in LPS-exposed PVSMCs. Therefore, we performed long-time cell culture experiments with 72 hours of stimulation for the investigation of drug effects. We provided evidence that inhibition of ETBR activity leads to the downregulation of constitutive sCD14 secretion by PVSMCs. Cytokine expression induced by long LPS forms depend on sCD14 in PVSMCs. Therefore, sCD14 reduction caused by ETBR inactivation is a reasonable mechanistic explanation for the suppression of LPS-induced cytokine release by dual and ETBR-specific inhibitors. In support of this, we demonstrated clear effects of bosentan and BQ788 on IL-8 release induced by S-LPS, but did not find significant effects of these ERAs on IL-8 release induced by Re-LPS, which activates TLR4 independent from CD14 (Huber et al., 2006). Thus, a putative utility of ERAs in infection-induced exacerbations could depend on the bacteria involved; for example, ERAs could be useful for infections with P. aeruginosa, which synthesizes long LPS forms (Bantroch et al., 1994; King et al., 2009), rather than for infections with nontypeable Haemophilus influenzae, which exclusively produces short LPS forms (Bantoch et al., 1994; King et al., 2009), rather than for infections with nontypeable Haemophilus influenzae, which exclusively produces short LPS forms (Bantoch et al., 1994; King et al., 2009), rather than for infections with nontypeable Haemophilus influenzae, which exclusively produces short LPS forms (Bantoch et al., 1994; King et al., 2009), rather than for infections with nontypeable Haemophilus influenzae, which exclusively produces short LPS forms (Bantoch et al., 1994; King et al., 2009), rather than for infections with nontypeable Haemophilus influenzae, which exclusively produces short LPS forms (Bantoch et al., 1994; King et al., 2009), rather than for infections with nontypeable Haemophilus influenzae, which exclusively produces short LPS forms (Bantoch et al., 1994; King et al., 2009), rather than for infections with nontypeable Haemophilus influenzae, which exclusively produces short LPS forms (Bantoch et al., 1994; King et al., 2009), rather than for infections with nontypeable Haemophilus influenzae, which exclusively produces short LPS forms (Bantoch et al., 1994; King et al., 2009), rather than for infections with nontypeable Haemophilus influenzae, which exclusively produces short LPS forms (Bantoch et al., 1994; King et al., 2009), rather than for infections with nontypeable Haemophilus influenzae, which exclusively produces short LPS forms (Bantoch et al., 1994; King et al., 2009), rather than for infections with nontypeable Haemophilus influenzae, which exclusively produces short LPS forms (Bantoch et al., 1994; King et al., 2009), rather than for infections with nontypeable Haemophilus influenzae, which exclusively produces short LPS forms (Bantoch et al., 1994; King et al., 2009), rather than for infections with nontypeable Haemophilus influenzae, which exclusively produces short LPS forms (Bantoch et al., 1994; King et al., 2009), rather than for infections with nontypeable Haemophilus influenzae, which exclusively produces short LPS forms (Bantoch et al., 1994; King et al., 2009), rather than for infections with nontypeable Haemophilus influenzae, which exclusively produces short LPS forms (Bantoch et al., 1994; King et al., 2009).
required to facilitate an LPS-induced release of IL-6 at ~3 ng/ml in VSMCs of venous origin, which do not express CD14 mRNA, sCD14, and mCD14 but TLR4 (Loppnow et al., 1995). Therefore, the low sCD14 amounts produced by PVSMCs might be close to the minimum threshold concentration required for detectable responses to S-LPS. However, considering that bosentan and BQ788 only slightly reduce sCD14 by ~25–29%, just the fact that PVSMCs secrete low sCD14 amounts might be a prerequisite for the clear suppressive effects of bosentan and BQ788 on cytokine production. Otherwise, if sCD14 would be produced in excess, these low reductive effects of ERAs on sCD14 would likely not result in significant consequences on LPS-induced cytokine expression. Nonetheless, this suggests that PVSMCs might have increased cytokine responses to long LPS forms in vivo, where additional exogenous sources of sCD14 are available, such as activated monocytes and macrophages that enter the lung tissue during acute bacterial infections. Therefore, future studies might investigate ERA effects on sCD14 production by monocytes/macrophages to provide more arguments for a utility of ERAs in infection-induced exacerbations of chronic lung diseases.

Limitations of the study are as follows. PVSMC preparations might contain fibroblasts and myofibroblasts, which also express endothelin receptors and cytokines and respond to LPS (Zhang et al., 2011; Ahmedat et al., 2013). We used SMA as a marker to exclude the presence of fibroblasts in our cell culture. SMA is expressed in a filamentous pattern in smooth muscle cells (SMCs) and myofibroblasts but, if any, in a different, diffuse pattern in some fibroblast populations (Hinz et al., 2007; Singh and Hall, 2008). The discrimination between PVSMCs and myofibroblasts in culture is difficult because there is a lack of suitable markers. Smoothelin is not expressed in myofibroblasts, definitely identifies terminally differentiated SMCs in tissue, but gets immediately lost when SMCs start to proliferate in culture (van der Loop et al., 1996). Myofibroblasts express other canonical SMC markers such as calponin, h-caldesmon, and desmin, and SMCs can express canonical fibroblast markers like vimentin, Thy-1, and D7-FIB (Skalli et al., 1989; Gabbiani, 1992; Singh and Hall, 2008). Although not completely unequivocal, SMMHC might be the best marker currently available for discrimination. Myofibroblasts associated with normal wound healing do not express SMMHC (Benzonana et al., 1988; Eddy et al., 1988; Gabbiani, 1992) but a chronic pathologic environment might induce SMMHC expression in myofibroblasts (Chiavegato et al., 1995). As we isolate PVSMCs from healthy, tumor-free tissue, it is unlikely to have SMMHC-positive myofibroblasts in our preparations. Moreover, if myofibroblasts are SMMHC positive, the staining is rather weak, but we observed strong staining in our cell cultures, which is characteristic for SMCs (Singh and Hall, 2008). Finally, cultured SMCs might dedifferentiate and gain characteristics of myofibroblasts (Singh and Hall, 2008). We exclusively used early passages and did not observe significant changes in cytokine responses to our stimulants when comparing different passages of one donor (unpublished observation). We conclude that by phenotyping with SMA and SMMHC and by using early passages, we have done the best that is currently possible to exclude the presence of significant amounts of (myo)fibroblasts in our PVSMC cultures.

In summary, we showed that ET_{A}R blockade reduces LPS-induced cytokine production in PVSMCs attributable to sCD14 suppression. Therefore, we provide the first evidence by means of data obtained from primary cell culture experiments that specific ET_{A}R blockade or dual blockers might attenuate inflammation in bacterial infection-induced exacerbations of chronic lung diseases. However, before this idea might be considered for clinical trials, it requires further experimental support, e.g., by investigating the sensitivity of additional cytokines to ERAs in different cell culture and animal models of bacterial infection and chronic lung diseases.

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