We evaluated the effectiveness of measuring active ghrelin compared with total ghrelin in response to oral glucose tolerance tests (OGTTs). Four healthy male volunteers (age range, 28–35 years; body mass index, 21.5–23.7 kg/m^2) were examined on 2 separate days (100 g of glucose administered on 1 day, and 50 g of glucose administered on the other day) at least 2 weeks apart in a randomized, crossover study. After the volunteers fasted overnight, 50 or 100 g of glucose was administered orally between 0930–1000. Blood samples were obtained at 0, 1, 2, 3, and 4 h after glucose ingestion. To each plasma sample was added 1 mol/L HCl (10% of plasma volume), which acidified the sample to pH ~4; samples were then treated with Sep-Pak C_{18} cartridges for ghrelin RIAs. After glucose ingestion, the mean plasma ghrelin concentrations as determined by N-RIA and C-RIA decreased to a nadir at 1 h (Fig. 1). At this point, 60.3% and 73.0% of the basal concentration was detected by the N-RIA and C-RIA, respectively, after the 100-g OGTT, and 64.2% and 78.7% of the basal concentration was detected after the 50-g OGTT. Plasma ghrelin values increased thereafter, although plasma ghrelin concentrations measured by the C-RIA were significantly lower for up to 2 h after the 100-g glucose load. The N-RIA for ghrelin could detect differences in the changes in ghrelin concentrations between the 50-g and 100-g OGTTs at 3 h. The ghrelin values observed with the C-RIA exhibited changes similar those in the N-RIA, but the changes were small and delayed. These effects may be attributable to the differential rates of metabolic turnover for octanoylated and desacylated ghrelin in circulating blood (see Fig. 2 in the online Date Supplement).

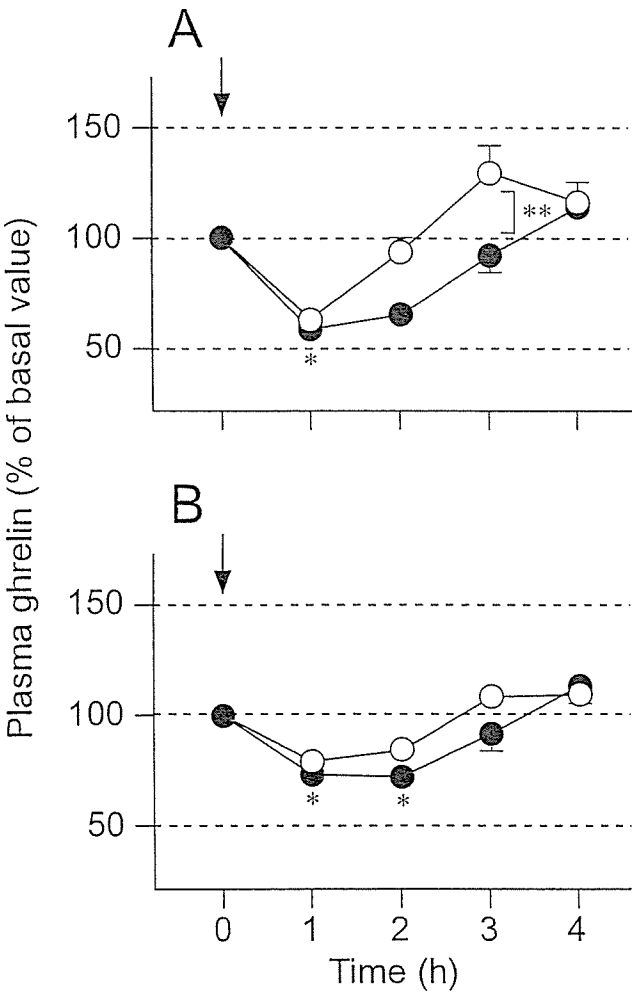

Fig. 1. Plasma ghrelin response to 50-g (○) and 100-g (●) OGTTs in four healthy individuals.

Plasma ghrelin concentrations assayed by N-RIA (*A*) and C-RIA (*B*) are given as the mean (SD; *error bars*) percentage change from basal values. *, $P < 0.05$ compared with basal values; **, $P < 0.05$ for difference in plasma ghrelin between 50-g and 100-g glucose loads.

The results for the plasma ghrelin response to the OGTTs show that measuring the concentration of active ghrelin is useful for studying plasma ghrelin changes over short time periods. Plasma concentrations of active ghrelin changed more rapidly and dynamically than those of total ghrelin immunoreactivity. Fasting led to markedly increased plasma ghrelin values as measured by N-RIA, and the values decreased in a clearer dose-dependent manner in rats after glucose injection compared with those measured by C-RIA *(16)*. The proportion of active ghrelin in plasma was 2–5% of total ghrelin in rodents. In this study, the quantity of active ghrelin was ~10% of the total ghrelin in human plasma (data not shown). These findings imply that inactive desacyl ghrelin circulates in the bloodstream at much higher concentrations than active ghrelin. Similar to previous studies in which ghrelin concentrations were measured by C-RIA *(17)*, desacyl ghrelin is relatively stable, and its stability is not altered by different storage conditions. An analogous situation has been reported for the activity of pancreatic beta cells, which secrete insulin and C-peptide in a 1:1 molar ratio. However, the half-life of C-peptide is much longer than that of insulin, leaving more C-peptide available in the circulation for quantification *(18, 19)*. Measurement of C-peptide provides an assessment of β-cell secretory activity. Similarly, desacyl ghrelin concentrations may serve as an indicator of ghrelin secretory function *(20)*.

To acquire accurate data on ghrelin concentrations, this study recommends a standard procedure for the collection of blood samples: (*a*) the collection of blood samples with EDTA–aprotinin is preferred; (*b*) blood samples should be chilled and centrifuged as soon as possible, at least within 30 min after collection; and (*c*) because acidification is the best method for the preservation of plasma ghrelin, 1 mol/L HCl (10% of sample volume) can be added to the plasma sample for adjustment to pH 4.

We thank H. Mondo and M. Miyazaki for technical assistance. This work was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan; the Ministry of Health, Labor and Welfare of Japan; the Promotion of Fundamental Studies in Health Science

from the Organization for Pharmaceutical Safety and Research of Japan; and the Takeda Science Foundation.

References

1. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 1999; 402:656–60.
2. Howard AD, Feighner SD, Cully DF, Arena JP, Liberator PA, Rosenblum CI, et al. A receptor in pituitary and hypothalamus that functions in growth hormone release. Science 1996;273:974–7.
3. Kanamoto N, Akamizu T, Hosoda H, Hataya Y, Ariyasu H, Takaya K, et al. Substantial production of ghrelin by a human medullary thyroid carcinoma cell line. J Clin Endocrinol Metab 2001;86:4984–90.
4. Hosoda H, Kojima M, Matsuo H, Kangawa K. Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. Biochem Biophys Res Commun 2000;279:909–13.
5. Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, et al. Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. Endocrinology 2000;141:4255–61.
6. Hosoda H, Kojima M, Mizushima T, Shimizu S, Kangawa K. Structural divergence of human ghrelin. Identification of multiple ghrelin-derived molecules produced by post-translational processing. J Biol Chem 2003;278: 64–70.
7. Nelesen RA, Dimsdale JE, Ziegler MG. Plasma atrial natriuretic peptide is unstable under most storage conditions. Circulation 1992;86:463–6.
8. Flower L, Ahuja RH, Humphries SE, Mohamed-Ali V. Effects of sample handling on the stability of interleukin 6, tumour necrosis factor-α and leptin. Cytokine 2000;12:1712–6.
9. Miki K, Sudo A. Effect of urine pH, storage time, and temperature on stability of catecholamines, cortisol, and creatinine. Clin Chem 1998;44(8 Pt 1):1759–62.
10. Evans MJ, Livesey JH, Ellis MJ, Yandle TG. Effect of anticoagulants and storage temperatures on stability of plasma and serum hormones. Clin Biochem 2001;34:107–12.
11. Reubsaet JL, Beijnen JH, Bult A, van Maanen RJ, Marchal JA, Underberg WJ. Analytical techniques used to study the degradation of proteins and peptides: chemical instability. J Pharm Biomed Anal 1998;17:955–78.
12. Reubsaet JL, Beijnen JH, Bult A, van Maanen RJ, Marchal JA, Underberg WJ. Analytical techniques used to study the degradation of proteins and peptides: physical instability. J Pharm Biomed Anal 1998;17:979–84.
13. Nabuchi Y, Fujiwara E, Kuboniwa H, Asoh Y, Ushio H. The stability and degradation pathway of recombinant human parathyroid hormone: deamidation of asparaginyl residue and peptide bond cleavage at aspartyl and asparaginyl residues. Pharm Res 1997;14:1685–90.
14. Strickley RG, Brandl M, Chan KW, Straub K, Gu L. High-performance liquid chromatographic (HPLC) and HPLC-mass spectrometric (MS) analysis of the degradation of the luteinizing hormone-releasing hormone (LH-RH) antagonist RS-26306 in aqueous solution. Pharm Res 1990;7:530–6.
15. Hoitink MA, Beijnen JH, Boschma MU, Bult A, van der Houwen OA, Wiese G, et al. Degradation kinetics of three gonadorelin analogues: developing a method for calculating epimerization parameters. Pharm Res 1998;15: 1449–55.
16. Ariyasu H, Takaya K, Hosoda H, Iwakura H, Ebihara K, Mori K, et al. Delayed short-term secretory regulation of ghrelin in obese animals: evidenced by a specific RIA for the active form of ghrelin. Endocrinology 2002;143:3341–50.
17. Groschl M, Wagner R, Dotsch J, Rascher W, Rauh M. Preanalytical influences on the measurement of ghrelin. Clin Chem 2002;48:1114–6.
18. Myrick JE, Gunter EW, Maggio VL, Miller DT, Hannon WH. An improved radioimmunoassay of C-peptide and its application in a multiyear study. Clin Chem 1989;35:37–42.
19. Horwitz DL, Starr JI, Mako ME, Blackard WG, Rubenstein AH. Proinsulin, insulin, and C-peptide concentrations in human portal and peripheral blood. J Clin Invest 1975;55:1278–83.
20. Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. Diabetes 2001;50:1714–9.

DOI: 10.1373/clinchem.2003.025841

Substitution of 3′-Phosphate Cap with a Carbon-Based Blocker Reduces the Possibility of Fluorescence Resonance Energy Transfer Probe Failure in Real-Time PCR Assays

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During the last decade, research and clinical use of real-time PCR applications has continued to grow in importance *(1)*. Many laboratories that use real-time PCR with fluorescent probes experience an unexplained loss of probe fluorescence at some stage, in particular with pairs of fluorescence resonance energy transfer (FRET) probes. Photobleaching is often assumed to be the cause. Structural integrity of the oligonucleotides is also a major factor, and its loss has been shown to correlate with repeated freeze–thaw cycles *(2)*. Laboratories guard against these two problems by aliquoting probes and protecting them from light. Despite these precautions, inexplicable FRET probe failures are still observed. In one recent such case, we were able to determine an additional mechanism for FRET probe failure: loss of the phosphate cap from the 3′ end of a probe. To our knowledge, this has not been described previously. Our studies revealed that this may be a common and important problem, intrinsic to 3′-phosphate-blocking chemistry. We also found that alternative terminating groups may be a preferable option to 3′-phosphate blocking.

