Protective effect of beraprost sodium, a stable prostacyclin analog, in the development of cigarette smoke extract-induced emphysema

Yan Chen,1,2 Masayuki Hanaoka,1 Ping Chen,2 Yunden Droma,1 Norbert F. Voelkel,3 and Keishi Kubo1

1First Department of Medicine, Shinshu University School of Medicine, Matsumoto, Nagano, Japan; 2Division of Respiratory Disease, Department of Internal Medicine, The Second Xiangya Hospital, Central-South University, Changsha, Hunan, China; 3Victoria Johnson Center for Obstructive Lung Diseases, Virginia Commonwealth University, Richmond, Virginia

Submitted 9 April 2008; accepted in final form 31 January 2009

Chronic obstructive pulmonary disease (COPD) is a major cause of chronic morbidity and mortality throughout the world. It is currently the fifth leading cause of death worldwide (27), and a further increase in its prevalence and mortality is predicted in the coming decades (19). The pathobiology of emphysema involves chronic inflammation of airways (5, 10), imbalance of proteolytic and anti-proteolytic activities (4, 6, 7), and oxidative stress (36). Recently, a role of the immune system in the pathogenesis of COPD has been proposed (2, 33), and several investigators reported apoptosis of structural cells in the lungs of patients with COPD (13, 20, 38) and experimental emphysema (3, 28, 36).

Prostaglandin (PGI2) is a major product of endothelial cells via the cyclooxygenase and PGI2 synthase (PGI2S) enzymes (31). PGI2 protects cells against apoptosis (8, 11), has anti-inflammatory properties (23), and partially prevents cigarette smoke extract (CSE)-induced apoptosis of the pulmonary endothelium in vitro and in a murine transgenic model of lung-specific PGI2 overexpression (24). Of interest, CSE suppressed PGI2S expression in human pulmonary microvascular endothelial cells (24).

Beraprost sodium (BPS) is a synthetic PGI2 analog that is chemically and metabolically more stable than PGI2. Here, we investigate whether the stable PGI2 analog BPS, by inhibiting alveolar cell apoptosis and other molecular pathogenesis pathways, protects against experimental emphysema.

MATERIALS AND METHODS

Preparation of CSE

CSE was prepared as previously reported (24) with some modifications. Briefly, one cigarette (Marlboro, Philip Morris Companies) was burned, and the smoke passed through a filter for removing particles and bacteria into a vessel containing PBS (1 ml/l cigarette) using a vacuum pump. The CSE-PBS solution was prepared fresh for each set of experiments.

Experimental Protocols

The animal protocol was approved by the Animal Care and Use Committee of Shinshu University. Six-week-old male Sprague-Dawley rats (weight range between 200 and 250 g) were randomly selected and divided into four groups (n = 6 in each group): 1) control group, 2) CSE group, 3) CSE + 100 μg·kg⁻¹·day⁻¹ of BPS (BPS-1 group), and 4) CSE + 200 μg·kg⁻¹·day⁻¹ of BPS (BPS-2 group). The control rats were injected intraperitoneally with 1 ml of vehicle (normal saline) on day 1, 8, and 15. One milliliter of the CSE-PBS solution was injected intraperitoneally on day 1, 8, and 15, and BPS (prepared in 2 ml of normal saline; Astellas Pharma, Tokyo, Japan) was administered daily via a gastric gavage for 21 days. The dosages of BPS applied to the current experiments were determined according to studies elsewhere (22, 37), and all rats tolerated well to the dosages. The BPS administration was started at the day of the first CSE injection. All rats were fed under the same conditions for 3 wk. At day 21 after starting the experiment, the rats were killed, and the blood samples and lung tissues were obtained as described below.

Sampling of Blood and Lung Tissues

At day 21 of the experiment, the rats were anesthetized with pentobarbital (50 mg/kg) intraperitoneally. The blood sample was
taken from the inferior vena cava via the opened chest, collected into a polypropylene tube containing clot activator, and centrifuged at 3,000 g for 15 min. The serum was separated and stored at −70°C until the samples were assayed.

