Soluble guanylyl cyclase expression is reduced in LPS-induced lung injury

Constantinos Glynos,1 Anastasia Kotanidou,1 Stylianos E. Orfanos,1,2 Zongmin Zhou,1 Davina C. M. Simoes,1 Christina Magkou,1 Charis Roussos,1 and Andreas Papapetropoulos1,3

1George P. Livanos and Marianthi Simou Laboratories, Evangelismos Hospital, 1st Department of Pulmonary and Critical Care, University of Athens, Athens, Greece; 2nd Department of Critical Care, Attikon University Hospital, University of Athens Medical School, Haidari (Athens); and 3Laboratory of Molecular Pharmacology, Department of Pharmacy, University of Patras, Patras, Greece

Submitted 22 May 2006; accepted in final form 28 December 2006

Glynos C, Kotanidou A, Orfanos SE, Zhou Z, Simoes DC, Magkou C, Roussos C, Papapetropoulos A. Soluble guanylyl cyclase expression is reduced in LPS-induced lung injury. Am J Physiol Regul Integr Comp Physiol 292: R1448–R1455, 2007. First published January 4, 2007; doi:10.1152/ajpregu.00341.2006.—Soluble guanylyl cyclase (sGC) is a cGMP-generating enzyme implicated in the control of smooth muscle tone that also regulates platelet aggregation. Moreover, sGC activation has been shown to reduce leukocyte adherence to the endothelium. Herein, we investigated the expression of sGC in a murine model of LPS-induced lung injury and evaluated the effects of sGC inhibition in the context of acute lung injury (ALI). Lung tissue sGC α1 and β1 subunit protein levels were determined by Western blot and immunohistochemistry, and steady-state mRNA levels for the β1 subunit were assessed by real-time PCR. LPS inhalation resulted in a decrease in β1 mRNA levels, as well as a reduction in both sGC subunit protein levels. Decreased α1 and β1 expression was observed in bronchial smooth muscle and endothelial cells. TNF-α was required for the LPS-triggered reduction in sGC protein levels, as no change in α1 and β1 levels was observed in TNF-α knockout mice. To determine the effects of sGC blockade in LPS-induced lung injury, mice were exposed to 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) prior to the LPS challenge. Such pretreatment led to a further increase in total cell number (mainly due to an increase in neutrophils) and protein concentration in the bronchoalveolar lavage fluid; the effects of ODQ were reversed by a cell-permeable cGMP analog. We conclude that sGC expression is reduced in LPS-induced lung injury, while inhibition of the enzyme with ODQ worsens lung inflammation, suggesting that sGC exerts a protective role in ALI.

endotoxin; guanosine 3′,5′-cyclic monophosphate; acute lung injury

LUNG INJURY IS A BROAD TERM that applies to conditions ranging from mild interstitial edema without cellular injury to massive and fatal destruction of the lung characterized by acute respiratory distress and severe oxygenation impairment. Acute lung injury (ALI) represents a pathological continuum that occurs as a consequence of the host’s exposure to a direct pulmonary insult (for example, gastric acid aspiration) or an indirect pulmonary insult (for example, extrapulmonary sepsis) (31, 37). Sepsis-induced acute lung injury remains an important clinical problem with significant morbidity, mortality, and use of health care resources (2, 31, 37). Irrespective of the initiating cause, the pathophysiology of ALI is characterized by endothelial and leukocyte activation, as well as upregulation of inflammatory agents. One of the mediators that increases severalfold in animal models of lung injury, as well as in humans with ALI, is nitric oxide (NO) (16). The majority of the published data favors the view that inducible NO synthase (iNOS)-derived NO contributes significantly to many of the ALI features, including protein-rich pulmonary edema, oxidant stress, and surfactant dysfunction [(16) and references therein]; however, protective roles for NO have also been observed in animal models of acute lung injury (13, 36, 39).

