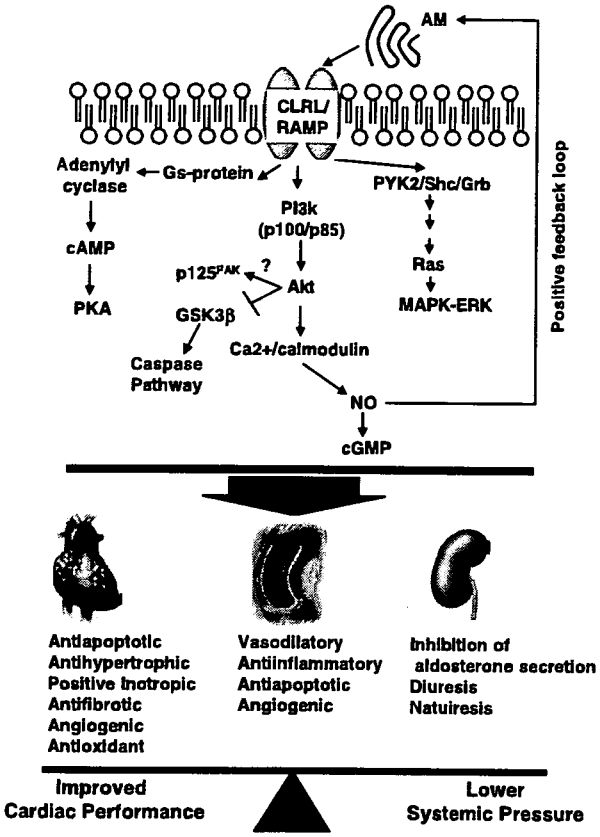

Fig. 1. Adrenomedullin (AM) signaling pathways and downstream consequences. Activation of three main signaling pathways has been identified thus far: cyclic adenosine monophosphate (cAMP), phosphoinositide 3-kinase (PI3K)/Akt and mitogen activated protein kinase (MAPK)-extracellular signal-regulated protein kinase (ERK). The downstream physiological effects are seen primarily in the vasculature, kidneys and heart, which results ultimately in a balance between improved cardiac performance and lower systemic blood pressure

3-kinase (PI3K)/Akt, and MAPK-ERK (Iwasaki, 1998, 2001; Shimekake, 1995) as summarized in Fig. 1.

The actions of AM are mediated by the 7 transmembrane G-protein-coupled calcitonin receptor-like receptor (CRLR), which co-assembles with subtypes 2 and 3 of a family of receptor-activity-modifying proteins (RAMPs) thus forming a receptor-co-receptor system (McLatchie, 1998). In rat hearts, CRLR and RAMP2 expression influences AM intracellular signaling, strongly suggesting that these are the functional receptors (Autelitano, 2001). The co-receptors RAMP2 and RAMP3 modify the effects of AM on CRLR, a G-protien-coupled receptor. Interestingly, AM and CRLR are both up-regulated under hypoxic conditions in microvascular endothelial cells (Kinitenko, 2003). The promoter for CRLR is at least partially regulated by HIF-1, which has also been shown to regulate the expression of AM under hypoxic conditions. Therefore, CRLR shares a common transcriptional mechanism with AM.

The PI3K and downstream serine-threonine kinase Akt (or protein kinase B) pathway is a well-characterized protective signaling program (Matsui, 1999; Fujio, 2000). AM has a potent protective, anti-apoptotic role through the PI3K/Akt pathway (Kim, 2002). Adenovirus-mediated gene delivery of AM has been shown to protect the myocardium from apoptosis in a rat model of myocardial infarction whereas this effect was blocked in dominant-negative Akt mice (Yin, 2004). GSK-3β is a downstream protein kinase of Akt, which when phorphorylated, causes inactivation and reduced caspase signaling. In hypoxic and reoxygenated cardiomyocytes, the AM-mediated antiapoptotic effect is associated with increased GSK-3β signaling. The angiogenic effect of AM is mediated by activation of Akt as well as mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) 1/2 and focal adhesion kinase (FAK) in endothelial cells (Kim, 2003).

The MAPK-ERK is also a well-characterized stress-induced protective signaling program whose expression is triggered in heart injury. In particular, ERK is part of an antiapoptotic and compensatory hypertrophic program (Baines, 2005). AM triggers smooth muscle cell proliferation at least in part via ERK (Shichiri, 2003). Following receptor binding in smooth muscle cells, AM triggers rapid ERK activation which likely limits acute myocardial injury (Iwasaki, 1998).

Binding of AM to CRLR can activate Gs, trigger cAMP accumulation and activate cyclic AMP-dependent protein kinase. cAMP/protein kinase in smooth muscle cells (SMCs) regulates the vasodilatory effects of AM (Ishizaka, 1994). AM strongly increases cAMP and Ca^{2+} concentration in bovine aortic endothelial cells (Shimekake, 1995) and it is 10-times more potent than CGRP in increasing the cAMP level in rat vascular SMC (Eguchi, 1994). In endothelial cells, vasodilation predominantly occurs by an eNOS/NO pathway (Hirata, 1995). Nishimatsu et al. (2001) have shown that AM induces Akt activation in the endothelium via the Ca^{2+}/calmodulin-dependent pathway. This is implicated in the production of nitric oxide, which in turn induced endothelium-dependent vasodilation. The increase in cAMP in SMCs by AM activates protein kinase A, resulting in decreased calcium content in SMCs (Ishizaka, 1994). The unique combination of the above signaling mechanisms results in a number of *in vivo* effects such as vasodilation, anti-apoptotosis, angiogenesis, and positive inotropy. An improved understanding

of the signaling pathways whereby AM mediates its physiologic effect may bring us closer to using AM as a therapeutic agent.

Adrenomedullin and the cardiovascular system

It is clear that AM expression is upregulated in patients with both acute and chronic cardiovascular disease and that such increases are associated with multiple host protective effects. Thus far, AM has been found to be increased in patients with essential hypertension (Ishimitsu, 1994), cardiac hypertrophy (Tsuruda, 2003), heart failure (Kreminski, 2002; Jougasaki, 1995; Nishikimi, 1995), acute myocardial infarction (Kobayashi, 1996; Miyao, 1998; Nagaya, 1999a), and peripheral arterial occlusive disease (Suzuki, 2004). In acute myocardial infarction, levels of AM expressed in patients correlates with the severity of illness (Miyao, 1998; Nagaya, 1999a). Tissue levels of AM peptide and mRNA are also markedly increased in the ischemic (Nagaya, 2000; Hofbauer, 2000) and failing heart (Cueille, 2002; Nishikimi, 2003; Tadokoro, 2003; Totsune, 2000). Thus, expression of AM is up-regulated both in serum and in the target tissue.

Even in a chronic setting, AM is increased in patients with end stage heart failure, a final common pathway for cardiomyopathies and ischemic heart disease, for which the only definitive treatment is heart transplantation. Notably, AM expression, as determined by immunohistochemistry on biopsy specimens, correlates with the severity of disease (Jougasaki, 1995; Nishikimi, 1995). Furthermore, evidence from patients with acute myocardial infarction shows plasma AM to be an independent prognostic indicator for mortality, although not for hemodynamic variables (Nagaya, 1999a; Katayama, 2004). Nagaya et al. (2000a) have shown that intravenous infusion of AM markedly increases cardiac index and improves hemodynamics, renal function and hormonal parameters in patients with left sided heart failure. Therefore, increase in AM above the endogenously mediated increase is of therapeutic benefit.