The left lung was inflated with 0.5% low melting agarose at a constant pressure of 25 cmH₂O and fixed in 10% formalin for 48 h. The paraffin-embedded sections from the same regions (upper and lower lobes) were used for histological examination. The right lung was homogenized immediately after harvest in a buffer containing 50 mM HEPES, 1 mM dithiothreitol, 0.1% Triton X-100, and 10% glycerol. The supernatant was separated after two cycles of centrifugation at 10,000 g for 10 min. The protein concentration in the supernatant was determined using the bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL).

**Morphological Assessment**

The paraffin sections of the left lung were cut (thickness 3.5 μm) and stained with hematoxylin and eosin (HE). Emphysematous changes were assessed by measurement of both the mean linear intercept (MLI) and the destructive index (DI). MLI is a measurement of mean interalveolar septal wall distance, which is widely used to examine alveolar space size. As previously described (9), the MLI was measured by dividing the length of a line drawn across the lung section by a total number of intercepts counted within this line at ×100 magnification. A total of 36 lines per each rat lung were drawn and measured. The DI was calculated to quantify the parenchymal destruction by using a microscopic point-count technique (29). The analysis was performed in duplication with more than 3,000 alveoli randomly counted from 50 HE sections in each rat at ×200 magnification. Destructive alveolus was defined if at least one of the following alveoli was observed: alveolar wall defects; intraluminal parenchymal rags in alveolar ducts; obviously abnormal morphology; and typically emphysematous changes. The DI was calculated by dividing the defined destructive alveolus by the total number of alveoli counted and represented as percentage of the destructive alveolus as a fraction of total alveoli. The morphological assessment was repeated on coded samples.

**Measurement of 6-Keto-PGF1α in Lung Tissues**

The 6-keto-PGF1α is produced by the non-enzymatic hydration of PGI₂ that has a half-life of only 2–3 min in buffer so that measurement of 6-keto-PGF1α is typically used to monitor the production of PGI₂. In the present experiment, the levels of 6-keto-PGF1α in the supernatants of lung tissue homogenates in all rats were measured by competitive immunoassay using the Correlate-EIA 6-keto-PGF1α kit (Assay Designs, Ann Arbor, MI) in duplication according to the manufacturer’s instructions. When the enzyme reaction was stopped, the colors generated were read on a microplate reader at 405 nm. The measured optical density was used to calculate the concentration of 6-keto-PGF1α.

**Apoptosis Assay**

**Measurement of the apoptotic index.** Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was performed to label the DNA-damaged cells in the lungs of experimental rats. The TACS 2 TdT DAB kit was purchased from Trevigen (Gaithersburg, MD) and used for this TUNEL experiment following the manufacturer’s instructions. The apoptotic index (AI) was calculated as the percentage of TUNEL-positive nuclei in a total of more than 3,000 nuclei randomly counted for each lung at ×400 magnification.

**Immunohistochemistry for activated caspase-3.** The expression of activated caspase-3, a marker of apoptosis, was determined using a rabbit polyclonal antibody against the cleaved caspase-3 (Cell Signaling Technology, Danvers, MA) by immunohistochemistry according to the manufacturer’s instructions.

**Western blotting.** The supernatant protein was electrophoresed on 14% SDS-PAGE (Invitrogen Life Technologies, Carlsbad, CA) at 125 V for 90 min and transferred electrophoretically on to a nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membrane was blocked and then rinsed and incubated with a rabbit polyclonal antibody against the cleaved caspase-3 (1:500, Cell Signaling Technology) overnight at 4°C. Following the incubation with the primary antibody, the membrane was carefully washed and reincubated for 1 h at room temperature with a secondary anti-rabbit antibody (1:1,000; R&D Systems, Minneapolis, MN). After washing, detection was performed using Quantity One 1-D Analysis Software (Bio-Rad Laboratories, Hercules, CA).

**Gelatin Zymography**

Matrix metalloproteinase (MMP)-2 and MMP-9 activities were determined by gelatin zymography (17). Proteins from each lung extract were electrophoresed on 10% SDS-Tris-glycine gels containing 1 mg/ml gelatin (Invitrogen, Carlsbad, CA). SDS was removed from gels by shaking in 2.5% Triton X-100 twice for 1 h. The gels were then incubated overnight at 37°C in substrate buffer (50 mM Tris-HCl, pH 7.5, 5 mM CaCl₂, 0.5 μM ZnCl₂). Gels were stained with Coomassie brilliant blue and destained with methanol. Proteolysis bands were visualized and quantified using Printgraph (ATTO, Tokyo, Japan).

