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

Shinsuke Murakami, 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.

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 pump (Alzet, Palo Alto, CA) was filled with either 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).

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Table 1. Physiological profiles of three experimental groups

<table>
<thead>
<tr>
<th></th>
<th>Sham group</th>
<th>Vehicle group</th>
<th>CNP group</th>
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<tbody>
<tr>
<td><strong>Low dose (0.02 U/mouse) of BLM</strong></td>
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<tr>
<td>n</td>
<td>7</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Body weight, g</td>
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<td>18.3±0.4*‡</td>
<td>20.2±0.3*‡</td>
</tr>
<tr>
<td>Lung weight/body weight, mg/g</td>
<td>6.1±0.1</td>
<td>12.9±0.5*‡</td>
<td>7.9±0.2*‡‡</td>
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<tr>
<td><strong>High dose (0.04 U/mouse) of BLM</strong></td>
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<tr>
<td>n</td>
<td>7</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>Body weight, g</td>
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<td>15.7±1.1*‡</td>
<td>19.9±0.6*‡</td>
</tr>
<tr>
<td>Lung weight/body weight, mg/g</td>
<td>6.1±0.1</td>
<td>18.4±2.0*‡</td>
<td>12.5±1.1*‡‡</td>
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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 cytopsin 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

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.
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. 1A). 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. Semi-quantitative assessment using Ashcroft score demonstrated that the degree of pulmonary fibrosis in the CNP group was lower than that in the vehicle group (Fig. 1B). 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 number of neutrophils was 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. 3C). However, the number of these cells 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. 3D). However, the number of these cells 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. 4A). 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. 4B). CNP infusion tended to inhibit the increase in BALF TNF-α level, but this was not significant (P = 0.058).
Representative photomicrographs showed that CNP infusion markedly inhibited macrophage infiltration compared with vehicle (Fig. 5A). Semi-quantitative analysis demonstrated that BLM injection significantly increased the number of macrophages (Fig. 5B). 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. 6A). 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. 6B).

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-1\( \beta \) 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-
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.

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

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.

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

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

GRANTS

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