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4-Aminopyridine Restores Impaired Hypoxic Pulmonary Vasoconstriction in Endotoxemic Mice

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Background: **Hypoxic pulmonary vasoconstriction (HPV) is impaired during inflammatory lung processes such as pneumonia or the acute respiratory distress syndrome. Voltage-gated potassium channels play a central role in mediating HPV. The aim of this study was to determine whether 4-aminopyridine (4-AP), a known voltage-gated potassium channel inhibitor, may restore HPV in sepsis.**

Methods: **The effects of 0.01, 0.1, and 1.0 mM 4-AP on HPV responsiveness were assessed in isolated lungs of untreated mice and of mice 18 h after lipopolysaccharide injection (20 mg/kg intraperitoneal *Escherichia coli* 0111:B4 lipopolysaccharide). HPV was quantified as the increase in perfusion pressure in response to hypoxic ventilation in percent of baseline perfusion pressure. Intrinsic pulmonary vascular resistance (R_0) and pulmonary vascular distensibility (α) were determined by nonlinear regression analysis of pulmonary vascular pressure–flow curves generated during normoxic and hypoxic ventilation, respectively.**

Results: **HPV was impaired in lungs isolated from lipopolysaccharide-challenged mice. Addition of 4-AP to the perfusate did not alter HPV responsiveness in untreated mice but dose dependently restored HPV in endotoxemic mice. Analysis of pulmonary vascular pressure–flow curves revealed that 4-AP (1) counteracted the observed lipopolysaccharide-induced changes in α and R_0 under normoxic conditions and (2) augmented the hypoxia-induced increase in R_0 in lungs of endotoxemic mice.**

Conclusions: **This study demonstrates that lipopolysaccharide-induced pulmonary vascular hyporesponsiveness to hypoxia can be restored by 4-AP in murine endotoxemia and, thus, may be a new therapeutic approach to treat patients with hypoxemia due to impaired HPV.**

IMPAIRED hypoxic pulmonary vasoconstriction (HPV) contributes to hypoxemia due to the mismatch of ventilation and perfusion in patients with pneumonia, sepsis, and acute respiratory distress syndrome,[1–3] as well as in animal models of endotoxemia.[2–9] Although a wide variety of inflammatory mediators induced during the course of endotoxemia have been shown to modulate pulmonary vascular tone,[8–14] there are no data available with regard to the mechanism of impaired HPV during endotoxemia.

There has been growing evidence for an important role of potassium channels in mediating vasoconstriction in response to hypoxia in the pulmonary circulation. After the first description of oxygen sensitivity of potassium channels in carotid body type I cells,[15] Post *et al.*[16] showed that the effect of hypoxia on pulmonary vascular tone could be mimicked by various potassium channel antagonists. Although the exact mechanism of oxygen sensing is still under debate,[17–20] the concept of voltage-gated potassium (K*v*) channels playing a critical role in the effector mechanism is now well established.[21–23] Under resting conditions, K*v* channels are in an activated state resulting in an efflux of potassium ions through the channels, which contributes to the negative resting membrane potential. Inhibition of K*v* channels, *i.e.*, by hypoxia or the K*v* channel inhibitor 4-amnopyridine (4-AP), causes membrane depolarization that in turn activates voltage-gated calcium channels resulting in vascular smooth muscle cell constriction.[17,21]

The main hypothesis of this study was that impaired HPV during sepsis is due to a decrease in K*v* channel sensitivity for hypoxia in the pulmonary vasculature. Here, we demonstrate that the K*v* channel inhibitor 4-AP dose-dependently augments HPV responsiveness in endotoxemic mice.

Materials and Methods

All animal experiments were conducted under protocols approved by the governmental animal care committee of Baden-Württemberg, Germany. Male C57BL/6 mice were obtained from RCC Ltd. (Füllinsdorf, Switzerland).

Isolated Perfused Mouse Lung

Male mice (body weight 20–35 g) received a lethal intraperitoneal injection of 200 mg/kg body weight pentobarbital sodium (Narcoren; Merial GmbH, Hallbergmoos, Germany) and were placed in a 37°C water-jacketed chamber (Isolated perfused lung size I; Hugo-Sachs-Elektronik, March-Hugstetten, Germany). After tracheostomy, volume-controlled ventilation (MiniVent 845; Hugo-Sachs-Elektronik) was initiated with a tidal volume of 10 ml/kg body weight, a positive end-expiratory pressure of 2 cm H_2O,

