may have some cell toxic effects and cell or membrane fusion can lead to labeling of neighboring cells in the target tissue. Second, the present study demonstrated that AM prolongs MNC survival through the PI3-kinase/Akt pathway and enhances neovascularization in a peri-infarcted area. However, further studies are necessary to examine the effect of AM on MNC differentiation into endothelial cells.

Autologous cell transplantation may be an alternative treatment for ischemic heart disease in the clinical setting. Because their use does not require immunosuppression, the clinical use of MNC for cellular cardiomyoplasty appears to be most advantageous. Administration of AM peptide is simple and relatively noninvasive. We and others (12, 16, 17) have reported the safety of AM infusion in humans. Thus combination therapy using AM infusion and MNC transplantation may be a new therapeutic strategy for the treatment of ischemic heart disease.

In conclusion, infusion of AM enhanced the angiogenic potency of MNC transplantation and improved cardiac function in rats with myocardial infarction. This beneficial effect may be mediated partly by the angiogenic property of AM itself and by its antiapoptotic effect on MNC. Thus combination therapy using AM infusion and MNC transplantation may be a new therapeutic strategy for the treatment of ischemic heart disease.

GRANTS

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C-type Natriuretic Peptide Ameliorates Monocrotaline-induced Pulmonary Hypertension in Rats

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C-type natriuretic peptide (CNP) has been shown to act as a local regulator of vascular tone and remodeling. We investigated whether CNP ameliorates monocrotaline (MCT)-induced pulmonary hypertension in rats. Rats received a continuous infusion of CNP or placebo. Significant pulmonary hypertension developed 3 weeks after MCT. However, infusion of CNP significantly attenuated the development of pulmonary hypertension and vascular remodeling. Neither systemic arterial pressure nor heart rate was altered. Interestingly, CNP enhanced Ki-67 expression, a marker for cell proliferation, in pulmonary endothelial cells and augmented lung tissue content of endothelial nitric oxide synthase. CNP significantly suppressed apoptosis of pulmonary endothelial cells, decreased the number of monocytes/macrophages, and inhibited expression of plasminogen activator inhibitor type 1, a marker for fibrinolysis impairment, in the lung. In addition, CNP significantly increased the survival rate in MCT rats. Finally, infusion of CNP after the establishment of pulmonary hypertension also had beneficial effects on hemodynamics and survival. In conclusion, infusion of CNP ameliorated MCT-induced pulmonary hypertension and improved survival. These beneficial effects may be mediated by regeneration of pulmonary endothelium, inhibition of endothelial cell apoptosis, and prevention of monocyte/macrophage infiltration and fibrinolysis impairment.

Keywords: monocrotaline; natriuretic peptides; pulmonary hypertension; vasoprotection

Primary pulmonary hypertension is a rare but life-threatening disease characterized by progressive pulmonary hypertension that leads to right ventricular (RV) failure and death (1). The common pathologic findings in primary pulmonary hypertension are endothelial cell injury, plexiform lesion, medial hypertrophy, infiltration of inflammatory cells, and thrombosis in small pulmonary arteries (2, 3). Endothelial dysfunction decreases the production of vasodilators such as prostacyclin and nitric oxide, whereas it increases that of vasoconstrictors, including thromboxane and endothelin-1 (4, 5). Infiltration of inflammatory cells, which release many cytokines and growth factors, contributes to the development of pulmonary vascular remodeling (6–8). Thrombosis obstructs small pulmonary arteries, which exaggerates pulmonary hypertension (4). Thus, a therapeutic strategy against these abnormalities may be effective for the treatment of primary pulmonary hypertension.

C-type natriuretic peptide (CNP), the third member of the natriuretic peptide family consisting of 22 amino acids (9), is secreted by vascular endothelial cells (10). CNP binds to natriuretic peptide receptor B, which bears a guanylate cyclase, induces generation of cGMP (11), and acts as a local regulator of vascular tone and remodeling (12). Its vasodilatory effect is much less potent than those of atrial natriuretic peptide and brain natriuretic peptide (9, 13). Nevertheless, CNP inhibits the proliferation of vascular smooth muscle cells (14) and has antiinflammatory and antithrombotic effects in blood vessels (15). Moreover, CNP has been shown to induce endothelial regeneration in the injured vasculature (14, 16, 17). These findings raise the possibility that CNP may improve pulmonary hypertension through multiple vasoprotective effects.

Thus, the purpose of this study was to investigate whether continuous infusion of CNP ameliorates monocrotaline (MCT)-induced pulmonary hypertension in rats.

METHODS

Animals

All protocols were performed in accordance with the guidelines of the Animal Care Ethics Committee of the National Cardiovascular Center Research Institute. Male Wistar rats weighing 80 to 100 g were used in this study. Rats were randomly given a subcutaneous injection of either 60-mg/kg MCT or 0.9% saline and assigned to receive a continuous infusion of CNP or placebo. This protocol resulted in the creation of three groups: sham rats given placebo (sham group, n = 8), MCT rats given placebo (placebo group, n = 8), and MCT rats treated with CNP (CNP group, n = 8). Another 10 rats were used to evaluate the acute hemodynamic effect of CNP. An additional 24 rats were used to examine the effect of CNP on established pulmonary hypertension. Finally, 48 rats were used to investigate the effect of CNP on survival in MCT rats.

Experimental Protocol

After the rats were anesthetized by intraperitoneal injection of pentobarbital (30 mg/kg), they were given a subcutaneous injection of either MCT or saline. Then, a micro-osmotic pump (Alzet) was filled with either CNP to deliver a dose of 0.75 μg/hour or 5% glucose vehicle and implanted subcutaneously between the scapulae. Two weeks after implantation, the pump was exchanged under anesthesia. The animals were maintained on standard rat chow.

Hemodynamic studies were performed on Day 21. A polyethylene catheter was inserted into the right carotid artery to measure mean arterial pressure and heart rate. A polyethylene catheter was inserted into the RV to measure RV pressure. After completion of the previously mentioned measurements, the ventricles and lungs were excised, dissected

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free, and weighed. The ratio of RV weight to body weight, the ratio of RV weight to left ventricular plus septal weight, and the ratio of left ventricular plus septal weight to body weight were calculated as indexes of ventricular hypertrophy.

Morphometric Analysis of Pulmonary Arteries

We analyzed the medial wall thickness of the pulmonary arteries in the middle region of the right lung as described previously (18). The external diameter and the medial wall thickness were measured in 20 muscular arteries (ranging in external diameter from 25 to 100 μm) per rat. The medial wall thickness was expressed as follows: percentage of wall thickness = ([medial thickness × 2]/external diameter) × 100.

Immunohistochemical Analysis

Paraffin sections 4 μm thick were obtained from the right lung on Days 7 and 21 from individual rats for comparison among the three groups. To investigate whether CNP induces endothelial regeneration, tissue sections were stained for Ki-67, a marker for cell proliferation, using monoclonal anti–Ki-67 antibody (Dako, Copenhagen, Denmark). Paraffin sections were also stained with a rabbit polyclonal antibody raised against factor VIII (Dako), a mouse monoclonal antibody raised against rat monocyte/macrophage (ED1; Serotec, Oxford, UK), and a rabbit polyclonal antibody raised against plasminogen activator inhibitor type 1 (PAI-1) (Santa Cruz Biotechnology, Santa Cruz, CA). To detect apoptosis in pulmonary endothelial cells 1 week after MCT injection, terminal dUTP nick-end labeling assays were performed using a commercially available kit (ApopTag Plus; Intergen, New York, NY). The number of Ki-67–positive endothelial cells per mm^2 was determined under light microscopy. The numbers of alveoli and factor VIII-positive capillaries (< 100 μm in diameter) were counted. Capillary density was expressed as the number of capillaries per 100 alveoli. The number of ED1-positive cells was determined in 10 randomly chosen high-power fields (×400). The percentage of PAI-1–positive endothelial cells was calculated (number of PAI-1–positive endothelial cells/total number of endothelial cells × 100) in 10 randomly chosen high-power fields (×400). The number of terminal dUTP nick-end labeling–positive endothelial cells per section was calculated. Histologic analysis was performed in a blinded fashion by two observers.

Western Blot Analysis

To identify endothelial nitric oxide synthase, Western blotting was performed using a mouse monoclonal antibody raised against endothelial nitric oxide synthase (Transduction Laboratories, Lexington, KY) as previously described (19). Western blot analysis using a mouse polyclonal antibody against β-actin (Santa Cruz) was used as a protein loading control. Peripheral samples of lung tissue were obtained on Day 21 from individual rats for comparison among the three groups (sham, placebo, and CNP groups, n = 8 each). Endothelial nitric oxide synthase protein was shown as the percentage of the level expressed in sham rats.

Acute Hemodynamic Study

To investigate the acute hemodynamic effects of CNP and atrial natriuretic peptide, CNP (0.05 μg/kg/min) or atrial natriuretic peptide (0.05 μg/kg/min) was intravenously administered at 3 weeks after MCT injection (n = 5 each). Hemodynamics were measured at 15-minute intervals before, during, and after infusion, and the effect of CNP was compared with that of atrial natriuretic peptide.

Delayed Therapy

To investigate the effect of CNP on established pulmonary hypertension, 24 rats were randomly given an injection of either MCT or saline. Three weeks after MCT injection, the animals received continuous infusion of CNP or placebo for 1 week (sham, placebo, and CNP groups, n = 8 each). These rats were evaluated on Day 28.

Survival Analysis

To evaluate the effect of CNP on survival in MCT rats, 24 rats received continuous infusion of CNP (n = 12) or placebo (n = 12) immediately after MCT injection. Another 24 rats received continuous infusion of CNP (n = 12) or placebo (n = 12) 3 weeks after MCT injection. Survival was estimated from the date of MCT injection to the death of the rat or 8 weeks after MCT injection.

Statistical Analysis

All data were expressed as mean ± SEM unless otherwise indicated. Comparisons of parameters among the three groups were made by one-way analysis of variance, followed by Newman-Keul's test. Survival curves were derived by the Kaplan-Meier method and compared by log-rank test. A value of p less than 0.05 was considered statistically significant.

RESULTS

Physiologic and Morphologic Assessment

The physiologic profiles of the three experimental groups are summarized in Table 1. Body weight was significantly lower in MCT rats than in sham rats. The ratio of RV weight to body weight was significantly increased after MCT injection (Figure 1A). However, CNP infusion significantly attenuated the increase in the ratio of RV weight to body weight compared with placebo.

Hemodynamics

RV systolic pressure was significantly increased 3 weeks after MCT injection (Figure 1B). However, CNP infusion significantly attenuated the increase in RV systolic pressure compared with placebo. There was no significant difference in mean arterial pressure or heart rate among the three groups (Table 1).

Morphometric Analysis of Pulmonary Arteries

Representative photomicrographs showed that CNP infusion significantly inhibited hypertrophy of the pulmonary vessel wall compared with placebo (Figure 1C). Quantitative analysis of peripheral pulmonary arteries demonstrated a significant increase in percentage wall thickness after MCT injection, but the increase in the CNP group was significantly inhibited compared with that in the placebo group (Figure 1D).

Endothelial Regeneration

The number of Ki-67–positive endothelial cells was significantly increased in the CNP group compared with the placebo group (Figures 2A–2D). The number of terminal dUTP nick-end labeling–positive pulmonary endothelial cells was significantly increased 1 week after MCT injection (Figure 2E). CNP infusion significantly decreased the number of terminal dUTP nick-end labeling–positive pulmonary endothelial cells. Although the capillary density was significantly decreased after MCT injection, CNP significantly increased the capillary density (Figures 3A–3D). Western blot analysis showed that lung tissue content of endothelial nitric oxide synthase protein was significantly decreased after MCT injection (Figures 3E and 3F). However, CNP infusion increased lung tissue content of endothelial nitric oxide synthase protein in MCT rats.

Monocyte/Macrophage Infiltration

Representative photomicrographs showed that CNP infusion markedly inhibited monocyte/macrophage infiltration into the alveolar spaces compared with placebo (Figures 4A–4C). Quantitative analysis demonstrated a significant increase in the number of monocytes/macrophages after MCT injection, but the increase in the CNP group was markedly inhibited compared with that in the placebo group (Figure 4D).

PAI-1 Expression

Representative photomicrographs demonstrated that CNP infusion markedly inhibited PAI-1 expression in pulmonary endothelial cells

TABLE 1. PHYSIOLOGIC PROFILES OF EXPERIMENTAL GROUPS

	Sham	Placebo	CNP
n	8	8	8
BW, g	195 ± 4	173 ± 8*	179 ± 3*
Heart rate, bpm	431 ± 14	455 ± 15	447 ± 13
MAP, mm Hg	124 ± 3	122 ± 4	123 ± 4
RV systolic pressure, mm Hg	35 ± 3	66 ± 4*	51 ± 3*†
RV/BW, g/kg body weight	0.55 ± 0.01	0.95 ± 0.03*	0.74 ± 0.03*†
RV/LV + S, g/g	0.25 ± 0.02	0.40 ± 0.02*	0.31 ± 0.01*†
LV + S/BW, g/kg body weight	2.21 ± 0.04	2.42 ± 0.05	2.36 ± 0.04

Definition of abbreviations: bpm = beats per minute; BW = body weight; CNP = C-type natriuretic peptide; LV + S/BW = ratio of left ventricular plus septal weight to body weight; MAP = mean arterial pressure; RV = right ventricular; RV/BW = ratio of RV weight to body weight; RV/LV + S = ratio of RV weight to left ventricular plus septal weight.