**Measurements of TNFα and IL-1β in Lung Tissues**

TNFα and IL-1β concentrations in the supernatants of lung homogenates were measured in duplication using commercially available ELISA kits (Quantikine Rat TNFα kit and Quantikine Rat IL-1β kit, R&D Systems, respectively) according to the manufacturer’s instructions.

**Measurements of Antioxidant Activity in Blood**

The serum antioxidant activity was measured using the bioantioxidant power (BAP) test using the Free Radical Elective Evaluation FRAS4 system (Diacron International, Grosseto, Italy). The BAP test was performed in each rat according to the analysis procedure of the manufacturer’s instructions. Briefly, 10 μl of serum was dissolved in a solution, previously prepared by mixing FeCl₃ with a thiocyanate derivative solution. After a short incubation (5 min) at 37°C, this solution lost color, and the intensity of this chromic change was directly proportional to the serum reductive activity. Photometric reading was employed to assess the intensity of discoloration to evaluate the effective reduction activity or antioxidant potential. A lyophilized human control serum with known antioxidant activity (μmol/l) was used for periodically calibrating the FRAS4 system. The results of the BAP test provided the serum biological antioxidant potential, which was expressed in μmol/l.

**Measurements of cAMP in Lung Tissues**

To observe the cAMP, a ubiquitous second messenger involved in various cellular activations in many cell and tissue types, the cAMP levels were measured in duplication using commercially available ELISA kits (Quantikine Rat cAMP kit, R&D Systems) according to the manufacturer’s instructions.

**Statistical Analysis**

A software package (SPSS 15.0; SPSS, Chicago, IL) was used to perform all statistical analyses. Continuous data were expressed as means ± SD. Any differences among the four groups were evaluated by a one-way ANOVA. The Pearson’s correlation was used to analyze the correlations of AI with the concentrations of serum TNFα and IL-1β separately. A P value less than 0.05 was considered statistically significant.
RESULTS

Morphological Findings

We confirmed a previously reported finding that intraperitoneal injection of CSE in rats caused emphysematous destruction of the lung within 3 wk (35). Compared with the normal alveolar architecture (control group, Fig. 1Aa), once a week injection of CSE for 3 wk induced enlargement of alveolar air spaces and destruction of lung parenchyma (CSE group, Fig. 1Ab). The MLI and DI were significantly increased in the CSE group compared with the values in the control group (MLI: 108.7 ± 6.8 μm vs. 69.8 ± 6.6 μm, P < 0.0001; DI: 62.2 ± 7.0% vs. 13.9 ± 2.7%, P < 0.0001, respectively), indicating air space enlargement as a consequence of the CSE injection (Fig. 1B). In contrast, BPS markedly inhibited the air space enlargement and the parenchyma destruction (Fig. 1Ac and Ad). The MLI and DI were significantly reduced in the lungs in the BPS-1 group (MLI: 83.9 ± 10.5 μm, P < 0.0001; DI: 49.3 ± 7.5%, P = 0.002) and the BPS-2 group (MLI: 77.0 ± 7.1 μm, P < 0.0001; DI: 47.1 ± 6.9%, P = 0.001) compared with those in the CSE group (Fig. 1B). However, the MLI and DI were not completely recovered to the values as those in control rats (P < 0.05 for MLI and DI in BPS-1 group and P < 0.05 for DI in BPS-2 group, compared with controls). There were no significant differences of the MLI and DI between the BPS-1 and BPS-2 groups (P > 0.05). In this regard, BPS only partially protected the lungs against alveolar space destruction as assessed by measuring the MLI and DI.