NO exerts its effects in the body through cGMP-dependent and independent pathways; the former are mediated by soluble guanylyl cyclase (sGC) activation and are generally considered to contribute to the beneficial properties of NO (15), while the latter are mostly associated with the deleterious effects of this labile messenger molecule and are mediated through protein tyrosine nitration, S-nitrosylation, interactions with transition metals, free radicals and lipids (5). Soluble guanylyl cyclase (sGC) is a heterodimeric cGMP-forming enzyme composed of two subunits that serve as a receptor for NO (8, 29). Although two variants for each subunit (α1, α2, and β1, β2) have been identified so far, the available experimental evidence points toward the existence of only two sGC isoforms, α1/β1 and α2/β1/8. In most tissues, including the lung, the α1/β1 sGC heterodimer is the predominant isoform (17). sGC expression and activity have been shown to be regulated by both transcriptional, posttranscriptional, and posttranslational mechanisms (29). NO, cyclic nucleotides, and cytokines, all of which are upregulated in ALI, are among the agents that reduce sGC state-state mRNA levels (7, 23, 24, 34). On the other hand, sGC subunit mRNA half-life is increased through the interaction with the RNA-binding protein HuR (10). Once, synthesized, the sGC protein is regulated through protein-protein interactions with members of the heat-shock family of proteins (hsp90 and hsp70), chaperonin-containing t-complex polypeptide, Arf-GAP with GFP-binding protein-like, ankyrin repeat and pleckstrin homology domains 1, and postsynaptic density 95 (3, 9, 19, 26, 32); these interactions affect both the activity and the localization of sGC.

sGC has been mostly studied in the context of smooth muscle relaxation, regulation of platelet aggregation, and non-adrenergic noncholinergic neurotransmission (15, 21). However, evidence for the involvement of sGC in inflammation-related processes also exists (1, 40). The overall purpose of our study was to determine whether sGC subunit expression is altered in a murine model of LPS-induced lung injury and to determine the effects of sGC inhibition in the context of this pathology.

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Reagents. Reagents for SDS-polyacrylamide gel electrophoresis and Western blot analysis were obtained from Bio-Rad (Hercules, CA). The Supersignal Chemiluminescent Substrate was obtained from Pierce (Rockford, IL). X-ray film was obtained from Eastman Kodak (Rochester, NY). TRIZol, SuperScript First-Strand Synthesis System for RT-PCR, DNase I, dNTPs, and platinum Taq polymerase were purchased from Invitrogen (Carlsbad, CA). Rabbit polyclonal antibodies for α1 and α2-actin were purchased from Sigma-Aldrich (St. Louis, MO); the β1 antibody was obtained from Cayman Chemicals (Ann Arbor, MI). The anti-rabbit horseradish peroxidase-labeled secondary antibody for Western blot analysis was purchased from NEN (Ann Arbor, MI). The anti-rabbit biotinylated secondary antibody for immunohistochemistry, as well as the 3,3′-diaminobenzidine (DAB) substrate kit were obtained from Vector Laboratories (Burlingame, CA); permutant was purchased from Fisher Scientific (Pittsburgh, PA). All other reagents, including H1-[1,2,4]oxadiazolo[4,3-a]quinolin-1-one (ODQ), 8pCPT-cGMP, Pseudomonas aureginosa serotype 10, and SYBR Green I were obtained from Sigma-Aldrich.

Study design. Male C57BL/6 and TNF-α−/− mice (Fleming Institute, Vari, Greece), weighing 16–24 g were used; the study protocol was approved by the local Animal Studies Committee. Animals were maintained in a virus-free facility on a 12:12-h light-dark cycle, with food and water provided ad libitum. The following groups of mice were used: control, LPS, ODQ, ODQ+LPS, and ODQ+LPS+8pCPT-cGMP. The control group was exposed to aerosolized DMSO/saline solution (50 μl DMSO in 5 ml saline); 30 min later, mice were exposed to nebulized saline (3 ml). The ODQ group received an aerosolized solution (5 mg ODQ diluted in 50 μl DMSO and then further dissolved in 5 ml saline) 30 min before nebulized saline (3 ml) at time 0. The LPS group was exposed to the vehicle solution 30 min before the challenge with the nebulized P. aeruginosa endotoxin (LPS was prepared as a 10 mg/ml saline solution and was sonicated for 30 s with a microtip sonicator before administration). The ODQ+LPS group inhaled the ODQ solution 30 min before receiving the aerosolized endotoxin. When 8pCPT-cGMP (10 mg diluted in 50 μl DMSO) and then further dissolved in 5 ml saline) was used, it was administered at the same time as LPS, that is, 30 min after the ODQ. All agents and vehicled were administered over a 20-min period in a nebulization chamber for seven mice under continuous oxygen flow.