*$p < 0.05$ vs. sham.

†$p < 0.05$ vs. placebo.

These measurements were performed on Day 21. Data are mean ± SEM.

compared with placebo (Figures 5A–5C). Semiquantitative analysis demonstrated a significant increase in the number of plasminogen activator inhibitor type 1 (PAI-1)–positive endothelial cells after MCT injection (Figure 5D). However, the increase in PAI-1–positive cells was significantly inhibited by CNP infusion.

Acute Hemodynamic Effect

Infusion of atrial natriuretic peptide significantly decreased RV systolic pressure and mean arterial pressure (Figure 6). In contrast, CNP did not significantly alter any hemodynamic parameters.

Delayed Therapy

Delayed CNP therapy slightly but significantly attenuated the increases in the ratio of RV weight to body weight and RV systolic pressure compared with placebo (Figures 7A and 7B). There was no significant difference in mean arterial pressure or heart rate among the three groups (data not shown). Morphometric analysis of pulmonary arteries demonstrated that delayed CNP therapy significantly attenuated hypertrophy of the medial wall (Figures 7C and 7D).

Survival Analysis

Kaplan-Meier survival curves demonstrated that rats treated with CNP immediately after MCT injection had a markedly higher survival rate than those given placebo (50% vs. 0% in 8-week survival, log-rank test, $p < 0.001$; Figure 8A). In addition, delayed CNP therapy also increased the survival rate in MCT rats compared with placebo (25% vs. 0% in 8-week survival, $p < 0.01$; Figure 8B).

DISCUSSION

In this study, we demonstrated that (*1*) continuous infusion of CNP ameliorated MCT-induced pulmonary hypertension and vascular remodeling and that (*2*) CNP infusion improved survival in MCT rats without definite adverse effects. We also dem-

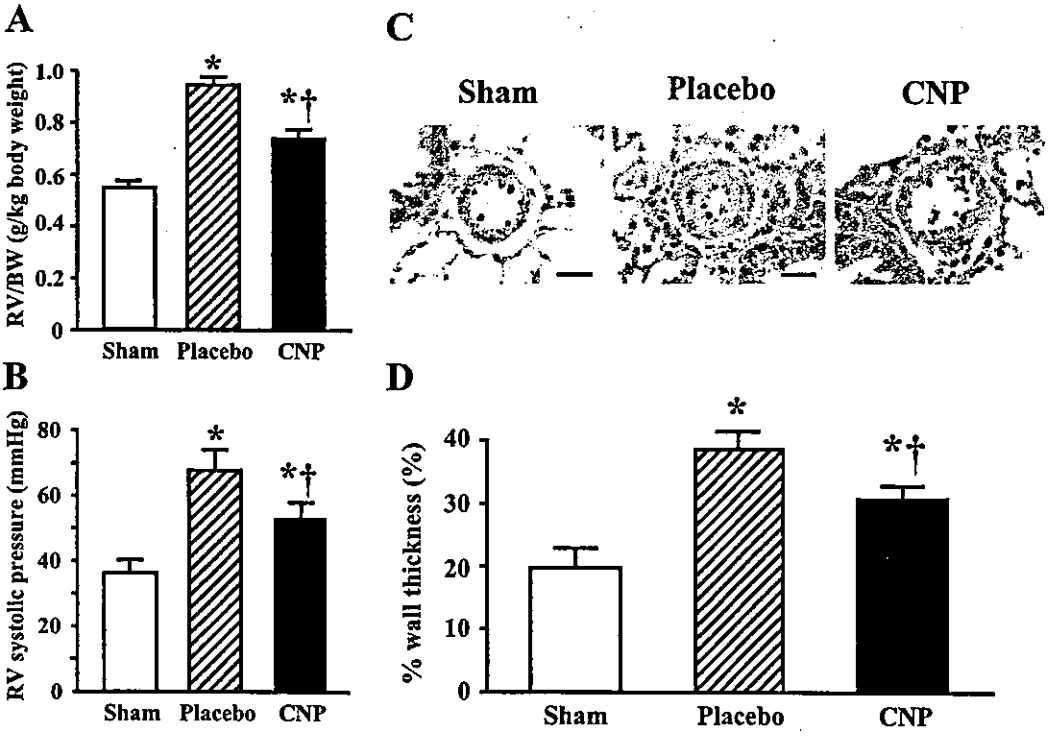

Figure 1. Effects of C-type natriuretic peptide (CNP) infusion on developing pulmonary hypertension. Continuous infusion of CNP was initiated immediately after monocrotaline (MCT) injection. (*A*) Right ventricular (RV) weight to body weight (RV/BW). (*B*) RV systolic pressure. (*C*) Representative photomicrographs of peripheral pulmonary arteries. *Scale bars* = 20 μm. (*D*) Quantitative analysis of percentage wall thickness in peripheral pulmonary arteries. Data are mean ± SEM. *$p < 0.05$ versus sham; †$p < 0.05$ versus placebo.

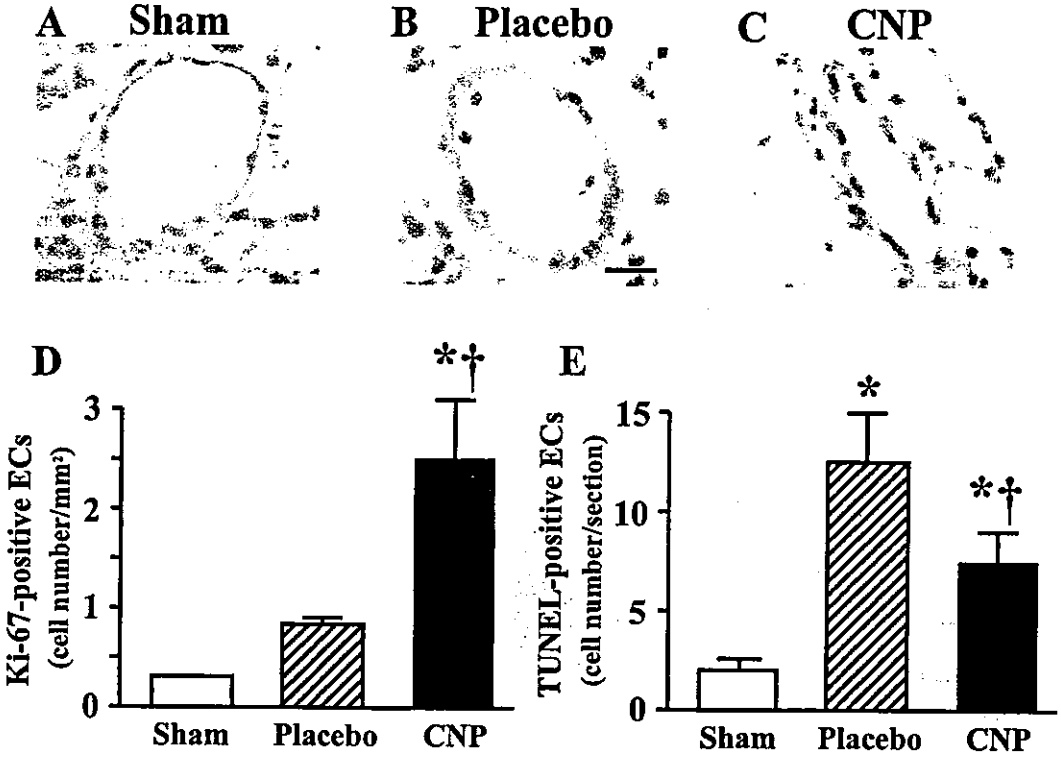

Figure 2. (*A–C*) Immunohistochemical demonstration of Ki-67 antigen, a marker for cell proliferation, in pulmonary endothelial cells (ECs) on Day 7. *Scale bars* = 20 μm. (*D*) Quantitative analysis of Ki-67–positive ECs in pulmonary vessels. The number of Ki-67–positive ECs in the CNP group was significantly increased compared with that in the placebo group. (*E*) Quantitative analysis of terminal dUTP nick-end labeling (TUNEL)-positive ECs in lungs on Day 7. Data are mean ± SEM. *p < 0.05 versus sham; †p < 0.05 versus placebo.

onstrated that (*3*) these effects of CNP may be attributable to regeneration of pulmonary endothelial cells, inhibition of pulmonary endothelial cell apoptosis, and prevention of monocyte/macrophage infiltration and PAI-1 expression.

Endothelial cell injury caused by MCT activates platelets and vasoconstrictive factors, resulting in pulmonary hypertension and vascular remodeling (20). We demonstrated that CNP infusion significantly attenuated the increases in RV systolic pressure and the ratio of RV weight to body weight, suggesting that CNP infusion ameliorates MCT-induced pulmonary hypertension. CNP has been shown to be less expressed than atrial natriuretic peptide (21). Nevertheless, continuous infusion of CNP had benefi-

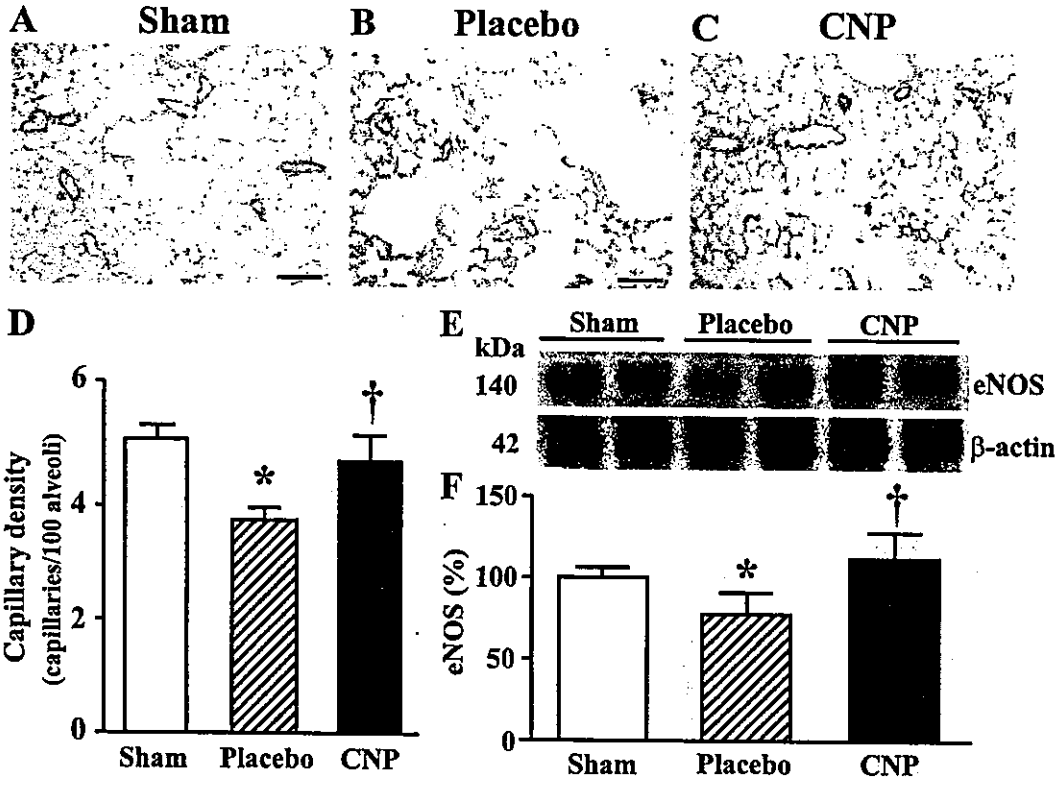

Figure 3. (*A–C*) Immunohistochemical demonstration of factor VIII antigen in lungs on Day 21. *Scale bars* = 100 μm. (*D*) Quantitative analysis of capillary density. (*E*) Representative Western blotting for endothelial nitric oxide synthase (eNOS) and β-actin (protein loading control) in lungs on Day 21. (*F*) Quantitative analysis of lung tissue content of eNOS. eNOS protein is shown as the percent of the level expressed in sham rats. CNP infusion significantly increased eNOS protein compared with placebo. Data are mean ± SEM. *p < 0.05 versus sham; †p < 0.05 versus placebo.

