Full Paper

Vulnerability of Gastric Mucosa to Prednisolone in Rats Chronically Exposed to Cigarette Smoke

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Abstract. We examined gastric mucosal vulnerability in a rat model of chronic obstructive pulmonary disease (COPD). Male Wistar rats were exposed to cigarette smoke for 12 weeks (CSE rats), and on the last 4 days of exposure, prednisolone was given to induce gastric mucosal injury. Histopathology, pulmonary function, arterial blood gases, and levels of lipid peroxides (LPO), prostaglandin E₂ (PGE₂), hypoxia-inducible factor 1 alpha subunit (HIF-1 α), and vascular endothelial growth factor (VEGF) in gastric mucosa were examined. We also tested the effect of rebamipide on prednisolone-induced gastric lesions. In CSE rats, although no gastric lesions were detected, LPO, PGE₂, HIF-1 α , and VEGF levels were higher than in control rats. Prednisolone induced gastric hemorrhagic lesions more readily in CSE rats than controls, with concomitant decrease in PaO₂ and increased levels of LPO, HIF-1 α , and VEGF. Rebamipide reversed gastric lesions without affecting any parameters examined. CSE rats were found to be a useful animal model of COPD, and COPD appeared to render the gastric mucosa vulnerable to prednisolone.

Keywords: chronic obstructive pulmonary disease (COPD), cigarette smoke, gastric mucosal injury, prednisolone, rebamipide

Introduction

Cigarette smoking is now widely recognized as an important risk factor for a variety of diseases including cardiovascular, neoplastic, and pulmonary disorders (1–3), although the mechanisms by which it causes these diseases are largely unknown. Among such disorders, COPD is a chronic airway inflammatory disease associated with cough, increased sputum, and shortness of breath. An epidemiological survey in 2001 reported that the prevalence of COPD was 8.5% in Japan (4, 5). WHO predicts that the prevalence of COPD will increase in the coming years, to become the fifth-most common cause of morbidity and the third-most common chronic disease worldwide. COPD will thus be one of the most important health care problems in the near future.

COPD has been shown to be complicated by heart disease, malnutrition, osteoporosis, depression, and aortic aneurysm. Some studies have also suggested that peptic ulcer disease is a complication of COPD (6–9). However, because the most important causative factors of peptic ulcer disease, such as *Helicobacter pylori* infection and non-steroidal anti-inflammatory drug (NSAID) use, have not been included in such studies, the causal relationship between COPD and peptic ulcer disease remains unclear.

Corticosteroids have been used to treat pulmonary diseases and are thought to increase the risk for a number of adverse gastrointestinal events. However, results of recent studies indicate that the independent effect of corticosteroids on peptic disease is small, and it is currently believed that concomitant use of NSAIDs with corticosteroids (10) and super-maximal administration of corticosteroids (11) increase the risk of gastrointestinal events. It is thus conceivable that corticosteroids render the gastric mucosa vulnerable to injury initiated by irritant medications.

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The aim of this study was to determine gastric mucosal vulnerability in COPD using cigarette smoke-exposed rats. Since hypoxia is one of the characteristic features of patients with COPD, we assessed gastric mucosal lipid peroxidation and parameters of gastric mucosal ischemia in this model. To evaluate vulnerability to gastric mucosal lesions, we used prednisolone as an inducer of gastric mucosal injury. We finally tested the effects of rebamipide, which has been shown to inhibit the generation of free radicals and lipid peroxidation, on gastric lesions (12–15).

Materials and Methods

Animals

Male Wistar rats weighing 80–110 g were purchased from Japan SLC, Inc. (Shizuoka), and housed at 19°C–25°C with 12-h lighting per day. All of the experimental procedures were approved by the Institutional Animal Care and Use Committee of Kobuchisawa Research Laboratories, Fuji Biomedix Co., Ltd. (Yamanashi).

Chemicals

Prednisolone was purchased from Sigma Chemical Company (St. Louis, MO, USA). Peace® cigarettes were purchased from Japan Tobacco, Inc. (Tokyo). Rebamipide was donated by Otsuka Pharmaceutical Co., Ltd. (Tokyo).

Cigarette smoke-exposed (CSE) rats

Rats were exposed to a diluted mainstream of cigarette smoke from 40 cigarettes per day for a period of 12 consecutive weeks using a smoking machine (Model INH06-CIG01A; Medical Interface Project Station, Osaka). A conscious rat was placed in an exposure holder, which was fixed to the exposure chamber connected to the smoking machine. Puffs of smoke (35 ml) generated from commercial filterless cigarettes (Peace®, 2.3 mg nicotine and 28 mg tar per cigarette) were diluted with 280 ml of room air and delivered to the exposure chamber. Each cigarette was puffed 40 times with a suction volume of 1 l/min. To provide a control group with sham exposure, normal rats were exposed to smoke-free air.