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and a respiratory rate of 90 breaths/min using an inspired gas mixture of 21% O_2, 5% CO_2, and 74% N_2 (Messer Griesheim GmbH, Ludwigshafen, Germany). After right parasternal thoracotomy, lungs were exposed, and a suture was placed around the ascending aorta and the main pulmonary artery. After injection of 10 U heparin into the right ventricle, a stainless steel cannula (1 mm ID) was inserted into the pulmonary artery *via* the right ventricle, and the suture was tightened. Another stainless steel cannula (1 mm ID) was advanced into the left atrium *via* the apex of the left ventricle across the mitral valve to drain pulmonary venous efflux. Left atrial pressure was maintained at 2 mm Hg. Lungs were perfused at a constant flow of 50 ml · kg body weight^{-1} · min^{-1} using a roller pump (Ismatec Laboratoriumstechnik GmbH, Wertheim-Monfeld, Germany) in a nonrecirculating system at 37°C. Perfusate flow was adjusted using a small vessel flow probe connected to a flowmeter (T106; Transonic Systems Inc., Ithaca, NY). The perfusate used was a Hanks' Balanced Salt Solution (Life Technologies Ltd., Paisley, Scotland) with 5% bovine serum albumin (Serva, Heidelberg, Germany) and 5% dextran (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) added to prevent pulmonary edema.[13,14] Indomethacin, 30 mM (Sigma-Aldrich Chemie GmbH), and 1 mM of the nonselective nitric oxide synthase inhibitor N^G-nitro-L-arginine methylester (Sigma-Aldrich Chemie GmbH) were added to the perfusate to inhibit endogenous prostaglandin and nitric oxide synthesis, respectively.[13,14] The partial pressure of oxygen of the perfusate was measured before and immediately after each experiment during normoxic ventilation and was found to range between 120 and 155 mm Hg. Pilot experiments revealed that variations in partial pressure of oxygen within this range do not affect subsequent pulmonary vasoconstrictor responses to hypoxia. Lungs were included in this study if perfusion pressure was stable and below 10 mm Hg during the first 5 min of an initial 10-min baseline perfusion period and if lungs had a homogenous white appearance with no signs of atelectasis, hemostasis, or edema. Because of these criteria, approximately 15% of lung preparations in each group were discarded before further measurements.

Pulmonary artery pressure (PAP) and left atrial pressure were measured using saline-filled membrane pressure transducers (Medex Medical GmbH & Co KG, Klein-Winterheim, Germany) connected to a side port of the inflow and outflow cannula, respectively. Pressure transducers as well as the flowmeter were connected to a transbridge amplifier (TBM4M; World Precision Instruments, Berlin, Germany). Data were recorded at 150 Hz per channel on a personal computer using an analog-to-digital interface and a data acquisition software system (DI-220/222; Dataq Instruments, Akron, OH). The system was calibrated before each experiment.

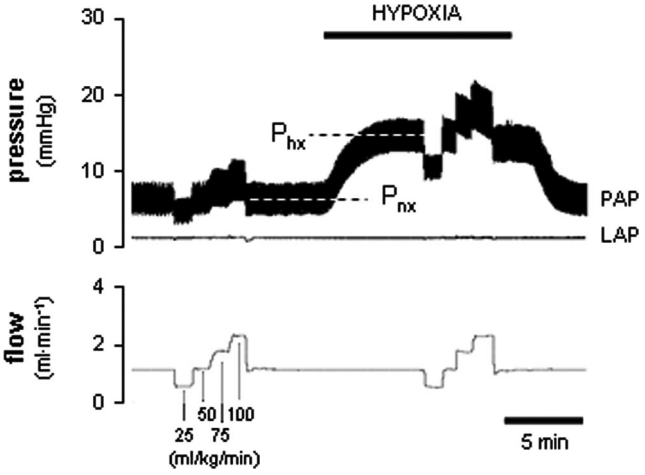

Fig. 1. Original recording of pressures in the main pulmonary artery (PAP; perfusion pressure) and in the left atrium (LAP) as well as perfusion flow during a typical experiment. After initial baseline perfusion period with a flow of 50 ml · kg^{-1} · min^{-1}, pressures at four different flows (25, 50, 75, and 100 ml · kg^{-1} · min^{-1}) were measured. After setting back the flow to 50 ml · kg^{-1} · min^{-1}, giving baseline perfusion pressure under normoxic ventilation (P_{nx}), ventilation was switched to hypoxia (1% oxygen), and perfusion pressure 6 min later (P_{hx}) was measured to calculate the vasoconstrictor response to hypoxia as the increase in perfusion pressure as percent of baseline perfusion pressure under normoxic conditions; thus: PAP = ($P_{hx} - P_{nx}$)/P_{nx} × 100. Then, a second pressure–flow curve was obtained during hypoxic ventilation before ventilation was switched back to normoxia.