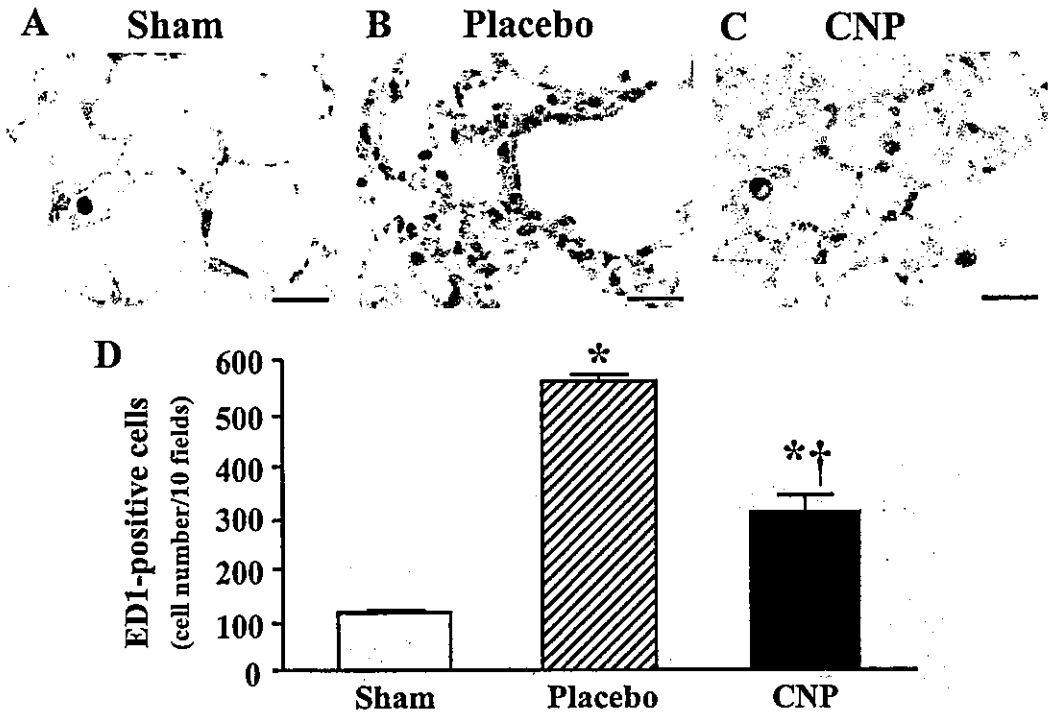

Figure 4. (*A–C*) Immunohistochemical demonstration of ED1 antigen, a marker for rat monocytes/macrophages, in lungs on Day 21. *Scale bars* = 50 μm. (*D*) Quantitative analysis of ED1-positive cells in lungs. The number of ED1-positive cells was significantly decreased in the CNP group compared with the placebo group. Data are mean ± SEM. *$p < 0.05$ versus sham; †$p < 0.05$ versus placebo.

cial effects in MCT rats, even if endogenous CNP had little physiologic significance under the condition of pulmonary hypertension. Earlier studies have shown that the vasodilator effect of CNP is much less potent than those of atrial natriuretic peptide (approximately 1:100) (9, 13, 21). In fact, unlike atrial natriuretic peptide, CNP infusion did not alter any hemodynamic parameters. These findings suggest that the pharmacologic effects of CNP are attributable to vasoprotective effects rather than to vasodilator activity.

MCT induces pulmonary endothelial cell injury and decreases the number of pulmonary capillaries (20, 22), which contributes to the development of MCT-induced pulmonary hypertension. A recent study has demonstrated that transplantation of endothelial progenitor cells attenuates MCT-induced pulmonary hypertension (23), suggesting that endothelial regeneration has beneficial effects on pulmonary hemodynamics. CNP has been shown to induce endothelial regeneration in an ischemic hindlimb model through the cGMP/cGMP-dependent protein kinase pathway (17). In this study, CNP infusion enhanced the expression of Ki-67, a marker for cell proliferation, in pulmonary endothelial cells. In addition, CNP increased the number of pulmonary capillaries in MCT rats. Interestingly, we demonstrated that CNP infu-

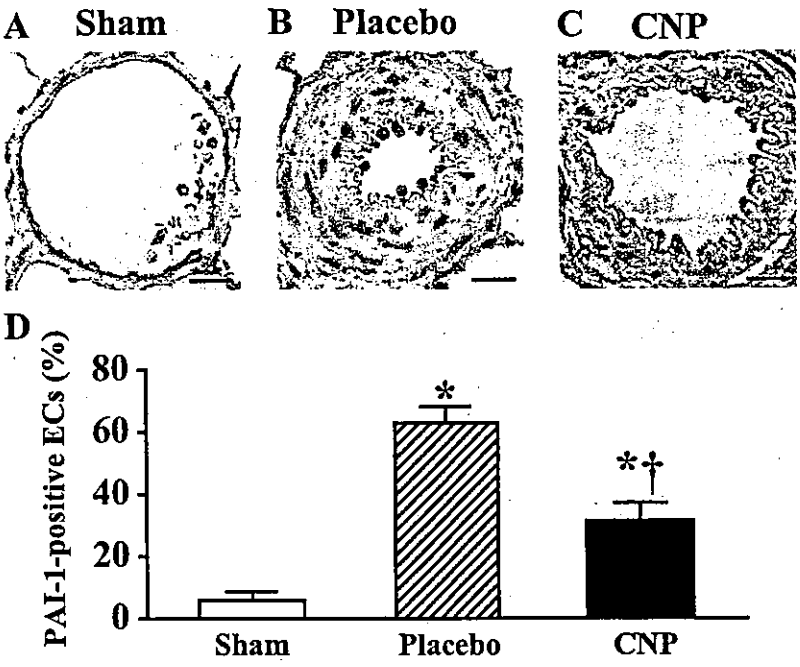

Figure 5. (*A–C*) Immunohistochemical demonstration of plasminogen activator inhibitor type 1 (PAI-1) expression in pulmonary ECs on Day 21. *Scale bars* = 20 μm. (*D*) Semiquantitative analysis of PAI-1–positive ECs. The percentage of PAI-1–positive ECs was calculated as (number of PAI-1–positive ECs/total number of ECs) × 100. Data are mean ± SEM. *$p < 0.05$ versus sham; †$p < 0.05$ versus placebo.

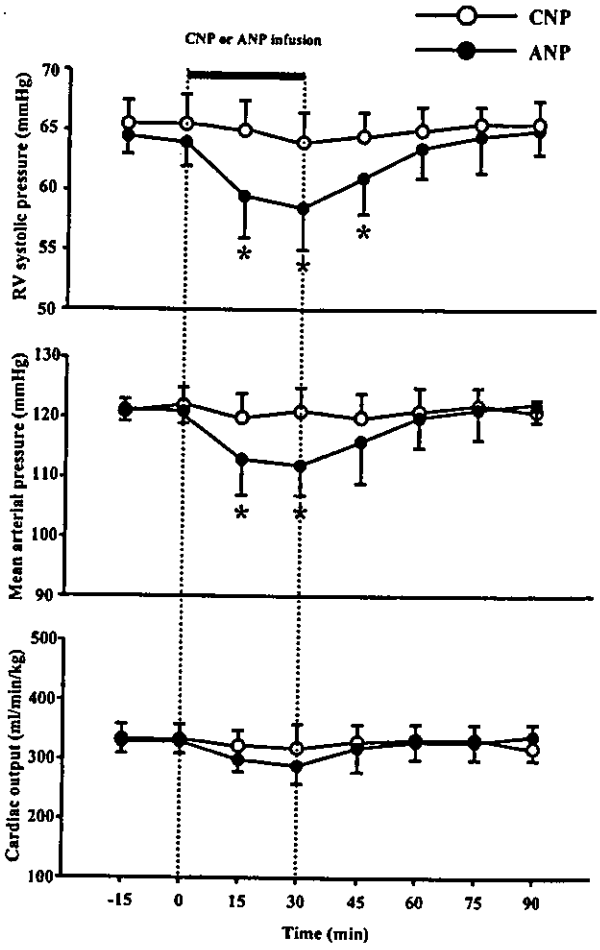

Figure 6. Acute hemodynamic responses to infusion of CNP (*open circles*) or arterial natriuretic peptide (ANP; *closed circles*) in MCT rats. Data are mean ± SEM. *p < 0.05 versus time 0.

sion significantly augmented lung tissue content of endothelial nitric oxide synthase protein. Endothelial nitric oxide synthase is an enzyme that produces nitric oxide in vascular endothelial cells (24, 25), which has a pivotal role in the regulation of pulmonary vascular tone (26). In fact, Champion and colleagues have demonstrated that intratracheal gene transfer of endothelial nitric oxide synthase to the lung attenuates hypoxia-induced pulmonary hypertension in mice (27). Thus, the therapeutic effects of CNP on pulmonary hypertension may be mediated by regeneration of pulmonary endothelium and improvement in nitric oxide bioavailability in MCT rats.

MCT induces apoptosis of pulmonary endothelial cells *in vivo* and *in vitro* (28–30). In fact, in this study, MCT injection increased the number of apoptotic pulmonary endothelial cells. Recent studies have shown that inhibition of pulmonary endothelial apoptosis attenuates MCT-induced pulmonary hypertension (29, 30). Interestingly, CNP infusion decreased the number of apoptotic cells in the lung of MCT rats. Thus, not only an increase in cell proliferation but also a decrease in cell apoptosis may contribute to improvement in pulmonary hemodynamics by CNP therapy.

Inflammatory cells, including macrophages, neutrophilis, lymphocytes, and mast cells, are observed in pulmonary arteries under the condition of pulmonary hypertension in animals and humans (6–8, 31). Particularly, monocyte/macrophage infiltration has a pivotal role in the development of MCT-induced pulmonary hypertension in rats (32, 33). In this study, CNP infusion inhibited monocyte/macrophage infiltration in the lungs, as indicated by a marked decrease in ED1-positive cells in pulmonary arterioles. These findings suggest that inhibition of monocyte/macrophage infiltration by CNP contributes to the improvement in pulmonary hemodynamics.

PAI-1, the principle inhibitor of the plasminogen system, irreversibly inactivates both tissue and urokinase plasminogen activators (34). PAI-1 is secreted by endothelial cells (35), smooth muscle cells, and macrophages (36). Inhibition of plasminogen activation by PAI-1 impairs fibrinolysis and thereby promotes thrombosis (37). It has been reported that the fibrino-

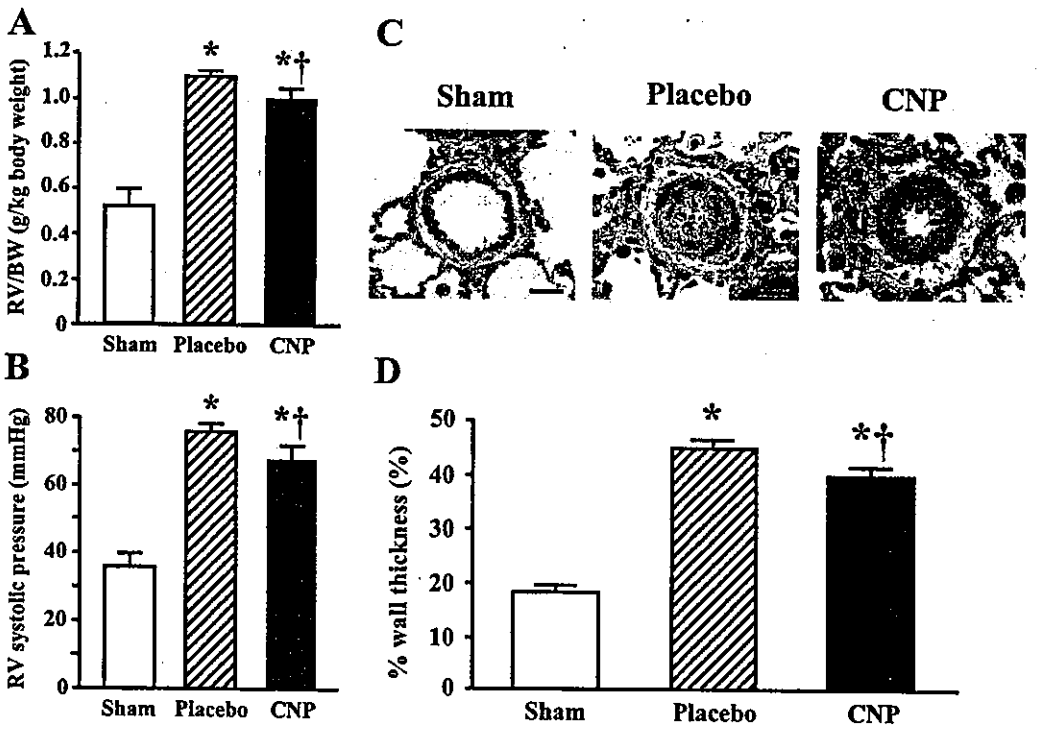

Figure 7. Effects of CNP infusion on established pulmonary hypertension. A continuous infusion of CNP was started 3 weeks after MCT injection. (*A*) RV/BW. (*B*) RV systolic pressure. (*C*) Representative photomicrographs of peripheral pulmonary arteries. *Scale bars* = 20 μm. (*D*) Quantitative analysis of percentage wall thickness in peripheral pulmonary arteries. Data are mean ± SEM. *p < 0.05 versus sham; †p < 0.05 versus placebo.