Levels of 6-Keto-PGF1α in Lung Tissues

We measured the stable metabolite of PGI2, 6-keto-PGF1α, in lung tissues (Fig. 2). As the amount of tissue in the emphysematous lung was decreased, the levels of 6-keto-PGF1α were expressed in relation to the quantity of protein in homogenates (μg/mg). The concentration of 6-keto-PGF1α was significantly reduced in the lung tissues of the rats with CSE-induced emphysema (40.6 ± 29.3 μg/mg) compared with those of control rats (173.2 ± 49.3 μg/mg, P = 0.001). BPS treatment markedly increased the level of 6-keto-PGF1α compared with CSE-injected rats. The levels of 6-keto-PGF1α were expressed in relation to the quantity of protein in homogenates (μg/mg). ∗P < 0.05 compared with control group; †P < 0.05 compared with CSE group; §P < 0.01 compared with BPS-1 group.
Effect of BPS on Apoptosis in the Lung of Rats with Emphysema Induced by CSE

The TUNEL-positive (TUNEL+) cells were frequently localized in the peribronchiolar, intra-alveolar, and septal structures in the CSE-injected rats (Fig. 3Aa) with apoptotic alveolar epithelial cells in dominance (Fig. 4). The TUNEL+ cells were quantified as AI by which the amount of DNA-damaged alveolar cells was identified to evaluate the apoptosis. The protective effect of BPS was related to a reduction in apoptosis as measured by AI (Fig. 3B). The DNA-damaged cells were very rare in the normal rats (AI: 2.9 ± 0.9%), whereas the TUNEL+ cells in CSE group were much more frequent (AI: 20.3 ± 0.9%, P < 0.0001). BPS greatly reduced the number of TUNEL+ cells in the lungs of CSE-treated emphysematous rats (AI: 8.9 ± 2.5% in BPS-1 group and 7.8 ± 1.3% in BPS-2 group, P < 0.0001 vs. controls for each). Again, BPS treatment did not provide complete protection against apoptosis of the alveolar cells in rats treated with CSE.

Caspase-3, a marker of apoptosis, was analyzed by immunohistochemistry and Western blotting. Rats injected with CSE showed a large number of caspase-3+ alveolar septal cells (arrows in Fig. 5Ab) compared with the cells in the control (Fig. 5Aa) and BPS-treated rats (Fig. 5Ac and d). The caspase-3 protein in the lung tissue measured by Western blot was expressed as a relative density of the caspase-3 to β-actin by the densitometry analysis. The protein levels of the cleaved form of caspase-3 were increased in the rats with CSE-induced emphysema (270.1 ± 34.7%) compared with those of control rats (146.3 ± 20.4%, P = 0.012) (Fig. 5B). In contrast, BPS treatment markedly reduced the protein level of cleaved caspase-3 compared with that in CSE-injected rats (BPS-1: 162.7 ± 53.3%, P = 0.023; BPS-2: 154.0 ± 32.8%, P = 0.016).

Effect of BPS on MMP-2 and MMP-9 Activity in the Lungs of Rats with Emphysema Induced by CSE

Prior studies showed an increased secretion and/or activity of MMP-2 and MMP-9 in bronchoalveolar lavage fluid (5), induced sputum (7), lung tissue (26), and peripheral blood (21) in patients suffering from COPD, consistent with the concept that the proteolytic activity of MMPs is related to pulmonary inflammation, airway remodeling, and alveolar destruction. In accordance with these reports, rats injected with CSE showed increased MMP-2 and MMP-9 activities in their lung tissue homogenates (MMP-2: 215.2 ± 33.5%, P = 0.002; MMP-9: 190.5 ± 60.0%, P = 0.016) compared with the activity in the control rats (MMP-2: 98.2 ± 10.2; MMP-9: 88.3 ± 12.5).
38.4%, MMP-9: 84.1 ± 29%) (Fig. 6, A and B). MMP-2 and MMP-9 activities were reduced in the two BPS treatment groups, but statistical significance occurred in MMP-2 activity of both low- and high-dose BPS treatment rats (BPS-1: 148.1 ± 45.3%, \( P = 0.049 \); BPS-2: 116.8 ± 53.3%, \( P = 0.007 \), respectively) and in MMP-9 activity of only high-dose BPS treatment rats (97.3 ± 40.6%, \( P = 0.031 \)).