Pulmonary function analysis

On the day after final exposure to cigarette smoke, animals were anesthetized with ketamine hydrochloride (60 mg/kg; Ketalar®, Sankyo Co., Ltd., Tokyo) and xylazine hydrochloride (8 mg/kg, Seractal® 2%; Bayer Co., Ltd., Leverkusen, Germany) for cannulation into the trachea. Pulmonary function was measured using a pulmonary maneuver system (Biosystem Maneuvers;

Buxco Electronics, Inc., Sharon, CT, USA). Parameters measured included functional residual capacity (FRC), residual volume (RV), and total lung capacity (TLC). Software was used to calculate RFC using Boyle's law, with pressure and thoracic displacement data collected after occluding the airway at the end of expiration. RV was calculated by subtracting the vital capacity from the TLC determined by pressure-volume maneuvers.

Arterial blood gas analysis

After measurement of pulmonary function, animals were sacrificed and blood samples were drawn from the abdominal artery to determine PaO₂ and PaCO₂ using an i-STAT® blood gas analyzer (Abbott Laboratories, Abbott Park, IL, USA).

Histopathological examination

The trachea, bronchi, lungs, and stomach were fixed in 10% neutral-buffered formalin, and hematoxylin and eosin-stained specimens were prepared for histological examination. Thickness of the trachea, changes in goblet cells in the bronchi, and alveolar infiltration by macrophages were microscopically assessed using scales from 0 to 4 for severity of change (0, no change; 1, very slight change; 2, slight change; 3, moderate change; and 4, marked change). To assess gastric mucosal damage, the gastric mucosa was photographed with a digital camera after fixation. The pictures were analyzed with an image analyzer (DP70; Olympus Corporation, Tokyo), with area of injury represented as a lesion index (mm²).

Levels of lipid peroxides (LPO), prostaglandin E₂, hypoxia-inducible factor 1 α (HIF-1 α), and vascular endothelial growth factor (VEGF) in the gastric mucosa

The gastric mucosa was scraped off with a glass slide and then homogenized in buffer solution with a teflon stick. Small portions of each sample were subsequently measured for LPO, HIF-1 α , and VEGF according to the instruction manuals. The commercial kits used in this study were as follows: Lipid Hydroperoxide Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA) for gastric mucosal lipid peroxides; Prostaglandin E₂ Immunoassay (R&D Systems, Inc., Minneapolis, MN, USA); DuoSet IC Human/Mouse HIF-1 α Kit (R&D Systems, Inc.) for HIF-1 α ; and Quantikine Rat VEGF Immunoassay (R&D Systems, Inc.) for VEGF. Protein concentrations were determined with the BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

Prednisolone-induced gastric damage

Prednisolone was subcutaneously administered to the control and CSE rats at doses of 12.5, 25, and 50 mg/kg at 1 h before exposure to smoke once daily for the last 4

Fig. 1. Histological findings in trachea (A), bronchi (B), lungs (C), and gastric mucosa (D) in control rats and those in CSE rats (E–H). The mucosal layer was thickened and goblet cells were increased in CSE rats. Breakdown of alveolar walls and alveolar expansion as well as infiltration by macrophages were observed in CSE rats. Gastric mucosa was unchanged by exposure to cigarette smoke.

consecutive days of the smoke-exposure period (16). Pulmonary function tests and gastric mucosal damage were assessed as above.

Statistical analyses

SAS® (Release 8.2 for Windows; SAS Institute, Inc., Cary, NC, USA) was used for statistical analysis. Histopathological scores were examined with Wilcoxon's

Table 1. Changes in histological scores for trachea and lungs

Group	Thickness of mucosal layer in trachea	Number of goblet cells in trachea	Macrophages infiltrating into alveoli
Control (n = 12)	0	0	0
CSE (n = 14)	0.9 **	0.9 **	1.1 **
+50 mg/kg of prednisolone (n = 14)	0.9	0.7	1.6
+30 mg/kg of rebamipide (n = 14)	0.9	0.9	0.7
+prednisolone + rebamipide (n = 14)	1.0	0.7	0.9

Values are means of scores. ** $P < 0.01$.