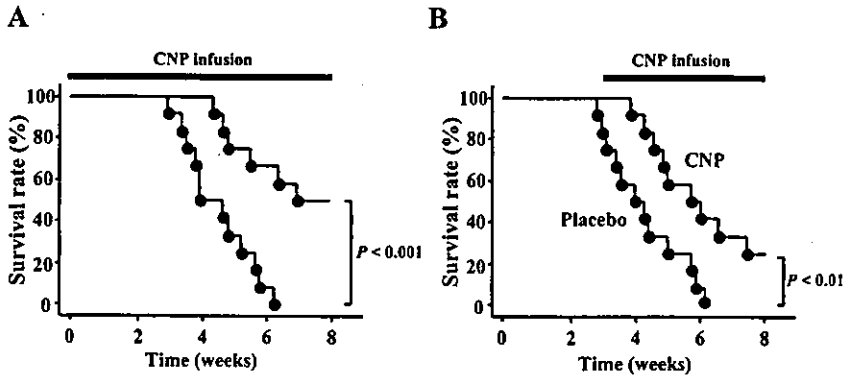

Figure 8. Kaplan-Meier survival curves. (*A*) Continuous infusion of CNP was initiated immediately after MCT injection. (*B*) CNP infusion was started 3 weeks after MCT injection.

lytic activity of lung tissue is decreased in MCT rats (38). These findings raise the possibility that PAI-1 may have a role in the development of MCT-induced pulmonary hypertension. In fact, immunohistologic examination demonstrated that MCT injection increased PAI-1 expression in pulmonary vessels. Recently, CNP has been shown to suppress PAI-1 expression in vascular smooth muscle cells and endothelial cells through an elevation of cGMP *in vitro* (39, 40). In this study, CNP infusion inhibited the MCT-induced increase in PAI-1 expression in pulmonary vessels. These findings suggest that CNP infusion ameliorates MCT-induced pulmonary hypertension at least in part through inhibition of fibrinolysis impairment.

CNP infusion also attenuated the increase in medial wall thickness of peripheral pulmonary arteries. CNP has also been shown to suppress the growth of vascular smooth muscle cells through an elevation of cGMP *in vitro* and inhibit the development of vascular remodeling of injured arteries *in vivo* (14). Thus, direct inhibitory effects of CNP on smooth muscle cell proliferation may contribute to inhibition of vascular remodeling.

Finally, we examined the effect of CNP on established pulmonary hypertension. CNP administration was started 3 weeks after MCT injection. CNP slightly but significantly attenuated the development of MCT-induced pulmonary hypertension. Importantly, CNP infusion improved survival not only in developing pulmonary hypertension but also in established pulmonary hypertension. Thus, continuous infusion of CNP may be a promising treatment for severe pulmonary hypertension.

This study includes some study limitations. First, the histology shown in the MCT model involves only smooth muscle hypertrophy. In contrast, the histopathology of pulmonary arterial hypertension in humans includes endothelial proliferation and fibrosis in addition to smooth muscle hypertrophy (2, 3). Thus, the results obtained from the MCT model may not be predictive of response to therapy in humans. Therefore, the initial success of CNP therapy reported here should be confirmed by further studies using other animal models of pulmonary hypertension before clinical trials. Second, the effects of CNP on pulmonary hemodynamics were modest. Unlike other vasodilators, however, CNP did not decrease systemic arterial pressure, which may be appropriate in the management of pulmonary hypertension. The improvement in pulmonary hypertension by CNP was mediated by multiple vasoprotective effects rather than by vasodilator activities. Thus, addition of CNP to conventional vasodilator therapy may be beneficial effects in patients with pulmonary hypertension. Finally, because the pathophysiologic role of CNP in cardiovascular disease is less well understood in humans, further studies are necessary to confirm the therapeutic potential of CNP in patients with pulmonary hypertension.

In conclusion, continuous infusion of CNP ameliorated MCT-induced pulmonary hypertension and improved survival in rats. These beneficial effects may be mediated by regeneration of pulmonary endothelium, inhibition of endothelial cell apoptosis, and prevention of monocyte/macrophage infiltration and fibrinolysis impairment after MCT injection.

Conflict of Interest Statement: T.I. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; N.N. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; S.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; T.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; T.I. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; H.I.-U. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; C.Y. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.Y. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; H.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; K.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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C-type natriuretic peptide attenuates bleomycin-induced pulmonary fibrosis in mice

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[1]*Department of Internal Medicine, National Cardiovascular Center, Osaka 565-8565;* [2]*Second Department of Internal Medicine, Nara Medical University, Nara 634-8522;* [3]*Department of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute, Osaka 565-8565; and* [4]*Department of Cardiac Physiology and* [5]*Department of Biochemistry, National Cardiovascular Center Research Institute, Osaka 565-8565, Japan*

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Murakami, Shinsuke, Noritoshi Nagaya, Takefumi Itoh, Takafumi Fujii, Takashi Iwase, Kaoru Hamada, Hiroshi Kimura, and Kenji Kangawa. C-type natriuretic peptide attenuates bleomycin-induced pulmonary fibrosis in mice. *Am J Physiol Lung Cell Mol Physiol* 287: L1172–L1177, 2004. First published July 30, 2004; doi:10.1152/ajplung.00087.2004.—C-type natriuretic peptide (CNP) has been shown to play an important role in the regulation of vascular tone and remodeling. However, the physiological role of CNP in the lung remains unknown. Accordingly, we investigated whether CNP infusion attenuates bleomycin (BLM)-induced pulmonary fibrosis in mice. After intratracheal injection of BLM or saline, mice were randomized to receive continuous infusion of CNP or vehicle for 14 days. CNP infusion significantly reduced the total number of cells and the numbers of macrophages, neutrophils, and lymphocytes in bronchoalveolar lavage fluid. Interestingly, CNP markedly reduced bronchoalveolar lavage fluid IL-1β levels. Immunohistochemical analysis demonstrated that CNP significantly inhibited infiltration of macrophages into the alveolar and interstitial regions. CNP infusion significantly attenuated BLM-induced pulmonary fibrosis, as indicated by significant decreases in Ashcroft score and lung hydroxyproline content. CNP markedly decreased the number of Ki-67-positive cells in fibrotic lesions of the lung, suggesting antiproliferative effects of CNP on pulmonary fibrosis. Kaplan-Meier survival curves demonstrated that BLM mice treated with CNP had a significantly higher survival rate than those given vehicle. These results suggest that continuous infusion of CNP attenuates BLM-induced pulmonary fibrosis and improves survival in BLM mice, at least in part by inhibition of pulmonary inflammation and cell proliferation.

inflammation; fibroblast; survival

PULMONARY FIBROSIS IS a life-threatening disease characterized by progressive dyspnea and worsening of pulmonary function (5). Most patients with pulmonary fibrosis are refractory to conventional therapy. The common pathological features observed in pulmonary fibrosis are infiltration of inflammatory cells, including activated macrophages and fibroblast proliferation with increased amounts of extracellular matrix (2). Thus a therapeutic strategy against these abnormalities may be effective for the treatment of pulmonary fibrosis.

C-type natriuretic peptide (CNP), the third member of the natriuretic peptide family consisting of 22 amino acid residues, is secreted by vascular endothelial cells (22, 24). CNP binds to natriuretic peptide receptor B, which bears a guanylate cyclase, induces generation of cGMP, and acts as a local regulator of vascular tone and remodeling (8, 23). Recently, CNP has been shown to suppress inflammation through inhibition of macrophage infiltration in injured carotid arteries of rabbits (20). Interestingly, CNP has been shown to directly inhibit cardiac fibroblast proliferation through a guanylate cyclase-B-mediated cGMP-dependent pathway (7). These findings suggest that CNP plays an important role in regulation of the cardiovascular system. However, the physiological role of CNP in the lung remains unknown. CNP mRNA and protein have been shown to be localized in bronchial airways and the alveolar epithelium (14). The respiratory epithelium has been shown to express a CNP-specific receptor (4). These findings raise the possibility that CNP may have protective effects against pulmonary inflammation and fibroblast proliferation, both of which are responsible for pulmonary fibrosis.

Thus the purposes of this study were *1*) to investigate whether continuous infusion of CNP attenuates bleomycin (BLM)-induced pulmonary fibrosis in mice, *2*) to investigate whether CNP infusion improves survival in BLM-treated mice, and *3*) to examine the underlying mechanisms responsible for the effects of CNP on pulmonary fibrosis.

METHODS

Animals. We used specific pathogen-free 10-wk-old female C57BL/6 mice weighing 18–20 g. The mice were randomly given an intratracheal injection of either BLM (Nippon Kayaku, Tokyo, Japan) or 0.9% saline and assigned to receive continuous infusion of CNP or vehicle. This protocol resulted in the creation of three groups: sham mice given vehicle (Sham group, $n = 27$), BLM mice given vehicle (vehicle group, $n = 55$), and BLM mice treated with CNP (CNP group, $n = 55$). All protocols were performed in accordance with guidelines of the Animal Care Ethics Committee of the National Cardiovascular Center Research Institute (Osaka, Japan).

Experimental protocol. After the mice were anesthetized by intraperitoneal injection of pentobarbital sodium, they were given an intratracheal injection of either BLM (0.02 or 0.04 U/mouse) dissolved in 50 μl of 0.9% sterile saline or saline alone. Then, an osmotic minipump (Alzet, Palo Alto, CA) was filled with either CNP to deliver a dose of 0.06 μg/h or 5% glucose vehicle throughout the experiment and implanted subcutaneously on the back, slightly caudal to the scapulae. The mice were maintained under standard conditions with free access to food and water.

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Table 1. *Physiological profiles of three experimental groups*

	Sham group	Vehicle group	CNP group
Low dose (0.02 U/mouse) of BLM			
n	7	8	9
Body weight, g	21.2±0.3	18.3±0.4*	20.2±0.3†
Lung weight/body weight, mg/g	6.1±0.1	12.9±0.5*	7.9±0.2*†
	Sham group	Vehicle group	CNP group
High dose (0.04 U/mouse) of BLM			
n	7	7	9
Body weight, g	21.2±0.3	15.7±1.1*	19.9±0.6†
Lung weight/body weight, mg/g	6.1±0.1	18.4±2.0*	12.5±1.1*†

Values are means ± SE. Measurements were performed 14 days after bleomycin (BLM) injection. Sham group, sham mice given vehicle; vehicle group, BLM mice given vehicle; CNP group, BLM mice treated with C-type natriuretic peptide. *$P < 0.05$ vs. Sham group; †$P < 0.05$ vs. vehicle group.

Mice treated with 0.02 units of BLM were used to assess the antifibrotic effects of CNP. Histological examination and measurement of lung hydroxyproline content were performed at 14 days. Cell proliferation detected by Ki-67 was also evaluated in mice given 0.02 units of BLM. Survival rate in mice given 0.02 units of BLM was relatively high (80%). On the other hand, mice treated with 0.04 units of BLM were used to assess the effects of CNP on pulmonary inflammation and survival. After anesthesia with pentobarbital sodium, bronchoalveolar lavage (BAL) was performed at 1, 3, 7, and 14 days. Macrophage infiltration was also evaluated 14 days after injection of 0.04 units of BLM. The wet lung weight was measured, and the wet lung weight-to-body weight ratio was then calculated at 14 days in mice not subjected to BAL.

Survival analysis. To evaluate the effect of CNP on survival in BLM mice, 60 mice received continuous infusion of CNP ($n = 30$) or vehicle ($n = 30$) for 14 days. Survival was estimated from the date of BLM injection to the death of the mouse or 14 days after injection.

BAL analysis. BAL was performed through a tracheal cannula with 1 ml of saline solution ($n = 5$ each). This procedure was repeated three times. 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 800 *g* for 10 min at 4°C. We counted the total number of cells using a standard hemocytometer. We examined cell differentiation by counting at least 200 cells on a smear prepared using cytospin and Wright-Giemsa staining.

Enzyme-linked immunosorbent assay. The supernatant of BALF was immediately stored at −80°C until the assay. We measured BALF TNF-α and BALF IL-1β levels with a mouse TNF-α ELISA kit (Pierce Chemical, Rockford, IL) and mouse IL-1β ELISA kit (Biosource International, Camarillo, CA), respectively ($n = 5$ each).

Histological examination. The right lung was fixed with 4% paraformaldehyde and embedded in paraffin ($n = 5$ each). Sections 4-μm thick were stained with hematoxylin-eosin. The Ashcroft score was used for semi-quantitative assessment of fibrotic changes (1). The severity of fibrotic changes in each histological section of the lung was assessed as the mean score of severity from observed microscopic fields. Thirty fields in each section were analyzed. Grading was done in a blinded fashion by two observers, and the mean was taken as the fibrosis score.

Measurement of hydroxyproline content. To quantify lung collagen content as an indicator of pulmonary fibrosis, the hydroxyproline content in the lung was measured in each group according to the previously described method ($n = 5$ each) (15). The left lung was frozen and kept at −80°C until the assay. After the lung was homogenized, the suspension was hydrolyzed with 0.5 ml of 12 N hydrochloric acid for 20 h at 100°C. After neutralization, a 0.1-ml aliquot of supernatant was mixed in 1.5 ml of 0.3 N lithium hydroxide solution. The hydroxyproline content was analyzed by high-performance chromatography.