Experimental Protocol for Quantification of HPV Responsiveness and Pulmonary Vascular Pressure–Flow Curves

An original recording of a typical experiment is shown in figure 1. After an initial 10-min baseline perfusion period, lungs were perfused with a flow of 25, 50, 75, and 100 ml · kg body weight^{-1} · min^{-1} in randomized order for 30 s each to generate a four-point pressure–flow (P-Q) curve under normoxic conditions. At each flow step, left atrial pressure was readjusted to 2 mm Hg, and PAP was measured at the end of each period. Then, flow was set to 50 ml · kg body weight^{-1} · min^{-1}, and, after another 3 min of baseline perfusion, the lungs were ventilated with a hypoxic gas mixture containing 1% O_2, 5% CO_2, and 94% N_2 (Messer Griesheim GmbH). Pilot experiments (n = 5) revealed that the maximal hypoxic pressure response was reached between 5 and 7 min followed by a slow decline in PAP (data not shown). Therefore, the hypoxic pulmonary vasoconstrictor response (ΔPAP) was defined as the increase in PAP 6 min after initiation of hypoxic ventilation in percent of baseline PAP. Then, a second P-Q curve was generated during hypoxia as described above. Finally, perfusate flow was reset to 50 ml · kg body weight^{-1} · min^{-1}, normoxic ventilation was reestablished, and PAP was allowed to return to baseline values. In all experiments, perfusion pressure at the end of the experiment did not differ from baseline pressure by more than 20%.

Analysis of Pulmonary Vascular P-Q Curves

The resulting four-point P-Q curves generated during normoxic and hypoxic ventilation for each experiment were analyzed (Statistica for Windows; StatSoft Inc., Tulsa, OK) based on the distensible vessel model of Linehan *et al.*[24] According to this model, the properties of the pulmonary circulation consist of a "static" component (R_0, intrinsic vascular resistance) and a "dynamic" component (α, vessel distensibility).[13] Linehan *et al.* used the following nonlinear regression analysis of the general form (see Linehan *et al.*,[24] equation 12)

$$PAP = \frac{[(1 + \alpha LAP)^5 + 5\alpha R_0 Q]^{15} - 1}{\alpha},$$

where R_0 describes the pulmonary vascular resistance that would exist if the vessels were at their respective diameter at zero vascular pressure, and α is the vascular "distensibility factor" describing the relation between vessel diameter and pressure (P) when the diameter (D_i) is normalized to the diameter at zero pressure (D_{0i}) given by (equation 5 in Linehan *et al.*[24])

$$\frac{D_i}{D_{0i}} = 1 + \alpha P.$$

LAP is the mean left atrial (outflow) pressure, which in our analysis was set to 2 mm Hg.

We decided to use this nonlinear regression model rather than the more often used linear regression analysis based on the collapsible vessel model (ohmic-Starling resistor model) of Permutt and Riley,[25] because several limitations in interpreting linear regression parameters (slope and intercept of the linear regression line) have been raised.[26,27] In a recent study,[13] we were able to demonstrate a differential modulation of the HPV response by nitric oxide synthase isoform-specific endogenous nitric oxide production only because we used the nonlinear regression analysis according to Linehan *et al.*[24]

Effects of Lipopolysaccharide and Kv Channel Inhibition on HPV

Mice were injected intraperitoneally with 20 mg/kg *Escherichia coli* 0111:B4 lipopolysaccharide (Difco Laboratories, Detroit, MI) dissolved in saline 18 h before lung perfusion experiments. Untreated mice served as controls.

To study the role of K*v* channels in lipopolysaccharide-induced impairment of HPV, lungs obtained from lipopolysaccharide-treated and untreated control mice were perfused with the K*v* channel specific inhibitor 4-AP (Sigma Aldrich GmbH) added to the perfusate at doses of 0.01, 0.1, and 1.0 mM, respectively.[28–31] Resulting data for ΔPAP and P-Q relations were compared with data obtained in untreated and lipopolysaccharide-treated mice in the absence of 4-AP (total of eight groups with n = 7 each).

Effects of Kv Channel Inhibition on Angiotensin II–induced Pulmonary Vasoconstriction after Lipopolysaccharide Treatment

We further investigated whether 4-AP specifically augments HPV or rather acts as a nonspecific modulator of pulmonary vascular responsiveness during endotoxemia. Therefore, we studied the pulmonary vasoconstrictor response to angiotensin II in lungs of untreated and lipopolysaccharide-treated mice with and without addition of 1 mM 4-AP to the perfusate (n = 7 per group). After 10 min of baseline perfusion, three doses of angiotensin II (0.01, 0.1, and 1 μg) were consecutively injected into the perfusion line proximal to the roller pump in a randomized order. After each injection, 10 min of perfusion was allowed for PAP to return to baseline. The vasoconstrictor response (ΔPAP) to angiotensin II was measured as the maximal increase of PAP in percent of baseline PAP.

Lung Kv Channel RNA Expression

To study potential mechanisms underlying 4-AP–induced effects on HPV in lipopolysaccharide-treated mice, we analyzed up-regulation of 4-AP and oxygen-sensitive K*v* channels, such as K*v*1.2, K*v*1.5, K*v*2.1, and K*v*3.1[28,32–34] in lipopolysaccharide-treated *versus* control mice by reverse-transcriptase polymerase chain reaction (PCR).