**Effect of BPS on Inflammation in the Lungs of Rats with Emphysema Induced by CSE**

To examine whether the emphysema-protective effect of BPS was associated with an anti-inflammatory effect on the rats after CSE injection, we measured the levels of TNF-\( \alpha \) and IL-1\( \beta \) in the lung tissues. As the amount of tissue in the emphysematous lung

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**Fig. 6.** Expression of MMP-2 and MMP-9 activities in the lungs of rats. **A:** representative gelatin zymogram of supernatant of lung tissues showing the effects of BPS treatment on inducible MMP-9 and MMP-2 activities by CSE injection. **B:** relative density of the zymogram showing gelatinolytic activity. *\( P < 0.05 \) compared with control group; †\( P < 0.05 \) compared with CSE group.
was decreased, the levels of TNFα and IL-1β were expressed in relation to the quantity of protein in homogenates (pg/mg). As shown in Fig. 7A, the levels of TNFα and IL-1β were increased in the CSE group (11.4 ± 1.4 pg/mg, \( P < 0.0001 \) and 214.4 ± 52.7 pg/mg, \( P = 0.022 \), respectively) compared with those in controls (8.3 ± 1.0 pg/mg and 131.4 ± 36.2 pg/mg, respectively). And high-dose (200 \( \mu g \cdot kg^{-1} \cdot day^{-1} \)) BPS treatment significantly reduced the levels of TNFα and IL-1β in the lungs of CSE-treated emphysematous rats. As shown in Fig. 7B, the levels of TNFα and IL-1β were positively correlated with the AI in the lungs of all rats including the control rats and emphysema rats induced by CSE injection (\( r = 0.657, P < 0.0001 \) and \( r = 0.530, P = 0.008 \), respectively).

**Effect of BPS on Antioxidant Activity in the Serum of Rats with Emphysema Induced by CSE**

We also examined whether the protective effect of BPS of rats after CSE injection was associated with a reduction in biological antioxidant activity (Fig. 8). The serum antioxidant activity was significantly decreased in the CSE-treated rats (1,653.4 ± 504.7 \( \mu \)mol/l, \( P = 0.01 \)) compared with the control rats (2,438.9 ± 235.6 \( \mu \)mol/l). BPS treatment significantly prevented the reduction of antioxidant activity in rats injected with CSE (BPS-1: 2,420 ± 434.4 \( \mu \)mol/l, \( P = 0.011 \); BPS-2: 2,344.8 ± 664.6 \( \mu \)mol/l, \( P = 0.026 \), compared with CSE group).

**DISCUSSION**

The most remarkable finding of this study is that the stable PGI2 analog BPS prevented the development of emphysema in...
Pulmonary endothelial PGI2S expression was decreased in the emphysematous rats and that BPS treatment significantly increased elastolytic activity in the lung, and development of alveolar destruction in rats within 3 wk.

Although we did not conduct a detailed exploration, DNA damage of alveolar cells and caspase-3 expression, either as a direct effect of noxious components of CSE and/or as a consequence of inflammation, were decreased in the emphysematous lungs of rats treated with BPS. A recent in vitro study demonstrated that cigarette smoke suppressed PGI2S and that pulmonary endothelial PGI2S expression was decreased in the arteriolar endothelium in emphysema, and lung-specific overexpression of PGI2S resulted in a decrease in caspase-3 activity after long-term smoke exposure in a transgenic murine model (24). Aoshiba et al. (3) also reported that intratracheal administration of active caspase-3 resulted in epithelial apoptosis, enhanced elastolytic activity in the lung, and development of emphysematous changes in mice. Our results revealed that 6-keto-PGF1α was significantly decreased in the lungs of emphysematous rats and that BPS treatment significantly increased the levels of 6-keto-PGF1α that is a production of endogenous PGI2 by non-enzymatic hydration. The high-dose BPS raised 6-keto-PGF1α levels to a supernormal level, probably due to a nonlinear correlation of the 6-keto-PGF1α with the endogenous PGI2. Administration of BPS, a PGI2 analog, may supply PGI2 to emphysematous rats that are in deficiency in PGI2. It is also possible that the BPS prevented apoptosis of PGI synthase-expressing cells that might have been damaged by CSE through activating the PGI2 receptor. The present results demonstrated that the BPS protected against emphysema formation and decreased apoptosis and expression of caspase-3 activity. The inhibition of apoptosis by BPS treatment is consonant with the evidence supporting that PGI2 has antiapoptotic effects in other organ systems (8, 11).