Bronchoalveolar lavage fluid. Animals were killed 12 or 24 h after treatment. After exsanguination, the trachea was cannulated with a 20-gauge plastic catheter. Lungs were lavaged by infusing 1 ml warm (37°C) saline, three sequential times. The recovered fluid was filtered through a single layer of gauze to remove mucus. Recovery of instilled lavage fluid averaged 2.4 ± 0.06 ml (80% of the original 3 ml), being similar among groups. The resulting bronchoalveolar lavage fluid (BALF) fluid was centrifuged (300 g, 4°C, 10 min), supernatants were collected, cells resuspended in 1 ml PBS, and stored at −70°C for further analysis. Total BALF cell counts were performed, and aliquots (5 × 10⁶ cells/slide) were pelleted on glass slides by cytocentrifugation. Differential counts were performed on Giemsa-stained cytopsin, and percentages of eosinophils, lymphocytes, neutrophils, and macrophages were determined by hemocyte subtype in 400 cells. To obtain the absolute number of each leukocyte subtype in BALF, these percentages were multiplied by the total number of cells recovered from BALF. Values for cell counts and protein content in the BALF, as well as values from the densitometric analysis of sGC subunits in control animals did not differ between 12 h and 24 h, and numbers were therefore pooled; the pooled group was then used for statistical comparisons with the LPS or ODQ+LPS groups at each time point. Similarly, as values for the above-mentioned parameters did not differ between the two time points in ODQ-treated animals, values were also pooled in this case.

Western blot and immunohistochemical detection of GC subunits. After the mice were killed, lung tissue was frozen in liquid nitrogen and stored at −80°C until used. One lobe was homogenized in 10 volumes (wt/vol) of a lysis buffer containing 1% Triton-X, 1% SDS, 150 mM NaCl, 50 mM NaF, 1 mM Na3VO4, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1 mM EGTA and protease inhibitors (10 μg/ml aprotinin, 10 μg/ml pepstatin, and 20 μg PMSF). Samples were subjected to SDS-PAGE followed by blotting with antibodies against the α1 (1:5,000) or β1 (1:2,000) and visualized using a chemiluminescent substrate. For immunohistochemical detection of the sGC subunits, lung sections (4 μm) were deparaffinized, rehydrated, and fixed in 2% parafomaldehyde; they were then treated with avidin-biotin complex, blocked with 10% donkey/horse serum, and incubated overnight at 4°C with rabbit polyclonal antibodies for sGC α1 (1:500) or β1 (1:400). Next day, sections were incubated with a secondary biotinylated anti-rabbit antibody. Subunits were visualized using the DAB substrate kit, which produces a dark brown color. Sections were counterstained briefly with hematoxylin before mounting.

Statistical analysis. Results are presented as means ± SE of the number of observations. Statistical comparisons between groups were made by unpaired t-test or Mann-Whitney U-test, using SPSS software. Differences were considered significant when P < 0.05.

RESULTS

Steady-state mRNA and protein levels for the sGC subunits are decreased in the lungs of mice treated with nebulized endotoxin. LPS inhalation caused interstitial and intra-alveolar leukocyte infiltration, focal thickening of alveolar membranes, and congestion (data not shown). To determine whether sGC expression is downregulated in mice treated with LPS, steady-state mRNA levels of sGC subunits were determined 12 h after LPS inhalation. Under the conditions used, β1 mRNA levels were found to be reduced to 33.2 ± 5.7% of control (n = 5). To ascertain whether LPS-induced ALI also affects sGC sub-
unit expression at the protein level, lung homogenates were analyzed by Western blot analysis. Representative blots from these experiments are shown in Fig. 1A. Densitometric analysis revealed that expression of α1 and β1 was significantly reduced at both time points tested (Fig. 1, B and C). sGC α1 decreased to 39 ± 16% of control at 12 h and 42 ± 17% of control at 24 h. Similarly, sGC β1 was reduced to 18.3 ± 9.3% and 36.8 ± 16.4% at 12 h and 24 h, respectively. To evaluate the cell type in which sGC was downregulated, we performed IHC staining of lung sections (Fig. 2). In control lung, α1 and β1 were ubiquitously present at high levels. (Fig. 2, A and C). In agreement with our results obtained in total lysates, expression of both subunits was reduced in mice exposed to aerosolized LPS, with lower sGC levels being detected in most cell types of the lung, including bronchial smooth muscle cells and epithelial cells (Fig. 2, B and D).

