Adrenomedullin ameliorates lipopolysaccharide-induced acute lung injury in rats

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Itoh T, Obata H, Murakami S, Hamada K, Kangawa K, Kimura H, Nagaya N. Adrenomedullin ameliorates lipopolysaccharide-induced acute lung injury in rats. Am J Physiol Lung Cell Mol Physiol 293: L446–L452, 2007. First published June 8, 2007; doi:10.1152/ajplung.00412.2005.—Adrenomedullin (AM), an endogenous peptide, has been shown to have a variety of protective effects on the cardiovascular system. However, the effect of AM on acute lung injury remains unknown. Accordingly, we investigated whether AM infusion ameliorates lipopolysaccharide (LPS)-induced acute lung injury in rats. Rats were randomized to receive continuous intravenous infusion of AM (0.1 μg·kg⁻¹·min⁻¹) or vehicle through a microosmotic pump. The animals were intratracheally injected with either LPS (1 mg/kg) or saline. At 6 and 18 h after intratracheal instillation, we performed histological examination and bronchoalveolar lavage and assessed the lung wet/dry weight ratio as an index of acute lung injury. Then we measured the numbers of total cells and neutrophils and the levels of tumor necrosis factor (TNF)-α and cytokine-induced neutrophil chemoattractant (CINC) in bronchoalveolar lavage fluid (BALF). In addition, we evaluated BALF total protein and albumin levels as indexes of lung permeability. LPS instillation caused severe acute lung injury, as indicated by the histological findings and the lung wet/dry weight ratio. However, AM infusion attenuated these LPS-induced abnormalities. AM decreased the numbers of total cells and neutrophils and the levels of TNF-α and CINC in BALF. AM also reduced BALF total protein and albumin levels. In addition, AM significantly suppressed apoptosis of alveolar wall cells as indicated by cleaved caspase-3 staining. In conclusion, continuous infusion of AM ameliorated LPS-induced acute lung injury in rats. This beneficial effect of AM on acute lung injury may be mediated by inhibition of inflammation, hyperpermeability, and alveolar wall cell apoptosis.

apoptosis; hyperpermeability; inflammation

ACUTE RESPIRATORY DISTRESS syndrome (ARDS) is a life-threatening disease characterized by diffuse lung injury that leads to respiratory failure and death (2, 12). Its mortality remains high despite recent advances in intensive care (4, 38). Therefore, a novel therapeutic strategy for ARDS is desirable. Potential mechanisms that induce ARDS include lung inflammation and hyperpermeability (4, 36). Lung inflammation induces the production of various molecules that mediate lung injury such as arachidonic acid metabolites (2, 16), proteases (59), and free radicals (10, 40). Lung hyperpermeability contributes to the development of pulmonary edema, resulting in abnormal gas exchange. Furthermore, apoptosis of several cell types, including neutrophils, alveolar epithelial cells, and endothelial cells, is involved in the pathogenesis of acute lung injury in ARDS (9, 24, 26). Thus a therapeutic strategy against these abnormalities may be effective for the treatment of ARDS.

Adrenomedullin (AM) is an endogenous peptide that was originally isolated from human pheochromocytoma (20). It has been shown to have a variety of protective effects on the cardiovascular system in addition to vasodilator activity (6, 18, 27–29, 32). It has been shown to inhibit inflammatory cytokine production (13, 14, 53). AM also has been reported to reduce endothelial hyperpermeability through a cyclic adenosine 3′,5′-monophosphate-dependent mechanism (11). Furthermore, AM has been reported to protect against apoptosis through a phosphatidylinositol 3-kinase/Akt-dependent pathway (15, 19, 34, 39, 45). Considering that AM has been shown to attenuate organ injury in sepsis models (7, 41, 51), it may have protective effects against inflammation, hyperpermeability, and cell apoptosis, which are responsible for acute lung injury in ARDS. However, the effects and mechanisms of AM in acute lung injury remain unknown.