Table 2. Effects of prednisolone and rebamipide on pulmonary function

Group	Functional residual capacity (ml)	Total lung capacity (ml)	Residual volume (ml)
Control (n = 12)	3.09 ± 0.11	10.46 ± 0.45	1.65 ± 0.27
CSE (n = 14)	4.20 ± 0.23 **	10.82 ± 0.62	3.42 ± 0.31 **
+50 mg/kg of prednisolone (n = 14)	3.92 ± 0.19	9.86 ± 0.54	2.83 ± 0.29
+30 mg/kg of rebamipide (n = 14)	3.96 ± 0.22	9.96 ± 0.54	3.20 ± 0.25

Values are means $\pm$ S.E.M. ** $P < 0.01$.

Table 3. Effects of prednisolone on arterial blood gases

Group	PaCO ₂ (mmHg)	PaO ₂ (mmHg)
Control (n = 7)	45.63 ± 1.57	55.25 ± 3.28
+12.5 mg/kg of prednisolone (n = 7)	51.13 ± 0.91 *	46.33 ± 0.76 *
+25 mg/kg of prednisolone (n = 7)	52.25 ± 0.96 **	42.33 ± 2.30 **
+50 mg/kg of prednisolone (n = 7)	54.07 ± 1.31 ***	41.00 ± 1.79 ***
CSE (n = 7)	54.25 ± 3.28	41.25 ± 4.11
+12.5 mg/kg of prednisolone (n = 7)	55.15 ± 0.68	42.00 ± 3.27
+25 mg/kg of prednisolone (n = 7)	55.45 ± 1.12	34.17 ± 3.75
+50 mg/kg of prednisolone (n = 7)	57.12 ± 0.94	34.00 ± 1.76

Values are means $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$.

rank-sum test. Comparisons of numerical values and categorical data were performed by Student's *t*-test and Fisher's exact test, respectively. The dose-dependency of effects of prednisolone was assessed by Dunnett's test. All statistical tests were two-tailed, with a significance level of 5%, and *P*-values < 0.05 were considered significant.

Results

Histological changes in trachea, bronchi, pulmonary alveoli, and gastric mucosa

In the trachea and bronchi, increases in number of goblet cells and mucosal thickening were apparent in CSE rats, compared to control rats. In the lung parenchyma, normal architecture was disrupted; break-

down of alveolar walls and resultant alveolar expansion were observed in parallel with macrophage infiltration into the alveoli (Fig. 1 and Table 1). On the other hand, the gastric mucosa exhibited no significant changes in CSE rats (Fig. 1).

Pulmonary function and arterial blood gas analysis

The FRC in CSE rats was 4.20 ± 0.23 ml, and significantly higher than the value of 3.09 ± 0.11 ml in the control rats ($P < 0.05$) (Table 2). Likewise, RV values in CSE rats and control rats were 3.42 ± 0.31 and 1.65 ± 0.27 ml, respectively ($P < 0.05$). TLC did not differ between CSE and control rats. Arterial blood gas analysis showed that PaO₂ in control rats was 55.25 ± 3.28 mmHg, but significantly lower, at 41.25 ± 4.11 mmHg, in CSE rats ($P < 0.05$). On the other hand, PaCO₂

Table 4. Parameters of gastric mucosal lipid peroxidation and hypoxia

Group	LPO (nmol/mg protein)	PGE ₂ (pg/mg protein)	HIF-1 α (pg/mg protein)	VEGF (pg/mg protein)
Control (n = 12)	0.06 $\pm$ 0.03	1634.86 $\pm$ 281.18	13.90 $\pm$ 1.91	16.46 $\pm$ 1.26
CSE (n = 14)	0.12 $\pm$ 0.05	2204.00 $\pm$ 459.70	19.55 $\pm$ 2.80	20.08 $\pm$ 3.07
+50 mg/kg of prednisolone (n = 14)	0.26 $\pm$ 0.05	1304.98 $\pm$ 315.48	41.18 $\pm$ 5.82	32.87 $\pm$ 1.87
+30 mg/kg of rebamipide (n = 14)	0.18 $\pm$ 0.05	2395.95 $\pm$ 332.45	20.29 $\pm$ 2.28	15.60 $\pm$ 1.18

Values are means $\pm$ S.E.M. * P <0.05, ** P <0.01.

Fig. 2. Vulnerability of gastric mucosa in CSE rats. Area of erosions in the gastric mucosa induced by prednisolone was larger in CSE than in control rats. * P <0.05: Significantly different from control rats of the CSE group. # P <0.05: Significantly different from the control group treated with the same dose of prednisolone.

was 45.63 ± 1.57 mmHg in control rats, but 54.25 ± 3.28 mmHg (P <0.05) after 12 weeks of exposure to smoking (Table 3).