Immunohistochemistry. Paraffin sections 4-μm thick were obtained from the lung ($n = 5$ each). To investigate whether CNP inhibits macrophage infiltration, tissue sections were stained for F4/80, a

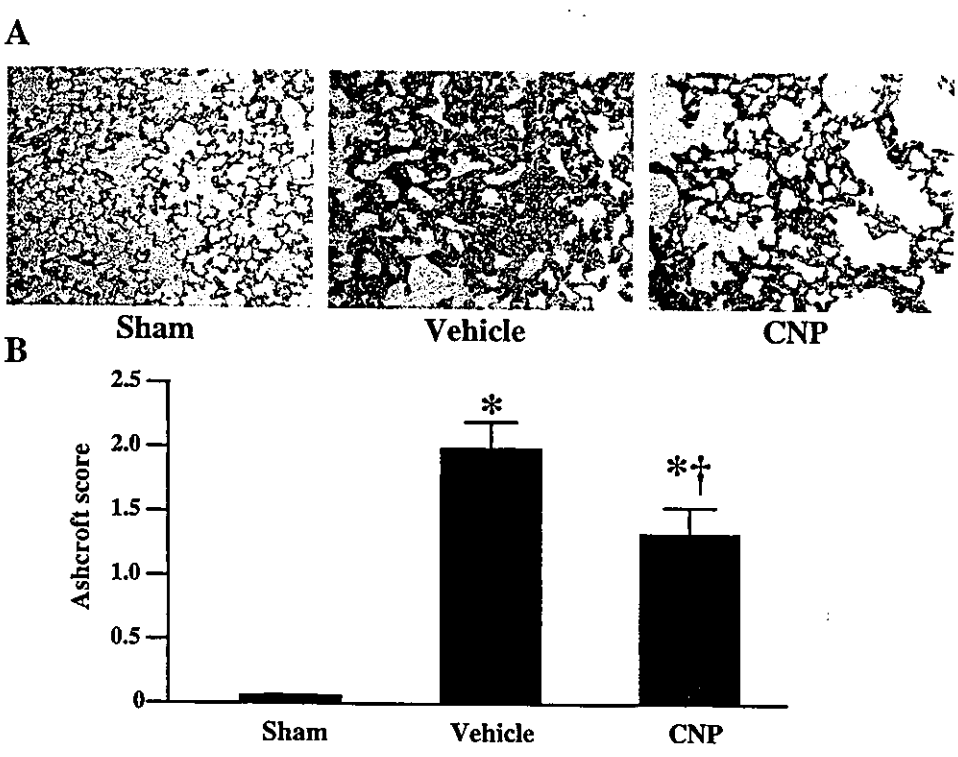

Fig. 1. *A*: representative photomicrographs of lung tissues stained with hematoxylin-eosin. Histological sections were taken from the same general regions of the lungs in each group. Intratracheal injection of bleomycin (BLM) induced pulmonary fibrosis in mice (vehicle group) compared with mice given vehicle (Sham group). C-type natriuretic peptide (CNP) infusion attenuated pulmonary fibrosis in BLM mice (CNP group). Magnification, ×100. *B*: semi-quantitative analyses of lung tissues using the Ashcroft score, a marker for pulmonary fibrosis. The score was significantly decreased in the CNP group. Data are means ± SE. *$P < 0.05$ vs. Sham group; †$P < 0.05$ vs. vehicle group.

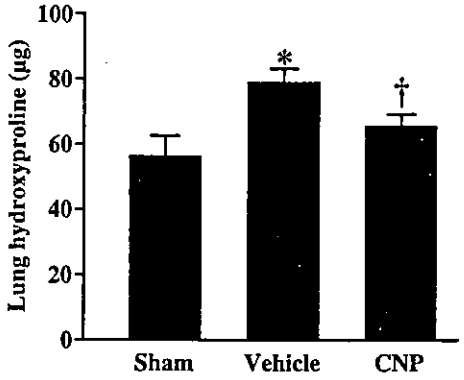

Fig. 2. Effects of CNP infusion on hydroxyproline content in left lung of BLM mice (n = 5 each). BLM injection increased lung hydroxyproline content (vehicle group). CNP infusion significantly decreased hydroxyproline content in BLM mice (CNP group). Data are means ± SE. *P < 0.05 vs. Sham group; †P < 0.05 vs. vehicle group.

murine monocyte/macrophage membrane antigen, using rat anti-mouse F4/80 IgG (Serotec, Oxford, UK). To investigate whether CNP inhibits cell proliferation of pulmonary fibrosis, we stained tissue sections for Ki-67, a marker for cell proliferation, using rat anti-mouse Ki-67 antibody (DAKO, Copenhagen, Denmark). The numbers of F4/80-positive cells and Ki-67-positive cells were determined in 10 randomly chosen fields (×400).

Statistical analysis. All data are expressed as means ± SE unless otherwise indicated. Comparisons of parameters among the three groups were made by one-way ANOVA, followed by Newman-Keuls test. Survival curves were derived by the Kaplan-Meier method and compared by log-rank test. A value of P < 0.05 was considered statistically significant.

RESULTS

Physiological profiles. The physiological profiles of the three experimental groups are shown in Table 1. Body weight was significantly lower in BLM mice given vehicle (vehicle group) than in normal mice given vehicle (Sham group) and in BLM mice treated with CNP (CNP group). However, there was no significant difference between the Sham and CNP groups. Wet lung weight-to-body weight ratio was significantly increased after BLM injection. However, the increase in the CNP group was significantly attenuated compared with that in the vehicle group.

Inhibition of pulmonary fibrosis by CNP. The normal alveolar structure was maintained in the Sham group (Fig. 1*A*). Fourteen days after BLM injection, the alveolar wall was thickened and the air spaces were collapsed in the vehicle group. In addition, focal fibrotic lesions were observed. In contrast to the findings in mice treated with BLM alone, fibrotic lesions were less focal in the CNP group. Semiquantitative assessment using Ashcroft score demonstrated that the degree of pulmonary fibrosis in the CNP group was lower than that in the vehicle group (Fig. 1*B*). The hydroxyproline content in the lung was significantly increased after BLM injection (Fig. 2). However, the content in the CNP group was significantly lower than that in the vehicle group.

Anti-inflammatory effects of CNP. The recovery rate of BALF was over 80% in all groups. The total number of cells and the number of macrophages were significantly increased at 3, 7, and 14 days after BLM injection (Fig. 3, *A* and *B*). However, the numbers of these cells in the CNP group were significantly lower than those in the vehicle group. The number of neutrophils was significantly increased at 1, 3, 7, and 14 days after BLM injection (Fig. 3*C*). However, the number of these in the CNP group was significantly lower than that in the vehicle group at 7 and 14 days after BLM injection. The number of lymphocytes was significantly increased at 3, 7, and 14 days after BLM injection (Fig. 3*D*). However, the number of these in the CNP group was significantly lower than that in the vehicle group at 3 and 7 days after BLM injection. The BALF IL-1β level was significantly increased at 3 and 14 days after BLM injection (Fig. 4*A*). However, CNP infusion markedly inhibited the increase in BALF IL-1β level. The BALF TNF-α level was significantly increased at 3 days after BLM injection (Fig. 4*B*). CNP infusion tended to inhibit the increase in BALF TNF-α level, but this was not significant (P = 0.058).

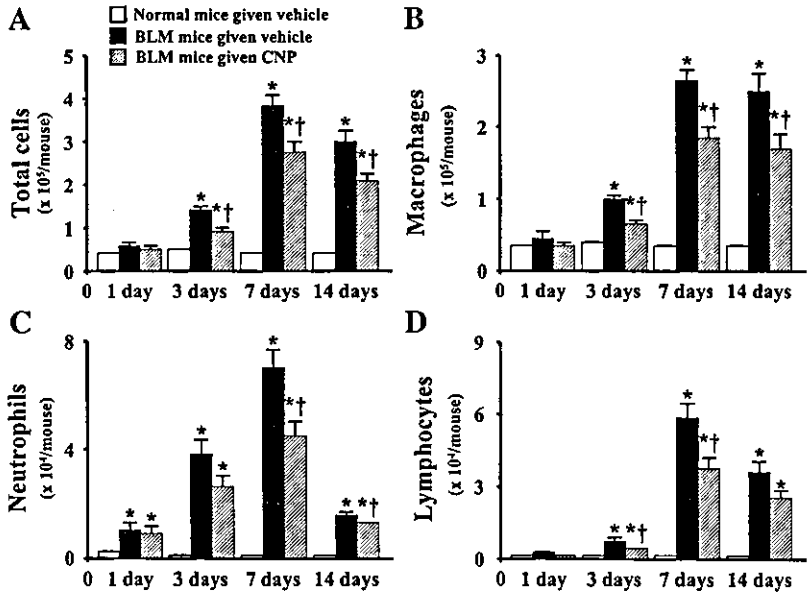

Fig. 3. Effects of CNP infusion on the numbers of total cells (*A*), macrophages (*B*), neutrophils (*C*), and lymphocytes (*D*) in bronchoalveolar lavage fluid (BALF). The total number of cells and the number of macrophages were significantly increased at 3, 7, and 14 days after BLM injection. However, the numbers of these cells in the CNP group were significantly lower than those in the vehicle group. The number of neutrophils was significantly increased at 1, 3, 7, and 14 days after BLM injection. However, the number of these in the CNP group was significantly lower than that in the vehicle group at 7 and 14 days after BLM injection. The number of lymphocytes was significantly increased at 3, 7, and 14 days after BLM injection. However, the number of these in the CNP group was significantly lower than that in the vehicle group at 3 and 7 days after BLM injection. Data are means ± SE. *P < 0.05 vs. Sham group, †P < 0.05 vs. vehicle group.

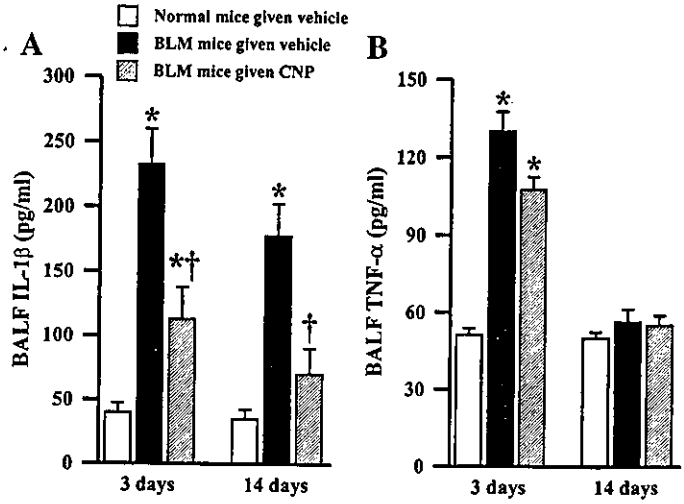

Fig. 4. Effects of CNP infusion on BALF IL-1β (*A*) and BALF TNF-α (*B*) levels at 3 and 14 days after BLM injection. CNP significantly decreased BALF IL-1β level. CNP tended to inhibit the increase in BALF TNF-α level, but this was not significant ($P = 0.058$). Data are means ± SE. *$P < 0.05$ vs. Sham group, †$P < 0.05$ vs. vehicle group.

Representative photomicrographs showed that CNP infusion markedly inhibited macrophage infiltration compared with vehicle (Fig. 5*A*). Semi-quantitative analysis demonstrated that BLM injection significantly increased the number of macrophages (Fig. 5*B*). However, the increase was markedly inhibited in the CNP group.

Antiproliferative effects of CNP. Unlike sham mice, Ki-67-positive cells were frequently observed mainly in fibrotic lesions 14 days after BLM injection (Fig. 6*A*). Interestingly, CNP infusion markedly decreased Ki-67-positive cells in the fibrotic lesions. Semi-quantitative analysis demonstrated that the number of Ki-67-positive cells was significantly decreased in the CNP group compared with that in the vehicle group (Fig. 6*B*).

Survival analysis. Kaplan-Meier survival curves demonstrated that BLM mice treated with CNP had a significantly higher survival rate than those given vehicle (70% vs. 40% 14-day survival, log-rank test, $P < 0.01$, Fig. 7).

DISCUSSION

In the present study, we demonstrated that *1*) continuous infusion of CNP attenuated BLM-induced pulmonary fibrosis, as indicated by decreases in Ashcroft score and lung hydroxyproline content, *2*) CNP inhibited cellular infiltration in the lung and decreased BALF IL-1β levels in BLM mice, and *3*) infusion of CNP decreased the number of Ki-67-positive cells in fibrotic lesions of the lung. Finally, we demonstrated that *4*) CNP infusion increased the survival rate in BLM mice.