Lipopolysaccharide (LPS), a bacterial cell wall component, is a stimulus for the initiation of local acute inflammation. Intratracheal instillation of LPS in animals has gained wide acceptance as an experimental model of ARDS (5). Thus the purposes of this study were 1) to investigate whether AM infusion ameliorates acute lung injury and 2) to examine the underlying mechanisms responsible for the effects of AM on acute lung injury.

METHODS

Animals. All protocols were performed in accordance with the guidelines of the Animal Care Ethics Committee of the National Cardiovascular Center Research Institute. Adult male Sprague-Dawley rats weighing 180–200 g were used in this study. Rats were assigned to receive a continuous infusion of AM or vehicle and underwent intratracheal instillation of either LPS or 0.9% saline. This protocol resulted in the creation of four groups: sham rats given vehicle (Sham-Vehicle group; n = 34), sham rats treated with AM (Sham-AM group; n = 34), LPS rats given vehicle (LPS-Vehicle group; n = 34), and LPS rats treated with AM (LPS-AM group; n = 34).

Experimental protocol. After the rats were anesthetized by intraperitoneal injection of pentobarbital (30 mg/kg), they were given a...
continuous intravenous infusion of either AM or saline vehicle via a microosmotic pump (2001D; Alzet, Palo Alto, CA). Briefly, an osmotic pump was filled with either AM or saline and was set to deliver a dose of 0.1 μg·kg⁻¹·min⁻¹, attached to a catheter (PE-60) placed in the left jugular vein, and implanted subcutaneously between the scapulae. The rats were allowed to recover from the anesthesia and were maintained on standard rat chow. Two hours after implantation, the rats were intratracheally injected with either 1 mg/kg LPS (Escherichia coli 055:B5; Sigma, St. Louis, MO) dissolved in 0.3 ml saline or vehicle (0.3 ml saline) under anesthesia. We measured the LPS content in the saline by using the Limulus amoebocyte lysate test (E-Toxate; Sigma). The saline used in this study contained <6 pg LPS/ml. After recovery from anesthesia, the animals were again maintained on standard rat chow. The animals showed no sign of distress with this procedure. All rats remained alive after intratracheal instillation of LPS.

The animals were killed with an overdose of pentobarbital, and the following parameters were analyzed. Bronchoalveolar lavage (BAL) was performed at 6 and 18 h after intratracheal instillation (n = 8 each). Histological examination was performed in another group of rats at 6 and 18 h (n = 5 each). To estimate the circulating level of AM, blood sampling was performed at 18 h. To evaluate the severity of acute lung injury, the lung wet/dry weight ratio was calculated at 18 h in the rats that were not subjected to BAL or histological examination (n = 8 each). The wet lung weight was measured immediately after dissection, and the dried lung weight was estimated after oven drying at 60°C for 72 h. The experimental design is summarized in Fig. 1.

Preparation of AM. Recombinant human AM was obtained from Shionogi (Osaka, Japan). The homogeneity of AM was confirmed by reverse-phase high-performance liquid chromatography and amino acid analysis. AM was stored at −80°C until the time of preparation for infusion.

Measurement of AM. Blood was immediately transferred into a chilled glass tube containing disodium EDTA (1 mg/ml) and aprotinin (500 U/ml) and was centrifuged immediately at 4°C. Plasma samples were frozen and stored at −80°C. Human AM was measured by using a specific immunoradiometric assay kit (AM RIA; Shionogi) (33). Rat AM was also measured by using this assay kit with some modifications, as reported previously (31).

BAL analysis. BAL was performed through a tracheal cannula with 5 ml saline solution. This procedure was performed twice. A 500-μl aliquot of BAL fluid (BALF) was reserved for determination of the total number of cells and cell differentiation, and the remainder was centrifuged immediately at 700 × g for 5 min at 4°C. The supernatant of BALF was immediately stored at −80°C before assays. The total number of cells was counted by using a standard hemocytometer. Cell differentiation was examined by counting at least 200 cells on a smear prepared by using cytopsin and Wright-Giemsa staining.