RNA was isolated from mouse lungs using the Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA), and complementary DNA was generated with MMLV reverse transcriptase (Promega, Madison, WI) and random primers (Promega, Madison, WI). Quantitative reverse-transcriptase PCR was performed with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using specific primers for K*v*1.2 (TGCGGTTCGAAACTCAGCTAA, GCGGTTGCGATCAAAAAAGTAC), K*v*1.5 (TCTTTTCCCCCGTTACCCTT, TGCGTCATGGAATGCGATA), K*v*2.1 (AGTTCCATGAAGACCCACAACC, GAGGATCGTGATACAAGCCCA), K*v*3.1 (CTTTGCCTCCCTCTTCTTCATC, CG-TTCTCGATTTCGGTCTTGTT), and SYBR® Green PCR Master Mix (Applied Biosystems). Postamplification dissociation curves were performed to verify the presence of a single amplification product in the absence of DNA contamination. 18S ribosomal RNA was detected with 18S VIC MGB primers (Applied Biosystems) and Taqman® Universal PCR Master Mix (Applied Biosystems). Changes in K*v* channel gene expression were determined using the Ct method with normalization 18S ribosomal RNA.

Lung Wet/Dry Weight Ratio

At the end of all experiments, lungs were excised without hilar structures and immediately weighed. Thereafter, lungs were dried in an oven at 100°C overnight and then reweighed. Lung wet-to-dry weight ratios were calculated as described previously.[14]

Statistical Analysis

Nonlinear regression analysis was performed for P-Q data obtained under normoxic and hypoxic conditions to yield α and R_0 values for each single experiment, respectively. All data are expressed as mean ± SD. To compare groups, a two-way analysis of variance followed by a *post hoc* least significant difference test for planned comparisons was performed (Statistica for Windows). Statistical significance was assumed at $P < 0.05$.

Results

Eighteen hours after lipopolysaccharide injection, mice showed lethargy, piloerection, and diarrhea. The mortality rate was approximately 10% at 18 h after lipopolysaccharide injection. Lung wet-to-dry weight ratios measured after completion of lung perfusion experiments did not differ between any of the studied groups (data not shown).

Pulmonary Vascular Response to Hypoxic Ventilation after Lipopolysaccharide Challenge

Baseline perfusion pressure under normoxic ventilation did not differ between lipopolysaccharide-pretreated and untreated mice (table 1). However, nonlinear regression analysis revealed that lipopolysaccharide treatment caused a significant increase in intrinsic vascular resistance (R_0) that was accompanied by an increase in vessel distensibility (α) (fig. 2).

As described previously,[13] ventilation of lungs of untreated mice with a hypoxic gas mixture increased PAP by 10.1 ± 3.2 mm Hg (ΔPAP 134 ± 37%; $P < 0.001$; fig. 3), which was associated with an increase in intrinsic resistance R_0 (*i.e.*, a decrease in total pulmonary vessel diameter at zero pressure), whereas vessel distensibility (α) did not change from baseline (fig. 2). In contrast, the hypoxia-induced ΔPAP was markedly reduced in lungs of lipopolysaccharide-pretreated mice (26 ± 27%; $P <$ 0.001 *vs.* untreated control; fig. 3). This lipopolysaccharide-induced reduction in the vasoconstrictor response to hypoxia was attributable to a completely abolished increase in R_0 (fig. 2A).

Table 1. Perfusion Pressure (in mm Hg) with and without 4-Aminopyridine Added to the Perfusate

	Control		LPS	
	Normoxia	Hypoxia	Normoxia	Hypoxia
w/o 4-AP	7.5 ± 0.8	17.6 ± 3.8	7.7 ± 0.7	9.8 ± 1.2†
0.01 mM 4-AP	7.0 ± 0.6	17.3 ± 2.7	7.3 ± 0.2	11.8 ± 1.7*†
0.1 mM 4-AP	7.4 ± 0.8	19.5 ± 3.7	7.3 ± 1.0	14.5 ± 5.4*†
1.0 mM 4-AP	7.4 ± 1.3	20.4 ± 4.3	8.3 ± 1.1	20.6 ± 6.0*

Data are presented as mean ± SD; n = 7 per group.

* $P < 0.05$ *vs.* w/o 4-AP. † $P < 0.05$ *vs.* control.

4-AP = groups in which lungs were perfused with 4-aminopyridine; control = data in lungs obtained from untreated control mice; hypoxia = values obtained during hypoxic (1% O_2) ventilation; LPS = data in lungs obtained from mice treated with 20 mg/kg *Escherichia coli* lipopolysaccharide 18 h before lung perfusion experiments; normoxia = values obtained during normoxic ventilation; w/o 4-AP = groups in which no 4-aminopyridine was added to the perfusate during lung perfusion.