BLM, an antineoplastic antibiotic, has been reported to induce pulmonary fibrosis dose dependently when intratracheally injected in experimental animals (21). In fact, in the present study, intratracheal administration of BLM induced fibrotic changes in the lung, as indicated by histological findings (Ashcroft score) and lung hydroxyproline content. These findings were consistent with the results from earlier studies (17, 27). Because acute lung injury induced by a high dose of BLM (0.04 U/mouse) was too severe for mice to survive, a low dose of BLM (0.02 U/mouse) was used to evaluate the antifibrotic effect of CNP. Interestingly, 14-day infusion of CNP significantly decreased Ashcroft score and lung hydroxyproline content. Thus, in the present study, we first demonstrated that CNP infusion attenuated BLM-induced pulmonary fibrosis. However, the underlying mechanisms still remain unclear. Earlier studies have shown that pulmonary inflammation and fibroblast proliferation are responsible for pulmonary fibrosis in BLM-treated animals and humans (6, 21). Thus we inves-

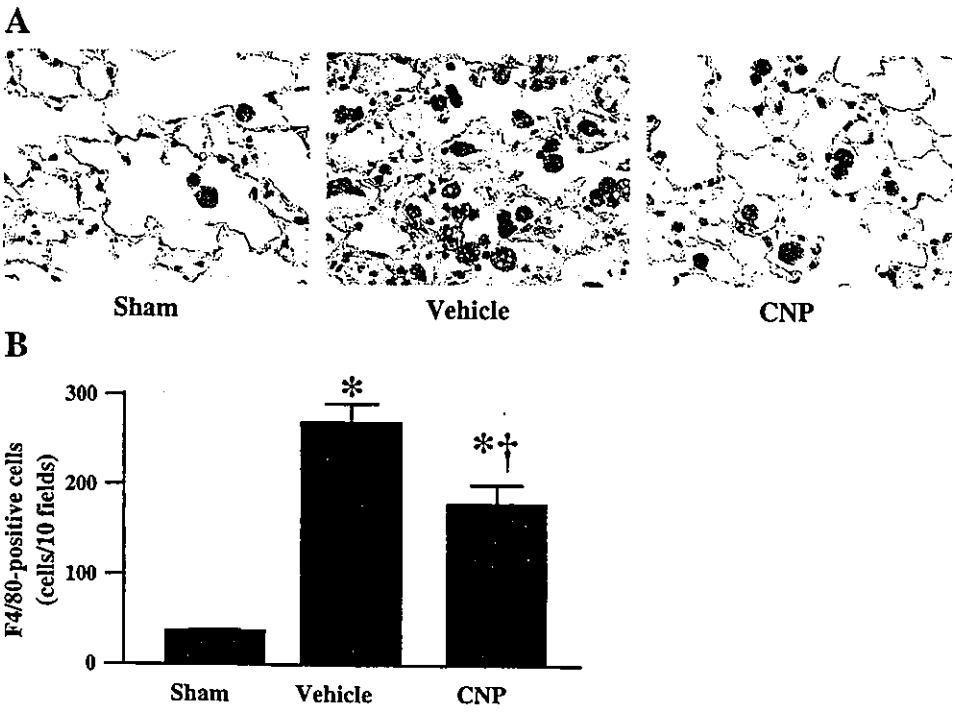

Fig. 5. *A*: immunohistochemical analysis of F4/80 antigen, a marker for monocytes/macrophages, 14 days after BLM injection. Histological sections were taken from the same general regions of the lungs in each group. Monocytes/macrophages were frequently observed in BLM mice given vehicle (vehicle group). CNP infusion markedly inhibited macrophage infiltration (CNP group). Magnification, ×400. *B*: semi-quantitative analyses of F4/80-positive cells in the lung. The number of F4/80-positive cells in the CNP group was significantly lower than that in the vehicle group. Data are means ± SE. *$P < 0.05$ vs. Sham group; †$P < 0.05$ vs. vehicle group.

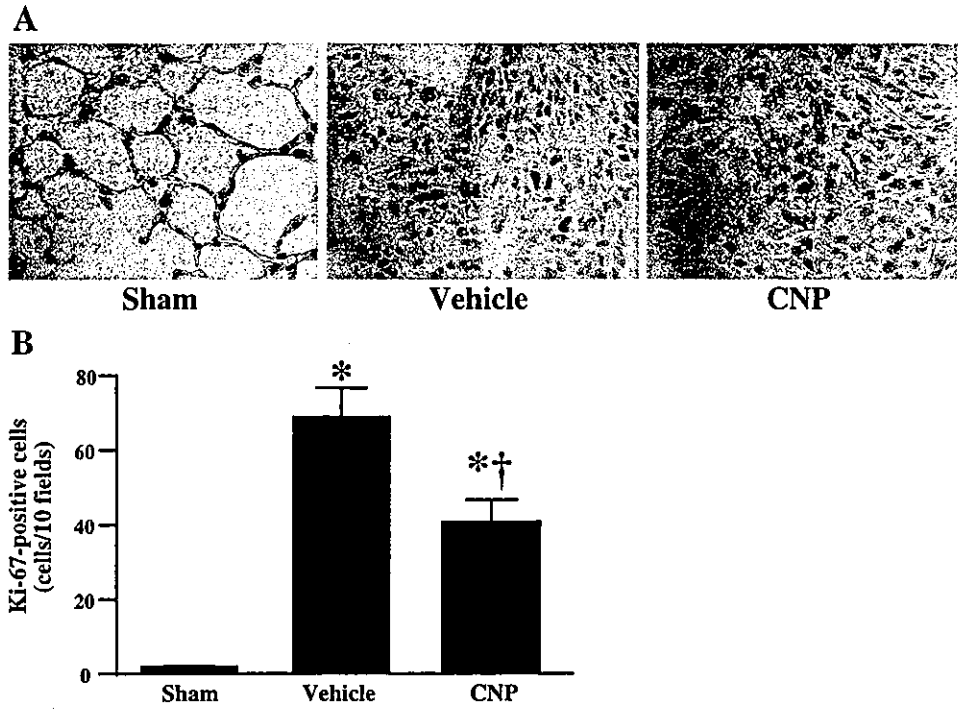

Fig. 6. *A*: immunohistochemical analysis of Ki-67 antigen, a marker for cell proliferation. Histological sections were taken from the same general regions of the lungs in each group. Ki-67-positive nuclei were detected mainly in fibrotic lesions 14 days after BLM injection (vehicle group). CNP infusion decreased the number of Ki-67-positive cells. Magnification, ×400. *B*: semi-quantitative analysis also demonstrated that the number of Ki-67-positive cells in the fibrotic lesions was significantly decreased in the CNP group. Data are means ± SE. *$P < 0.05$ vs. Sham group; †$P < 0.05$ vs. vehicle group.

tigated whether CNP infusion inhibits pulmonary inflammation and fibroblast proliferation in vivo.

Several proinflammatory cytokines including IL-1β and TNF-α are involved in pulmonary inflammation and the subsequent development of pulmonary fibrosis in a mouse model of BLM-induced pulmonary fibrosis (3, 11, 18). A previous study showed that continuous infusion of an IL-1 receptor antagonist prevented BLM-induced pulmonary fibrosis (19). Moreover, BLM-stimulated alveolar macrophages released IL-1β, which can serve as a fibroblast growth factor (25). These findings implicate IL-1β as a key mediator in BLM-induced pulmonary fibrosis. In the present study, CNP infusion markedly inhibited the increase in BALF IL-1β levels after BLM injection, together with a significant decrease in the number of inflammatory cells in BALF. Immunohistochemical examination also demonstrated that CNP infusion significantly inhibited infiltration of macrophages into the alveolar and interstitial regions. A recent study has shown that CNP suppresses the expression of monocyte chemoattractant protein-1, which induces migration and activation of macrophages (16). Considering that IL-1β is mainly produced by activated alveolar macrophages, it is interesting to speculate that CNP inhibits IL-1β production via inactivation of macrophages. Neutrophils have been shown to induce lung parenchymal injury by producing toxic radical oxygen species and a variety of proteolytic enzymes in BLM-induced fibrosis (12, 13, 26). The recruitment of lymphocytes has been shown to precede the development of pulmonary fibrosis (10). In the present study, CNP significantly attenuated the increase in the numbers of neutrophils and lymphocytes in BALF. Thus CNP infusion may attenuate BLM-induced pulmonary fibrosis in part through inhibition of pulmonary inflammation.

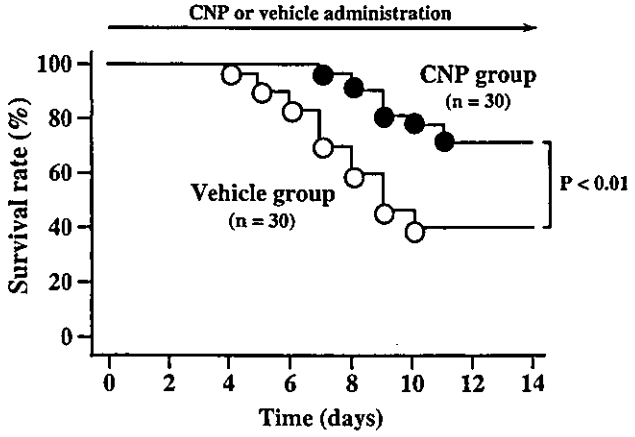

Fig. 7. Kaplan-Meier survival curves. BLM mice treated with CNP (●) had a significantly higher survival rate than those given vehicle (○) (log-rank test, $P < 0.01$).

Fibroblasts in fibrotic lesions have been considered to be the cells responsible for deposition of matrix (9). In addition, fibroblasts have been found to be significant sources of several cytokines, including transforming growth factor-β, a well-established key fibrogenic mediator, and monocyte chemoattractant protein-1 (28, 29). Thus pulmonary fibroblasts play an important role in the development of fibrosis in the lung. The present study demonstrated that BLM injection enhanced cell proliferation in the lung, as indicated by an increase in the number of Ki-67-positive cells in the fibrotic lesions. Interestingly, CNP infusion markedly inhibited Ki-67-positive cells in fibrotic lesions of the lung. An in vitro study showed that CNP directly inhibited proliferation of cardiac fibroblasts through a guanylate cyclase-B-mediated cGMP-dependent pathway (7). Thus it is possible that the reduction of pulmonary fibrosis by CNP infusion may be mediated by a direct antiproliferative effect of CNP on fibroblasts in the lung.

In the present study, continuous infusion of CNP significantly improved survival in BLM mice. Infusion of CNP

inhibited the development of pulmonary fibrosis and inflammation. As a result, CNP may have beneficial effects on survival in BLM mice. Considering that most patients with pulmonary fibrosis are refractory to conventional therapy, this therapy may be an alternative approach for severe pulmonary fibrosis. However, the initial success of CNP administration reported here should be confirmed by long-term experiments, and extensive toxicity studies in animals are needed before clinical trials.

In conclusion, continuous infusion of CNP attenuated BLM-induced pulmonary fibrosis and improved survival in BLM mice. These beneficial effects of CNP may be mediated at least in part by inhibition of pulmonary inflammation and cell proliferation. Thus CNP supplementation may be a new therapeutic strategy for the treatment of pulmonary fibrosis.

GRANTS

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Adrenomedullin Infusion Attenuates Myocardial Ischemia/Reperfusion Injury Through the Phosphatidylinositol 3-Kinase/Akt-Dependent Pathway

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Background—Infusion of adrenomedullin (AM) has beneficial hemodynamic effects in patients with heart failure. However, the effect of AM on myocardial ischemia/reperfusion remains unknown.

Methods and Results—Male Sprague-Dawley rats were exposed to a 30-minute period of ischemia induced by ligation of the left coronary artery. They were randomized to receive AM, AM plus wortmannin (a phosphatidylinositol 3-kinase [PI3K] inhibitor), or saline for 60 minutes after coronary ligation. Hemodynamics and infarct size were examined 24 hours after reperfusion. Myocardial apoptosis was also examined 6 hours after reperfusion. The effect of AM on Akt phosphorylation in cardiac tissues was examined by Western blotting. Intravenous administration of AM significantly reduced myocardial infarct size (28±4% to 16±1%, $P<0.01$), left ventricular end-diastolic pressure (19±2 to 8±2 mm Hg, $P<0.05$), and myocardial apoptotic death (19±2% to 9±4%, $P<0.05$). Western blot analysis showed that AM infusion accelerated Akt phosphorylation in cardiac tissues and that pretreatment with wortmannin significantly attenuated AM-induced Akt phosphorylation. Moreover, pretreatment with wortmannin abolished the beneficial effects of AM: a reduction of infarct size, a decrease in left ventricular end-diastolic pressure, and inhibition of myocardial apoptosis after ischemia/reperfusion.

Conclusions—Short-term infusion of AM significantly attenuated myocardial ischemia/reperfusion injury. These cardioprotective effects are attributed mainly to antiapoptotic effects of AM via a PI3K/Akt-dependent pathway. (***Circulation.* 2004;109:242-248.**)

Key Words: peptides ■ reperfusion ■ apoptosis ■ myocardial infarction ■ hemodynamics

Coronary revascularization has been established as the most effective treatment for coronary artery disease. However, reperfusion can elicit a number of adverse reactions that may limit its beneficial actions. Although it has been attempted to reduce ischemia/reperfusion injury in many basic or clinical studies, few agents are clinically available for ischemia/reperfusion injury.

Adrenomedullin (AM) is a potent vasodilatory peptide that was originally isolated from human pheochromocytoma.[1] We have shown that AM peptide and mRNA are distributed in the heart[2,3] and that plasma and cardiac AM markedly increase after acute myocardial infarction.[4,5] AM has been shown to be a possible endogenous suppressor of myocyte hypertrophy[6] and fibroblast proliferation.[7] In addition, intravenous infusion of AM has beneficial hemodynamic effects in patients with heart failure.[8] These findings suggest that AM induces cardioprotective effects not only as a circulating factor but also as a paracrine and/or autocrine factor.

Recently, AM has been shown to activate the Akt pathway in vascular endothelial cells.[9] Interestingly, the Akt activation has been reported to lead to the prevention of myocardial injury after transient ischemia in vivo through antiapoptotic effects.[10] However, whether AM, a potent Akt activator, attenuates myocardial ischemia/reperfusion injury remains unknown.