Levels of LPO, PGE₂, HIF-1 α , and VEGF

In CSE rats, the level of LPO in gastric mucosa was higher than that in control rats (0.12 ± 0.05 and 0.06 ± 0.03 nmol/mg proteins in CSE and control rats, respectively), although this difference was not significant (Table 4). Similar trends toward higher levels of PGE₂, HIF-1 α , and VEGF were observed in CSE rats, although again they were not significant (Table 4).

Effects of prednisolone on gastric mucosa, pulmonary function, arterial blood gas analysis, and LPO, PGE₂, HIF-1 α , and VEGF levels in CSE rats

Prednisolone induced gastric mucosal damage in 50% of control rats at a dose of 50 mg/kg (Fig. 2), with an affected area of 3.33 ± 2.45 mm². As shown in Table 3, prednisolone decreased PaO₂ and increased PaCO₂ (55.25 ± 3.28 and 41.00 ± 1.79 mmHg of PaO₂, 45.63 ± 1.57 and 54.07 ± 1.31 mmHg of PaCO₂, in the absence

and presence of 50 mg/kg of prednisolone, respectively) (P <0.05).

In contrast, gastric mucosal damage occurred in 100% of CSE rats at 50 mg/kg and could be observed at a dose as low as 12.5 mg/kg in 1 of 6 rats. When 50 mg/kg of prednisolone was administered, the affected area was 10.83 ± 2.65 mm² and mucosal damage appeared to have been aggravated (Figs. 2 and 3). Neither FRC nor RV was affected by administration of prednisolone, as shown in Table 2 (4.20 ± 0.23 and 3.92 ± 0.19 for FRC, 3.42 ± 0.31 and 2.83 ± 0.29 for RV, in the absence and presence of prednisolone, respectively). Although both PaO₂ and PaCO₂ were substantially altered (41.25 ± 4.11 and 34.00 ± 1.76 mmHg for PaO₂ and 54.25 ± 3.28 and 57.12 ± 0.94 mmHg for PaCO₂, in the absence and presence of 50 mg/kg of prednisolone, respectively), the differences observed were not significant.

In the case of administration of prednisolone to CSE rats, significant increases in LPO, HIF-1 α , and VEGF levels were observed (0.12 ± 0.05 and 0.26 ± 0.05 nmol/mg protein for LPO, 19.55 ± 2.80 and 41.18 ± 5.82 pg/mg protein for HIF-1 α , and 20.08 ± 3.07 and 32.87 ± 1.87 pg/mg protein for VEGF, in the absence and presence of 50 mg/kg of prednisolone, respectively) (Table 4) (P <0.05). In contrast, the concentration of PGE₂ was decreased (2204.00 ± 459.70 and 1304.98 ± 315.48 pg/mg protein, in the absence and presence of 50 mg/kg of prednisolone, respectively).

Effects of rebamipide on prednisolone-induced gastric mucosal damage

Treatment with rebamipide at 30 mg/kg significantly suppressed prednisolone-induced gastric mucosal damage (Fig. 3 and Table 5). While gastric damage was observed in 6 of 7 CSE rats without rebamipide, it occurred in only 1 of 7 rats with rebamipide (Table 5) (P <0.05). Rebamipide had no effect on findings of the histopathologic examination, pulmonary function, or parameters of mucosal ischemia (Tables 1, 2, and 4).

Fig. 3. Gastric mucosa in CSE rats treated with prednisolone in the absence or presence of rebamipide. No pathological changes were observed in CSE rats (A and D). Prednisolone induced hemorrhagic lesions (B and E). Rebamipide inhibited gastric mucosal injury (C and F).

Table 5. Effects of prednisolone and rebamipide on gastric mucosa

Group	Gastric erosive area (mm ²)	Number of hemorrhagic animals
CSE (n = 7)	0 ± 0	0/7
+50 mg/kg of prednisolone (n = 7)	42.51 ± 9.92	6/7
+30 mg/kg of rebamipide (n = 7)	0 ± 0	0/7
+prednisolone + rebamipide (n = 7)	13.49 ± 6.06	1/7

Values are means ± S.E.M. **P*<0.05.

Discussion

COPD is a multi-factorial disease, and indeed, only a subgroup of smokers, approximately 15%–20% of those who smoke heavily, develop COPD; genetic susceptibility, aging, and environmental factors such as air pollution appear to be additional causative factors in the development of COPD (17). This complexity hampers establishment of animal models of COPD.