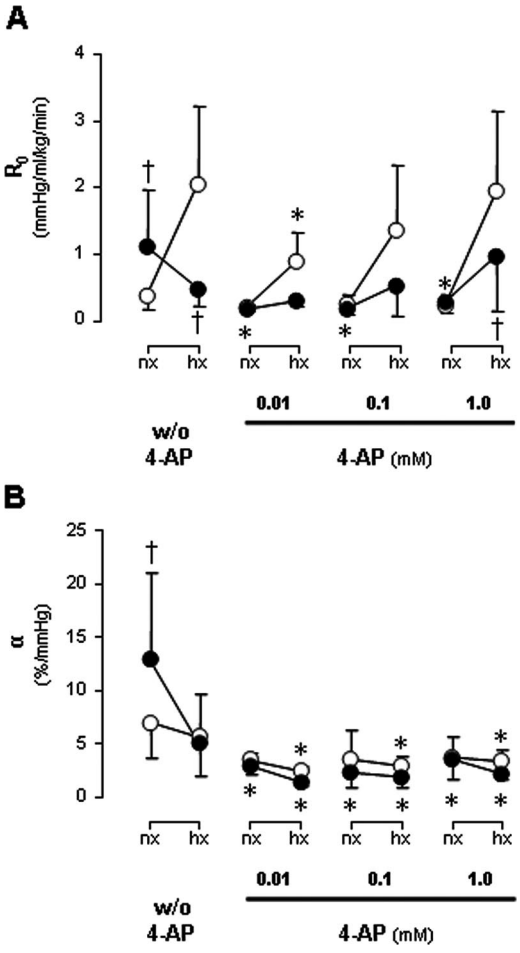

Fig. 2. Effects of lung perfusion with 0.01, 0.1, and 1.0 mM 4-aminopyridine (4-AP) on intrinsic vascular resistance (R_0; *A*), representing pulmonary vascular resistance that would exist if the vessels were at their respective diameter at zero vascular pressure, and on vessel distensibility (α; *B*) according to nonlinear regression analysis of the pulmonary vascular pressure–flow relation in lungs obtained from lipopolysaccharide-treated (●) and untreated control (○) mice (n = 7 per group). hx = hypoxic ventilation; nx = normoxic ventilation; w/o 4-AP = no 4-aminopyridine was added to the perfusate. * $P < 0.05$ *versus* w/o 4-AP. † $P < 0.05$ *versus* control. Data are presented as mean ± SD.

Effects of 4-AP on Pulmonary Vascular Tone and HPV after Lipopolysaccharide Challenge

In untreated control mice, inhibition of K*v* channels by 4-AP at doses of 0.01, 0.1, and 1.0 mM did not affect baseline PAP (table 1) or any of the parameters describing the pulmonary vascular P-Q relation (fig. 2) under normoxic conditions.

In contrast, in lipopolysaccharide-challenged mice, 4-AP restored the changes in pulmonary vascular P-Q relation observed during normoxic conditions: Intrinsic vascular resistance (R_0) was reduced by 4-AP to values

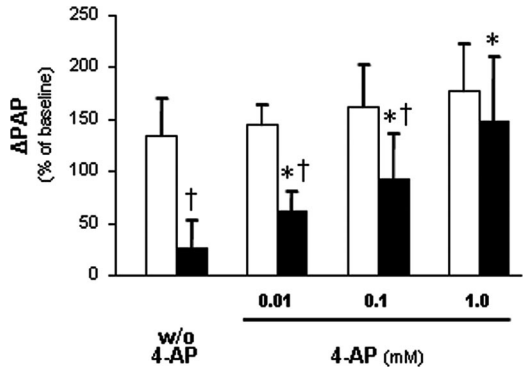

Fig. 3. Effects of lung perfusion with 0.01, 0.1, and 1.0 mM 4-aminopyridine (4-AP) on hypoxia induced vasoconstriction expressed as increase in percent of baseline perfusion pressure (ΔPAP) in lungs obtained from lipopolysaccharide-treated (■) and untreated control (□) mice (n = 7 per group). w/o 4-AP = no 4-aminopyridine was added to the perfusate. * $P < 0.05$ *versus* w/o 4-AP. † $P < 0.05$ *versus* control. Data are presented as mean ± SD.

comparable to those measured in untreated control mice (fig. 2A). Moreover, the observed lipopolysaccharide-induced increase in vessel distensibility (α) under normoxic resting conditions was abolished by perfusion with 4-AP (fig. 2B). These effects of 4-AP were only detectable by analyzing pulmonary vascular P-Q relations because baseline perfusion pressures (PAP) did not differ significantly between isolated lungs of lipopolysaccharide-treated animals perfused with or without 4-AP (table 1).

The magnitude of the pulmonary vascular response to 1% oxygen (ΔPAP) was not affected by 4-AP in untreated control mice (fig. 3). However, vessel distensibility (α) was lower during hypoxic ventilation in lungs of control animals perfused with 4-AP as compared with lungs without the K*v* channel inhibitor (fig. 2B).