Although the precise mechanism of AM upregulation is unclear, the expression of proinflammatory cytokines TNF-α and IL-1β as well as IFN-γ and NO can directly trigger increased AM expression in cultured endothelial cells, myocytes and SMCs (Hofbauer, 2002; Sugo, 1994, 1995; Ihara, 2000). Upregulation may occur via the hypoxia-inducible factor-1 (HIF-1) as observed under hypoxic conditions (Garaoya, 2000). Considering the lack of AM stores, transcriptional control is considered rather important for AM regulation. However, the precise mechanism of AM transcriptional upregulation is incompletely understood.

The potential functions of AM include vasodilator, natriuretic, diuretic, antiapoptotic and pro-survival roles (Fig. 1). Also, the angiogenic and inflammatory modulating properties of AM have been recently described. Experimental and clinical evidence for the cardiovascular effects of AM will now be examined in greater detail.

Positive inotropic effects

Considerable evidence exists for a positive inotropic effect of AM. AM increases cardiac cAMP, which is known to mediate the positive inotropic action of β-adrenergic stimulants. Szokodi et al. (1998) have shown that AM produces positive inotropic action through cAMP-independent Ca^{2+} release. A recent binding study demonstrated the presence of abundant binding sites for AM in the ventricular myocardium (Owji, 1995). Furthermore, our laboratory has demonstrated that infusion of AM markedly increases cardiac index and stroke volume index in patients with congestive heart failure (Nagaya, 2000b, 2002). Considering the strong vasodilator effect of AM, a decrease in mean arterial pressure may be partially responsible for increased cardiac index during infusion. Oya et al. (2000) reported that AM administration increased cardiac index and stroke volume index in patients. However, some reports are indicative of negative inotropy or no effect on contractility following AM treatment in cultured cardiomyocytes and *in vivo* (Ikenouchi, 1997; Perret, 1993; Stangl, 2000). In primary cultured cardiocytes, AM induced a biphasic acute increase and decrease of myocyte shortening and Ca^{2+} transients (Mittra, 2004). Thus, it is possible that timing of AM administration *in vivo* is an important determinant of pump function. The particular circumstances for a positive inotropic effect of AM remain to be determined.

Vasodilation

AM was originally characterized as a potent vasodilatory molecule (Kitamura, 1993). The vasodilatory effect of AM is mediated by cAMP (Ishizaka, 1994) and nitric oxide/cGMP-dependent mechanisms (Nakamura, 1997; Hayakawa, 1999). AM increases cAMP thus activating protein kinase A, resulting in decreased calcium content. Vasodilatation and natiuresis is NO- and cGMP-dependent as E-4021, a cGMP-specific phosphodiesterase inhibitor, blocked these effects (Hayakawa, 1999). In patients with congestive heart failure, intravenous infusion

of AM decreases mean arterial pressure but to a lesser extent than in normal patients (Nagaya, 2000). The fall in mean arterial pressure is associated with a significant increase in heart rate (Nagaya, 2000). Infusion of AM causes a greater and more prolonged reduction of mean arterial pressure than that of an equimolar amount of atrial natriuretic peptide (Oya et al., 2000). In patients with pulmonary hypertension, AM administration also decreases pulmonary vascular resistance and is a potential new treatment for this condition (Nagaya, 2004).

Diuresis and natiuresis

In vivo studies in sheep have shown that intravenously administered AM causes diuresis, in an experimental model of heart failure (Rademaker, 1997). Natriuretic activity that is characterized by increased glomerular filtration and decreased sodium reabsorption was also demonstrated in a NO-dependent manner (Elhawary, 1995; Majid, 1996). Even in normal animals, AM causes diuresis and natriuresis as well as vasodilation (Majid, 1996; Parkes, 1997). Systemically administered AM increases urine volume and urinary sodium excretion in patients with congestive heart failure, consistent with results obtained from earlier animal studies (Majid, 1996; Rademaker, 1997; Nagaya, 1999).

Inhibition of aldosterone production

The renin-angiotensin-aldosterone system is excessively activated in patients with heart failure, leading to adverse effects related to hypertension. In this setting, infusion of AM significantly and selectively decreases plasma aldosterone in patients with congestive heart failure, although there is no significant change in plasma renin (Nagaya, 2000a). *In vitro* studies showed that AM inhibits Ang II-induced secretion of aldosterone from dispersed rat adrenal zona glomerulosa cells (Yamaguchi, 1995). Thus, we speculate that AM may play a compensatory role in the pathophysiology of heart failure by inhibiting the augmented production of aldosterone thus preventing sodium retention.

Antihypertrophic, anti-apoptotic and antifibrotic effects

AM has direct cardioprotective effects in reducing ventricular remodeling following MI in rats in the absence of changes in mean arterial pressure (Nakamura, 2002). In this regard, it is a possible endogenous suppressor of myocyte hypertrophy and fibroblast proliferation (Tsuruda, 1998, 1999). AM inhibits collagen synthesis and cardiac fibroblast proliferation, and AM+/− mice have increased fibrosis compared to wildtype littermates (Nishikimi, 2005; Niu, 2004). As well, it confers an anti-apoptotic effect on cardiomyocytes via the PI3K/Akt pathway (Okumura, 2004). Inhibition of vascular endothelial cell apoptosis and induction of angiogenesis by AM also occurs through the PI3K/Akt pathway (Kim, 2002; Tokunaga, 2004). Thus, AM has protective effects in the myocardium and vasculature, which may have beneficial effects in patients with congestive heart failure.

Angiogenesis

An angiogenic effect of AM could partly explain the protective effect of AM seen following myocardial infarction. Recent studies using homozygous AM knockout mice highlight the importance of AM in vascular morphogenesis (Caron, 2001; Imai, 2001; Shindo, 2001). AM signaling is of particular significance in endothelial cell biology since the peptide protects cells from apoptosis (Kato, 1997), promotes angiogenesis (Kim, 2003; Oehler, 2002) and affects vascular tone (Ishizaki, 1994). AM activates the PI3K/Akt-dependent pathway in vascular endothelial cells which is considered to regulate multiple critical steps in angiogenesis, including endothelial cell survival, proliferation, migration, and capillary-like structure formation (Jiang, 2000; Nishimatsu, 2001). *In vitro*, AM enhances endothelial cell migration and neovessel formation, a function that is attenuated by PI3K and ERK inhibition (Kim, 2003). Similarly, AM promotes re-endothelialization in an injury model via PKA and PI3K-dependent Akt activation in HUVECs (Miyashita, 2003). Interestingly, AM inhibits the proliferation and migration of vascular SMCs (Horio, 1995; Kano, 1996).