A 3-nmol/L synthesis-scale LightCycler™ hybridization probe set was purchased from Idaho Technology Inc. Biochem in April 2003. As is common practice, the manufacturer produced a large-scale synthesis and, after shipping our order, archived the remainder for a possible future reorder. The first half of the batch (α-probe set) was sent immediately, whereas the second half (β-probe set) was stored lyophilized for 6 months at −20 °C and then shipped with our next order.

Oligonucleotides from the first shipment were used in PCR reactions in a LightCycler with satisfactory results. PCR conditions were as follows: 1× LightCycler FastStart DNA Master Hybridization Probe Mix (Roche Diagnostics), $MgCl_2$ (final concentration, 3.5 mM), 0.5 μM each of the forward (5′-GGCCTTTCTGAAGCAAG-3′) and reverse (5′-GACGATTTCTTATTTCACAGCTCC-3′) primers, 0.2 μM each of the donor (5′-GGACGCAGAGGGGATGG-FITC-3′, where FITC is fluorescein isothiocyanate) and acceptor (LCRed640-GTGTATGGGACCCGCCAG-phosphate) probes, and 2 μL of cDNA template mixture. The final reaction volume was 10 μL. The reaction started with an initial melting step at 95 °C for 10 min followed by 45 cycles of 95 °C for 2 s, 57 °C for 10 s, and 72 °C for 5 s.

Loss of fluorescence activity was first observed when we received the β-probe set. PCR reactions were carried out under the same conditions, but the amplification

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

(*Received in original form April 2, 2004; accepted in final form August 25, 2004*)

Supported by grants from New Energy and Industrial Technology Development Organization, the Mochida Memorial Foundation for Medical and Pharmaceutical Research, and the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research of Japan.

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Am J Respir Crit Care Med Vol 170. pp 1204–1211, 2004
Originally Published in Press as DOI: 10.1164/rccm.200404-455OC on August 27, 2004
Internet address: www.atsjournals.org

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-

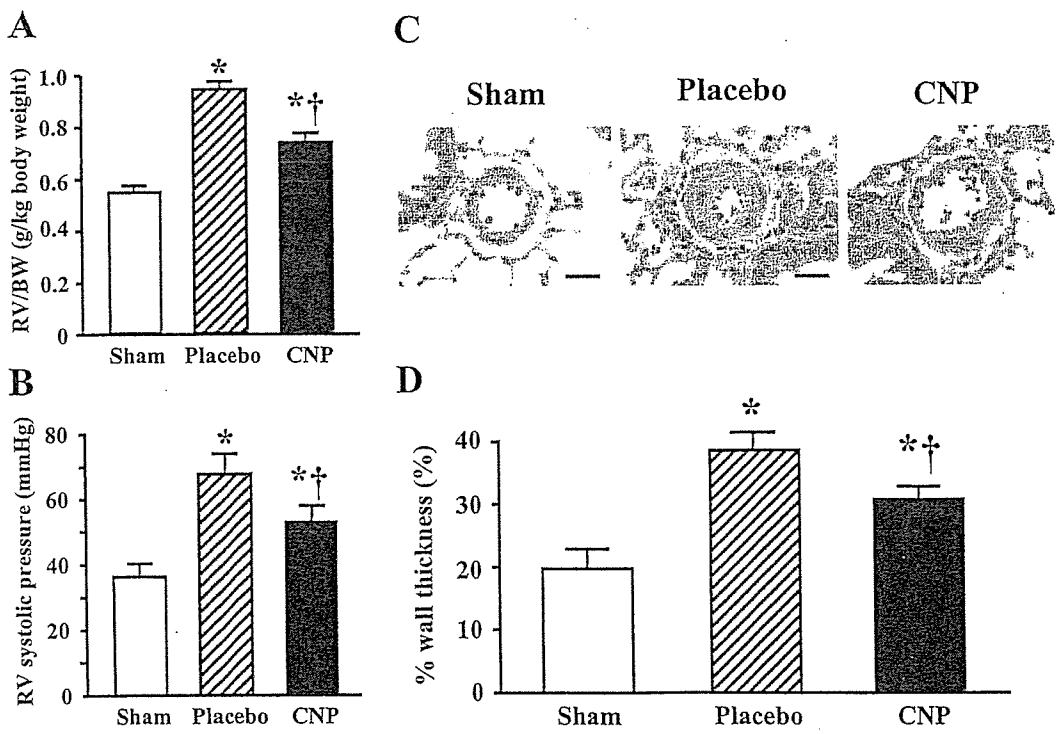

Figure 1. Effects of C-type natriuretic peptide (CNP) infusion on developing pulmonary hypertension. Continuous infusion of CNP was initiated immediately after monocrotaline (MCT) injection. (*A*) Right ventricular (RV) weight to body weight (RV/BW). (*B*) RV systolic pressure. (C) Representative photomicrographs 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.

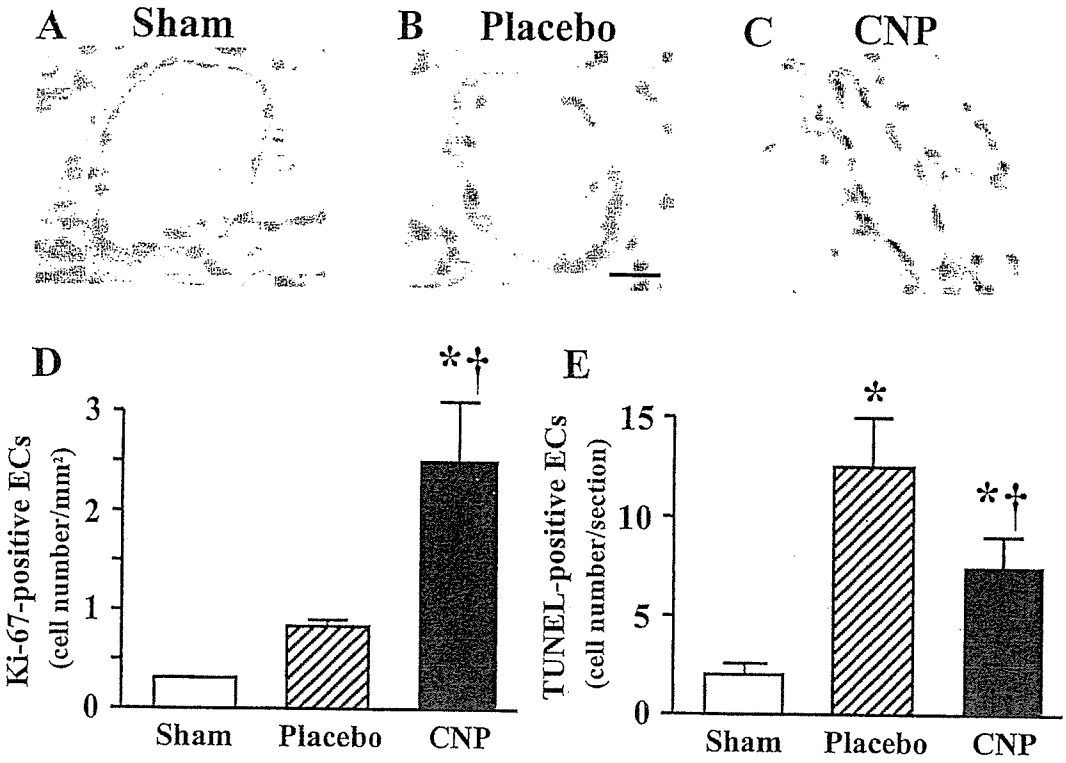

Figure 2. (*A–C*) Immunohistochemical demonstration of Ki-67 antigen, a marker for cell proliferation, in pulmonary endothelial cells (ECs) on Day 7. *Scale bars* = 20 μm. (*D*) Quantitative analysis of Ki-67–positive ECs in pulmonary vessels. The number of Ki-67–positive ECs in the CNP group was significantly 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-

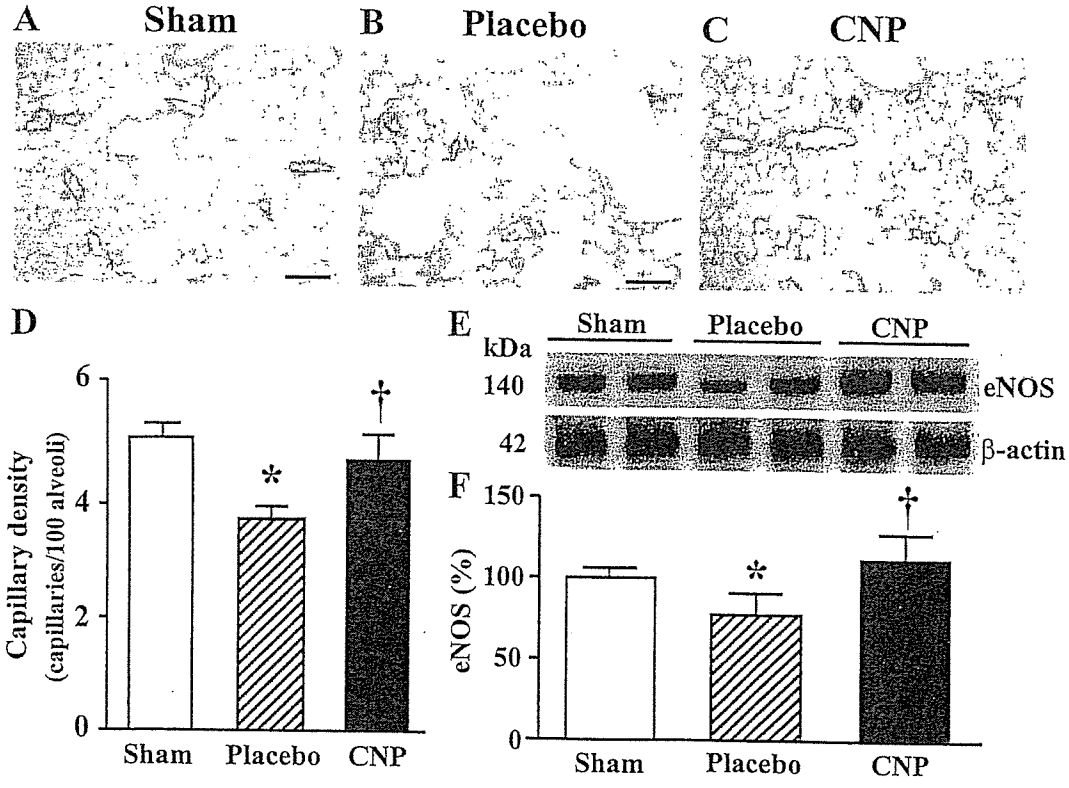

Figure 3. (*A–C*) Immunohistochemical demonstration of factor VIII antigen in lungs on Day 21. *Scale bars* = 100 μm. (*D*) Quantitative analysis of capillary density. (*E*) Representative Western blotting for endothelial nitric oxide synthase (eNOS) and β-actin (protein loading control) in lungs on 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.