4-Aminopyridine augmented the hypoxia-induced ΔPAP in lipopolysaccharide-challenged mice in a dose-dependent manner (fig. 3). At the highest concentration of 4-AP (1 mM), ΔPAP in response to hypoxia did not differ between lungs isolated from untreated and lipopolysaccharide-treated mice. This effect of 4-AP on HPV was associated with a dose-dependent restoration of the hypoxia-induced change in R_0 in endotoxemic mice (ΔR_0: -0.64 ± 0.81 mm Hg · ml^{-1} · kg^{-1} · min^{-1} without 4-AP *vs.* 0.11 ± 0.07, 0.36 ± 0.40, and 0.69 ± 0.83 mm Hg · ml^{-1} · kg^{-1} · min^{-1} with 0.01, 0.1, and 1.0 mM 4-AP, respectively; $P < 0.05$ for 0.1 and 1.0 mM 4-AP *vs.* without 4-AP). We found low doses of 4-AP (0.01 mM) to decrease vessel distensibility significantly in lipopolysaccharide-pretreated mice under normoxic conditions counteracting the observed lipopolysaccharide-induced distensibility increase, whereas there was no further decrease in distensibility by hypoxic ventilation in lungs perfused with higher concentrations of 4-AP (fig. 2B).

Pulmonary Vascular Response to Angiotensin II after Lipopolysaccharide Challenge with and without Kv Channel Inhibition

To evaluate whether the ability of 4-AP to restore the pulmonary vascular response in lipopolysaccharide-treated mice is limited to hypoxia, we measured the pulmonary vasoconstrictor response (ΔPAP) to boluses of 0.01, 0.1, and 1.0 μg angiotensin II with and without 1.0 mM 4-AP added to the perfusate in lungs of untreated and lipopolysaccharide-challenged mice. Angiotensin II caused a dose-dependent vasoconstriction that was significantly reduced by lipopolysaccharide pretreatment (fig. 4A). In contrast to HPV, perfusion of lungs with 4-AP augmented the vasoconstrictor response to angiotensin II in lungs of both lipopolysaccharide-treated and untreated animals, whereas the difference between lipopolysaccharide-treated and control mice in the vasoconstrictor response to 0.1 and 1.0 μg angiotensin II persisted (fig. 4B).

Pulmonary Kv Channel Expression after Lipopolysaccharide Treatment

Several K*v* channel subtypes, namely K*v*1.2, K*v*1.5, K*v*2.1, and K*v*3.1, have been shown to be oxygen sensitive and have been proposed to play a key role in mediating HPV. To test whether up-regulation of these K*v* channels during endotoxemia may account for (1) the reduced HPV response and (2) the above demonstrated augmentation of HPV responsiveness by 4-AP, we analyzed K*v* channel expression on the messenger RNA (mRNA) level. As shown in figure 5, expression of K*v*1.5, K*v*2.1, and K*v*3.1 mRNA did not differ between un-

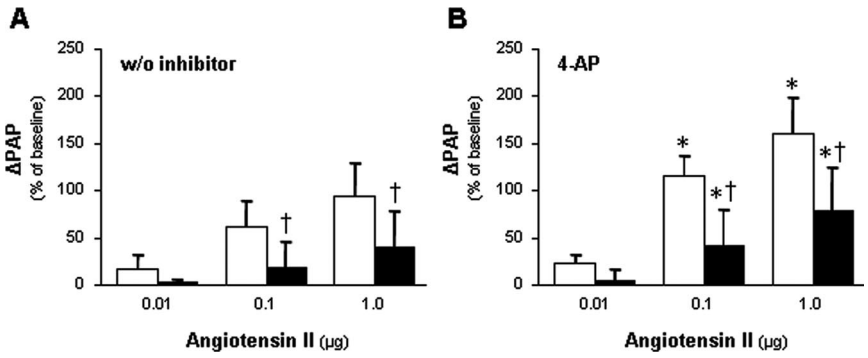

Fig. 4. Pulmonary vasoconstriction by bolus injections of 0.01, 0.1, and 1.0 μg angiotensin II in random order expressed as perfusion pressure increase in percent of baseline (ΔPAP) in lungs obtained from lipopolysaccharide-treated (■) and untreated control (□) mice with (*B*; 4-AP) and without (*A*; w/o 4-AP) 1.0 mM 4-aminopyridine (4-AP) added to the perfusate (n = 7 per group). * $P < 0.05$ *versus* w/o 4-AP. † $P < 0.05$ *versus* control. Data are presented as mean ± SD.