A recent study demonstrated that heterozygous AM+/− mice show significantly less blood flow recovery with less collateral capillary development than their wild-type counterparts (Iimuro, 2004). AM gene transfer promotes blood flow recovery and capillary formation in a murine model of chronic hind limb ischemia (Tokunaga, 2004). Taken together, these findings raise the possibility that AM plays a role in modulating angiogenesis and neovascularization.

Antioxidant effects

Oxidative stress injury is induced by reactive oxygen species (ROS), the most common being free radicals and anions containing reactive oxygen atoms. Damage by

excess oxidative stress is a major metabolic abnormality in atherosclerosis as well as myocardial infarction. Oxidative stress caused by H_2O_2 in endothelial cells can trigger AM transcription and increased expression (Chun, 2000). Such expression may be protective as angiotensin II-stimulated intracellular reactive oxygen species generation was directly blocked by AM, possibly in a cAMP-dependent manner (Yoshimoto, 2005).

Pro- and anti-inflammatory effects of AM

The effects of AM on inflammation are still unclear and appear to differ based on the disease model. For instance, ocular inflammation is increased with AM whereas acetic acid-induced colonitis is attenuated (Ashizuka, 2005; Clementi, 1999). In a cultured macrophage cell line, AM increased secretion of IL-6 but slightly reduced TNF-α levels (Wong, 2005). Furthermore, AM can induce expression of the cell surface adhesion molecules E-selection, VCAM-1, and ICAM-1, critical for leukocyte migration, on cultured endothelial cells (Hagi-Pavli, 2004). However, Kim et al. (2003) showed that AM inhibits VEGF-induced expression of these adhesion molecules. Thus, it appears that AM can act to both promote and inhibit inflammation depending on the cell type and disease of interest.

Conclusion and future studies

Since its discovery, there has been great interest in AM as a promising endogenous peptide for the treatment of cardiovascular diseases. Considering that AM is an endogenous neurohormonal peptide, it may be more readily accepted as a therapeutic agent. Its vasodilatory, inotropic, antiapoptotic, natuiretic and diuretic actions afford this molecule a significant potential clinical advantage in terms of cardiac injury and vascular alterations seen in cardiac diseases. However, it is still unclear which combination of effects of AM is triggered to produce the observed beneficial effects in experimental models and in patients. Several clinical studies have tested the effect of AM in hypertension, ischemic heart disease and pulmonary hypertension. Although their data looks promising, a prospective randomized control trial is needed to reinforce the pharmaceutical prospects of AM.

The optimum timing, amount and method of AM delivery are still under investigation. Although AM has a relatively short half-life compared to other peptides, it is still vulnerable to rapid degradation by renal neural endopeptidases *in vivo*. Thus, in a clinical setting, AM is likely to have an optimal benefit in acute diseases. To address this point, Nagaya et al. (2004) have investigated the efficacy of three types of AM delivery systems: intravenous administration, inhalation, and cell-based gene transfer. Interestingly, ionically-linked DNA-gelatin complexes were able to block chronic hind limb ischemia when delivered by direct muscular injection (Tokunaga, 2004).

Despite unanswered questions regarding the precise mechanism and optimum method of delivery of AM in a clinical setting, the data thus far fully warrants the excitement felt about its potential benefit as a treatment for cardiac diseases. We greatly look forward to the prospects of clinical trials using AM in the setting of cardiovascular illnesses.

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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 $\mu g \cdot kg^{-1} \cdot min^{-1}$) 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

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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 $\mu g \cdot kg^{-1} \cdot min^{-1}$, 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 cytospin and Wright-Giemsa staining.

Tumor necrosis factor-α and cytokine-induced neutrophil chemoattractant assays. BALF tumor necrosis factor (TNF)-α and cytokine-induced neutrophil chemoattractant (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 bromcresol 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.

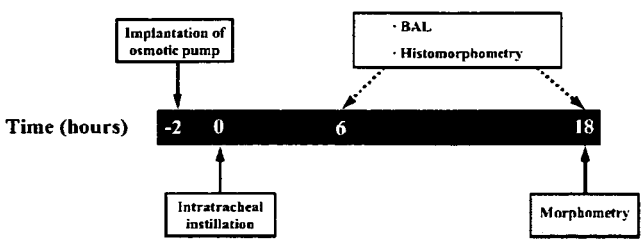

Fig. 1. Study protocol timeline. Implantation of osmotic pump, intratracheal instillation, bronchoalveolar lavage (BAL), histomorphometry, and morphometry were performed.

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. 2*A*). 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. 2*B*). 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

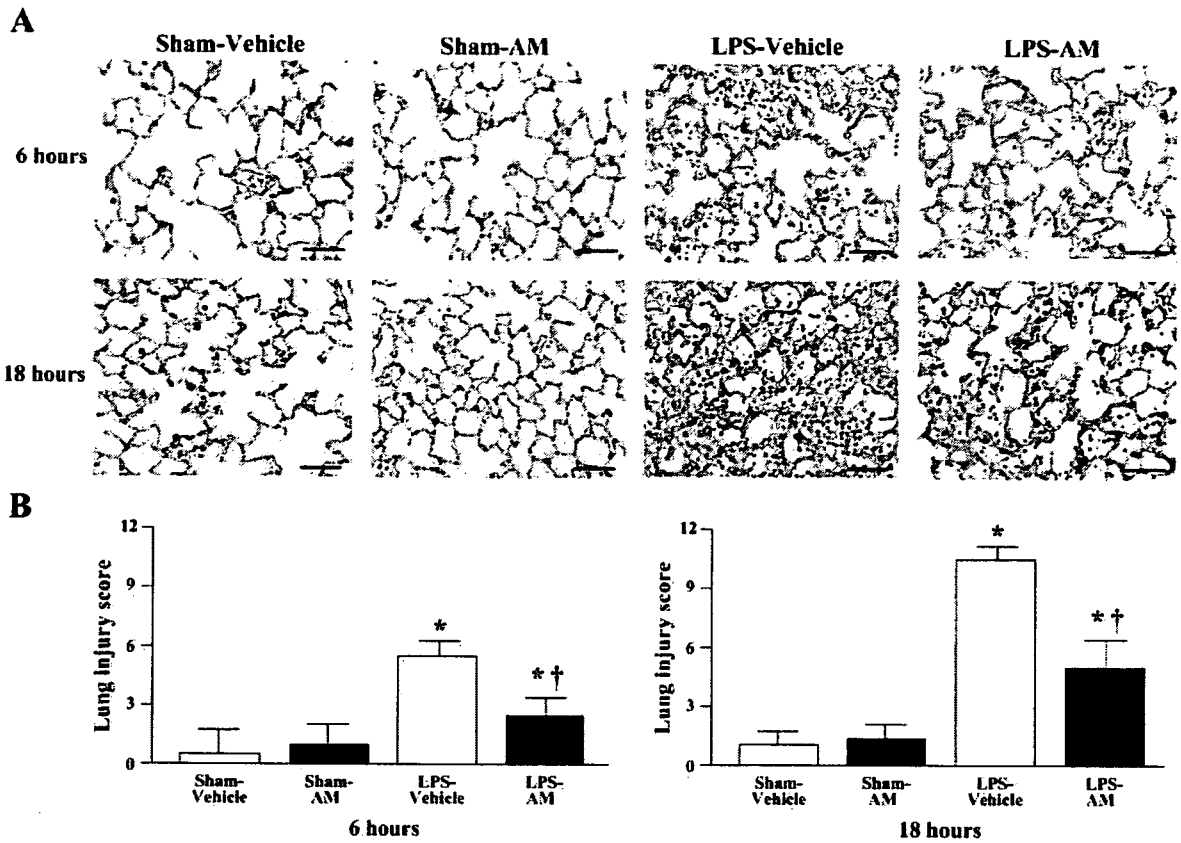

Fig. 2. *A*: representative photomicrographs of lung tissues stained with hematoxylin and eosin at 6 and 18 h after lipopolysaccharide (LPS) instillation. Intratracheal instillation of LPS caused infiltration of inflammatory cells into lung interstitium and alveolar spaces, alveolar wall thickening, 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; $\dagger P < 0.05$ vs. LPS-Vehicle.