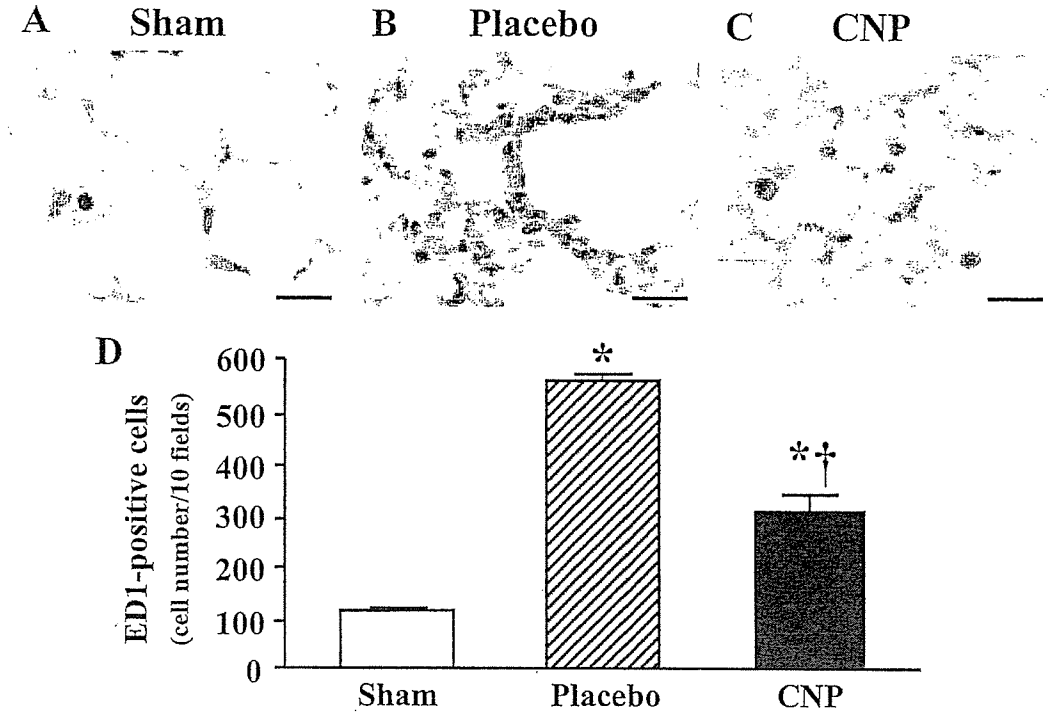

Figure 4. (*A–C*) Immunohistochemical demonstration of ED1 antigen, a marker for rat monocytes/macrophages, in lungs on Day 21. *Scale bars* = 50 μm. (*D*) Quantitative analysis of ED1-positive cells in lungs. The number of ED1-positive cells was significantly decreased in the CNP group compared with 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-

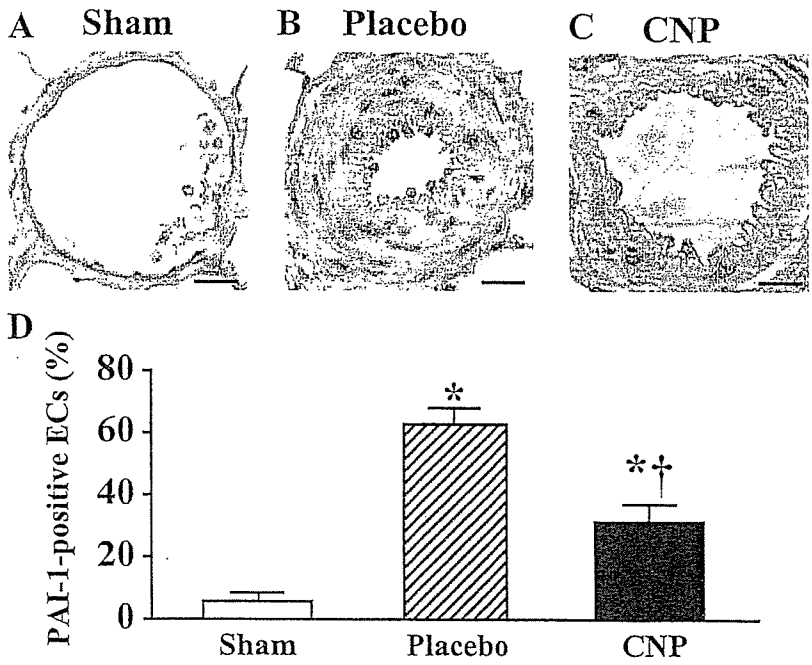

Figure 5. (*A–C*) Immunohistochemical demonstration of plasminogen activator inhibitor type 1 (PAI-1) expression in pulmonary ECs on Day 21. *Scale bars* = 20 μm. (*D*) Semiquantitative analysis of PAI-1–positive ECs. The percentage of PAI-1–positive ECs was calculated as (number of PAI-1–positive ECs/total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

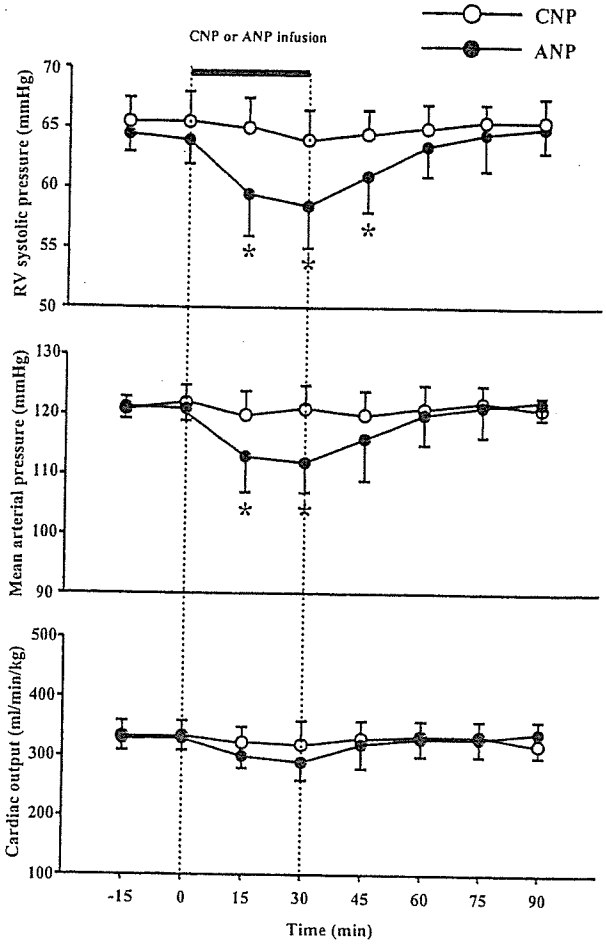

Figure 6. Acute hemodynamic responses to infusion of CNP (*open circles*) or arterial natriuretic peptide (ANP; *closed circles*) in MCT rats. Data are mean ± SEM. *p < 0.05 versus time 0.

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-

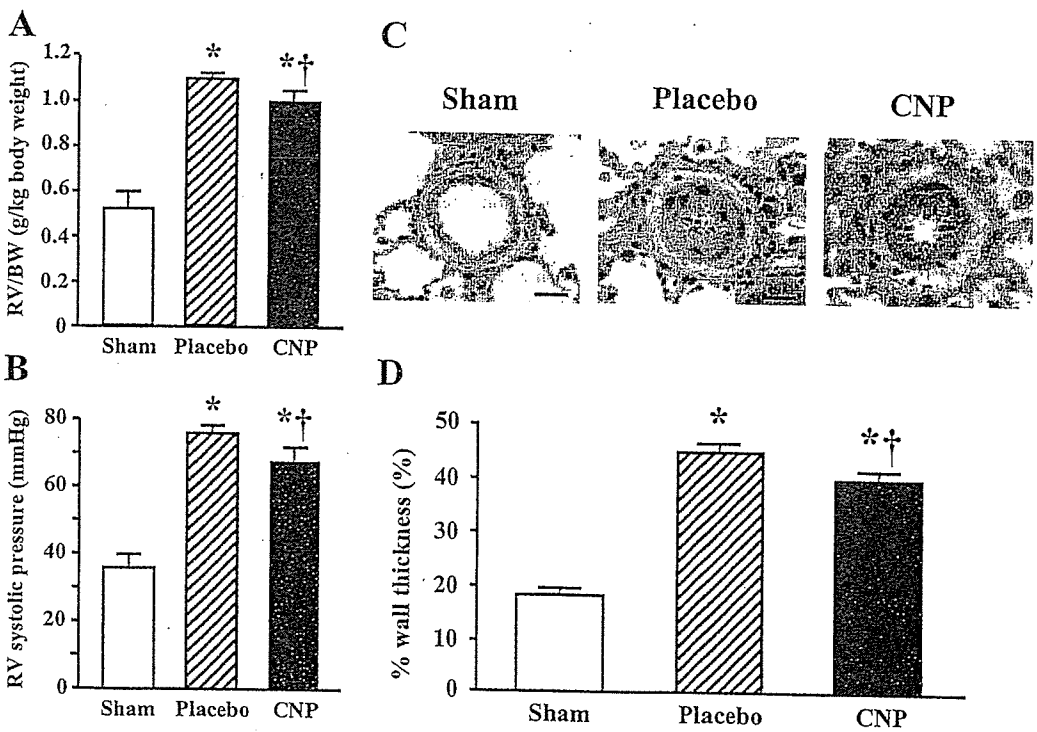

Figure 7. Effects of CNP infusion on established pulmonary hypertension. A continuous infusion of CNP was started 3 weeks after MCT injection. (*A*) RV/BW. (*B*) RV systolic pressure. (*C*) Representative photomicrographs of peripheral pulmonary arteries. *Scale bars* = 20 μm. (*D*) Quantitative analysis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

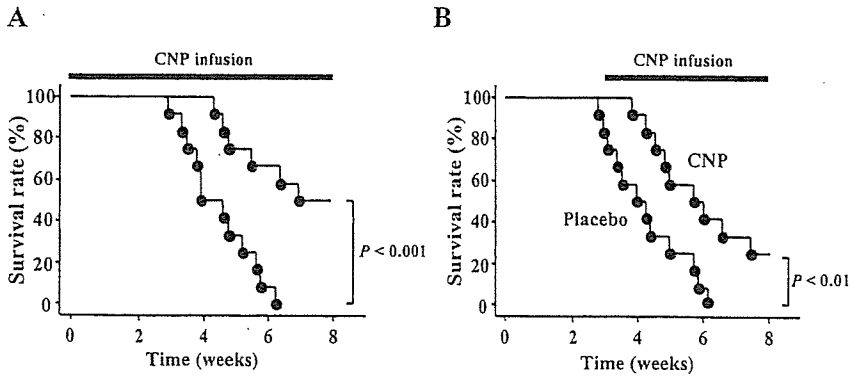

Figure 8. Kaplan-Meier survival curves. (*A*) Continuous infusion of CNP was initiated immediately after MCT injection. (*B*) CNP infusion was started 3 weeks after MCT injection.

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.