Mechanisms of LPS-triggered sGC downregulation. To study the possible mediators that contribute to the downregulation in sGC expression in the context of lung injury, experiments with inhaled LPS were repeated in mice with targeted disruption of the TNF-α locus. Similarly to what was observed before, α1 and β1 were reduced in wild-type mice 12 h after LPS (Fig 3, A and B). However, no reduction in sGC protein was observed in TNF-α knockout mice, suggesting that this cytokine mediates the effects of LPS on sGC levels (Fig. 3, A and C).

Effect of ODQ on LPS-induced increase BALF cell number. To determine the effects of sGC inhibition during LPS-induced ALI, animals were pretreated with vehicle or ODQ and then challenged with nebulized LPS before white blood cell determination in the BALF. When intracellular cGMP levels were measured in leukocytes recovered from the BALF 24 h after LPS inhalation, they were found to be reduced in the ODQ+LPS vs. LPS group (0.57 ± 0.1 vs. 1.28 ± 0.1 pmol/mg protein, respectively; n = 3 P < 0.05). In LPS-treated mice, total BALF cell number was markedly increased at 12 h (1.2 ± 0.3 × 10^6 cells/ml) and 24 h (0.8 ± 0.2 × 10^6/ml), mainly because of a neutrophil influx (Fig. 4, A and B). Animals treated with ODQ only did not exhibit an increase in total cell number in the BALF. In contrast, inhaled ODQ pretreatment significantly increased LPS-induced BALF neutrophil counts at 12 h (2.1 ± 0.3 × 10^6/ml) and 24 h (1.8 ± 0.4 × 10^6/ml), compared with the LPS group, suggesting that sGC inhibition enhances leukocyte infiltration in the lung in this model of ALI. Exposure of animals to the cGMP-dependent protein kinase activator 8pCPTcGMP partially reversed the effect of ODQ reducing BALF cell number (Fig. 4C).

Effect of ODQ on LPS-induced increase BALF protein content. To evaluate whether sGC inhibition also contributes to barrier dysfunction, protein content was measured in the BALF in the presence and absence of ODQ pretreatment. ODQ treatment did not affect BALF protein concentration, suggesting that under baseline conditions, sGC inhibition does not lead to increased leakage in the lung. On the other hand, inhalation of LPS increased BALF protein content at both time points tested compared with the control group (0.08 ± 0.03 and 0.15 ± 0.05 mg/ml, at 12 and 24 h, respectively, vs. undetectable in control). Similar to what was observed with the cell counts in the BALF, ODQ pretreatment enhanced the LPS-

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**Fig. 1.** Protein levels of sGC subunits are decreased in mice exposed to nebulized LPS. Control mice or mice treated with LPS were killed at the indicated time after treatment. Lung lysates were prepared and subjected to SDS-PAGE. A: representative Western blots for the α1, β1, and actin. Blots were quantified by densitometry at 12 h (B) and 24 h (C). Expression for each subunit normalized for actin was set at 100% for control mice. Values are expressed as means ± SE; n = 6 or 7; *P < 0.05 from control.
stimulated increase in BALF protein content (Fig. 5A), and the PKG activator attenuated the effect of ODQ on albumin leakage (Fig. 5B).

**DISCUSSION**

In the present study, we have investigated the changes in expression and the biological role of sGC in a murine model of LPS-induced lung injury. Consistently with many reports in the literature, LPS inhalation led to neutrophilic lung infiltration and increased cellularity and protein leakage in the BALF, all of which are consistent with the changes observed in humans with ALI/ARDS. Our major findings are 1) steady-state sGC subunit mRNA and protein levels are decreased in animals following LPS-induced ALI, 2) the reduction in sGC levels in LPS-induced lung injury. Consistently with many reports in the literature, LPS inhalation led to neutrophilic lung infiltration and increased cellularity and protein leakage in the BALF, all of which are consistent with the changes observed in humans with ALI/ARDS. Our major findings are 1) steady-state sGC subunit mRNA and protein levels are decreased in animals following LPS-induced ALI, 2) the reduction in sGC levels in

**Fig. 2.** sGC expression is reduced in smooth muscle and epithelial cells after the LPS challenge. Representative photomicrographs of lung sections from control animals (A and C) or animals treated with LPS (B and D) for 24 h are shown after immunohistochemistry staining with soluble guanylyl cyclase (sGC) antibodies. White arrows point to epithelial cells and black arrows to smooth muscle cells. Hematoxylin was used as a counterstain. ×400 magnification.