Thus, the purposes of this study were (1) to investigate whether short-term infusion of AM reduces myocardial infarct size, inhibits myocyte apoptosis, and thereby improves cardiac function after ischemia/reperfusion and (2) to determine whether the underlying mechanisms are associated with

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the phosphatidylinositol 3-kinase (PI3K)/Akt-dependent pathway.

Methods

Reperfusion Model

We used male Sprague-Dawley rats (Japan SLC Inc, Hamamatsu, Japan) weighing 180 to 220 g. Ligation of the left coronary artery was performed as described previously.[11] In brief, under anesthesia with pentobarbital sodium (30 mg/kg) and artificial ventilation, the heart was exposed via left thoracotomy, and the left coronary artery was ligated 2 to 3 mm from its origin between the pulmonary artery conus and the left atrium with a 6-0 Prolene suture. The heart was subjected to regional ischemia for 30 minutes, followed by coronary reperfusion through release of the tie. After ligation of the left coronary artery, AM (0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$), AM plus wortmannin (16 μg/kg intravenous injection 15 minutes before AM infusion; a PI3K inhibitor),[12] or placebo (0.9% saline) was administered for 60 minutes through a catheter inserted into the left jugular vein. Sham-operated rats only underwent left thoracotomy. The chest wall was then closed, and the animal was allowed to recover. This protocol resulted in the creation of 4 groups: sham-operated rats (sham group, n=12), placebo-treated rats with ischemia/reperfusion (I/R-placebo group, n=19), AM-treated rats with ischemia/reperfusion (I/R-AM group, n=19) and AM plus wortmannin–treated rats with ischemia/reperfusion (I/R-Wo+AM group, n=15).

All animal experiments were conducted in accordance with the principles and procedures outlined in the *National Cardiovascular Center Guide for the Care and Use of Laboratory Animals*, which adheres strictly to the National Institutes of Health animal experimental guidelines, with the approval of the National Cardiovascular Center Animal Experimental Committee.

Hemodynamic Studies

We performed hemodynamic measurements 24 hours after ischemia/reperfusion. A 1.5F micromanometer-tipped catheter 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 with an ECG.

Measurement of Plasma AM Level

Blood samples were obtained from the right carotid artery during 0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$ AM infusion. Plasma AM level was measured by immunoradiometric assay, as described previously.[8,11]

Assessment of Infarct Size

After hemodynamic measurements, the heart was removed and perfused with a Langendorff apparatus for 10 minutes to wash out the blood and then fixed with 10% neutral buffered formalin. The heart was sliced transversely from the apex to the atrioventricular groove in 2.5-mm thicknesses and weighed separately. Within 24 hours after fixation, each section was embedded in paraffin. Serial 5-μm myocardial sections were cut with microtome and mounted on siliconized slides. After Masson trichrome staining, infarct size of each slice was analyzed by microscopy. Myocardial coagulation necrosis could be distinguished from viable myocardium as a definite alteration of staining, and then the infarct area was outlined and measured by planimetry. Infarct weight was determined with the following equation: % infarct area × weight of each slice, as described previously.[13] Finally, we determined percent infarct size as total infarct weight divided by total left ventricular (LV) weight.

TUNEL Staining

Hearts were isolated from each group (n=5) 6 hours after reperfusion for the terminal dUTP nick-end labeling (TUNEL) assay. After the blood and the fixation were washed out, the heart was also sliced transversely in 2.5-mm thicknesses. Paraffin-embedded, 5-μm-thick myocardial sections were used as described previously.[14] In brief, after deparaffinization and enzyme-mediated antigen retrieval, TUNEL staining was performed with a commercially available kit (Apop Tag Plus, Intergen). Samples were incubated with monoclonal anti-desmin antibody (Sigma) followed by tetramethylrhodamine isothiocyanate-conjugated rabbit anti-mouse antibody (DAKO). Counterstaining was performed with propidium iodide. Finally, these slides were mounted with Vector Shield (Vector Laboratories) containing an antifade reagent. We measured the number of TUNEL-positive nuclei in myocytes by means of confocal microscopy (Olympus, Fluoview 500). Quantitative analysis was performed on 60 high-power fields (magnification ×600) with at least 10 randomly selected fields used per section. We counted the number of cardiomyocytes at least $>10^4$ cells per heart.

DNA Ladder Assay

We used 10 additional rats for the DNA ladder assay (sham group, n=2; I/R-placebo group, n=4; I/R-AM group, n=4). Rats were killed, and the heart was excised 24 hours after ischemia/reperfusion. Immediately before heart isolation, 1% Evans blue was infused slowly into the left ventricle to delineate the risk area after coronary religation. Then, 40 mg of myocardium in the posterolateral border zone between the nonrisk area and the risk area was resected. Each specimen was frozen in liquid nitrogen and stored at −80°C until DNA extraction. DNA extraction and electrophoresis were performed with a commercially available kit (Apoptosis Ladder Detection Kit, WAKO).

Immunohistochemical Analysis

To assess localization of calcitonin receptor-like receptor (CRLR), a receptor for AM, in cardiac tissues, we performed immunohistochemical analysis using rabbit anti-rat CRLR antibody (Zymed). Localization of Akt phosphorylation was examined with rabbit anti-rat phospho-Akt antibody (Cell Signaling).

Western Blot Analysis

To identify Akt phosphorylation in myocardial tissues after AM infusion, Western blotting was performed with a commercially available kit (PhosphoPlus Akt [Ser 473] antibody kit, Cell Signaling). Myocardial tissues were obtained from rats treated with intravenous AM (0.01, 0.05, and 0.25 $\mu g \cdot kg^{-1} \cdot min^{-1}$), AM (0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$) plus wortmannin (16 μg/kg intravenous injection 15 minutes before AM infusion), or saline for 60 minutes during ischemia/reperfusion. These samples were homogenized on ice in a 0.1% Tween 20 homogenization buffer with a protease inhibitor (Complete, Roche). After centrifugation for 20 minutes at 4°C, the clear supernatant was used for Western blot analysis. Protein concentration was measured by Bradford's method (Bio-Rad). Fifty micrograms of each protein extract were transferred in sample buffer, loaded on 7.5% SDS-polyacrylamide gel, and blotted onto nitrocellulose membrane (Bio-Rad) with a wet blotting system. After being blocked for 60 minutes, the membranes were incubated with primary antibodies in blocking buffer (1:500) at 4°C overnight. Antibodies were used at the manufacturer's recommended dilution (Cell Signaling). The membranes were incubated with secondary antibodies, which were conjugated with horseradish peroxidase (Cell Signaling), at a final dilution of 1:2000. Signals were detected with LumiGLO chemiluminescence reagents (Cell Signaling).

Statistical Analysis

All data are expressed as mean±SEM unless otherwise indicated. Comparisons of parameters among the 3 or 4 groups were made by 1-way ANOVA for repeated measures, followed by Scheffé test. A probability value <0.05 was considered to indicate statistical significance.

Results

Reduction of Myocardial Infarct Size After AM Infusion

Moderate to large infarcts were observed in Masson trichrome–stained myocardial sections 24 hours after ische-

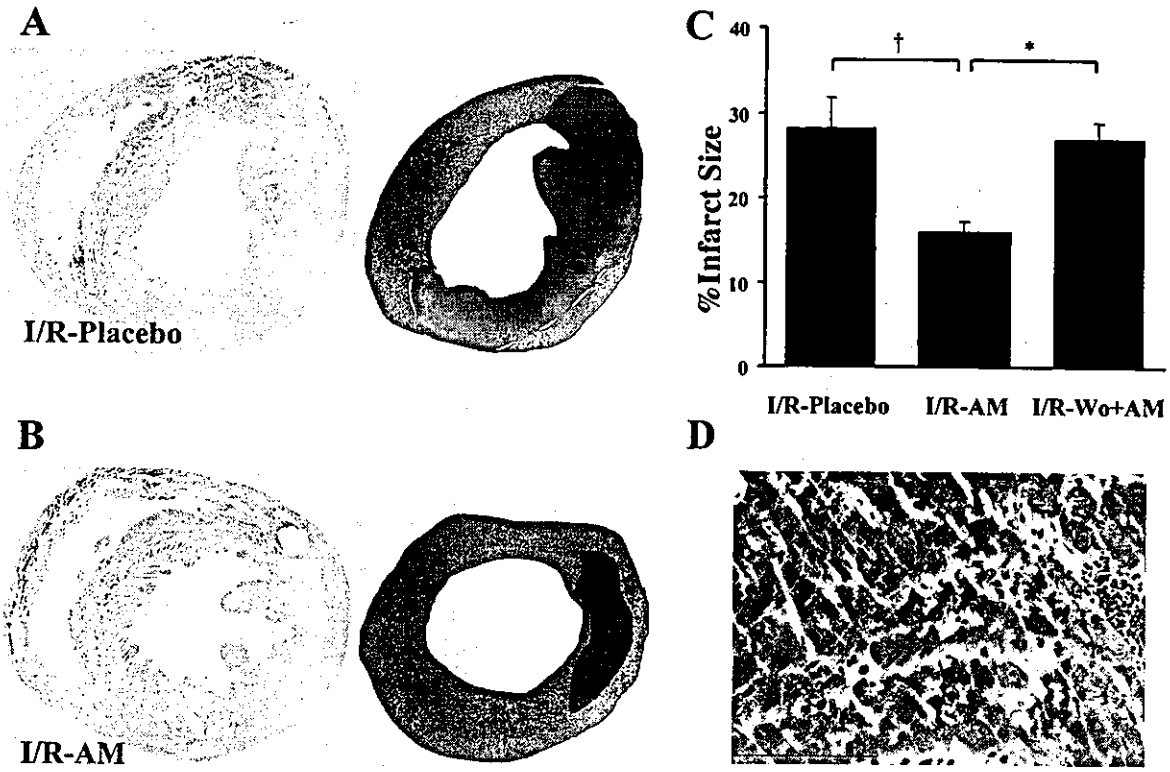

Figure 1. Effect of AM on myocardial infarct size 24 hours after ischemia/reperfusion. A and B, Photomicrographs show representative myocardial sections stained with Masson trichrome in I/R-placebo (A) and I/R-AM groups (B). Light red area indicates coagulation necrosis (right). C, Quantitative analysis demonstrated that AM infusion decreased infarct size after ischemia/reperfusion. However, pretreatment with wortmannin attenuated effect of AM. D, Typical reperfusion injury was observed in all groups on high-power field. Bar=100 μm. Data are mean±SEM. *$P<0.05$, †$P<0.01$.

mia/reperfusion (Figures 1A and 1B). Quantitative analysis revealed that 60-minute infusion of AM (0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$) significantly reduced myocardial infarct size compared with placebo infusion (16±1 versus 28±4%, $P<0.01$; Figure 1C). Infusion of AM markedly increased plasma AM level (from 10±2 fmol/mL at baseline to 96±13 fmol/mL at 60 minutes), which suggests that the plasma AM level was pharmacologically high. Pretreatment with wortmannin reversed the reducing effects of AM on myocardial infarct size (from 16±1% to 27±2%, $P<0.05$ versus I/R-AM group; Figure 1D). Although typical reperfusion injury, including contraction bands, hemorrhage, myocardial cell coagulation, and inflammatory cell infiltration, was observed after ischemia/reperfusion (Figure 1D), there were no histological differences among the 3 groups.

Hemodynamic Effects of AM

Twenty-four hours after ischemia/reperfusion, LV end-diastolic pressure (LVEDP) showed a marked elevation in the I/R-placebo group (19±2 mm Hg); the elevation was significantly attenuated in the I/R-AM group (8±2 mm Hg, $P<0.05$; Figure 2A). Pretreatment with wortmannin attenuated the reducing effects of AM on LVEDP (from 8±2 to 17±2 mm Hg, $P<0.05$ versus I/R-AM group; Figure 2A) 24 hours after ischemia/reperfusion. LV dP/dt_{max} tended to be higher in the I/R-AM group than in the I/R-placebo group (5285±285 versus 4524±247 mm Hg/s), and LV dP/dt_{min} tended to be lower in the I/R-AM group than in the I/R-placebo group (−4700±303 versus −3695±165 mm Hg/s; Figure 2B). Furthermore, pretreatment with wortmannin reversed the effects of AM on LV dP/dt_{max} and LV dP/dt_{min} after ischemia/reperfusion (5285±285 to 4570±239 mm Hg/s, −4700±303 to −3843±227 mm Hg/s, respectively; Figure 2B). These results suggest that AM infusion improved LV systolic and diastolic function after ischemia/reperfusion through the PI3K pathway. Interestingly, heart rate was significantly higher in the I/R-placebo and I/R-AM groups than in the sham group (Table). Although mean aortic pressure was significantly lower in the I/R-placebo group than in the sham group, a significant decrease in mean aortic pressure was not observed in the I/R-AM group. Right ventricular systolic pressure was significantly lower in the I/R-AM group than in the I/R-placebo group.