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. 4*C*). Similarly, the BALF CINC level was significantly increased after LPS instillation (Fig. 4*D*). AM infusion significantly attenuated the increases in BALF TNF-α and CINC levels. AM infusion did not significantly alter BAL data in sham rats.

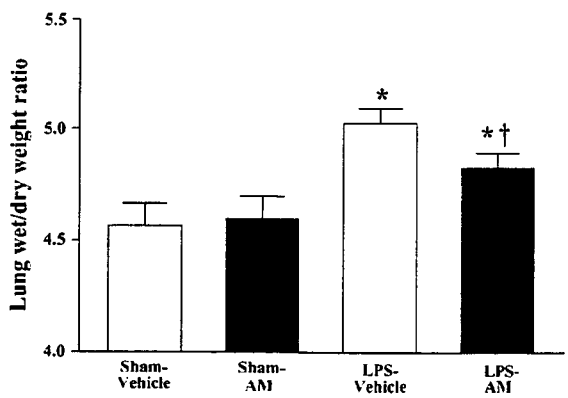

Fig. 3. Effect of AM infusion on lung weight after LPS instillation. Data are means ± SE. $*P < 0.05$ vs. Sham-Vehicle; $\dagger P < 0.05$ vs. LPS-Vehicle.

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. 6*A*). 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. 6*B*). AM infusion did not significantly change the percentage of cleaved caspase-3-positive inflammatory cells compared with vehicle infusion (3 ± 2 vs. 4 ± 1%).

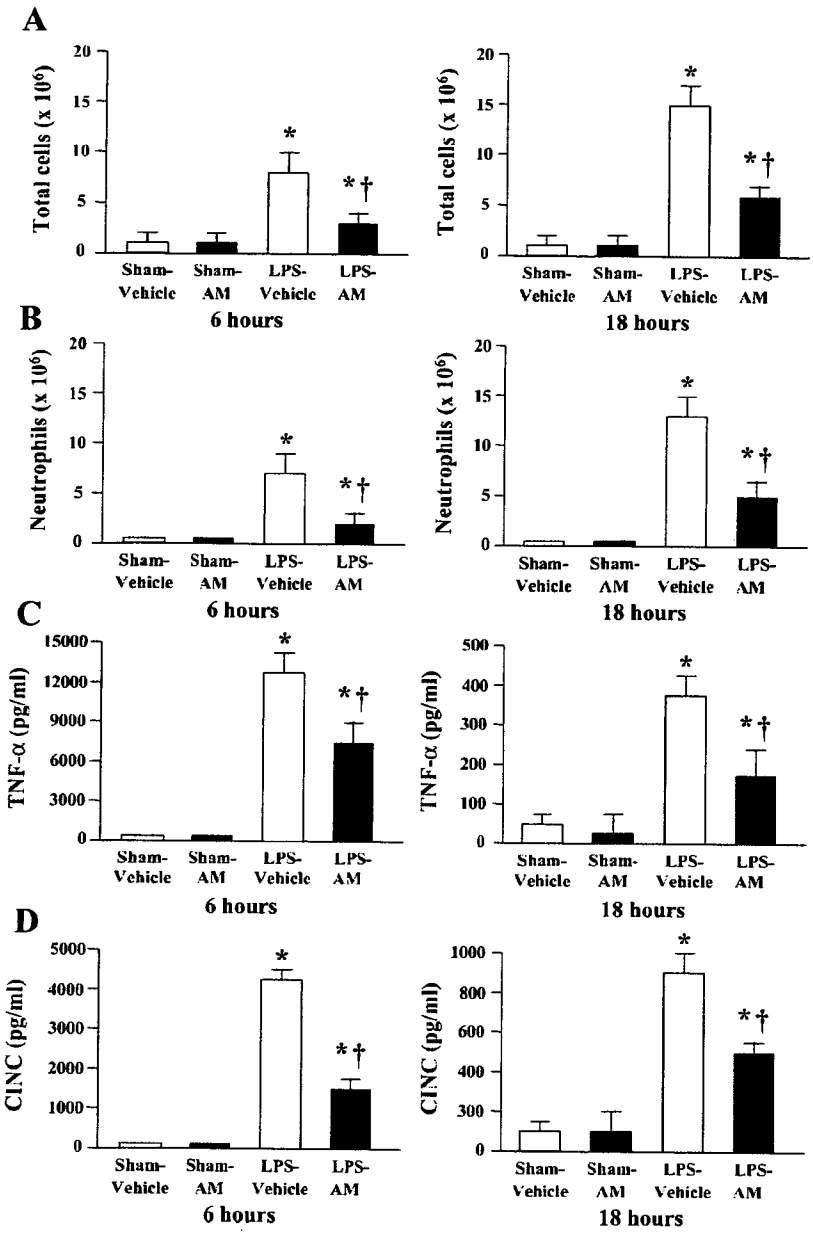

Fig. 4. Effects of AM infusion on numbers of total cells (*A*) and neutrophils (*B*) and levels of tumor necrosis factor (TNF)-α (C) and cytokine-induced neutrophil chemoattractant (CINC; D) in BAL fluid (BALF) at 6 and 18 h after LPS instillation. Numbers of total cells and neutrophils were significantly increased at 6 and 18 h after LPS instillation. However, numbers of these cells in LPS-AM group were significantly lower than those in LPS-Vehicle group. AM infusion significantly decreased BALF TNF-α and CINC levels. Data are means ± SE. $*P < 0.05$ vs. Sham-Vehicle; $\dagger P < 0.05$ 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.