BPS, the stable PGI2 analog, causes relaxation of vascular smooth muscle in most blood vessels (14, 23, 37) and inhibits platelet aggregation (12, 15). The properties of vasodilatation and antithrombosis may help to protect against pathological changes in the small pulmonary arteries of patients with pulmonary hypertension (14, 23, 37). Beyond the vasodilatation, BPS also modulates tissue remodeling by affecting fibroblast-mediated contraction of extracellular matrix protein production in fibroblasts (16) and human cultured mesangial cells (18), and diminishes the amounts of cytokines such as IL-1, IL-6, and TNFα produced by alveolar macrophages in the development of pulmonary hypertension (23). Our present in vivo study similarly demonstrates significant suppressions of MMP-9 and MMP-2 activities and reductions in TNFα and IL-1β in the BPS-treated lungs with emphysema. Attenuation of proteolytic enzymes, such as MMP-2 and MMP-9, may inhibit one of the mechanisms of lung tissue remodeling in COPD, whereby MMPs degrade collagen and elastin, regulate extracellular matrix deposition, and contribute to cell migration and proliferation (4, 6). Kitahara and colleagues (18) demonstrated that the prostacyclin inhibits the production of MMP-9 induced by phorbol ester through protein kinase A activation in human cultured mesangial cells. Similarly, reduction of the inflammatory cytokines, for example, TNFα and IL-1β, may dampen the inflammation contributing to the development of COPD (10). In addition, we found a normal biological antioxidant activity in the serum of CSE-treated rats after administration of BPS. Ohmori et al. (25) demonstrated that BPS decreases the polymorphonuclear leukocyte-induced superoxide anion (O2−) production and serum superoxide dismutase activity in spontaneously hypertensive rats, suggesting that BPS had merit for preventing the O2−-related organ damage. Adderley and Fitzgerald (1) showed that the oxidative damage of cardiomyocytes is limited by extracellular regulated kinases 1/2-mediated induction of cyclooxygenase-2. Shimura et al. (30) reported that prostacyclin attenuates oxidative damage of myocytes by opening mitochondrial ATP-sensitive K+ channels via the EP3 receptor. In our case, we suspect that the normal biological antioxidant activity after administration of BPS is probably related to the inhibitory effect of BPS on inflammation in this model. However, it is important to note that we were only able to show a protective, but not a curative, effect of BPS in this experimental rat model.

BPS is a potent vasodilator whose biological effects are believed to be mediated by cAMP (14, 32). PGI2 and its stable analogs activate a specific cell-surface receptor (IP receptor, IPR), which is coupled to adenyl cyclase through Gs protein. Elevation of cAMP levels has been considered to be a key cellular event to trigger blood vessel relaxation by IP agonists. BPS, an IP agonist, elicited a vascular relaxation by activating...
IPR through a cAMP-dependent pathway. Moreover, BPS modulates tissue remodeling by affecting fibroblast-mediated contraction of extracellular matrix through activating the cAMP-dependent protein kinase A pathway (16). In this emphysema model of rats induced by injection of CSE, BPS significantly improved the emphysematous molecular pathologies with an enhancement of cAMP levels in the lung tissues. It seems likely that, similar to the signaling events in relaxation of blood vessels (14, 32), modulation of tissue remodeling (16), and reduction of thrombolic events (15), BPS generates the identified molecular preventive events in emphysematous lungs through activating cAMP downstream signaling.

In conclusion, we have shown in this study that BPS, an orally PGI2 analog, protects against the development of CSE-induced emphysema in rats by attenuating apoptosis, inhibiting proteolytic enzyme activities, reducing inflammatory cytokines, and augmenting serum antioxidant activity. BPS may represent a new therapeutic option in the prevention of emphysema in humans in prospect.

ACKNOWLEDGMENTS

We thank Hitomi Imamura for valuable technical assistance. Beraprost was kindly provided by Astellas Pharma (Tokyo, Japan).

GRANTS

This study was supported in part by the Japan-China Sasakiaka Medical Fellowship, the Grant-in-Aid for Scientific Research (C), No. 19590887, from the Japan Society for the Promotion of Science, and the Victoria Johnson Center for Obstructive Lung Disease Research.

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