References

1. Rich S, Dantzker DR, Ayres SM, Bergofsky EH, Brundage BH, Detre KM, Fishman AP, Goldring RM, Groves BM, Koerner SK, *et al.* Primary pulmonary hypertension: a national prospective study. *Ann Intern Med* 1987;107:216–223.
2. Tuder RM, Groves B, Badesch DB, Voelkel NF. Exuberant endothelial cell growth and elements of inflammation are present in plexiform lesions of pulmonary hypertension. *Am J Pathol* 1994;144:275–285.
3. Rich S, Brundage BH. Pulmonary hypertension: a cellular basis for understanding the pathophysiology and treatment. *J Am Coll Cardiol* 1989; 14:545–550.
4. Rich S, Archer S. Primary pulmonary hypertension: a vascular biology and translational research "Work in progress." *Circulation* 2000;102: 2781–2791.
5. Davie N, Haleen SJ, Upton PD, Polak JM, Yacoub MH, Morrell NW, Wharton J. ET(A) and ET(B) receptors modulate the proliferation of human pulmonary artery smooth muscle cells. *Am J Respir Crit Care Med* 2002;165:398–405.
6. Tuder RM, Voelkel NF. Pulmonary hypertension and inflammation. *J Lab Clin Med* 1998;132:16–24.
7. Dorfmuller P, Zarka V, Durand-Gasselin I, Monti G, Balabanian K, Garcia G, Capron F, Coulomb-Lhermine A, Marfaing-Koka A, Simonneau G, *et al.* Chemokine RANTES in severe pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2002;165:534–539.
8. Balabanian K, Foussat A, Dorfmuller P, Durand-Gasselin I, Capel F, Bouchet-Delbos L, Portier A, Marfaing-Koka A, Krzysiek R, Rimaniol AC, *et al.* CX(3)C chemokine fractalkine in pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2002;165:1419–1425.
9. Sudoh T, Minamino N, Kangawa K, Matsuo H. C-type natriuretic peptide (CNP): a new member of natriuretic peptide family identified in porcine brain. *Biochem Biophys Res Commun* 1990;168:863–870.
10. Suga S, Nakao K, Itoh H, Komatsu Y, Ogawa Y, Hama N, Imura H. Endothelial production of C-type natriuretic peptide and its marked augmentation by transforming growth factor-beta: possible existence

of "vascular natriuretic peptide system." *J Clin Invest* 1992;90:1145–1149.
11. Suga S, Nakao K, Hosoda K, Mukoyama M, Ogawa Y, Shirakami G, Arai H, Saito Y, Kambayashi Y, Inouye K, *et al.* Receptor selectivity of natriuretic peptide family, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide. *Endocrinology* 1992;130:229–239.
12. Komatsu Y, Nakao K, Itoh H, Suga S, Ogawa Y, Imura H. Vascular natriuretic peptide. *Lancet* 1992;340:622.
13. Igaki T, Itoh H, Suga SI, Hama N, Ogawa Y, Komatsu Y, Yamashita J, Doi K, Chun TH, Nakao K. Effects of intravenously administered C-type natriuretic peptide in humans: comparison with atrial natriuretic peptide. *Hypertens Res* 1998;21:7–13.
14. Doi K, Ikeda T, Itoh H, Ueyama K, Hosoda K, Ogawa Y, Yamashita J, Chun TH, Inoue M, Masatsugu K, *et al.* C-type natriuretic peptide induces redifferentiation of vascular smooth muscle cells with accelerated reendothelialization. *Arterioscler Thromb Vasc Biol* 2001;21:930–936.
15. Qian JY, Haruno A, Asada Y, Nishida T, Saito Y, Matsuda T, Ueno H. Local expression of C-type natriuretic peptide suppresses inflammation, eliminates shear stress-induced thrombosis, and prevents neointima formation through enhanced nitric oxide production in rabbit injured carotid arteries. *Circ Res* 2002;91:1063–1069.
16. Ohno N, Itoh H, Ikeda T, Ueyama K, Yamahara K, Doi K, Yamashita J, Inoue M, Masatsugu K, Sawada N, *et al.* Accelerated reendothelialization with suppressed thrombogenic property and neointimal hyperplasia of rabbit jugular vein grafts by adenovirus-mediated gene transfer of C-type natriuretic peptide. *Circulation* 2002;105:1623–1626.
17. Yamahara K, Itoh H, Chun TH, Ogawa Y, Yamashita J, Sawada N, Fukunaga Y, Sone M, Yurugi-Kobayashi T, Miyashita K, *et al.* Significance and therapeutic potential of the natriuretic peptides/cGMP/cGMP-dependent protein kinase pathway in vascular regeneration. *Proc Natl Acad Sci USA* 2003;100:3404–3409.
18. Nagaya N, Yokoyama C, Kyotani S, Shimonishi M, Morishita R, Uematsu M, Nishikimi T, Nakanishi N, Ogihara T, Yamagishi M, *et al.* Gene transfer of human prostacyclin synthase ameliorates monocrotaline-induced pulmonary hypertension in rats. *Circulation* 2000;102:2005–2010.
19. Shimizu Y, Nagaya N, Teranishi Y, Imazu M, Yamamoto H, Shokawa T, Kangawa K, Kohno N, Yoshizumi M. Ghrelin improves endothelial dysfunction through growth hormone-independent mechanisms in rats. *Biochem Biophys Res Commun* 2003;310:830–835.
20. Rosenberg H, Rabinovitch M. Endothelial injury and vascular reactivity in monocrotaline pulmonary hypertension. *Am J Physiol* 1988;255:H1484–H1491.
21. Klinger JR, Siddiq FM, Swift RA, Jackson C, Pietras L, Warburton RR, Alia C, Hill NS. C-type natriuretic peptide expression and pulmonary vasodilation in hypoxia-adapted rats. *Am J Physiol* 1998;275:L645–L652.
22. Schraufnagel DE, Schmid A. Pulmonary capillary density in rats given monocrotaline: a cast corrosion study. *Am Rev Respir Dis* 1989;140:1405–1409.
23. Nagaya N, Kangawa K, Kanda M, Uematsu M, Horio T, Fukuyama N, Hino J, Harada-Shiba M, Okumura H, Tabata Y, *et al.* Hybrid cell-gene therapy for pulmonary hypertension based on phagocytosing action of endothelial progenitor cells. *Circulation* 2003;108:889–895.
24. Knowles RG, Moncada S. Nitric oxide synthases in mammals. *Biochem J* 1994;298:249–258.
25. Palmer RMJ, Ashton DS, Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* 1988;333:664–666.
26. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 1987;327:524–526.
27. Champion HC, Bivalacqua TJ, D'Souza FM, Ortiz LA, Jeter JR, Toyoda K, Heistad DD, Hyman AL, Kadowitz PJ. Gene transfer of endothelial nitric oxide synthase to the lung of the mouse in vivo: effect on agonist-induced and flow-mediated vascular responses. *Circ Res* 1999;84:1422–1432.
28. Thomas HC, Lame MW, Dunston SK, Segall HJ, Wilson DW. Monocrotaline pyrrole induces apoptosis in pulmonary artery endothelial cells. *Toxicol Appl Pharmacol* 1998;151:236–244.
29. Campbell AI, Zhao Y, Sandhu R, Stewart DJ. Cell-based gene transfer of vascular endothelial growth factor attenuates monocrotaline-induced pulmonary hypertension. *Circulation* 2001;104:2242–2248.
30. Zhao YD, Campbell AI, Robb M, Ng D, Stewart DJ. Protective role of angiopoietin-1 in experimental pulmonary hypertension. *Circ Res* 2003;92:984–991.
31. Voelkel NF, Tuder RM, Bridges J, Arend WP. Interleukin-1 receptor antagonist treatment reduces pulmonary hypertension generated in rats by monocrotaline. *Am J Respir Cell Mol Biol* 1994;11:664–675.
32. Sugita T, Hyers TM, Dauber IM, Wagner WW, McMurtry IF, Reeves JT. Lung vessel leak precedes right ventricular hypertrophy in monocrotaline-treated rats. *J Appl Physiol* 1983;54:371–374.
33. Kimura H, Kasahara Y, Kurosu K, Sugito K, Takiguchi Y, Terai M, Mikata A, Natsume M, Mukaida N, Matsushima K, *et al.* Alleviation of monocrotaline-induced pulmonary hypertension by antibodies to monocyte chemotactic and activating factor/monocyte chemoattractant protein-1. *Lab Invest* 1998;78:571–581.
34. Loskutoff DJ, Sawdey M, Mimuro J. Type 1 plasminogen activator inhibitor. *Prog Hemost Thromb* 1989;9:87–115.
35. Hekman CM, Loskutoff DJ. Endothelial cells produce a latent inhibitor of plasminogen activators that can be activated by denaturants. *J Biol Chem* 1985;260:11581–11587.
36. Simpson AJ, Booth NA, Moore NR, Bennett B. Distribution of plasminogen activator inhibitor (PAI-1) in tissues. *J Clin Pathol* 1991;44:139–143.
37. Krishnamurti C, Alving BM. Plasminogen activator inhibitor type 1: biochemistry and evidence for modulation of fibrinolysis in vivo. *Semin Thromb Hemost* 1992;18:67–80.
38. Schultze AE, Roth RA. Fibrinolytic activity in blood and lungs of rats treated with monocrotaline pyrrole. *Toxicol Appl Pharmacol* 1993;121:129–137.
39. Bouchie JL, Hansen H, Feener EP. Natriuretic factors and nitric oxide suppress plasminogen activator inhibitor-1 expression in vascular smooth muscle cells: role of cGMP in the regulation of the plasminogen system. *Arterioscler Thromb Vasc Biol* 1998;18:1771–1779.
40. Pawlowska Z, Jerczynska H, Szemraj J, Baranska P, Swiatkowska M, Cierniewski CS. Natriuretic peptides reduce plasminogen activator inhibitor-1 expression in human endothelial cells. *Cell Mol Biol Lett* 2002;7:1153–1157.

Elevated Plasma Ghrelin Level in Underweight Patients with Chronic Obstructive Pulmonary Disease

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Ghrelin, a novel growth hormone–releasing peptide, has been shown to cause a positive energy balance by reducing fat use and stimulating food intake. This study investigated whether plasma ghrelin is associated with clinical parameters in patients with chronic obstructive pulmonary disease. Plasma ghrelin was measured in 50 patients and 13 control subjects, together with anabolic and catabolic factors. Patients were divided into two groups based on body mass index: underweight patients (n = 26) or normal weight patients (n = 24). Plasma ghrelin was significantly higher in underweight patients than in normal weight patients and healthy control subjects. Circulating tumor necrosis factor-α, interleukin-6, and norepinephrine were significantly higher in underweight patients than in normal weight patients. Plasma ghrelin correlated negatively with body mass index and correlated positively with catabolic factors such as tumor necrosis factor-α and norepinephrine. In addition, plasma ghrelin correlated positively with percent predicted residual volume and residual volume-to-total lung capacity ratio. In conclusion, plasma ghrelin was elevated in underweight patients with chronic obstructive pulmonary disease, and the level was associated with a cachectic state and abnormality of pulmonary function.