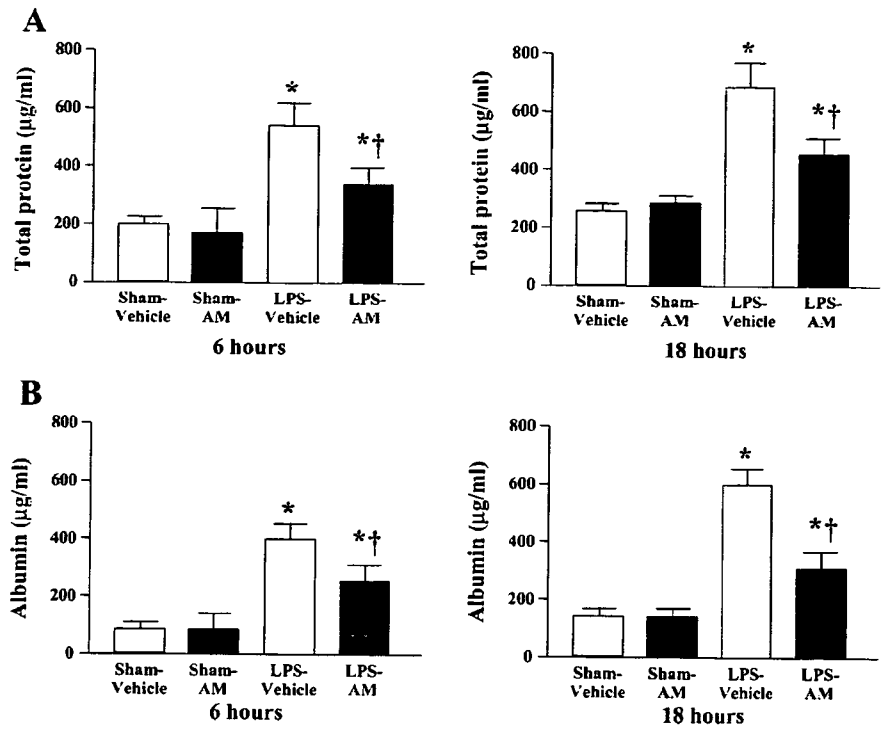

Fig. 5. Effects of adrenomedullin (AM) infusion on BALF total protein (*A*) and albumin (*B*) at 6 and 18 h after LPS instillation. AM infusion significantly reduced BALF total protein and albumin levels. Data are means ± SE. $^*P < 0.05$ vs. Sham-Vehicle; $\dagger P < 0.05$ vs. LPS-Vehicle.

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

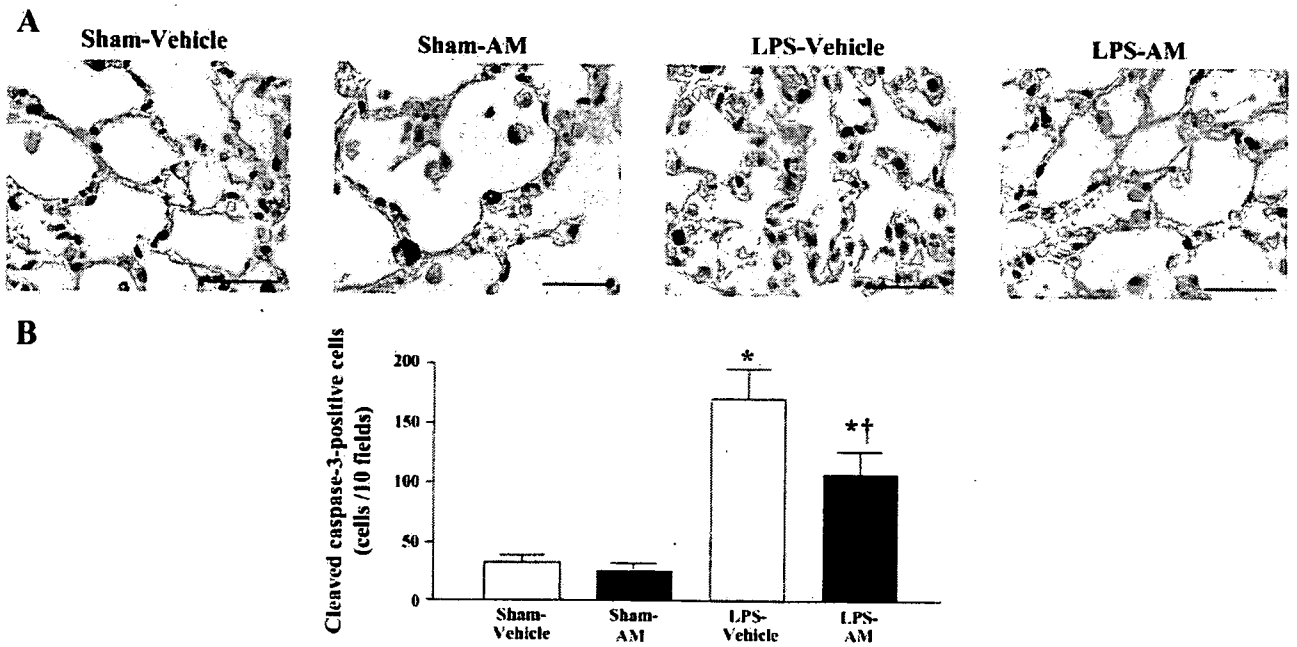

Fig. 6. *A*: immunohistochemical demonstration of cleaved caspase-3 antigen, a marker for cell apoptosis, in lungs at 6 h after LPS instillation. Scale bars, 20 μm. *B*: semiquantitative analysis of cleaved caspase-3-positive alveolar wall cells. Number of cleaved caspase-3-positive alveolar wall cells was significantly decreased in LPS-AM group compared with LPS-Vehicle group. Data are means ± SE. $^*P < 0.05$ vs. Sham-Vehicle; $\dagger P < 0.05$ 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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Infusion of adrenomedullin improves acute myocarditis *via* attenuation of myocardial inflammation and edema

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Abstract

Objective: Our aim was to assess whether adrenomedullin (AM), a potent vasodilator peptide with a variety of cardioprotective effects, has a therapeutic potential for the treatment of acute myocarditis in a rat model.
Methods: One week after myosin injection, rats received a continuous infusion of AM or vehicle for 2 weeks, and pathological and physiological investigations were performed.
Results: AM treatment significantly reduced the infiltration of inflammatory cells in myocarditic hearts, and decreased the expressions of macrophage chemoattractant protein-1, matrix metalloproteinase-2 and transforming growth factor-β. Myocardial edema indicated by increased heart weight to body weight ratio and wall thickness was attenuated by AM infusion (5.7±0.5 *vs.* 6.5±0.4 g/kg, and 1.9±0.3 *vs.* 2.8±0.5 mm, respectively). Infusion of AM significantly improved left ventricular maximum dP/dt and fractional shortening of myocarditic hearts (4203±640 *vs.* 3450±607 mm Hg/s, and 21.3±4.1 *vs.* 14.7±5.1%, respectively).
Conclusion: Infusion of AM improved cardiac function and pathological findings in a rat model of acute myocarditis. Thus, infusion of AM may be a potent therapeutic strategy for acute myocarditis.

Keywords: Autoimmune myocarditis; Adrenomedullin; Angiogenesis; Inflammation

1. Introduction

Acute myocarditis is a non-ischemic heart disease characterized by myocardial inflammation and edema. This disease is associated with rapidly progressive heart failure, arrhythmias and sudden death [1]. Although early evidence for efficacy of immunoglobulin and interferon therapy appears promising, these results have yet to be demonstrated in randomized or controlled clinical trials [2]. Current therapeutic options are restricted to supportive care for heart failure or arrhythmias [3]. The lack of specific treatment and the potential severity of the illness emphasize the importance of novel and effective therapeutic strategies for myocarditis.

