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Effect of cigarette smoke on ethanol-induced gastric mucosal lesions: The role of nitric oxide and neutrophils

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Abstract

The roles of neutrophil aggregation, inducible nitric oxide synthase activation and chemoattractant, leukotriene B_4, in potentiation of the cigarette smoke effect on ethanol-induced gastric mucosal damage were studied. Smoke exposure markedly increased gastric lesion formation following ethanol administration and this was accompanied by substantial increase in gastric mucosal leukotriene B_4 concentration, myeloperoxidase and inducible nitric oxide synthase activities. Antineutrophil serum or aminoguanidine pretreatment significantly attenuated both gastric mucosal lesion formation and inducible nitric oxide synthase activity. The increased myeloperoxidase activity was abolished by antineutrophil serum but not by aminoguanidine. These data indicated that both neutrophil mobilization and inducible nitric oxide synthase activation in the gastric mucosa play an important role in the potentiating action of cigarette smoke on ethanol-induced gastric mucosal lesion formation. Increased synthesis of nitric oxide from inducible nitric oxide synthase during gastric damage may be secondary to neutrophil infiltration in the gastric mucosa. Chemoattractant leukotriene B_4 could also contribute to neutrophil recruitment in the tissue. © 1998 Elsevier Science B.V.

Keywords: Ethanol; Myeloperoxidase; Leukotriene B_4; Neutrophil; Nitric oxide (NO) synthase, inducible; Cigarette smoking; Gastric mucosal damage

1. Introduction

Cigarette smoking has been associated with gastric ulcers in humans (Kato et al., 1992) and in animals (Iwata et al., 1995). Also, smokers are more susceptible to gastric ulcer formation than non-smokers (Friedman et al., 1974). The healing rate of ulcers was found to be delayed in smokers (Korman et al., 1981). Indeed, a previous study (Chow et al., 1996) showed that passive smoking potentiated ethanol-induced gastric mucosal lesion formation. However, the exact mechanisms involved in such adverse reactions have not been defined.

Neutrophils have been implicated in the development of inflammation and injury in the gastric mucosa (Ninemann, 1988). During inflammation, both neutrophil adherence and aggregation to the vascular endothelium have been demonstrated and these pathological changes could occlude mucosal blood flow (Tauber and Babior, 1985). In addition, the activated neutrophils discharge tissue-damaging substances, such as superoxide anion, hydrogen peroxide, myeloperoxidase and proteases (Harlan, 1985) which further disrupt mucosal integrity in the stomach (Anderson et al., 1991; Chow et al., 1997). Neutrophil aggregation could be triggered by some chemoattractants such as leukotrienes. Leukotriene B_4, isolated initially from incubation mixtures of rabbit peritoneal polymorphonuclear leukocytes and arachidonic acid (Borgeat and Samuelsson, 1979), plays a significant role in the mediation of inflammatory reactions (Samuelsson, 1983). It has also been shown to be a potent granulocyte chemotactic factor which promotes leukocyte migration both in vitro and in vivo (Ford-Hutchinson et al., 1980; Goetzl and Pickett, 1980; Bray et al., 1981; Thureson-Klein et al., 1986). We therefore suggest that cigarette smoking could induce leukotriene B_4 synthesis and activate neutrophil infiltration in the gastric mucosa.

Nitric oxide (NO) has also been demonstrated in different physiological and pathological conditions in animals (Moncada et al., 1991). Two isoforms of nitric oxide synthase were identified, the Ca^{2+}-dependent constitutive isoform synthesizes small amounts of NO which partici-

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pates in neuronal transmission (Bredt and Snyder, 1989) and vasodilatation (Palmer et al., 1987) and the Ca^{2+}-independent inducible isoform produces high levels of NO which is implicated in the pathogenesis of inflammatory- and immunological-mediated diseases (Mulligan et al., 1991). The relationship between the inducible NO and neutrophil aggregation during the course of inflammation is still unknown. It could be a dependent or independent process during ulceration in the gastrointestinal tract.