Keywords: cachexia; ghrelin; hormone; pulmonary disease, chronic obstructive

Patients with chronic obstructive pulmonary disease (COPD) often show a certain degree of cachexia. Cachexia is an independent risk factor for mortality in such patients (1–3). Studies have shown that changes in endocrine hormones such as orexin and leptin have close relationships with cachexia associated with COPD (4–6). Growth hormone (GH) and its mediator, insulin-like growth factor (IGF)-I, are anabolic hormones that are essential for skeletal growth and metabolic homeostasis (7, 8). GH treatment has been shown to increase muscle mass in patients with COPD (9), although it has adverse effects including edema and abnormal glucose tolerance. These findings suggest a role of the GH/IGF-I axis in cachexia associated with COPD.

Ghrelin, a novel endogenous GH-releasing peptide, was isolated from the stomach (10). Ghrelin stimulates the secretion of GH through a mechanism independent from that of hypothalamic GH-releasing hormone. Ghrelin has been shown to cause a positive energy balance by reducing fat utilization through GH-independent mechanisms (11). In addition, both intracerebroventricular and peripheral administration of ghrelin have been shown to elicit potent, long-lasting stimulation of food intake via activation of neuropeptide Y neurons in the hypothalamic arcuate nucleus in animals (12–14). The plasma ghrelin level has been reported to be elevated in cachectic states (15, 16). However, little information is available regarding the pathophysiology of ghrelin in COPD.

Thus, the purposes of this study were to investigate (1) whether the plasma ghrelin level is elevated in patients with COPD, and (2) whether the plasma ghrelin level is related to a cachectic state and pulmonary function in patients with COPD.

METHODS

Subjects

We studied 50 patients with COPD (46 men and 4 women; mean age, 71 years; range, 41 to 83 years). COPD was diagnosed according to Global Initiative for Chronic Obstructive Lung Disease criteria. All patients were clinically stable at the time of evaluation. This study included 13 control subjects who had normal pulmonary function. The age and sex of the control subjects were similar to those of the 50 patients. The Institutional Review Board of Nara Medical University (Nara, Japan) approved this study. All subjects provided informed consent.

Patients with COPD were divided into two groups based on body mass index (BMI): underweight patients (BMI < 20, n = 26), or normal weight patients (BMI ≥ 20, n = 24). There was no significant difference in age, sex, smoking history, disease severity, or medication use between underweight and normal weight patients with COPD (Table 1). The mean smoking history was significantly higher in patients with COPD than in control subjects.

Fat-free mass (lean body mass) was measured by bioelectrical impedance analysis to investigate the relationship between plasma ghrelin and body composition in a subsample of 16 patients (underweight patients, n = 8; normal weight patients, n = 8). Lean body mass was significantly lower in underweight patients than in normal weight patients (39.3 ± 1.4 versus 46.5 ± 2.1 kg, $p < 0.05$).

Pulmonary Function Testing

Lung volumes were measured by the helium gas dilution method, and forced expiratory flow rates were measured with a mass flow anemometer (FUDAC 70; Fukuda Denshi, Tokyo, Japan). Carbon monoxide transfer factor was measured by the single-breath method. Pulmonary function values were expressed as a percentage of predicted values (17). Arterial blood gases were measured at rest with a blood gas analyzer (ABL 720; Radiometer, Brønshøj, Denmark).

Blood Sampling and Analysis

Blood samples were taken from the antecubital vein in the morning between 7:00 and 8:00 A.M. after an overnight fast. The blood was centrifuged immediately at 4°C and stored at −80°C. Plasma ghrelin was measured by radioimmunoassay as described previously (18).

Serum IGF-I was measured by radioimmunoassay (Somatomedin CII Bayer; Bayer Medical, Tokyo, Japan). Serum tumor necrosis

(*Received in original form October 14, 2003; accepted in final form July 20, 2004*)

Supported by the Mochida Memorial Foundation for Medical and Pharmaceutical Research and by grants from the Japan Cardiovascular Research Foundation, the New Energy and Industrial Technology Development Organization (NEDO), the Organization for Pharmaceutical Safety and Research (OPSR) of Japan (Promotion of Fundamental Studies in Health Science), and the Research Committee, Intractable Respiratory Failure, Ministry of Health, Labor, and Welfare of Japan.

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Am J Respir Crit Care Med Vol 170. pp 879–882, 2004
Originally Published in Press as DOI: 10.1164/rccm.200310-1404OC on July 21, 2004
Internet address: www.atsjournals.org

TABLE 1. PATIENT CHARACTERISTICS

	Control (*n* = 13)	COPD	
		Normal Weight (*n* = 24)	Underweight (*n* = 26)
Age, yr	69 ± 2	71 ± 2	71 ± 2
Sex, male/female	11/2	23/1	23/3
Body mass index, kg/m²	24.2 ± 0.7	24.2 ± 0.6	18.0 ± 0.3*,†
Smoking history, pack-years	17.6 ± 5.8	69.4 ± 6.2*	53.9 ± 5.9*
Severity stage, n			
I	NA	6	2
II	NA	5	7
III	NA	9	11
IV	NA	4	6
Medication use, n			
Anticholinergics	NA	11	16
β-Agonists	NA	15	14
Inhaled corticosteroids	NA	8	7
Xanthines	NA	14	14
Pulmonary function			
FEV_1, % predicted	93.0 ± 3.9	47.7 ± 4.4*	47.6 ± 3.5*
FEV_1/FVC, %	84.0 ± 2.3	41.4 ± 2.5*	41.7 ± 2.8*
VC, % predicted	95.7 ± 2.0	90.6 ± 5.5	84.8 ± 3.4
RV, % predicted		132.3 ± 7.8	152.0 ± 9.2
TLC, % predicted		101.2 ± 2.9	105.6 ± 3.9
RV/TLC, %		48.7 ± 2.3	52.8 ± 1.7
D_{LCO}, % predicted		61.6 ± 7.8	41.9 ± 5.5†
Pa_{O_2}, mm Hg		72.1 ± 1.7	71.4 ± 2.0
Pa_{CO_2}, mm Hg		44.5 ± 1.4	44.1 ± 1.6

Definition of abbreviations: D_{LCO} = diffusing capacity of the lung for carbon monoxide; NA = not applicable; RV = residual volume; RV/TLC, RV-to-TLC ratio; TLC = total lung capacity; VC = vital capacity.

Data represent means ± SEM.

*$p < 0.05$ versus control.

†$p < 0.05$ versus normal weight.

factor-α, interleukin-6, and insulin were measured by enzyme immunoassay (Quantikine HS [R&D Systems, Minneapolis, MN]; TFB kit [TFB, Tokyo, Japan]; and AIA-PACK IRI [Tosoh, Tokyo, Japan], respectively). Plasma epinephrine and norepinephrine were measured by high-performance liquid chromatography (HLC8030; Tosoh). Serum testosterone in male subjects was measured by radioimmunoassay (DPC testosterone kit; DPC, Los Angeles, CA). Serum prealbumin, retinol-binding protein, and transferrin were measured by nephelometry (Dade Behring, Deerfield, IL).

Statistical Analysis

Data are expressed as means ± SEM. Comparisons of parameters between the two groups were made by Fishes exact test or unpaired Student *t* test. Comparisons of parameters among three groups were made by one-way analysis of variance followed by the Scheffé multiple comparison test. Five groups (control subjects and patients with Stage I, II, III, and IV COPD) were compared by one-way analysis of variance followed by the Scheffé multiple comparison test. Independent relations between plasma ghrelin and pulmonary function parameters were examined by multivariate regression analyses. A p value less than 0.05 was considered statistically significant.

RESULTS

Biochemical Factors

Serum total protein and total cholesterol were significantly lower in underweight patients with COPD than in control subjects (Table 2). In addition, serum triglyceride, prealbumin, retinol-binding protein, and transferrin were significantly lower in underweight patients than in normal weight patients and control subjects.

TABLE 2. CIRCULATING LEVELS OF HORMONAL AND BIOCHEMICAL FACTORS

	Control (*n* = 13)	COPD	
		Normal Weight (*n* = 24)	Underweight (*n* = 26)
Total protein, g/dl	7.5 ± 0.1	7.2 ± 0.1	7.0 ± 0.1*
Albumin, g/dl	4.6 ± 0.1	4.5 ± 0.1	4.4 ± 0.1
Total cholesterol, mg/dl	221 ± 7	206 ± 6	192 ± 5*
Triglyceride, mg/dl	119 ± 9	106 ± 11	68 ± 4†,‡
Fasting glucose, mg/dl	109 ± 6	99 ± 4	97 ± 3
Prealbumin, mg/dl	30.3 ± 1.8	27.2 ± 0.8	23.0 ± 0.7†,‡
Retinol-binding protein, g/dl	4.6 ± 0.4	3.9 ± 0.2	3.0 ± 0.1†,‡
Transferrin, mg/dl	262 ± 7	228 ± 7*	202 ± 7†,§
Tumor necrosis factor-α, pg/ml	1.4 ± 0.1	4.3 ± 0.3†	6.8 ± 0.8†,‡
Interleukin-6, pg/ml	1.6 ± 0.3	2.3 ± 0.5	4.2 ± 0.7*,§
Epinephrine, pg/ml	43 ± 8	45 ± 6	59 ± 7
Norepinephrine, pg/ml	308 ± 19	674 ± 76*	982 ± 97†,§
Insulin-like growth factor-1, ng/ml	107 ± 6	137 ± 7*	114 ± 6§
Insulin, μU/ml	8.2 ± 0.8	7.0 ± 0.8	3.9 ± 0.6†,‡
Testosterone, ng/dl	419 ± 32	421 ± 19	484 ± 24

Data represent means ± SEM.

*$p < 0.05$ versus control.

†$p < 0.01$ versus control.

‡$p < 0.01$ versus normal weight.

§$p < 0.05$ versus normal weight.

Plasma Ghrelin and Cachectic State in Patients with COPD

The plasma ghrelin level was significantly higher in patients with COPD than in control subjects (237 ± 13 versus 157 ± 10 fmol/ml, $p < 0.01$). In particular, the plasma ghrelin level was higher in underweight patients than in normal weight patients and control subjects (272 ± 20 versus 195 ± 11 and 157 ± 10 fmol/ml, respectively, $p < 0.01$; Figure 1). The level did not significantly differ between normal weight patients and control subjects. The plasma ghrelin level correlated negatively with BMI ($r = -0.38$, $p < 0.01$; Figure 2). In addition, plasma ghrelin level correlated negatively with fat-free mass (lean body mass) ($r = -0.49$, $p < 0.05$) in a subsample of 16 patients.

Circulating levels of catabolic factors such as tumor necrosis factor-α and norepinephrine were significantly higher in both COPD groups than in control subjects (Table 2). Furthermore, the increases in these catabolic factors were marked in underweight patients compared with normal weight patients. On the other hand, circulating levels of anabolic factors such as IGF-I and insulin were significantly lower in underweight patients than in normal weight patients, although these anabolic factors in normal weight patients ware increased (IGF-I) or unchanged (insulin) compared with those in control subjects. The plasma ghrelin level correlated positively with serum tumor necrosis

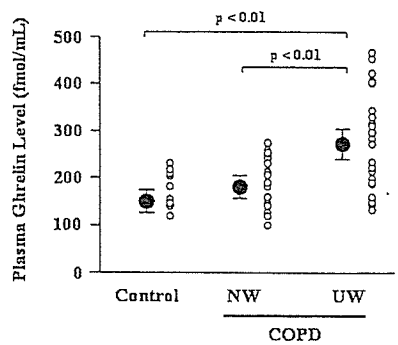

Figure 1. Plasma level of ghrelin in control subjects (Control), normal weight patients with chronic obstructive pulmonary disease (COPD) (NW), and underweight patients with COPD (UW).

