INTRODUCTION

Sepsis-induced acute lung injury has been associated with significant morbidity and mortality (1, 2). An important feature of acute lung injury is the mismatch of ventilation and perfusion leading to intrapulmonary shunting and systemic hypoxemia. This is caused by an impairment of hypoxic pulmonary vasoconstriction (HPV), a mechanism that diverts regional blood flow away from poorly ventilated toward better ventilated lung regions to preserve oxygenation. Although the mechanisms of HPV have been incompletely understood, several lines of evidence have implicated nitric oxide (NO) to be involved in the development of sepsis-induced pulmonary and systemic vascular hyporesponsiveness (3) We have previously shown that acute pharmacological inhibition of endogenous NO production at 18 h after LPS injection restored the blunted HPV in part, whereas congenital NOS-deficiency completely protected mice from impairment of HPV after LPS injection (4). These data have suggested that NO may counteract HPV not only by its abundant production in the later course of endotoxemia as being a vasodilator, but may also affect the mechanism of HPV itself at an earlier stage of endotoxemia.

NO which is produced excessively in response to lipopolysaccharides (LPS) or cytokines by the inducible isoform of nitric oxide synthase (NOS2) (5) acts mainly by stimulating soluble guanylate cyclase (sGC) to produce guanosine cyclic 3',5'-monophosphate (cGMP) from guanosine triphosphate (GTP). The second messenger cGMP activates protein kinase which phosphorylates several intracellular targets resulting in smooth muscle relaxation (6). Many of the actions, including vasodilation and platelet actions, are mediated by this NO-cGMP pathway (7). In addition, there are cGMP-independent mechanisms by which NO may cause vasodilation. Peroxynitrite, for example, which can be produced from NO and superoxide, may induce vasodilation by activating membrane potassium channels (8, 9). Moreover, NO or peroxynitrite can directly activate cyclooxygenases and therefore promote the production of vasoactive prostaglandins (10).

The present study was therefore designed to determine whether the effects of NO on the development of HPV during endotoxemia are mediated via the NO-cGMP-pathway. We hypothesized that NO causes an impairment of HPV during endotoxemia mainly by activation of the NO-cGMP pathway that may, therefore, be counteracted by administration of 1H-

KEY WORDS: cyclic GMP, endotoxemia, guanylate cyclase, lung, mice, nitric oxide, vasoconstriction
(1,2,4)oxadiazole(4,3-α)quinoxaline-1-one (ODQ), a specific antagonist of sGC (11), early in the course of endotoxemia.

MATERIAL AND METHODS

All experiments were approved by the governmental animal care committee of Baden-Württemberg, Germany. Male C57BL/6 mice (body weight 20-35 g) were obtained from Charles River GmbH, Sulzfeld, Germany.

Inhibition of guanylate cyclase after LPS challenge on HPV

Eighteen hours before lung perfusion experiments, mice were injected intraperitoneonally with E. coli 0111:B4 lipopolysaccharide (Difco Lab., Detroit, USA) at a dose of 20 mg/kg. Untreated mice served as controls.

To study the effects of inhibition of soluble guanylate cyclase after LPS challenge on HPV, three hours after LPS challenge, mice were injected intraperitoneally with the selective guanylate cyclase inhibitor 1 H-(1,2,4)oxadiazole(4,3-α)quinoxaline-1-one (ODQ) solved in dimethyl sulfoxide (DMSO) 30%, at a dose of 2, 10, and 20 mg/kg, respectively. Previous studies have suggested that inhibition of soluble guanylate cyclase may have beneficial effects on vasopressor response (12) and survival (13) if administered in an early stage of endotoxemia.

Data for APAP and P/Q relationships in mice treated with ODQ and LPS were compared with data from untreated and LPS treated mice in the absence of ODQ (total of five groups with n = 7 each).

In addition, to study the effect of ODQ on lungs of mice not treated with LPS, one group (n=5) of animals received 20 mg/kg ODQ 15 hours before the experiments.