Tumor necrosis factor-α and cytokine-induced neutrophil chemotactant assays. BALF tumor necrosis factor (TNF)-α and cytokine-induced neutrophil chemotactant (CINC) levels were measured by using a rat TNF-α ELISA kit (BioSource International, Camarillo, CA) and a rat Gro/CINC-1 kit (Amersham Biosciences, Piscataway, NJ), respectively.

Total protein and albumin assays. To investigate the effect of AM on lung permeability, BALF total protein and albumin levels were measured by using a Bradford assay (Bio-Rad, Tokyo, Japan) and a bromocresol green assay (Sigma), respectively.

Histological examination. The lungs were fixed with 4% paraformaldehyde and were embedded in paraffin. Paraffin sections 4-μm thick were stained with hematoxylin and eosin for examination by light microscopy. Lung injury was graded from 0 (normal) to 4 (severe) in four categories: interstitial inflammation, neutrophil infiltration, congestion, and edema (42). Lung-injury score was calculated by adding the individual scores for each category. Grading was performed by a blinded pathologist. Lung-injury score for each animal was calculated as the mean of four lung sections. Paraffin sections were obtained from individual rats at 6 or 18 h after intratracheal instillation (n = 5 per group).

Immunohistochemical study. To investigate the effect of AM on lung apoptosis, tissue sections were stained for cleaved caspase-3, a key executor of apoptosis, by using a rabbit polyclonal anti-cleaved caspase-3 antibody (Cell Signaling Technology, Beverly, MA). The number of cleaved caspase-3-positive alveolar wall cells was determined in 10 randomly chosen fields (×400) per section. The percentage of cleaved caspase-3-positive inflammatory cells was calculated (number of cleaved caspase-3-positive inflammatory cells/total number of inflammatory cells × 100) in 10 randomly chosen fields (×400) per section, as previously described (21). The mean of four sections per animal was determined in a blinded manner. Paraffin sections 4-μm thick were obtained from the lungs at 6 h after intratracheal instillation (n = 5 per group).

Statistical analysis. All data are expressed as means ± SE. All data have been tested for normality by using the Shapiro-Wilk normality test and were determined to have a normal distribution. Homogeneity of variance was tested by using Bartlett’s test. When Bartlett’s test indicated that the group comparisons had equal variance, one-way ANOVA and Newman-Keuls’ test were used. When the group data showed unequal variance, nonparametric statistical analysis was used. A value of P < 0.05 was considered statistically significant.

RESULTS

Inhibition of LPS-induced acute lung injury by AM. Photomicrographs showed that intratracheal instillation of LPS caused infiltration of inflammatory cells into the lung interstitium and alveolar spaces, alveolar wall thickening, and intra-alveolar exudation at 6 and 18 h after LPS instillation. (Fig. 2A). However, AM infusion attenuated these histological changes. Semiquantitative assessment using lung-injury score demonstrated that the degree of lung injury in the LPS-AM group was lower than that in the LPS-Vehicle group at 6 and 18 h after LPS instillation (Fig. 2B). The lung wet/dry weight ratio was significantly increased at 18 h after LPS instillation (Fig. 3). AM infusion significantly attenuated the increase in the lung wet/dry weight ratio compared with vehicle. AM infusion did not induce any changes in lung histology and the lung wet/dry weight ratio in Sham rats. AM infusion tended to decrease systemic blood pressure but did not cause severe hypotension in LPS rats (121 ± 8 to 114 ± 10 mmHg).

Plasma AM level. Plasma AM level was significantly higher in LPS rats than in Sham rats (10 ± 1 vs. 3 ± 1 fmol/ml, P < 0.05). Furthermore, the level was markedly increased in LPS

Fig. 1. Study protocol timeline. Implantation of osmotic pump, intratracheal instillation, bronchoalveolar lavage (BAL), histomorphometry, and morphometry were performed.
rats treated with AM (32 ± 3 fmol/ml) compared with in those given vehicle (P < 0.01). These results suggest that the administered AM reached pharmacological levels.