Adrenomedullin (AM) is a potent vasodilator peptide that was originally isolated from human pheochromocytoma [4]. Earlier studies have shown that AM has beneficial hemodynamic effects on failing hearts *via* its vasodilatory action and diuretic effects [5,6]. Furthermore, AM has direct cardioprotective effects such as anti-inflammatory effects [7], inhibition

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of apoptosis [8], induction of angiogenesis [9] and attenuation of myocardial hypertrophy [10]. Interestingly, AM has also been shown to decrease endothelial hyperpermeability in the heart [11]. These findings raise the possibility that infusion of AM may attenuate myocardial inflammation and edema in acute myocarditis. Although previous findings have demonstrated that infusion of AM is effective for heart failure, its therapeutic effects in acute myocarditis are still unknown.

Experimental autoimmune myocarditis can be induced in rats by immunizing them with cardiac myosin, providing a model that resembles human giant cell myocarditis [12,13]. Although the majority of acute myocarditis is linked to a viral infection such as coxsackievirus B3, this viral infection can in some cases cause an autoimmune myocarditis with chronic myocardial inflammation without viral persistence, due to the exposure of autoantigens such as cardiac myosin to the immune system [14,15].

Thus, the purposes of this study were 1) to investigate whether infusion of AM improves cardiac function and pathological findings including myocardial inflammation and edema in rats with myosin-induced myocarditis, and 2) to investigate the underlying mechanisms responsible for the effects of AM.

2. Methods

2.1. Experimental autoimmune myocarditis

Purified cardiac myosin from the ventricular muscle of pig hearts was prepared according to a procedure described previously [16]. The antigen was dissolved at a concentration of 20 mg/ml in phosphate-buffered saline (PBS) containing 0.3 M KCl mixed with an equal volume of complete Freund's adjuvant (CFA) containing 11 mg/ml of *Mycobacterium tuberculosis* (Difco Laboratories, Sparks, MD, USA).

Male 10-week-old Lewis rats were used in the present study. Rats were anesthetized by intraperitoneal injection of pentobarbital (30 mg/kg) and were given an injection of either 0.2 ml of antigen–adjuvant emulsion or saline mixed with CFA into the footpad. One week after myosin injection, an osmotic pump (Alzet, Cupertino, CA, USA) was filled with either AM (0.05 μg/kg/min) or PBS for 2 weeks, and implanted subcutaneously between the scapulae. This protocol resulted in the creation of 3 groups ($n=11$ in each group): sham rats given PBS (sham group), myosin-treated rats given PBS (control group), and myosin-treated rats given AM (AM group). The dose of AM used in this study has anti-apoptotic effects without significant hypotension [8]. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.2. Histopathology

After completion of hemodynamic measurements on day 21 post-myosin injection, the heart was excised above the origin of the great vessels, and ventricular weight was recorded. Midventricular portions of the heart were formalin-fixed and embedded in paraffin, and 4 μm-sections were cut and stained with hematoxylin and eosin (H&E). H&E-stained sections were graded by a cardiovascular pathologist (H.I-U.) as described previously [17]. Briefly, coagulation necrosis, granulation, inflammation and edema were evaluated without knowledge of the experimental groups on the following scale: 0, no or questionable presence; 1, limited focal distribution less than 25% area of the section; 2, intermediate severity covering less than 50% area of the section; 3, intermediate severity covering greater than 50% and less than 75% area of the section; and 4, coalescent and extensive foci more than 75% area to the entirety of the transversely sectioned ventricular tissue (5 fields per rat, $n=8$ in each group).

2.3. Picrosirius red staining

Paraffin-embedded sections were submitted for picrosirius staining for total collagen distribution. Slides were hydrated, placed in Weigert's iron hematoxylin and in Bouin's fluid (70% saturated aqueous picric acid, 5% acetic acid, 25% formalin) for 10 min. The slides were rinsed in distilled water and placed in 0.025% picrosirius red solution overnight. The sections were rinsed, dehydrated, cleared, and mounted. Amount of collagen stain was quantitated using image analysis software on high-powered (×200) cross-sectional images (10 fields per rat, $n=5$ in each group).

2.4. Immunohistochemistry

Paraffin-embedded heart sections were washed in increasing concentrations of ethanol and then in PBS. Immunohistochemical staining of the sections was performed with antibodies raised against macrophage chemoattractant protein-1 (MCP-1) (BD Bioscience Pharmingen, San Jose, CA, USA) or CD68 (DakoCytomation, Glostrup, Denmark), a marker of monocytes and macrophages. The number of CD68-positive cells was counted with a light microscope (×200, 10 fields per rat, $n=6$ in each group). To detect capillary endothelial cells, immunohistochemical staining of the sections was performed with a rabbit polyclonal antibody raised against von Willebrand factor (vWF, DakoCytomation). The number of capillary vessels was counted using a light microscope (×200, 10 fields per rat, $n=6$ in each group).

2.5. Western blot analysis

Western blot was performed as previously described [18]. Briefly, LV tissues were homogenized in 0.1% Tween-20 with a protease inhibitor, loaded (40 μg) on a 7.5% sodium dodecyl sulfate-polyacrylamide gel, and blotted onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). After blocking for 2 h, membranes were incubated with MMP-2 (Laboratory Vision, Fremont, CA, USA) or MMP-9