Isolated perfused mouse lung

The isolated perfused mouse lung model was used as described previously (4). Briefly, mice were euthanized by an intraperitoneal injection of pentobarbital sodium (200 mg/kg body weight) and 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, March-Hugstetten, Germany) was initiated using an inspired gas mixture of 21% O₂, 5% CO₂ and 74% N₂ (Messner Griesheim GmbH, Ludwigshafen, Germany). A respiratory rate of 90/min, a tidal volume of 10 ml/kg body weight and a positive end-expiratory pressure of 2 cmH₂O were used. Lungs were exposed via a midline thoracotomy, 10 IU heparin were injected into the right ventricle, and the pulmonary artery was cannulated. The left atrium was cannulated via the apex of the left ventricle in order to drain pulmonary venous effluent. Left atrial pressure was maintained at 2 mmHg. Lungs were perfused at a constant flow of 50 ml/kg body weight/min using a roller pump (Ismatec Laboratoriumstechnik GmbH, Wertheim-Monfeld, Germany) in a non-recirculating system at 37 °C. The perfusate contained Hanks' Balanced Salt Solution (Life Technologies Ltd., Paisley, Scotland) with 5% bovine serum albumin (Serva, Heidelberg, Germany) 5% and dextran (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) added to prevent pulmonary edema (4, 14). To inhibit endogenous prostaglandin and nitric oxide synthesis, 30 mM of indomethacin (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) and 1 μM of the non-selective nitric oxide synthase (NOS) inhibitor, N’-nitro-L-arginine methyl ester (L-NAME, Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) were added to the perfusate (4, 14). Perfusate flow was measured by a flowmeter (T106, Transonic Systems Inc., Ithaca, NY, USA). The partial pressure of oxygen of the perfusate was measured before and immediately after each experiment and was found to range between 120 and 155 mmHg. Lungs were included in this study if they had a homogenous white appearance with no signs of atelectasis, hemostasis or edema and perfusion pressure was stable and below 10 mmHg during the first five minutes of perfusion. Consequently, approximately 15% of lung preparations in each group were discarded prior to further measurements.

Pulmonary artery pressure (PAP) and left atrial pressure (LAP) were measured using saline-filled membrane pressure transducers (Medex Medical GmbH & Co KG, Klein-Winterheim, Germany). Data were recorded at 150 Hz per channel on a personal computer using a data acquisition software system (DI-220/222, Dataq Instruments, Akron, OH, USA).

Quantification of HPV responsiveness and pulmonary vascular pressure-flow curves

After equilibration of the system, lungs were perfused with a flow of 25, 50, 75, and 100 ml/kg body weight/min in randomized order for 30 seconds each to generate a pressure-flow (P-Q) curve under normoxic conditions (10). LAP was readjusted to 2 mmHg at each flow step, and PAP was measured at the end of each period. After return to baseline perfusion (50 ml/kg body weight/min for three minutes), lungs were ventilated with a hypoxic gas mixture containing 1% O₂, 5% CO₂ and 94% N₂ (Messner Griesheim GmbH, Ludwigshafen, Germany). The hypoxic pulmonary vasoconstrctor response (APAP) was defined as the increase in PAP 6 minutes after initiation of hypoxic ventilation in percent of baseline PAP as described previously (4). A second P-Q curve was generated during hypoxia as described above.

Analysis of pulmonary vascular P/Q curves

Pulmonary vascular pressure-flow (P-Q) relationships during normoxic and hypoxic ventilation were analyzed for each experiment (Statistica for Windows, StatSoft Inc., Tulsa, OK, USA) based on the non-linear regression model proposed by Linehan et al. (15) as described previously (4). Briefly, according to this model, the properties of the pulmonary vasculature are described by ‘static’ component (R₀, intrinsic vascular resistance) and a ‘dynamic’ component (α, vessel distensibility). R₀ 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’.

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In addition, to study the effect of ODQ on lungs of mice not treated with LPS, one group (n=5) of animals received 20 mg/kg ODQ 15 hours before the experiments.
Lung wet/dry weight ratio

At the end of all experiments, lungs excluding their hilar structures were excised and immediately weighed. Lungs were dried in an oven at 100 °C overnight, and then re-weighed. Lung wet-to-dry weight ratios were calculated as described previously (14).

Statistical analysis

All data are reported as means ± standard deviation (SD). To compare groups, a two-way ANOVA followed by an appropriate post hoc comparison test was used. When significant differences were detected by ANOVA, a post hoc least significance difference (LSD) test for planned comparisons was used (Statistica for Windows, StatSoft Inc., Tulsa, OK, USA). Statistical significance was assumed at \( p < 0.05 \).