An animal model of COPD was first produced by Gross et al. in 1965 by intratracheal administration of papain in rats (18). Thereafter, development of animal models using rodents or gene-manipulated animals was attempted (19). It has been thought that emphysematous changes are much less strongly induced in rats than in guinea pigs and mice (20, 21); in many experimental studies using Sprague Dawley rats and Fisher-344 rats, long-term exposure to cigarette smoke was required for development of emphysematous changes. However, Escolá et al. recently reported that emphysematous changes could be induced by exposure to cigarette smoke for 12 weeks in Wistar rats (22), as in the present study. Chiba et al. (23) also reported successful induction of airway inflammation in Wistar rats by exposure to smoke for 2 weeks. Wistar rats could be more susceptible to cigarette smoke than SD rats or Fisher-344 rats since susceptibility to smoke in mice is strain-dependent (24). We therefore chose Wistar rats for use in this study.

We successfully produced rat COPD models by chronic exposure to cigarette smoke, confirming that cigarette smoking is clearly the most important risk factor for COPD. Although bronchoalveolar lavage fluid (BALF) was not examined in the present study, histological findings in the respiratory tract in CSE rats were consistent with those of pulmonary emphysema in humans. Furthermore, results of pulmonary function tests and arterial blood gas analyses were consistent with those in patients with COPD. Thus, the findings of the present study indicate that CSE rats are a practical and useful animal model of human COPD.

Although, PaO_2 was significantly decreased in CSE rats (Table 3), no pathological changes were found in the gastric mucosa (Fig. 1). Nakazawa et al. reported that gastric mucosal lesions were induced under hypoxic conditions in rat, accompanied by reduction of the gastric mucin component, endogenous prostaglandins, and gastric mucosal blood flow (25–27). However, the gastric mucosal PGE_2 levels in the present model were only slightly increased, as shown in Table 4. In addition, although we examined the gastric mucus with PAS staining (data not shown), no remarkable differences were recognized between control rats and CSE rats.

These discrepancies in findings may be explained by the duration of hypoxia. In contrast to the short periods of hypoxic challenge used by Nakazawa et al., we exposed the rats for 12 weeks. Although we did not measure gastric mucosal blood flow in this study, Tsugawa et al. reported (28) increased gastric mucosal blood flow under conditions of decreased PaO_2 in rats with portal hypertension. It may be that systemic hypoxia induces increased gastric mucosal blood flow.

Interestingly, all CSE rats treated with 50 mg/kg of prednisolone exhibited mucosal injury, while only half of control rats did. In addition, gastric mucosal injury in CSE rats was noted at a lower dose of prednisolone than in control rats, suggesting that the gastric mucosa in CSE rats was vulnerable to prednisolone-induced injury. It appeared that the gastric mucosa in CSE rats was exposed to persistent ischemia with low PaO_2 levels. Consistent with this, parameters indicative of chronic hypoxia, such as HIF-1 α and VEGF, were slightly increased. Furthermore, it was recently reported that free radicals are generated under conditions of chronic hypoxia (29). It may be that LPO increased as a result of the chronic hypoxia in CSE rats. Under these conditions, it is likely that stimuli that have the potential to break down the integrity of the gastric mucosa will readily cause gastric mucosal injury.

It is difficult to explain why the PGE_2 level was increased in CSE rats, although it may have functioned as a compensatory mechanism under conditions of ischemia, which may account for the maintenance of homeostasis of gastric mucosa in CSE rats.

On the other hand, it is possible that cigarette smoking contributed to this vulnerability; cigarette smoking has been found to promote generation of free radicals and, as a consequence, to increase myeloperoxidase (MPO) activity, activate xanthine oxidase (XO), and decrease superoxide dismutase (SOD) activity (30). Smoking has also been shown to reduce levels of various growth factors (31). It is thus likely that both cigarette smoking and chronic hypoxia decreased mucosal integrity and, as a result, mucosal injury was readily induced at lower dosages of prednisolone in CSE rats.

Rebamipide, which has been used as an anti-ulcer agent in Asian countries, successfully inhibited prednisolone-induced gastric injury in CSE rats. Since this agent has been demonstrated to promote gastric protection by preventing the production of free radicals and synthesis of prostaglandins (32), the finding that the gastric mucosal injury induced by prednisolone was reversed by rebamipide is reasonable. However, rebamipide affected neither mucosal hypoxia nor lipid peroxidation, suggesting that the effects of rebamipide may not be due primarily to elimination of free radicals.

In conclusion, we found that CSE rats are a useful animal model of COPD. COPD was not the sole cause of gastric mucosal injury. Prednisolone readily caused gastric mucosal injury in CSE rats, suggesting that COPD makes the gastric mucosa vulnerable to hypoxia. Our findings suggest that use of gastroprotective agents such as rebamipide may be beneficial in patients with COPD.

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