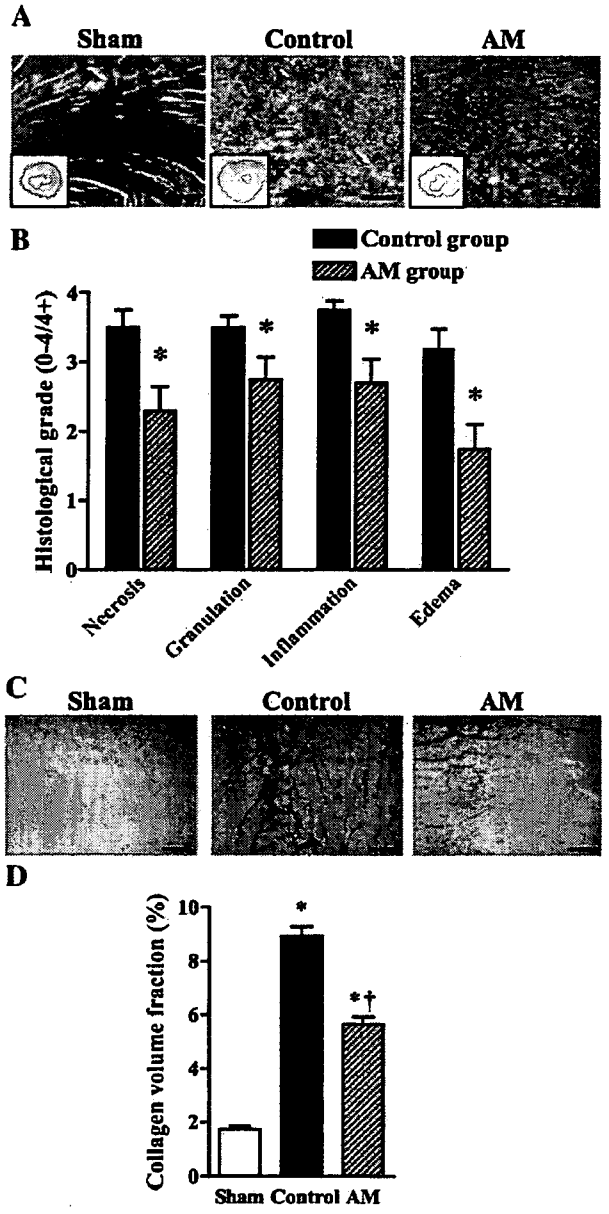

Fig. 1. Pathological findings in acute myocarditis after AM infusion. A: Representative H&E staining of myocardial sections showed markedly decreased inflammation and tissue necrosis in AM-treated hearts as compared to control hearts. Insets are transverse sections of myocardial freewall. B: Semi-quantitative histological grades for necrosis and tissue granulation as well as for inflammation and edema were significantly lower in AM-treated hearts as compared to control hearts ($n=8$ in each group). Sham tissues exhibited no measurable pathological changes. Data are mean±S.E. *, $P<0.05$ *vs.* control. C: Representative picrosirius staining showed decreased collagen deposition in AM-treated hearts as compared to control hearts. D: Collagen volume fraction in 10 random representative fields (×200) confirmed a significant decrease in AM-treated hearts *vs.* control hearts ($n=5$ in each group). Scale bars: 50 μm. Data are mean±S.E. *, $P<0.05$ *vs.* sham; †, $P<0.05$ *vs.* control.

(Chemicon, Temecula, CA, USA) rabbit polyclonal antibodies (1:200), then incubated with peroxidase labeled with secondary antibody (1:1000). Positive protein bands were visualized with an ECL kit (GE Healthcare, Piscataway, NJ, USA) and measured by densitometry. A mouse polyclonal antibody against β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a control ($n=5$ in each group).

2.6. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

Heart tissues ($n=5$ in each group) were homogenized with TissueLyser (Qiagen, Hilden, Germany). Total RNA was extracted using RNeasy Mini Kit (Qiagen), followed by reverse transcription into cDNA using the avian myeloblastosis virus transcriptase (Ambion, Austin, TX, USA), according to the manufacturers' protocol. PCR amplification was performed in 50 μl containing 1 μl of cDNA and 25 μl of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The following sequence-specific primers were used for TGF-β, as described previously [19]: forward, 5′-GTTCTTCAATACGTCAGACATTCG-3′; reverse, 5′-CATTATCTTTGCTGTCACAAGAGC-3′. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA amplified from the same samples was served as an internal control: forward, 5′-GAACATCATCCCTGCATCCA-3′; reverse, 5′-CCAGTGAGCTTCCCGTTCA-3′. After an initial denaturation at 95 °C for 10 min, a 2-step cycle procedure was used (denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min) for 40 cycles in a 7700 sequence detector (Applied Biosystems). The data were analyzed with Sequence Detection Systems software.

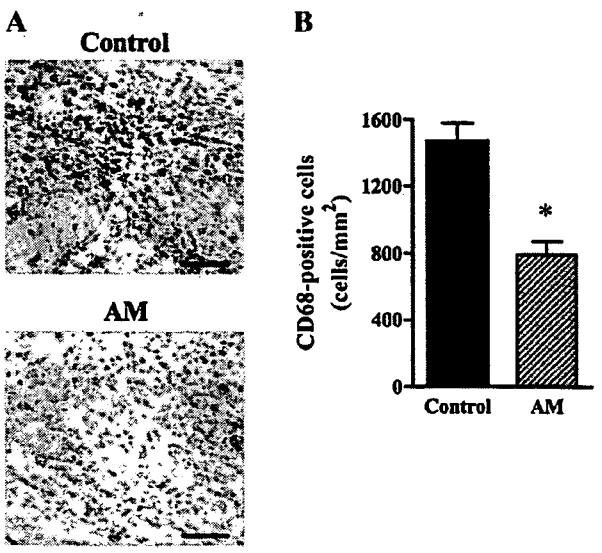

Fig. 2. Infiltration of inflammatory cells in myocardium. A: Immunohistochemical analysis of CD68-positive cell infiltration in myocardium. AM infusion markedly attenuated the increase in CD68-positive cells in myocarditic hearts. Scale bars: 50 μm. B: Semi-quantitative analysis of CD68-positive cell infiltration. CD68-positive cells in 10 random representative high-power fields (×200) confirmed a significant decrease in AM-treated hearts *vs.* control hearts ($n=6$ in each group). Data are mean±S.E. *, $P<0.05$ *vs.* control.

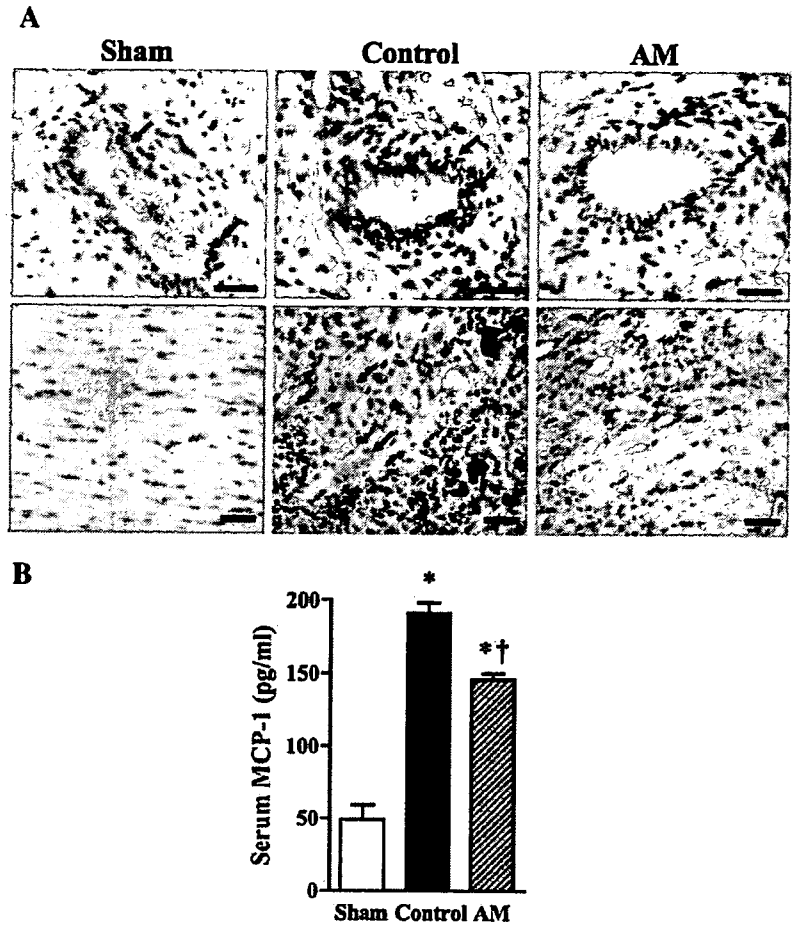

Fig. 3. Effects of AM infusion on MCP-1 expression. A: Representative myocardial sections immunohistochemically stained with anti-MCP-1 antibody showed increased vascular endothelial and myocyte staining of MCP-1 (arrows) and the presence of giant cells (arrowheads) in control hearts as compared to AM-treated hearts. Sham hearts showed subtle endothelial staining. Scale bars: 20 μm. B: Serum MCP-1 level was greatly increased in myocarditic rats. However, the increase in serum MCP-1 was significantly attenuated by AM infusion ($n=6$ in each group). Data are mean±S.E. *, $P<0.05$ *vs.* sham; †, $P<0.05$ *vs.* control.