**Fig. 3.** TNF-α mediates the effects of LPS on sGC levels during lung injury. Control or TNF-α −/− mice were treated with aerosolized LPS and killed 12 h after treatment. Lung lysates were prepared and subjected to SDS-PAGE. Representative Western blot analysis for the α1, β1, and actin are shown (A). Blots were quantified using densitometry (B and C); expression for each subunit was normalized for actin. Values are expressed as means ± SE; n = 4; *P < 0.05 from control.
the context of ALI is mediated by TNF-α, 3) decreased sGC expression is observed in epithelial and smooth muscle cells, 4) pharmacological inhibition of sGC in the context of LPS-induced lung injury led to an increase in the number of neutrophils in the BALF, and 5) sGC blockade promoted barrier dysfunction in mice with LPS-induced lung injury.

To ascertain whether development of lung injury following LPS administration is accompanied by changes in sGC expression, we measured β1 steady-state mRNA levels at 12 h post-LPS inhalation. Under the conditions used, β1 steady-state mRNA levels were drastically reduced. As sGC is an obligate heterodimer carrying a β1 subunit (4), decreased β1 levels—if also observed at the protein level—would indicate a decline in the amount of active enzyme. Our present findings extend observations made in vitro, showing that sGC subunit mRNA is downregulated in rat aortic or pulmonary artery smooth muscle cells stimulated with endotoxin (23, 33). To determine whether protein levels are also affected in tissues of mice with LPS-induced lung injury, we performed Western blot analyses in lung homogenates. These experiments revealed that both α1 and β1 levels were reduced in mice challenged with LPS 12 and 24 h after inhalation. As sGC is ubiquitously expressed, determination of protein levels in lung homogenates does not reveal the cell types in which sGC is decreased. To determine the cell types in which sGC protein levels are reduced, we stained lung sections with α1 and β1 antibodies; we observed a marked reduction in both subunits in bronchial smooth muscle and epithelial cells of mice with LPS-induced lung injury. Of note, we have shown recently that sGC expression is reduced in another inflammation-related disease that affects the lung, namely asthma, contributing to airway hyperreactivity (25).

Nitric oxide, TNF-α, and IL-1β are increased in ALI and have been reported to reduce sGC protein expression in cultured cells (7, 23, 34, 38). To determine whether TNF-α contributes to the downregulation in sGC expression observed in LPS-induced lung injury, experiments were performed in mice with targeted disruption of the TNF-α locus. α1/β1 lung tissue content was comparable in vehicle and LPS-treated α1/−/− mice, suggesting that in the absence of this cytokine, sGC expression remains elevated. To our knowledge, TNF-α is the first cytokine shown to affect sGC levels in vivo. Interestingly, TNF-α and IL-1β can inhibit sGC subunit expression in vitro via both NO-dependent and independent pathways; Takata et al. (34) demonstrated a reduction in α1 and β1 levels in pulmonary artery smooth muscle cells isolated from iNOS−/− exposed to a TNF-α/IL-1β cytokine mixture. At the molecular level, two mechanisms have been shown to
inhibit sGC subunit expression: one that is PKA/PKG-mediated and decreases mRNA stability by reducing the expression of the mRNA-stabilizing protein HuR and one that relies on JNK activation (11, 12). Further studies are required to unravel which of the above-mentioned mechanisms contribute to the inhibition of sGC expression in LPS-induced lung injury. It should also be kept in mind that, sGC activity is expected to be greatly reduced in ALI not only because of the low levels of sGC subunit expression, but also because of desensitization that occurs upon exposure of the enzyme to excessive amounts of NO (24).