RESULTS

Three hours after LPS injection, when ODQ or saline was injected, animals appeared clinically unchanged. Eighteen hours after LPS injection, both untreated mice and mice treated with ODQ showed lethargy, piloerection and diarrhea in equal measure. The mortality rate 18 hours after LPS injection was approximately 15% in all groups. 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 after LPS challenge

During normoxic ventilation LPS pretreatment did not change baseline perfusion pressures (LPS: 7.7±0.7 mmHg vs. control: 7.5±0.8 mmHg). In contrast, LPS pretreatment caused a significant increase in both intrinsic vascular resistance \( R_0 \) (1.40±0.66 vs. 0.37±0.19 mmHg·ml\(^{-1}\)·kg\(^{-1}\)·min\(^{-1}\), \( p < 0.05 \)) and vessel distensibility \( \alpha \) (0.13±0.06 vs. 0.07±0.03 mmHg\(^{-1}\), \( p < 0.05 \)) compared to untreated mice under normoxic conditions (Fig. 2 and 3).

Hypoxic ventilation significantly increased PAP (\( \Delta \)PAP 133.7±37.3 %) and \( R_0 \) (2.32±0.84 mmHg·ml\(^{-1}\)·kg\(^{-1}\)·min\(^{-1}\) vs. 0.37±0.19 mmHg·ml\(^{-1}\)·kg\(^{-1}\)·min\(^{-1}\), \( p < 0.05 \)), in untreated mice, while \( \alpha \) did not change significantly compared to normoxic ventilation (0.06±0.04 vs. 0.07±0.03 mmHg\(^{-1}\)). In LPS challenged mice, however, the hypoxia-induced increase in PAP was
significantly reduced (ΔPAP 26.4±27.1 %) as compared to untreated mice (ΔPAP 133.7±37.3 %, p < 0.05). LPS treatment abolished the increase in R0 during hypoxic ventilation (0.47±0.26 vs. 2.32±0.84 mmHg·ml⁻¹·kg⁻¹·min⁻¹), while did not change significantly during hypoxia compared to untreated mice (0.05±0.03 vs. 0.06±0.04 mmHg⁻¹).

Effects of ODQ on pulmonary vascular response

Inhibition of cGMP by 20 mg/kg ODQ did not change baseline PAP (6.9±0.4 vs. 7.5±0.8 mmHg) in untreated mice. In LPS treated animals, ODQ at a doses of 2, 10, and 20 mg/kg did not affect baseline PAP (Fig. 1). Pretreatment with 20 mg/kg ODQ attenuated the increase in PAP during hypoxia in untreated mice (ΔPAP 98.1±20.9 %) compared to animals not pretreated with ODQ (133.7±37.3 %, p < 0.05).

Inhibition of cGMP by 20 mg/kg ODQ caused no significant change in R0 or α during normoxia or hypoxia in untreated controls. In LPS-challenged mice, however, ODQ at a dose of 2, 10, and 20 mg/kg abolished the LPS-induced increase in R0 and α during normoxic ventilation (Fig. 2 and 3).

ODQ treatment at a dose of 20 mg/kg, but not at a dose of 2 or 10 mg/kg three hours following LPS injection augmented ΔPAP (Table 1). PAP during hypoxic ventilation in LPS challenged mice treated with 20 mg/kg ODQ was significantly higher than in mice not treated with ODQ (79.9±13.9 vs. 26.4±27.1 %, p < 0.05, Fig. 1) and did not differ from controls treated with 20 mg/kg ODQ (79.9±13.9 vs. 98.1±20.9 %). During hypoxic ventilation in LPS-challenged mice, ODQ at a dose of 2, 10, and 20 mg/kg caused no significant change in R0 and compared to endotoxemic mice not treated with ODQ (Fig. 2 and 3).

Table 1. Changes in pulmonary vasoconstrictor response (ΔPAP), static resistance (ΔR0), and vessel distensibility (Δα) during hypoxic ventilation compared to normoxia. LPS = lipopolysaccharide, ODQ = 1-H-(1,2,4)oxadiazole(4,3-α)quinoxaline-1-one. *p < 0.05 versus 0 mg/kg ODQ, †p < 0.05 versus control.