**Effects of AM on LPS-induced lung inflammation.** The recovery rate of BALF was >80% in all groups. The numbers of total cells and neutrophils were significantly increased at 6 and 18 h after LPS instillation (Fig. 4, A and B). However, the numbers of these cells in the LPS-AM group were significantly lower than those in the LPS-Vehicle group. The BALF TNF-α level was significantly increased at 6 and 18 h after LPS instillation (Fig. 4C). Similarly, the BALF CINC level was significantly increased after LPS instillation (Fig. 4D). AM infusion significantly attenuated the increases in BALF TNF-α and CINC levels. AM infusion did not significantly alter BAL data in sham rats.

**Effects of AM on LPS-induced lung hyperpermeability.** The BALF total protein and albumin levels, markers for lung permeability, were significantly increased at 6 and 18 h after LPS instillation (Fig. 5). AM infusion significantly attenuated the increases in BALF total protein and albumin levels.

**Effect of AM on LPS-induced alveolar wall cell apoptosis.** Cleaved caspase-3-positive cells were frequently observed in the alveolar wall at 6 h after LPS instillation (Fig. 6A). AM infusion markedly decreased cleaved caspase-3-positive cells in the alveolar wall. Semiquantitative analysis demonstrated a significant increase in the number of cleaved caspase-3-positive alveolar wall cells after LPS instillation, and the increase in the LPS-AM group was significantly attenuated compared with that in the LPS-Vehicle group (Fig. 6B). AM infusion did not significantly change the percentage of cleaved caspase-3-positive inflammatory cells compared with vehicle infusion (3 ± 2 vs. 4 ± 1%).

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**Fig. 2.** A: representative photomicrographs of lung tissues stained with hematoxylin and eosin at 6 and 18 h after lipopolysaccharide (LPS) instillation. Intratracheal instillation of LPS caused infiltration of inflammatory cells into lung interstitium and alveolar spaces, alveolar wall thickening, and intra-alveolar exudation. Scale bars, 50 μm. B: semiquantitative analysis of lung tissues by lung-injury score. Lung-injury score was significantly decreased in LPS-adrenomedullin (AM) group compared with LPS-Vehicle (saline) group. Data are means ± SE. *P < 0.05 vs. Sham-Vehicle; †P < 0.05 vs. LPS-Vehicle.

**Fig. 3.** Effect of AM infusion on lung weight after LPS instillation. Data are means ± SE. *P < 0.05 vs. Sham-Vehicle; †P < 0.05 vs. LPS-Vehicle.
DISCUSSION

In the present study, we demonstrated that AM infusion 1) ameliorated LPS-induced histological changes and attenuated the increase in lung weight after LPS instillation, 2) decreased the numbers of total cells and neutrophils and the levels of TNF-α and CINC in BALF, 3) reduced the levels of total protein and albumin in BALF, and 4) inhibited apoptosis of alveolar wall cells.

In the present study, intratracheal instillation of LPS was used to produce a model of ARDS in rats. Acute lung injury was histologically confirmed in rats subjected to LPS instillation. LPS instillation also increased the lung wet/dry weight ratio, an index of acute lung injury. AM infusion significantly attenuated these abnormalities, suggesting that AM ameliorates LPS-induced acute lung injury in rats. We also demonstrated that the circulating level of AM was significantly increased after intratracheal instillation of LPS, which is consistent with previous observations that AM expression is increased in animals and humans with acute lung injury (1, 46). In the present study, AM infusion caused a significant additional increase in the circulating level of AM in rats subjected to LPS instillation. Thus supplementation of AM may produce beneficial actions at pharmacological levels. However, the underlying mechanisms still remain unclear. Considering the variety of protective effects of AM, the present study investigated the effects of AM on lung inflammation, permeability, and cell apoptosis, all of which are responsible for acute lung injury.
LPS is known to induce severe lung inflammation through the migration and activation of inflammatory cells. In particular, neutrophils are considered to be responsible (54). The present study also showed that LPS instillation markedly increased the number of neutrophils in BALF. However, AM infusion significantly attenuated the increase in neutrophils. These findings suggest that AM infusion ameliorates LPS-induced lung inflammation at least in part through inhibition of neutrophil infiltration. Several investigations have identified that several cytokines play pivotal roles in the initiation and development of inflammation (44, 47, 57). LPS has been reported to induce the production of several cytokines in vivo.