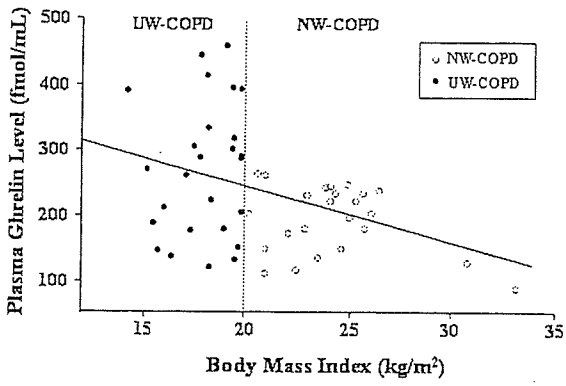

Figure 2. Correlation between plasma ghrelin level and body mass index in patients with COPD. Patients with COPD were divided into two groups: normal weight patients (NW-COPD) and underweight patients (UW-COPD). $r = -0.38$, $p < 0.01$.

factor-α ($r = 0.47$, $p < 0.01$) and plasma norepinephrine ($r = 0.40$, $p < 0.01$), but not serum IGF-I ($r = 0.12$, $p = 0.83$) and insulin ($r = -0.25$, $p = 0.27$). The plasma ghrelin level did not significantly differ between COPD patients with (n = 15) and without (n = 35) corticosteroid therapy (255 ± 27 versus 225 ± 14 fmol/ml, p = NS).

Plasma Ghrelin and Pulmonary Function in Patients with COPD

The plasma ghrelin level was higher in COPD patients with Stage IV disease than in control subjects (283 ± 31 versus 157 ± 10 fmol/ml, $p < 0.05$; Figure 3). Plasma ghrelin level tended to correlate negatively with percent predicted forced expiratory volume in one second ($r = -0.28$, $p = 0.07$), although the correlation did not reach statistical significance. Interestingly, plasma ghrelin level correlated positively with percent predicted residual volume ($r = 0.34$, $p < 0.05$) and residual volume-to-total lung capacity ratio ($r = 0.33$, $p < 0.05$) (Figure 4). Multiple regression analysis demonstrated that percent predicted residual volume or the residual volume-to-total lung capacity ratio was an independent determinant of plasma ghrelin level (each $p < 0.05$) even after adjustment for age, sex, and BMI. On the other hand, the plasma ghrelin level did not significantly correlate with any other pulmonary function parameters.

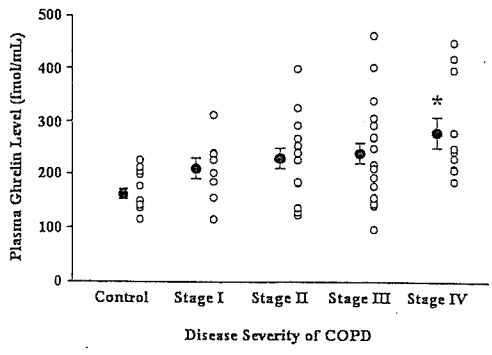

Figure 3. Plasma ghrelin level in patients with COPD according to disease severity based on Global Initiative for Chronic Obstructive Lung Disease guidelines. *$p < 0.05$ versus control.

DISCUSSION

In the present study, we demonstrated that (*1*) the plasma ghrelin level was elevated in underweight patients with COPD, and that (*2*) plasma ghrelin correlated negatively with BMI and correlated positively with circulating levels of tumor necrosis factor-α and norepinephrine. We also demonstrated that (*3*) the plasma ghrelin level was associated with indexes of hyperinflation including percent predicted residual volume and residual volume-to-total lung capacity ratio.

Ghrelin strongly stimulates GH release through a mechanism independent from that of hypothalamic GH-releasing hormone (10). Ghrelin has also been shown to cause a positive energy balance by reducing fat utilization and stimulating food intake (11–14). These findings suggest that ghrelin induces anabolic effects through GH-dependent and independent mechanisms. Thus, we investigated the pathophysiological significance of ghrelin in pulmonary cachexia. In the present study, we defined underweight as BMI < 20 kg/m². Some nutritional parameters including serum triglyceride, prealbumin, retinol-binding protein, and transferrin were also lower in underweight patients than in normal weight patients. These results suggest that "underweight" defined in the present study is accompanied by malnutrition. We demonstrated that plasma ghrelin level was higher in underweight patients than in normal weight patients. Furthermore, the plasma ghrelin level correlated negatively with BMI and lean body mass. These results suggest that the plasma ghrelin level is elevated in response to a cachectic state. Earlier studies have shown that hormonal changes and cytokine activation induce a catabolic state in patients with COPD, resulting in the development of cachexia (4–6, 19). In fact, some catabolic factors such as tumor necrosis factor-α, interleukin-6, and norepinephrine were significantly higher in underweight patients than in normal weight patients, whereas anabolic factors including IGF-I and insulin were significantly lower in underweight patients than in normal weight patients. The plasma ghrelin level correlated positively with catabolic factors such as tumor necrosis factor-α and norepinephrine. Considering the positive energy effects induced by ghrelin, increased ghrelin may represent a compensatory mechanism under catabolic–anabolic imbalance in cachectic patients with COPD. Unexpectedly, the serum IGF-I level was significantly higher in normal weight patients than in control subjects. Catabolic factors including tumor necrosis factor-α and norepinephrine were significantly higher in normal weight patients than in control subjects, although the increases were marked in underweight patients. These findings raise the possibility that increased IGF-I in normal weight patients may represent a compensatory mechanism under conditions of energy imbalance.

In the present study, the plasma ghrelin level showed significantly positive correlation with indexes of hyperinflation such as percent predicted residual volume and residual volume-to-total lung capacity ratio. In addition, the plasma ghrelin level tended to correlate negatively with percent predicted forced expiratory volume in 1 second. Thus, elevated ghrelin may be associated with abnormality of pulmonary function in patients with COPD. Because GH secretagogues receptor, a receptor for ghrelin, is expressed in the lung (20), further studies are to investigate a role of ghrelin in the lung. Although the present study demonstrated that body composition and indexes of hyperinflation were among the determinants of the plasma ghrelin level, further work will be required to determine the factors that contribute to the wide range of ghrelin levels among underweight patients with COPD.

In conclusion, the plasma ghrelin level was elevated in underweight patients with COPD, and the level was associated with a cachectic state and abnormality of pulmonary function.

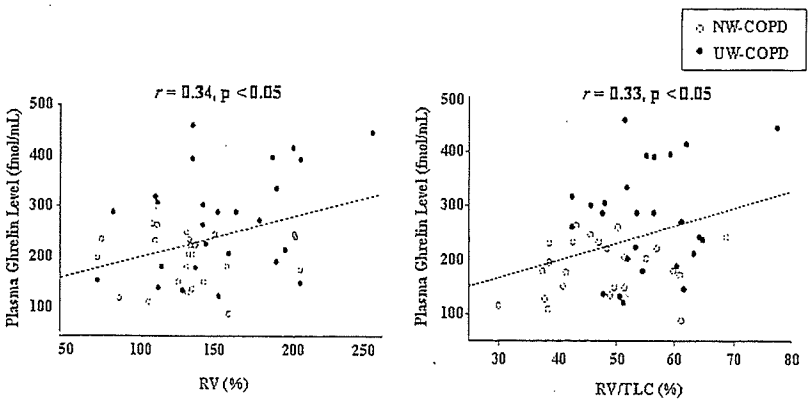

Figure 4. Correlations between plasma ghrelin level and percent predicted residual volume (RV, *left*), and between plasma ghrelin level and residual volume-to-total lung capacity ratio (RV/TLC, *right*) in patients with COPD. NW-COPD indicates normal weight patients with COPD; UW-COPD, underweight patients with COPD.

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; M.Y. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; A.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; H.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; Y.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; Y.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; H.O. 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.H. 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; H.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

References

1. Wilson DO, Rogers RM, Wright EC, Anthonisen NR. Body weight in chronic obstructive pulmonary disease: the National Institutes of Health Intermittent Positive Breathing Trial. *Am Rev Respir Dis* 1989; 139:1435–1438.
2. Gray-Donald K, Gibbons L, Shapiro SH, Macklem PT, Martin JG. Nutritional status and mortality in COPD. *Am J Respir Crit Care Med* 1996; 153:961–966.
3. Landbo C, Prescott E, Lange P, Vestbo J, Almdal TP. Prognostic value of nutritional status in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1999;160:1856–1861.
4. Takabatake N, Nakamura H, Minamihaba O, Inage M, Inoue S, Kagaya S, Yamaki M, Tomoike H. A novel pathophysiologic phenomenon in cachexic patients with chronic obstructive pulmonary disease: the relationship between the circadian rhythm of circulating leptin and the very low-frequency component of heart rate variability. *Am J Respir Crit Care Med* 2001;163:1314–1319.
5. Matsumura T, Nakayama M, Satoh H, Naito A, Kamahara K, Sekizawa K. Plasma orexin-A levels and body composition in COPD. *Chest* 2003; 123:1060–1065.
6. Debigare R, Marquis K, Cote CH, Tremblay RR, Michaud A, LeBlanc P, Maltais F. Catabolic/anabolic balance and muscle wasting in patients with COPD. *Chest* 2003;124:83–89.
7. Bark TH, McNurlan MA, Lang CH, Garlick PJ. Increased protein synthesis after acute IGF-I or insulin infusion is localized to muscle in mice. *Am J Physiol* 1998;275:E118–E123.
8. Amato G, Carella C, Fazio S, La Montagna G, Cittadini A, Sabatini D, Marciano-Mone C, Sacca L, Bellastella A. Body composition, bone metabolism, heart structure and function in growth hormone deficient adults before and after growth hormone replacement therapy at low doses. *J Clin Endocrinol Metab* 1993;77:1671–1676.
9. Burdet L, de Muralt B, Schutz Y, Pichard C, Fitting JW. Administration of growth hormone to underweight patients with chronic obstructive pulmonary disease: a prospective, randomized, controlled study. *Am J Respir Crit Care Med* 1997;156:1800–1806.
10. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone releasing acylated peptide from stomach. *Nature* 1999;402:656–660.
11. Tschop M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. *Nature* 2000;407:908–913.
12. Wren AM, Small CJ, Ward HL, Murphy KG, Dakin CL, Taheri S, Kennedy AR, Roberts GH, Morgan DG, Ghatei MA, *et al.* The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. *Endocrinology* 2000;141:4325–4328.
13. Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S. A role for ghrelin in the central regulation of feeding. *Nature* 2001;409:194–198.
14. Shintani M, Ogawa Y, Ebihara K, Aizawa-Abe M, Miyanaga F, Takaya K, Hayashi T, Inoue G, Hosoda K, Kojima M, *et al.* Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway. *Diabetes* 2001;50:227–232.
15. Nagaya N, Uematsu M, Kojima M, Date Y, Nakazato M, Okumura H, Hosoda H, Shimizu W, Yamagishi M, Oya H, *et al.* Elevated circulating level of ghrelin in cachexia associated with chronic heart failure: relationships between ghrelin and anabolic/catabolic factors. *Circulation* 2001;23:2034–2038.
16. Shimizu Y, Nagaya N, Isobe T, Imazu M, Okumura H, Hosoda H, Kojima M, Kangawa K, Kohno N. Increased plasma ghrelin level in lung cancer cachexia. *Clin Cancer Res* 2003;9:774–778.
17. Berglund E, Birath G, Bjure J, Grimby G, Kjellmer I, Sandqvist L, Soderholm B. Spirometric studies in normal subjects. *Acta Med Scand* 1963;173:185–191.
18. Hosoda H, Kojima M, Matsuo H, Kangawa K. Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. *Biochem Biophys Res Commun* 2000;279:909–913.
19. Eid AA, Ionescu AA, Nixon LS, Lewis-Jenkins V, Matthews SB, Griffiths TL, Shale DJ. Inflammatory response and body composition in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2001;164: 1414–1418.
20. Gnanapavan S, Kola B, Bustin SA, Morris DG, McGee P, Fairclough P, Bhattacharya S, Carpenter R, Grossman AB, Korbonits M. The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. *J Clin Endocrinol Metab* 2002;87:2988–2991.