2.7. Enzyme-linked immunosorbent assay (ELISA)

To investigate the effect of AM infusion on serum MCP-1 level, blood was drawn from the heart before excision ($n=6$ in each group). Blood was centrifuged and serum samples were frozen and stored at −80 °C. Serum MCP-1 level was measured by ELISA according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

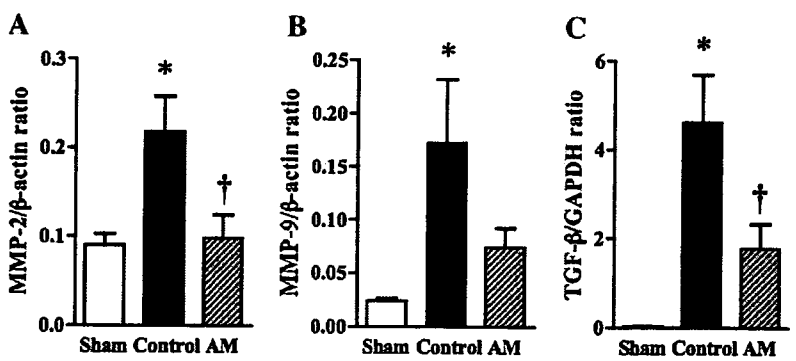

Fig. 4. Effects of AM infusion on MMP and TGF-β expression. A and B: Western blot analysis for MMP-2 (A) and -9 (B) expression. Levels of MMP-2 and -9 were significantly increased in control hearts. MMP-2 expression was markedly decreased by AM infusion, and MMP-9 expression tended to be decreased after AM infusion ($n=5$ in each group). C: Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) for TGF-β expression. Expression of TGF-β was increased in myocarditis and significantly decreased by AM treatment ($n=5$ in each group). Data are mean±S.E. *, $P<0.05$ *vs.* sham; †, $P<0.05$ *vs.* control.

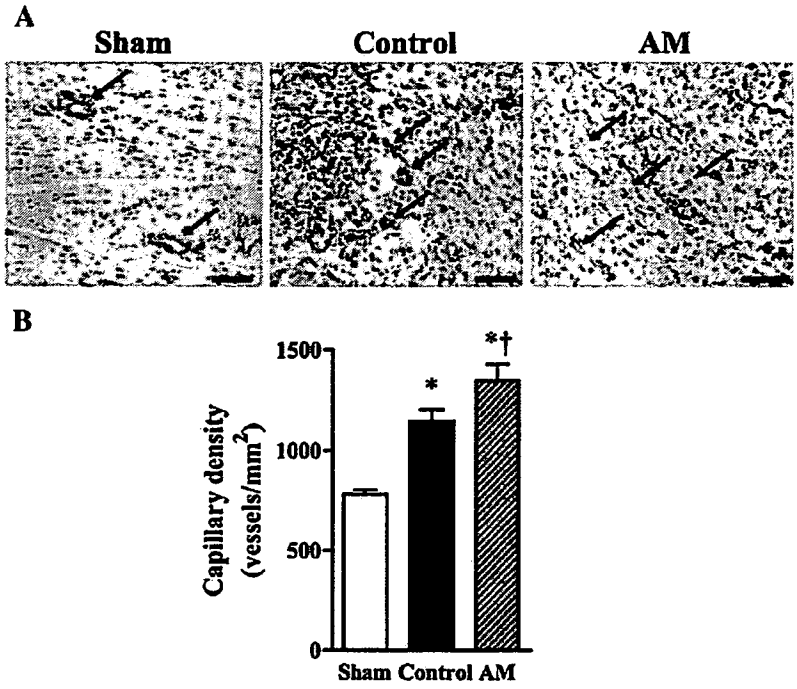

Fig. 5. Increased endothelial regeneration with AM infusion. A: Immunohistochemical demonstration of von Willebrand factor in myocardium. Arrows indicate microvasculature. Scale bars: 20 μm. B: Capillary density measured in 10 random representative high-power fields (×200) showed a significant increase in control hearts and a further increase in AM-treated hearts *vs.* sham hearts ($n=6$ in each group). Data are mean±S.E. *, $P<0.05$ *vs.* sham; †, $P<0.05$ *vs.* control.

2.8. Hemodynamic study

Hemodynamic measurements were taken on day 21 post-myosin injection ($n=7$ in each group). Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg) as a supplement to maintain mild anesthesia. A 1.5 Fr micromanometer-tipped catheter (Millar Instruments, Houston, TX, USA) was advanced into the left ventricle through the right carotid artery, and a polyethylene catheter (PE-50) was advanced into the right ventricle through the right jugular vein to measure right ventricular pressure. Heart rate was also monitored by electrocardiography. As hemodynamic indices, heart rate, mean arterial pressure, LV end-diastolic pressure, maximum dP/dt, and minimum dP/dt were used.

2.9. Echocardiography

Echocardiography was performed on day 21 post-myosin injection. A 12-MHz probe was placed in the left 4th intercostal space for M-mode imaging using 2D echocardiography (Sonos 5500, Philips, Bothell, WA, USA). M-mode tracings were obtained at the level of the papillary muscles. Anterior and posterior end-diastolic wall thickness, left ventricular (LV) end-diastolic and end-systolic dimension, LV fractional shortening (FS), and LV ejection fraction (EF) were measured in three consecutive cardiac cycles by the American Society for Echocardiology leading-edge method ($n=10$ in each group). EF and FS were calculated from the following formula, respectively:

$$EF = (\text{end-diastolic volume} - \text{end-systolic volume}) / \text{end-diastolic volume}$$

$$FS = (\text{end-diastolic diameter} - \text{end-systolic diameter}) / \text{end-diastolic diameter}$$

2.10. Statistical analysis

All data were expressed as mean±S.E. Comparisons of parameters among the groups were made by one-way

Table 1
Physiological profiles of three experimental groups

	Sham	Control	AM
Body weight, g	236±2	197±2*	199±2*
Ventricular weight, g	0.70±0.01	1.28±0.02*	1.15±0.03*†
Lung/body weight	4.9±0.4	4.9±0.5	5.0±0.8
Heart rate, bpm	432±10	373±11	393±6
MAP, mm Hg	103±3	77±5*	93±3†
LVSP, mm Hg	127±3	103±5*	117±3†
LVEDP, mm Hg	4±1	21±5*	14±3

Sham, sham rats given vehicle; Control, myosin-treated rats given vehicle; AM, myosin-treated rats given AM; MAP, mean arterial pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure. Data are mean±S.E. *$P<0.05$ *vs.* sham; †$P<0.05$ *vs.* control. $n=7$ in each group.