In this study, we used a smoking model (Chow et al., 1996) to demonstrate the role of neutrophil aggregation and inducible NO activation, and also the involvement of leukotriene B_4 in the potentiation of ethanol-induced gastric mucosal damage by smoke exposure in rats. We also investigated the temporal relationship between these mediators in lesion formation, using neutrophil antiserum and inducible NO synthase inhibitor, during the ulcerogenic process in the gastric mucosa.

2. Materials and methods

2.1. Animals and reagents

All studies were approved by the Committee on the Use of Live Animals for Teaching and Research of the University of Hong Kong. Male Sprague–Dawley rats (180–200 g) were reared on a standard laboratory diet (Ralston Purina Co., Chicago, IL) and given tap water to drink. They were kept in a room where temperature (22 ± 1°C), humidity (65–70%) and day/night cycle (12/12 h light/dark) were controlled. The rats were starved for 24 h but allowed free access to water until 1 h before the last cigarette smoke exposure.

2.2. Experimental protocol

Rats were divided into 3 groups of 6–10 rats each which were exposed to a smoke/air mixture (0, 2 or 4% v/v). Rats exposed to 0% of cigarette smoke served as controls and were exposed only to air. In separate experiments, rats exposed to the highest smoke concentration (i.e. 4%) were pretreated intravenously with either antineutrophil serum (0.4 ml/rat, 24 h before ethanol administration) which was raised from rabbits, or aminoguanidine (aminoguanidine hydrogen carbonate, Sigma Chemical Co., St. Louis, MO; 15 mg/kg, 90 min before ethanol administration).

In a time course study on the number of circulating neutrophils, rats were restrained and pretreated with a single i.v. injection of either normal serum or antineutrophil serum (0.4 ml/rat). Blood (50–100 μl) was then collected from the tail vein by venipuncture at 0, 1, 2, 4, 8 or 24 h and smeared on a glass slide. The neutrophils were stained with hematoxylin–eosin and counted under a microscope ($\times$100; Olympus BX-50, Olympus, Tokyo, Japan). The number of neutrophils per 100 white blood cells was based on counting of at least 5 consecutive microscopic fields.

2.3. Smoke exposure

The method for cigarette smoke exposure was described previously (Chow et al., 1996) and was shown not to disturb the normal physiological functions of the animals. In addition, the model did not impose any stress on the animals being tested. Commercial cigarettes (Camel, R.J. Reynolds, Winston-Salem, NC) were used throughout the experiment. Initially, residual air inside a balloon was emptied by means of a mechanical pump. The balloon was refilled with air with moisture removed by passage through a conical flask containing anhydrous calcium chloride pellets. The volume of air infused into the balloon was measured by a water displacement method in which the balloon was connected to a 5 l graduated bell-jar. A measured volume of cigarette smoke was infused into either the balloon (10 l) or the smoke chamber (20 l) so that a known concentration of smoke and air mixture was achieved. The chamber and the balloon were then connected by means of silicone tubing (96400-15, Masterflex, Cole-Parmer Instrument Co, Vernon Hills, IL). The smoke/air mixture in the balloon was delivered constantly into the chamber with a flow rate of 250 ml/min by a peristaltic pump (Masterflex, Cole-Parmer Instrument) attached to a pump head (# 7015, Masterflex). This procedure was necessary to maintain a constant supply of a fixed concentration of smoke/air mixture to the chamber throughout the three 1-h experimental periods during the 24 h starvation period with 8 h intervals between smoke exposure periods.

2.4. Gastric mucosal lesion induction

Rats were removed from the chamber after the last 1 h smoke exposure and given an oral dose of 70% (v/v) ethanol (British Drug House, Poole, England) in a volume of 10 ml/kg 15 min later. They were decapitated 30 min later. The stomachs were rapidly removed, opened along the greater curvature and examined for mucosal lesions. Lesion size was measured, using a grid (each grid square was 1 mm^2), by a person who was unaware of the treatment. Five petechiae were counted as 1 mm^2. The total lesion area was divided by the number of rats in each group and expressed as the mean lesion area (Poon et al., 1989). The stomachs were rinsed with ice-cold saline and the glandular mucosae were scraped from the underlying muscle using a glass slide, immediately frozen in liquid nitrogen and stored at -70°C for further enzyme assays.