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 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

Submitted 12 March 2004; accepted in final form 21 July 2004

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

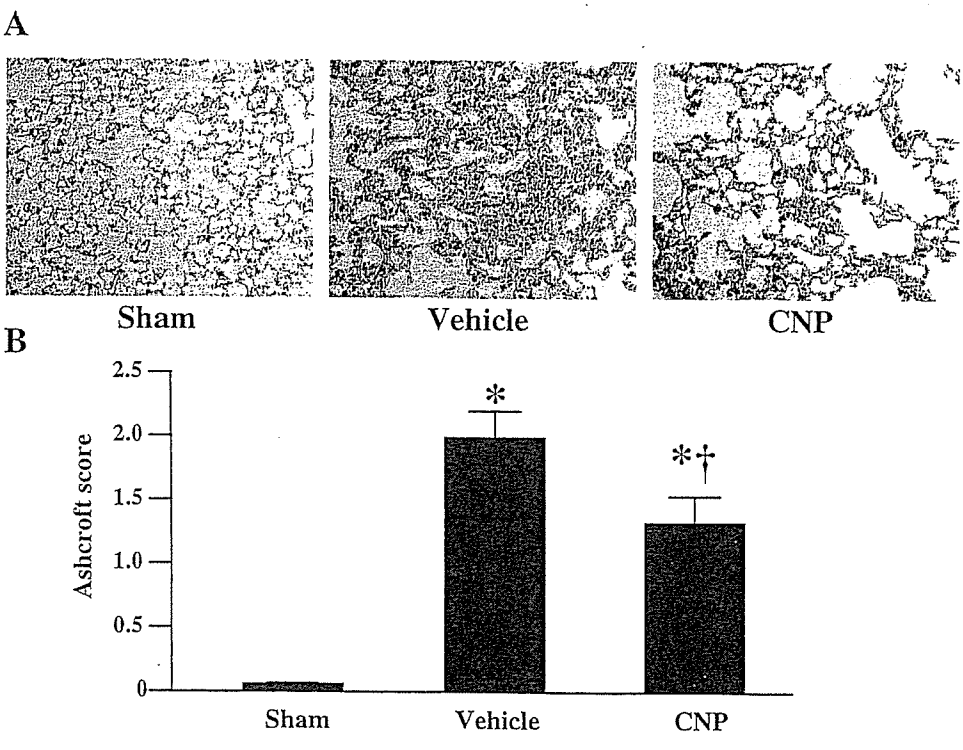

Fig. 1. *A*: representative photomicrographs of lung tissues stained with hematoxylin-eosin. Histological sections were taken from the same general regions of the lungs in each group. Intratracheal injection of bleomycin (BLM) induced pulmonary fibrosis in mice (vehicle group) compared with mice given 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.

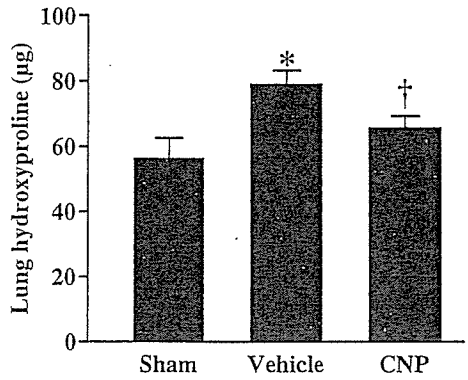

Fig. 2. Effects of CNP infusion on hydroxyproline content in left lung of BLM mice (n = 5 each). BLM injection increased lung hydroxyproline content (vehicle group). CNP infusion significantly decreased hydroxyproline content in BLM mice (CNP group). Data are means ± SE. *$P < 0.05$ vs. Sham group; †$P < 0.05$ 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 ($P = 0.058$).

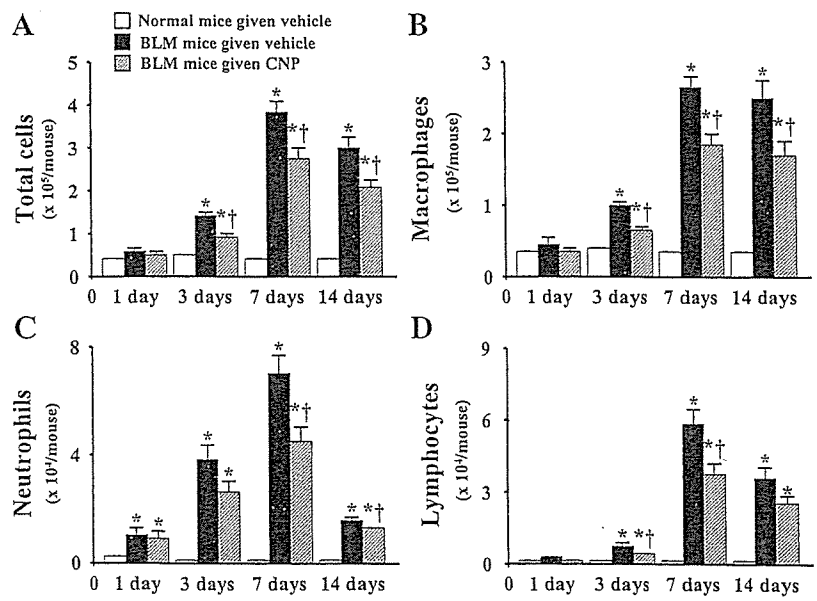

Fig. 3. Effects of CNP infusion on the numbers of total cells (*A*), macrophages (*B*), neutrophils (*C*), and lymphocytes (*D*) in bronchoalveolar lavage fluid (BALF). The total number of cells and the number of macrophages were significantly increased at 3, 7, and 14 days after BLM injection. However, 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.

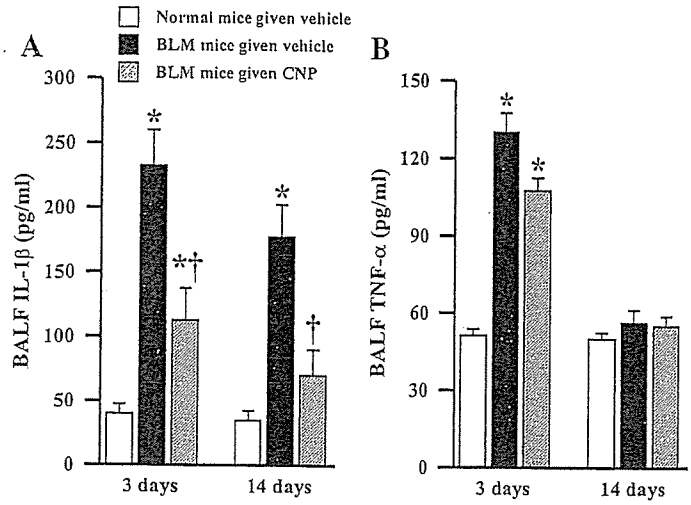

Fig. 4. Effects of CNP infusion on BALF IL-1β (*A*) and BALF TNF-α (*B*) levels at 3 and 14 days after BLM injection. CNP significantly decreased BALF IL-1β level. CNP tended to inhibit the increase in BALF TNF-α level, but this was not significant ($P = 0.058$). Data are means ± SE. *$P < 0.05$ vs.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

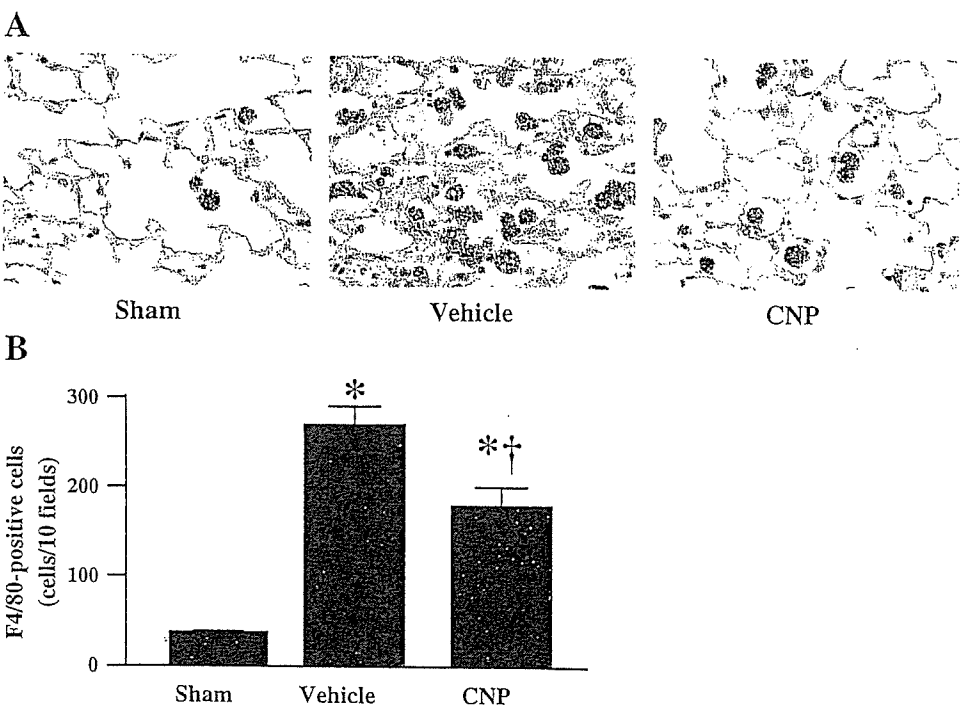

Fig. 5. *A*: immunohistochemical analysis of F4/80 antigen, a marker for monocytes/macrophages, 14 days after BLM injection. Histological sections were taken from the same general regions of the lungs in each group. Monocytes/macrophages were frequently observed in BLM mice given vehicle (vehicle group). 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.