Neutrophils represent a key cell type for ALI-associated tissue injury (6, 35). Neutrophils infiltrate in the lung parenchyma following pulmonary vascular sequestration, adhesion to the endothelium, and transendothelial migration. Neutrophil infiltration is facilitated by endothelial cell (EC) barrier dysfunction, but it also contributes to barrier dysfunction and ALI by releasing a variety of proinflammatory factors and by activating intracellular signaling cascades in the EC that promote vascular leakage (37). In our study, LPS inhalation was associated with a significant increase in BALF cellularity that was mainly due to an increase in the number of neutrophils, consistent with acute inflammation. To study the contribution of sGC to the leukocyte infiltration in the lung, we treated animals with inhaled ODQ prior to the LPS challenge. Such pretreatment increased LPS-induced total BALF cell number at 12 or 24 h, with the cell population once again consisting mainly of neutrophils, indicating an anti-inflammatory role for sGC/cGMP signaling in the lung of LPS-treated mice. However, ODQ has been shown to have off-target actions, inhibiting several heme-containing enzymes other than sGC. We, therefore, investigated whether PKG activation could reverse the effects of ODQ. Indeed, 8pCPT-cGMP inhibited the ODQ-induced increase in BALF cellularity in mice exposed to aerosolized LPS, suggesting that the observed effects of ODQ are due to sGC inhibition.

It would be tempting to speculate that the increase in neutrophil influx in the lungs of animals challenged with LPS only is aided by the decline of endogenous levels of sGC. Our results demonstrating a protective action of sGC in the context of ALI are in agreement with a recent report that the NO-independent sGC activator BAY 41-2272 reduced leukocyte rolling and adhesion in eNOS−/− mice (1). Moreover, in an experimental setting more relevant to ALI, sGC stimulation attenuated the IL-1β-induced increase in leukocyte adhesion (1). The anti-inflammatory effects of sGC activation were proposed to be mediated by inhibition of P-selectin expression and function. On the other hand, in a study in which anesthetized rats were treated with lipoteichoic acid/peptidoglycan or lipopolysaccharide as models for gram-positive and gram-negative sepsis, pretreatment with ODQ prevented multiple organ injury and dysfunction (40). In this latter study, histologic evaluation of the lung demonstrated that sGC inhibition attenuates lung injury. Several differences in the experimental conditions that may explain the differences in the outcomes of these studies, as well as the need for further research into the role of sGC in ALI, are described in the manuscript.
design might be responsible for the discrepancy in the findings reported by Zacharowski et al. (40) and the present study; these include the different species used (rats vs. mice), the use of anesthesia, the time point of analysis after the LPS challenge, the type of LPS administered, and the route of administration (systemic vs. inhaled).

The alveolar-capillary barrier is enforced by two components: the microvascular endothelium and the alveolar epithelium. During the acute phase of ALI, increased permeability of the alveolar-capillary barrier allows the influx of protein-rich fluid into the air spaces. The importance of endothelial injury leading to increased permeability and to the formation of pulmonary edema in ALI is well established (22, 37). Although modulation of cGMP levels in alveolar epithelial cells did not alter their barrier properties in vitro (30), existing evidence in the literature demonstrates that increased intracellular cGMP in the endothelium or administration of membrane-permeable analogs of cGMP, or cGMP-dependent protein kinase overexpression, reduces the permeability of cultured endothelial cells (14, 20, 27, 28). In our study, lavage fluid protein, a nonspecific marker of breakdown in the epithelial-endothelial barrier, was significantly elevated at 12 and 24 h after LPS exposure. Inhibition of sGC by ODQ deteriorated barrier function, as evidenced by the increase in BALF protein concentration, reinforcing the notion that sGC exerts beneficial actions in ALI. The protective effect of sGC in our model of ALI is consistent with observations made using pharmacological and genetic approaches. Inhibitors of the NO/cGMP pathway have been demonstrated to cause a deterioration in inflammation and vascular leakage in animal models of lung injury (16); moreover, transgenic mice overexpressing eNOS are more resistant to LPS-induced lethality and develop less severe ALI, characterized by reduced neutrophil infiltration, edema, and histologic injury after LPS administration (39).

In summary, we have shown that sGC expression is reduced in the mouse lung both at the mRNA and protein level after inhalation of endotoxin P. aeruginosa and that the decrease in sGC is TNF-α dependent. We have also observed that further attenuation of sGC activity by a selective sGC inhibitor was accompanied by more pronounced inflammation and tissue damage. The present study provides new insights into the pathophysiologic changes observed in ALI and offers evidence for a protective role of the sGC/cGMP pathway in this disease state.

GRANTS

This study was supported by grants from the Greek Secretariat of Research and Technology (AP, PENED 2001/01EΔ67), the University of Athens (SEO, 70/4/5918), an unrestricted grant by GlaxoSmithKline (to A. Papapetropoulos), and by the Thorax Foundation (Athens, Greece).

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