Fig. 5. Effects of adrenomedullin (AM) infusion on BALF total protein (A) and albumin (B) at 6 and 18 h after LPS instillation. AM infusion significantly reduced BALF total protein and albumin levels. Data are means ± SE. *P < 0.05 vs. Sham-Vehicle; †P < 0.05 vs. LPS-Vehicle.

Fig. 6. A: immunohistochemical demonstration of cleaved caspase-3 antigen, a marker for cell apoptosis, in lungs at 6 h after LPS instillation. Scale bars, 20 μm. B: semiquantitative analysis of cleaved caspase-3-positive alveolar wall cells. Number of cleaved caspase-3-positive alveolar wall cells was significantly decreased in LPS-AM group compared with LPS-Vehicle group. Data are means ± SE. *P < 0.05 vs. Sham-Vehicle; †P < 0.05 vs. LPS-Vehicle.
and in vitro (3, 8). In fact, in the present study, BALF TNF-α and CINC levels were markedly increased in rats with LPS instillation. TNF-α, a proinflammatory cytokine, participates in several important processes involved in the inflammatory response (23, 48–50, 55). On the other hand, CINC, a member of the CXC chemokine family, plays a pivotal role in neutrophil migration in rats (47, 56). These findings suggest that these cytokines are potentially important mediators of LPS-induced lung inflammation. It should be noted that AM infusion significantly decreased BALF TNF-α and CINC levels. Our results may be supported by earlier in vitro findings that AM reduces LPS-stimulated secretion of TNF-α and CINC from macrophages (17, 58). These findings suggest that AM infusion suppresses LPS-induced lung inflammation through inhibition of cytokine production.

Lung hyperpermeability is also involved in the pathogenesis of ARDS (4, 36). Intratracheal instillation of LPS has been shown to injure pulmonary endothelial and epithelial cell layers and to increase lung permeability, resulting in pulmonary edema (25). Recent studies have shown that a reduction of lung hyperpermeability protects against LPS-induced acute lung injury (35, 43). In the present study, LPS instillation significantly increased BALF total protein and albumin levels, markers for lung permeability. AM infusion attenuated the LPS-induced increases in BALF total protein and albumin levels. Recently, AM has been shown to reduce endothelial hyperpermeability through a cyclic adenosine 3’,5’-monophosphate-dependent mechanism in perfused rabbit lungs (11). Thus the therapeutic effects of AM on acute lung injury may be mediated by a reduction of lung hyperpermeability.

Apoptosis of several cell types, including neutrophils, alveolar epithelial cells, and endothelial cells, is involved in the pathogenesis of acute lung injury in ARDS (9, 24, 26). In fact, in the present study, LPS instillation significantly increased the number of apoptotic alveolar wall cells. AM infusion attenuated the LPS-induced increase in the number of apoptotic alveolar wall cells, although it did not affect inflammatory cell apoptosis. Cell apoptosis and survival in the alveolar wall play an important role in the maintenance of lung homeostasis. Several studies have shown that apoptosis inhibitors attenuate LPS-induced acute lung injury in animals (21, 52). Inhibition of alveolar wall cell apoptosis has been shown to be associated with the attenuation of LPS-induced acute lung injury (21, 30). In addition, AM has been shown to protect against apoptosis in vivo and in vitro (15, 19, 34, 39, 45). These findings suggest that AM infusion ameliorates LPS-induced acute lung injury at least in part through inhibition of alveolar wall cell apoptosis. Further studies are necessary to clarify whether the hemodynamic effect of AM influences LPS-induced acute lung injury.

In conclusion, continuous infusion of AM ameliorated LPS-induced acute lung injury in rats. This beneficial effect of AM may be mediated by inhibition of inflammation, hyperpermeability, and alveolar wall cell apoptosis.

GRANTS

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