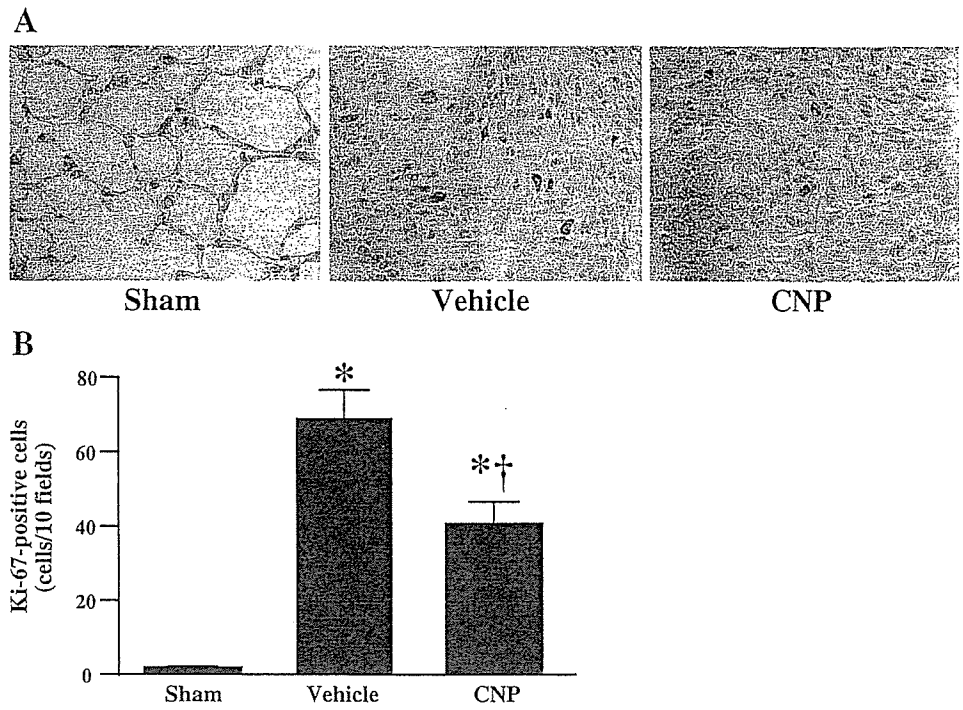

Fig. 6. *A*: immunohistochemical analysis of Ki-67 antigen, a marker for cell proliferation. Histological sections were taken from the same general regions of the lungs in each group. Ki-67-positive nuclei were detected mainly in fibrotic lesions 14 days after BLM injection (vehicle group). CNP infusion 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.

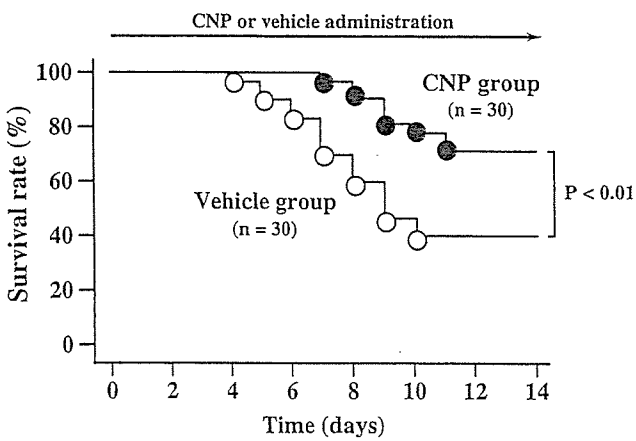

Fig. 7. Kaplan-Meier survival curves. BLM mice treated with CNP (●) had a significantly higher survival rate than those given vehicle (○) (log-rank test, $P < 0.01$).

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

This work was supported by Research Grant for Cardiovascular Disease 16C-6 from the Ministry of Health, Labour, and Welfare; Industrial Technology Research Grant Program in '03 from New Energy and Industrial Technology Development Organization of Japan; Health and Labor Sciences Research Grants-genome 005; and the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research of Japan.

REFERENCES

1. **Ashcroft T, Simpson JM, and Timbrell V.** Simple method of estimating severity of pulmonary fibrosis on a numerical scale. *J Clin Pathol* 41: 467–470, 1988.
2. **Crouch E.** Pathobiology of pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 259: L159–L184, 1990.
3. **Elias JA, Freundlich B, Kern JA, and Rosenbloom J.** Cytokine networks in the regulation of inflammation and fibrosis in the lung. *Chest* 97: 1439–1445, 1990.
4. **Geary CA, Goy MF, and Boucher RC.** Synthesis and vectorial export of cGMP in airway epithelium: expression of soluble and CNP-specific guanylate cyclases. *Am J Physiol Lung Cell Mol Physiol* 265: L598–L605, 1993.
5. **Gross TJ and Hunninghake GW.** Idiopathic pulmonary fibrosis. *N Engl J Med* 345: 517–525, 2001.
6. **Hay J, Shahzeidi S, and Laurent G.** Mechanisms of bleomycin-induced lung damage. *Arch Toxicol* 65: 81–94, 1991.
7. **Horio T, Tokudome T, Maki T, Yoshihara F, Suga S, Nishikimi T, Kojima M, Kawano Y, and Kangawa K.** Gene expression, secretion, and autocrine action of C-type natriuretic peptide in cultured adult rat cardiac fibroblasts. *Endocrinology* 144: 2279–2284, 2003.
8. **Komatsu Y, Nakao K, Itoh H, Suga S, Ogawa Y, and Imura H.** Vascular natriuretic peptide. *Lancet* 340: 622, 1992.
9. **Kuhn C and McDonald JA.** The roles of the myofibroblast in idiopathic pulmonary fibrosis. Ultrastructural and immunohistochemical features of sites of active extracellular matrix synthesis. *Am J Pathol* 138: 1257–1265, 1991.
10. **Kumar RK.** Quantitative immunohistologic assessment of lymphocyte populations in the pulmonary inflammatory response to intratracheal silica. *Am J Pathol* 135: 605–614, 1989.
11. **Maeda A, Hiyama K, Yamakido H, Ishioka S, and Yamakido M.** Increased expression of platelet-derived growth factor A and insulin-like growth factor-I in BAL cells during the development of bleomycin-induced pulmonary fibrosis in mice. *Chest* 109: 780–786, 1996.
12. **Mitsuhashi H, Asano S, Nonaka T, Hamamura I, Masuda K, and Kiyoki M.** Administration of truncated secretory leukoprotease inhibitor ameliorates bleomycin-induced pulmonary fibrosis in hamsters. *Am J Respir Crit Care Med* 153: 1369–1374, 1996.
13. **Nagai A, Aoshiba K, Ishihara Y, Inano H, Sakamoto K, Yamaguchi E, Kagawa J, and Takizawa T.** Administration of α1-proteinase inhibitor ameliorates bleomycin-induced pulmonary fibrosis in hamsters. *Am Rev Respir Dis* 145: 651–656, 1992.
14. **Nakanishi K, Tajima F, Itoh H, Nakata Y, Hama N, Nakagawa O, Nakao K, Kawai T, Torikata C, Suga T, Takishima K, Aurues T, and Ikeda T.** Expression of C-type natriuretic peptide during development of rat lung. *Am J Physiol Lung Cell Mol Physiol* 277: L996–L1002, 1999.
15. **Nakazawa K, Tanaka H, and Arima M.** Rapid, simultaneous and sensitive determination of free hydroxyproline and proline in human serum by high-performance liquid chromatography. *J Chromatogr A* 233: 313–316, 1982.
16. **Osawa H, Yamabe H, and Kaizuka M.** C-type natriuretic peptide inhibits proliferation and monocyte chemoattractant protein-1 secretion in cultured human mesangial cells. *Nephron* 86: 467–472, 2000.
17. **Phan SH, Thrall RS, and Williams C.** Bleomycin-induced pulmonary fibrosis. Effects of steroid on lung collagen metabolism. *Am Rev Respir Dis* 124: 428–434, 1981.
18. **Piguet PF, Collart MA, Grau GE, Kapanci Y, and Vassalli P.** Tumor necrosis factor/cachectin plays a key role in bleomycin-induced pneumopathy and fibrosis. *J Exp Med* 170: 655–663, 1989.
19. **Piguet PF, Vesin C, Grau GE, and Thompson RC.** Interleukin 1 receptor antagonist (IL-1ra) prevents or cures pulmonary fibrosis elicited in mice by bleomycin or silica. *Cytokine* 5: 57–61, 1993.
20. **Qian JY, Haruno A, Asada Y, Nishida T, Saito Y, Matsuda T, and Ueno H.** Local expression of C-type natriuretic peptide suppresses inflammation, eliminates shear stress-induced thrombosis, and prevents neointima formation through enhanced nitric oxide production in rabbit injured carotid arteries. *Circ Res* 91: 1063–1069, 2002.
21. **Snider GL, Hayes JA, and Korthy AL.** Chronic interstitial pulmonary fibrosis produced in hamsters by endotracheal bleomycin: pathology and stereology. *Am Rev Respir Dis* 117: 1099–1108, 1978.
22. **Sudoh T, Minamino N, Kangawa K, and Matsuo H.** C-type natriuretic peptide (CNP): a new member of natriuretic peptide family identified in porcine brain. *Biochem Biophys Res Commun* 168: 863–870, 1990.
23. **Suga S, Nakao K, Hosoda K, Mukoyama M, Ogawa Y, Shirakami G, Arai H, Saito Y, Kambayashi Y, Inouye K, and Imura H.** Receptor selectivity of natriuretic peptide family, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide. *Endocrinology* 130: 229–239, 1992.
24. **Suga S, Nakao K, Itoh H, Komatsu Y, Ogawa Y, Hama N, and Imura H.** Endothelial production of C-type natriuretic peptide and its marked augmentation by transforming growth factor-β. Possible existence of "vascular natriuretic peptide system." *J Clin Invest* 90: 1145–1149, 1992.
25. **Suwabe A, Takahashi K, Yasui S, Arai S, and Sendo F.** Bleomycin-stimulated hamster alveolar macrophages release interleukin-1. *Am J Pathol* 132: 512–520, 1988.
26. **Taooka Y, Maeda A, Hiyama K, Ishioka S, and Yamakido M.** Effects of neutrophil elastase inhibitor on bleomycin-induced pulmonary fibrosis in mice. *Am J Respir Crit Care Med* 156: 260–265, 1997.
27. **Yaekashiwa M, Nakayama S, Ohnuma K, Sakai T, Abe T, Satoh K, Matsumoto K, Nakamura T, Takahashi T, and Nukiwa T.** Simultaneous or delayed administration of hepatocyte growth factor equally represses the fibrotic changes in murine lung injury induced by bleomycin. A morphologic study. *Am J Respir Crit Care Med* 156: 1937–1944, 1997.
28. **Zhang K, Flanders KC, and Phan SH.** Cellular localization of transforming growth factor-β expression in bleomycin-induced pulmonary fibrosis. *Am J Pathol* 147: 352–361, 1995.
29. **Zhang K, Gharaee-Kermani M, Jones ML, Warren JS, and Phan SH.** Lung monocyte chemoattractant protein-1 gene expression in bleomycin-induced pulmonary fibrosis. *J Immunol* 153: 4733–4741, 1994.