<table>
<thead>
<tr>
<th>ODQ (mg/kg)</th>
<th>Control</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mg/kg ODQ</td>
<td>133.7 ± 37.3</td>
<td>26.4 ± 27.1</td>
</tr>
<tr>
<td>20 mg/kg ODQ</td>
<td>98.1 ± 20.9*</td>
<td>79.9 ± 13.9*</td>
</tr>
<tr>
<td>0 mg/kg ODQ</td>
<td>6.9 ± 5.2</td>
<td>-0.6 ± 0.2</td>
</tr>
<tr>
<td>20 mg/kg ODQ</td>
<td>6.8 ± 1.7</td>
<td>0.6 ± 0.5</td>
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DISCUSSION

The present study demonstrates that selective inhibition of guanylate cyclase restores the blunted HPV responsiveness in endotoxemic mice. In addition, our results suggest that inhibition of guanylate cyclase counteracts lipopolysaccharide-induced alterations of pulmonary vascular properties under normoxic conditions.

We used an isolated, perfused lung model to study the effect of ODQ on pulmonary vasoreactivity in response to alveolar hypoxia in mice challenged with a single dose of intraperitoneal LPS. This model is highly reproducible in yielding systemic inflammation and has been shown to be sensitive for proving impaired reactivity of the pulmonary vasculature in response to vasoactive drugs (14) and hypoxic stimuli (4, 16). In addition, in contrast to in vivo models, the isolated, perfused lung model allows to generate and study P/Q relationships that may provide more detailed information regarding the properties of the pulmonary vasculature that cannot be obtained by studying pulmonary artery pressures at a single given flow (11).

To prevent cGMP-independent mechanisms possibly interfering with HPV, we added indomethacin to the perfusate. The role of cyclooxygenase inhibitors in modulating HPV, however, is not clear without ambiguity. In a murine model, indomethacin treatment did not change HPV compared to
untreated animals (17). In contrast, in a canine model of oleic acid-induced acute lung injury, indomethacin increased HPV (18), whereas in sheep isolated pulmonary veins cyclooxygenase inhibition with indomethacin reduced HPV (19).

To study the role of the endogenous NO-cGMP pathway 18 hours after LPS treatment, we perfused lungs with L-NAME. L-NAME is an inhibitor of non-selective NO synthesis and counteracts excessive NO production causing systemic hypotension during septic shock (20). Previously, we have shown that congenital deficiency of NOS2, but not treatment of wild type mice with L-NAME restored HPV during endotoxemia suggesting that mechanisms other than simply pulmonary vasodilatation by NOS2-derived NO overproduction be responsible for impaired HPV during endotoxemia (4). We injected ODQ as a single dose 3 h after LPS pretreatment, because NO synthase 2 (NOS2) activity has been shown to vary over the course of endotoxemia with induction of mRNA in rat lungs being present as early as 15 minutes after LPS injection (21) and showing a maximal expression in murine lungs 6 h after LPS (22). In addition, inhibition of sGC by ODQ 4 h after LPS injection significantly improved survival at 24-144 h in mice (13).

Specific inhibition of soluble guanylate cyclase by ODQ had no effect on baseline perfusion pressure in control or LPS-pretreated mice in our study. This is consistent with the findings of other studies that showed no change in baseline perfusion pressure by nonspecific NOS inhibition by N\text{\textsuperscript{o}}-nitro-L-arginine methyl ester (L-NAME) or in mice with congenital deficiency of NOS2 (4, 23-25). In addition, as L-NAME ODQ had no effect on intrinsic vascular resistance $R_0$ and vessel distensibility $\alpha$ under normoxic conditions in controls.

We did not find an augmentation of HPV by ODQ in untreated animals. In our study, untreated mice showed a moderate attenuation in HPV after treatment with ODQ without significant alterations of intrinsic vascular resistance $R_0$ and vessel distensibility $\alpha$. Although ODQ has been shown to potentiate HPV in the perfused rabbit (26) and salt-perfused rat lung (27, 28), this effect was lost under conditions of preblocked lung NO synthesis by L-NAME (26). In our experiments, the effect of L-NAME which we used to prevent acute vasodilatory effects of NO during lung perfusion in both ODQ-treated and ODQ-untreated mice, may have outweighed a potential augmentation of HPV by ODQ. Moreover, in contrast to the abovementioned studies, we administered a single dose of ODQ early in the course of endotoxemia, so that at the time of lung preparation for perfusion 15 hours later tissue levels may not have been sufficient to block guanylate cyclase activation by endogenous NO. Our data are further supported by a study of Fernandes et al. showing beneficial effect of ODQ on restoration of vascular responsiveness to be most pronounced at early stages of endotoxemia (12, 29).