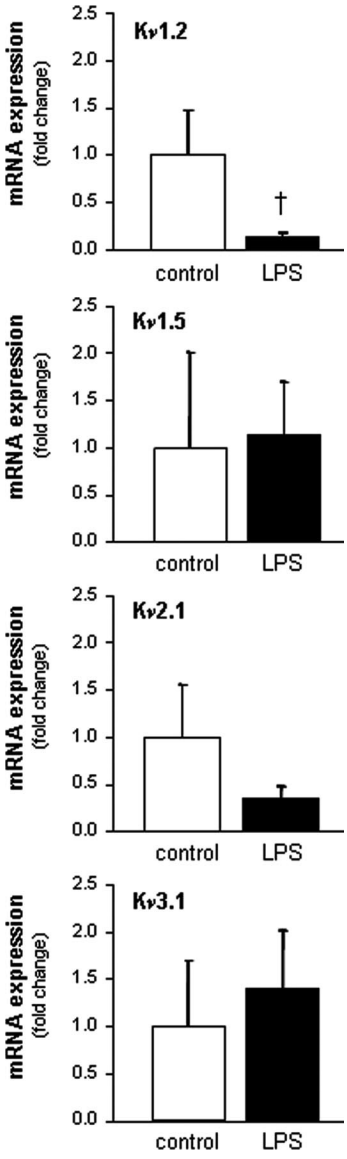

Fig. 5. Total lung messenger RNA (mRNA) expression of oxygen-sensitive K*v* channel subtypes K*v*1.2, K*v*1.5, K*v*2.1, and K*v*3.1 in lungs obtained from lipopolysaccharide (LPS)–treated (■) and untreated control (□) mice (n = 3 per group). † $P < 0.05$ *versus* control. Data are presented as mean ± SD.

treated and lipopolysaccharide-treated mouse groups. We only found a significant reduction in total lung mRNA expression of K*v* channel subtype K*v*1.2 in lungs from lipopolysaccharide-pretreated mice as compared with untreated controls.

Discussion

The main finding of this study is that impaired pulmonary vasoconstrictor responsiveness to hypoxia (HPV) induced by lipopolysaccharide-treatment in mice can be augmented, and ultimately fully restored, by the K*v* channel inhibitor 4-AP. Our results suggest that 4-AP (1) augments K*v* channel sensitivity to hypoxia in endotoxemic mice and (2) counteracts lipopolysaccharide-induced changes in pulmonary vascular wall properties.

Inhibition of K*v* channels by 4-AP has been shown to depolarize rat pulmonary artery smooth muscle cells and to constrict isolated pulmonary arteries.[35] Barman *et al.*[30] showed a small increase in perfusion pressure by 4-AP in isolated blood-perfused rat lungs, which was also evident as a trend in the current study, yet without reaching significance (table 1). Hasunuma *et al.* reported 4-AP to augment HPV in salt-perfused rat lungs.[36] In our study in isolated buffer-perfused lungs, 4-AP augmented HPV in lipopolysaccharide-treated mice (fig. 3). That 4-AP did not affect the magnitude of hypoxia-induced pressure response in untreated mice may be due to an already maximal vasoconstrictor stimulus by ventilating lungs with 1% oxygen. This notion is in accordance with the fact that maximal ΔPAP response to angiotensin II in the presence of 4-AP did not exceed the hypoxic pulmonary pressure response in untreated mice. It is further supported by a study by Littler *et al.*[37] showing that ventilating isolated mouse lungs with 0% oxygen increased perfusion pressure by approximately 6.5 mm Hg starting at a normoxic baseline of 8–12 mm Hg. Consistent with our results (fig. 3), these authors did not find 4-AP to augment the hypoxia-induced increase in perfusion pressure. However, evidence that there was indeed an effect of 4-AP on hypoxic vasoconstriction in control animals in the current study comes from pulmonary vascular P-Q relation analyses revealing significantly lower vessel distensibility (α) and a dose-dependent reduction in intrinsic vascular resistance (R_0) during hypoxic ventilation in lungs perfused with 4-AP as compared with perfusion without 4-AP (fig. 2B). Further analyses of the pulmonary vascular P-Q relation using the vessel distensibility model[24] revealed 4-AP to augment HPV in lipopolysaccharide-challenged mice in two ways. First, 4-AP perfusion restored the basal pulmonary vascular properties that were altered by lipopolysaccharide treatment: 4-AP normalized the lipopolysaccharide-induced increase in vessel distensibility α and the lipopolysaccharide-induced decrease in intrinsic vascular resistance R_0 under normoxic baseline conditions (fig. 2). Second, 4-AP in addition augmented the hypoxia-induced change in R_0 in a dose-dependent manner (fig. 2A). These findings cannot be directly attributed to the inhibitory effect of 4-AP on oxygen-sensitive K*v* channels, but rather suggest a sensitization of these channels to subsequent hypoxic stimulation. A potential mechanism underlying this unexpected finding may be that the effects of 4-AP under normoxic conditions in lipopolysaccharide-challenged mice are due to inhibition of non–oxygen-sensitive K*v* channels, which then "sensitizes" the pulmonary vasculature for a vasoconstrictor stimuli. This is further supported by our finding that these effects of 4-AP were not limited to the hypoxic vasoconstrictor response, but that 4-AP also augmented

pulmonary vasoconstriction in response to angiotensin II in both untreated and lipopolysaccharide-treated mice (fig. 4). It should be emphasized that the pulmonary vascular effects of 4-AP under normoxic conditions were only discovered because we studied pulmonary vascular P-Q curves by nonlinear regression analysis rather than only perfusion pressures.