Antiapoptotic Effect of AM in Cardiomyocytes

Representative photomicrographs showed that TUNEL-positive myocytes were more frequently observed in the I/R-placebo group than in the sham group. However, TUNEL-positive myocytes were less frequently observed in the I/R-AM group than in the I/R-placebo group (Figure 3). Although a typical DNA ladder indicating fragmented DNA in cardiomyocytes was also observed in the I/R-placebo group, it was attenuated in the I/R-AM group (Figure 4). Quantitative analyses demonstrated that the number of TUNEL-positive cardiomyocytes was significantly smaller in the I/R-AM group than in the I/R-placebo group (9±4%

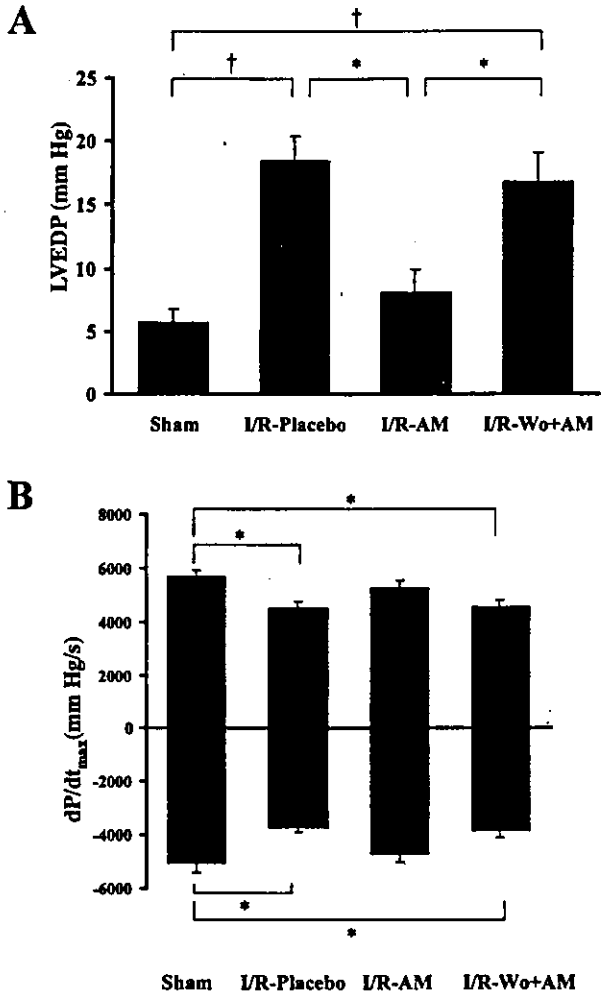

Figure 2. Effects of AM on LVEDP (A) and LV dP/dt (B) 24 hours after ischemia/reperfusion. AM infusion significantly inhibited increase in LVEDP compared with placebo infusion. AM infusion also improved LV dP/dt 24 hours after ischemia/reperfusion. Pretreatment with wortmannin attenuated effects of AM on LVEDP and LV dP/dt. Data are mean±SEM. *$P<0.05$; †$P<0.01$.

versus 19±2%, $P<0.05$; Figure 5). Furthermore, pretreatment with wortmannin abolished the AM-induced antiapoptotic effect in cardiomyocytes (from 9±4% to 20±1%, $P<0.05$; Figure 5). These results suggest that AM exerted antiapoptotic effects through the PI3K-dependent signal.

Summary of Hemodynamic Studies

	Sham (n=5)	I/R-Placebo (n=8)	I/R-AM (n=8)	I/R-Wo+AM (n=10)
Body weight, g	184±10	184±9	183±7	195±6
Heart rate, bpm	450±10	501±5*	494±9*	488±4
MAP, mm Hg	120±3	97±3*	105±4	99±7*
RAP, mm Hg	3±1	5±2	4±1	3±1
RVSP, mm Hg	32±1	47±1†	43±2†‡	48±2†

MAP indicates mean aortic pressure; RAP, right atrial pressure; and RVSP, right ventricular systolic pressure. Data are mean±SEM.

*$P<0.05$ vs sham group.

†$P<0.01$ vs Sham group.

‡$P<0.01$ vs I/R-placebo group.

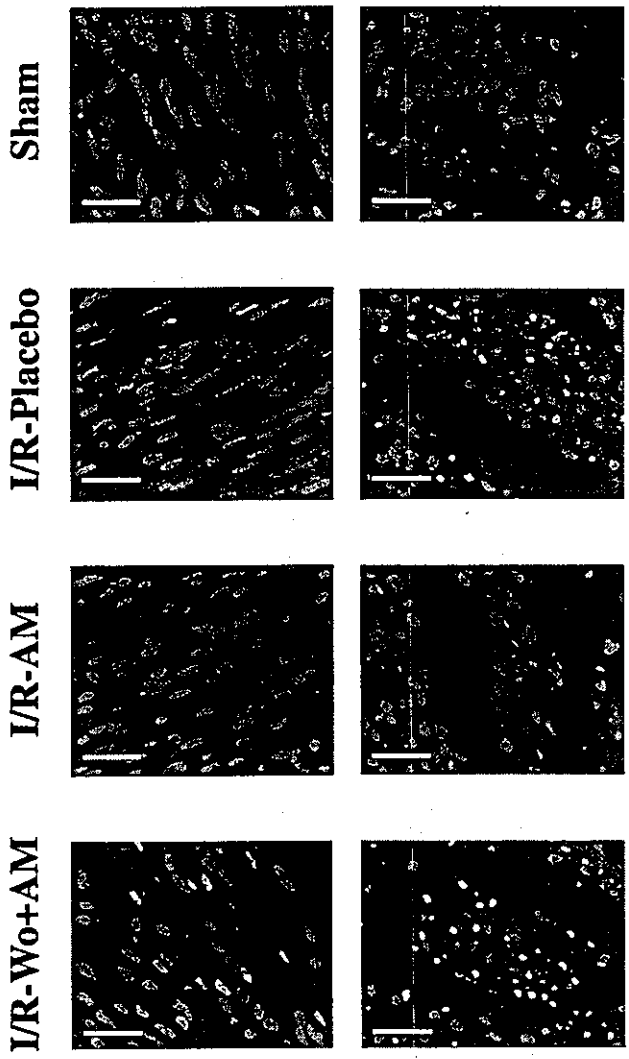

Figure 3. Representative photomicrographs of immunofluorescent staining for TUNEL-positive nuclei in sham, I/R-placebo, I/R-AM, and I/R-Wo+AM groups. Each left panel shows longitudinal myocytes, and each right panel shows short-axial myocytes. Yellow nuclei with red-stained myofilaments indicate TUNEL-positive myocytes. TUNEL-positive myocytes were less frequently observed in I/R-AM group than in I/R-placebo group. Pretreatment with wortmannin increased number of TUNEL-positive nuclei despite receipt of AM. Original magnification ×600. Bar=20 μm.

Akt Phosphorylation Induced by AM Infusion in Cardiac Tissue

Immunohistochemical analysis revealed that CRLR, a receptor for AM, was localized in cardiomyocytes and vascular endothelial cells (Figure 6). After 60-minute infusion of AM, Akt phosphorylation was detected in the nuclei of cardiomyocytes and vascular endothelial cells (Figures 7A and 7B). Western blot analyses also revealed that AM at 0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$ significantly phosphorylated Akt in cardiac tissue that was exposed to ischemia/reperfusion (Figure 7C). The effect of AM on Akt was inhibited by pretreatment with wortmannin. These results suggest that AM acts directly on myocardium and induces cardioprotective effects through the activation of PI3K/Akt-pathway.

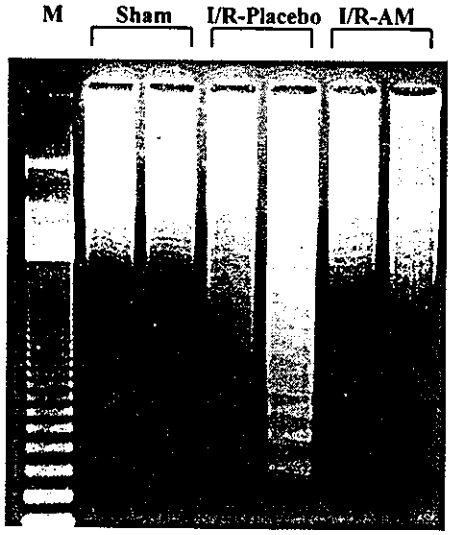

Figure 4. DNA ladder in sham, I/R-placebo, and I/R-AM groups. Although typical DNA ladder indicating fragmented DNA in cardiomyocytes was observed in I/R-placebo group, it was attenuated in I/R-AM group. M indicates molecular marker.

Discussion

In the present study, we demonstrated that short-term infusion of AM during the early phase of ischemia/reperfusion significantly reduced myocardial infarct size and inhibited myocyte apoptosis, and AM significantly decreased LVEDP and tended to improve LV dP/dt_{max} and dP/dt_{min}. We also demonstrated that AM enhanced Akt phosphorylation in cardiac tissue and that pretreatment with a PI3K inhibitor attenuated AM-induced cardioprotective effects against ischemia/reperfusion and inhibited AM-induced Akt phosphorylation.

Intravenous infusion of AM has beneficial hemodynamic and renal effects in patients with heart failure.[8] However, whether AM has direct cardioprotective effects in vivo remains unclear. In the present study, we demonstrated that short-term infusion of AM during the early phase of ischemia/reperfusion markedly reduced myocardial infarct size. Cardiomyocyte apoptosis is one of the major contributors to the development of myocardial infarcts,[15,16] which is related to the pathogenesis of heart failure. Thus, we examined whether AM has antiapoptotic effects in cardiomyocytes. Interestingly, short-term infusion of AM significantly reduced myocyte apoptosis after ischemia/reperfusion. This is the first study to demonstrate antiapoptotic effects of AM against myocardial ischemia/reperfusion injury, although AM has been shown to have antiapoptotic effects in vascular endothelial cells.[17,18] Given that cardiomyocyte apoptosis rather than necrosis contributes to myocyte death after ischemia/reperfusion, the antiapoptotic effects of AM may result in the reduced infarct size after ischemia/reperfusion.

In the present study, 60-minute infusion of AM improved cardiac function after ischemia/reperfusion, as indicated by a significant decrease in LVEDP and a tendency for an increase in LV dP/dt_{max} and a decrease in LV dP/dt_{min}. Previous studies have shown that the susceptibility to cardiac dysfunction

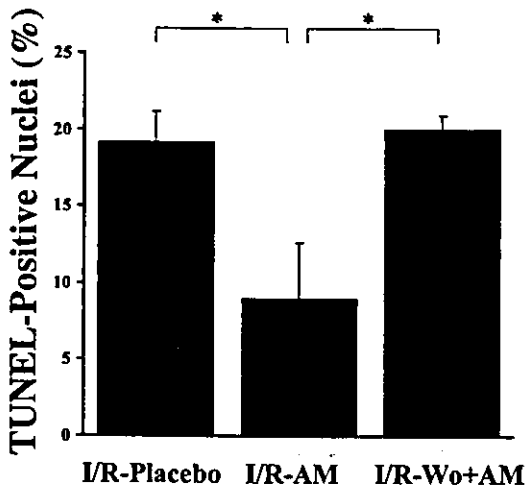

Figure 5. Quantitative analysis of TUNEL-positive nuclei in myocytes. Number of TUNEL-positive myocytes was lower in I/R-AM group than in I/R-placebo group. However, number of TUNEL-positive myocytes in I/R-Wo+AM group was as large as in I/R-placebo group. Data are mean±SEM. *$P<0.05$.

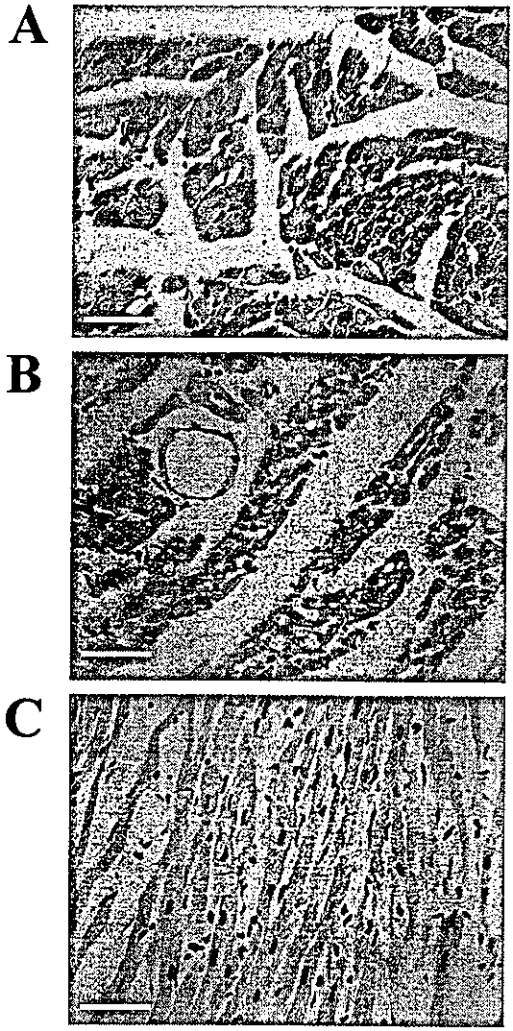

Figure 6. Immunohistochemistry for CRLR in rat cardiac tissue. Representative photomicrographs revealed that CRLR was localized in cardiomyocytes (A) and vascular endothelial cells (B). Negative control study (using mouse IgG) showed no positive staining in cardiac tissue (C). Original magnification ×400. Bar=20 μm.