This study shows prevention of the loss of HPV during endotoxemia by inhibition of guanylate cyclase. Consistently with previous studies (4, 30), we found that LPS-pretreatment in mice caused a significant attenuation of HPV that was accompanied by an increase in $R_0$ and $\alpha$ under normoxia. ODQ at a dose of 20 mg/kg, but not at a dose of 2 mg/kg or 10 mg/kg, restored HPV after LPS challenge. In addition, even low doses of ODQ (2 mg/kg) abolished the increase in $R_0$ and $\alpha$ in LPS-pretreated mice under normoxia, whereas ODQ at any dose had no effect on $R_0$ and $\alpha$ under hypoxia in LPS-challenged mice. These results suggest that ODQ primarily restores basal pulmonary vascular properties under normoxic conditions on the basis of which hypoxic ventilation elicits vasoconstriction in endotoxemic mice. These effects of ODQ were only detectable by analyzing pulmonary P-Q relations since LPS treatment did not change baseline perfusion pressure significantly. In line with our results, Zacharowski et al. showed in a rat model that ODQ at a low dose (2 mg/kg) was sufficient to reduce lung injury as assessed by light microscopy of lung tissue whereas it had no positive effect on the decrease in blood pressure caused by LPS (31). These and our data suggest that there may be an additional mechanism that leads to the restoration of HPV at higher doses of ODQ which we were not able to detect by analyzing P-Q relations.

In contrast to our results, administration of ODQ which was added to the recirculating perfusate did not restore the blunted HPV in isolated lungs from rats with cirrhosis (32). Based on our previous observations that suggested a role of NO in the development of attenuation of HPV (4) and a potential role of sGC inhibitors at an early stage of endotoxemia (12), however, we considered that an effect of ODQ on HPV was most likely to occur if ODQ was administered at a time point during endotoxemia when LPS-induced iNOS-activity would reach its peak, i.e. at 3 hours after the LPS-challenge.

It is unclear if early administration of ODQ can also limit the morphological lung injury after LPS challenge. Histological microscopic changes in lung tissue may not reflect the functional integrity of the pulmonary vasculature. Whereas the histological degree of lung injury was reduced six hours after LPS challenge in rats that had received ODQ two hours before LPS (31), inhalation of ODQ before inhalation of LPS caused a further increase in total cell number and protein concentration in the bronchoalveolar fluid of 12 and 24 hours after LPS treatment in mice (33). These studies, however, did not assess parameters of lung function. Lung dry/wet ratios in our experiments did not indicate a difference in lung edema between mice treated with ODQ compared to untreated mice. Further studies are needed to elucidate the effect of ODQ on lung integrity after endotoxemic lung injury.

Inhibition of sGC may be an effective way to block the effect of excessive NO production that is suggested to contribute to the impairment of pulmonary vascular responsiveness in patients with sepsis. Compared to specific inhibitors of inducible NOS, an inhibitor of sGC may be superior in terms of potential immunosuppressive effects, as NO-related antimicrobial effects are independent of the cGMP pathway (27). Methylene blue, an unspecific inhibitor of guanylate cyclase, has been used in clinical studies that showed a neutral (34) or even adverse effect (35) on arterial oxygenation. Since methylene blue, however, lacks specificity (36), may have only short-lasting effects (37) and has the capacity to generate free radicals (38), the use of a specific inhibitor of sGC in a clinical setting would help to determine if the positive effect of ODQ on HPV shown in the present study translate into a clinically relevant benefit.

In summary, the present study shows that inhibition of guanylate cyclase by ODQ restores impaired HPV in endotoxemic mice. Our data suggest that ODQ facilitates pulmonary vasoconstriction during hypoxia mainly by restoring pulmonary vascular properties during normoxia.

Acknowledgements: This study was supported by grant WE 2114/4-2 by the Deutsche Forschungsgemeinschaft to JW. We cordially thank Professor Martha M. Gebhard for her support during our studies in her facilities.

Conflict of interests: None declared.

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Received: July 2, 2008
Accepted: April 30, 2009

Author’s address: Jörg Weimann, M.D., D.E.A.A., Professor of Anaesthesia, Department of Anaesthesia and Intensive Care Medicine, Sankt Gertrauden Hospital, 10713 Berlin, Germany; Phone: +49-(0)30-8272-2242; Fax: +49-(0)30-8272-2112; e-mail: Joerg.Weimann@sankt-gertrauden.de