Hypoxic pulmonary vasoconstriction is intrinsic to pulmonary vascular smooth muscle cells. Although the exact mechanism by which low alveolar oxygen tension is sensed by the pulmonary vasculature is still under debate,[17–20] it is well accepted that modulation of the opening probability of K*v* channels setting vascular smooth muscle membrane potential is central to the effector mechanism of pulmonary vasoconstriction in response to hypoxia.[21–23] Nine families of K*v* channels have been described, of which multiple subtypes were shown to be present in the pulmonary vasculature in animals[18,28,33,38] and humans.[39] Of these subtypes, K*v*1.2, K*v*1.5, K*v*2.1, K*v*3.1, and K*v*9.3 seem to possess oxygen sensitivity.[23,40] Convincing evidence that K*v*1.5 has a critical role in mediating HPV has come from studies of K*v*1.5-deficient mice[34] and studies in which K*v*1.5 overexpression restored HPV in chronically hypoxic rats.[41] Dysfunction or decreased expression of K*v*1.5 and K*v*2.1 in the pulmonary circulation is associated with impaired HPV in rats exposed to chronic hypoxia[42] or in rats with nitrogen-induced congenital diaphragmatic hernia (K*v*1.2 and K*v*2.1).[43] Because 4-AP, which is conventionally known to act as a K*v* channel inhibitor, restored HPV responsiveness after lipopolysaccharide it could be speculated that lipopolysaccharide treatment may increase the expression of pulmonary vascular K*v* channels, which will result in an increased threshold for hypoxia-induced smooth muscle membrane depolarization (HPV hyporesponsiveness). Inhibiting these K*v* channels with 4-AP at a dose that alone—as in our model (table 1 and fig. 2)—does not induce vasoconstriction may "sensitize" these channels to reach the threshold for membrane depolarization in response to a hypoxic challenge. Yet surprisingly, we did not detect an up-regulation of K*v* channel mRNA expression (fig. 5), especially with regard to subtypes K*v*1.5 and K*v*2.1, which have been previously suggested to be involved in disease-related HPV hyporesponsiveness.[34,42] Solely mRNA levels of the K*v*1.2 subtype were found to be significantly reduced (and a trend was seen for K*v*2.1) in lungs from lipopolysaccharide-challenged mice as compared with untreated controls (fig. 5). Although this finding may suggest a role of K*v*1.2 and/or K*v*2.1 in lipopolysaccharide-induced HPV hyporesponsiveness during sepsis, reduced channel expression cannot explain the restoration of impaired HPV by 4-AP on the basis of its inhibitory action on K*v* channels. The current data rather suggest that 4-AP restores the impaired HPV response during endotoxemia by "sensitizing" pulmonary vascular K*v* channels to hypoxia by a novel, yet unidentified mechanism.

In summary, we found that 4-AP dose-dependently restored impaired HPV in endotoxemic mice. Impairment of HPV can result in critical hypoxemia due to ventilation–perfusion mismatching in patients with pneumonia, sepsis, or acute respiratory distress syndrome. Restoration of intact HPV by "sensitization" of pulmonary vascular K*v* channels may provide a new therapeutic approach in these patients. 4-AP may present a promising drug in this strategy because it is—also known as fampridine—currently already on the US Food and Drug Administration's compounding for compassionate use list for treatment of certain neuropathies, spinal cord injury, and multiple sclerosis. The concept of therapeutic pulmonary vasoconstriction for the treatment of hypoxemia due to acute lung injury has been tested before using drugs such as phenylephrine,[44] norepinephrine,[45] or almitrine[46] or their combination with inhaled pulmonary vasodilators,[47–49] but has not yielded beneficial results so far. A potential reason for this lack in effectiveness of these drugs may be that they induce a generalized increase in pulmonary vascular tone but do not alter the pulmonary vascular sensitivity to subsequent vasoactive stimuli. Here, we demonstrate that 4-AP augmented not only HPV but also the pulmonary vascular response to angiotensin II in endotoxemic mice. Analysis of pulmonary vascular P-Q curves suggests that this may be due to both counteracting the lipopolysaccharide-induced changes in pulmonary vascular properties under normoxic conditions, *i.e.*, vascular distensibility and basal vascular resistance, and augmenting the vasoconstrictor response. Therefore, 4-AP may "normalize" basal pulmonary vascular tone and pulmonary vascular wall properties during endotoxemia thereby restoring the pulmonary vasculature's ability to respond to various endogenous or exogenous vasoactive agents.

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