2.5. Preparation of antineutrophil serum

The preparation of antineutrophil serum was described previously (Simpson and Ross, 1971; Sandler et al., 1987).

Rat neutrophils were harvested from the peritoneal exudate 24 h after an intraperitoneal injection of 30 ml freshly prepared sterile 3% proteose peptone (Oxoid, London, England). Following isolation of neutrophils by density gradient centrifugation at room temperature using Histopaque 1077 and 1119 (Sigma Chemical Co.), the cells were briefly suspended in distilled water to hemolyse any red blood cells present, then washed twice in a Ca^{2+}- and Mg^{2+}-free Hank's buffer (pH 7.2). They were resuspended in the buffer at a concentration of 10^7 cells/ml. The suspension was mixed with an equal volume of Freund's complete adjuvant (Gibco Laboratories, Grand Island, NY). Two adult New Zealand albino rabbits weighing 3.5–4.5 kg were immunized subcutaneously with 50×10^6 neutrophils. This was repeated twice at 1-week intervals with 10^7 cells/ml of Hank's buffer. The rabbits were anesthetized with pentobarbitone (40 mg/kg, i.v.) and bled by carotid cannulation. Sera obtained by centrifuging clotted blood were inactivated at 56°C for 30 min and processed by absorption.

2.6. Absorption of antineutrophil serum

The procedure was described by Sandler et al. (1987). Hemagglutinins were eliminated by absorption to 0.1 ml of packed RBCs/ml antineutrophil serum. Anti-serum antibodies were removed by absorption with 0.05 ml rat serum/ml antineutrophil serum, and anti-lymphocyte antibodies by absorption against lymphocytes harvested from the rat spleen and thymus (twice, 8×10^6 lymphocytes/ml antineutrophil serum). All absorption was performed for 1 h at room temperature with shaking. After centrifugation, antiserum and normal serum were filtered through a Millipore membrane (pore size 0.22 μm; Millipore Corp., Bedford, MA), collected in microcentrifuge tubes and stored at -70°C until use.

2.7. Induction of neutropenia

Rats were made neutropenic by intravenous administration of absorbed antineutrophil serum at a dose of 0.4 ml per rat 24 h prior to ethanol administration. This amount of antineutrophil serum had been tested and shown to be enough to aggregate circulating neutrophils without affecting other cell types. Control animals were injected over a similar time course with an equal volume of normal serum. Neutropenia was confirmed by counting of circulating neutrophils histologically.

2.8. Measurement of leukotriene B_4 concentration in the gastric mucosa

The mucosal sample (100 mg) was homogenized in phosphate buffer (50 mM, pH 7.4) with indomethacin (28 μM) for 15 s. The homogenate was centrifuged (Beckman J2-21 centrifuge, Beckman Instrument, Fullerton, CA) at 3,000 r.p.m. for 15 min at 4°C. The supernatant was assayed using a leukotriene B_4 [^{3}H] radioimmunoassay kit (New England Nuclear Corp., Dupont, Boston, MA) based on the method developed by Karmeli et al. (1994). The samples were counted in a liquid scintillation counter (LS-6500, Beckman Instrument). A standard curve was obtained in the range of 0.0125–0.5 ng/tube. The protein content was assayed based on the method of Lowry et al. (1951) using bovine serum albumin as standard. The final values for the samples were expressed as picograms per mg of protein.

2.9. Measurement of myeloperoxidase activity in the gastric mucosa

Myeloperoxidase activity was determined by the method described by Bradley et al. (1982). with modifications. Samples of 100–150 mg were homogenized in 0.5% hexadecyltrimethylammonium bromide (Sigma Chemical Co.) in 50 mM potassium phosphate buffer, pH 6.0. The homogenates were freeze-thawed three times, followed by repeated sonication for 10 s each. Suspensions were centrifuged at 12,000 rpm for 30 min and the resulting supernatant was assayed as follows. 100 μl of the sample was mixed with 50 μl of 0.0005% hydrogen peroxide (Sigma Chemical Co.) and 2.45 ml of 0.167 mg/ml *o*-dianisidine (Sigma Chemical Co.) prepared in potassium phosphate buffer. The mixture was incubated at 25°C for 10 min followed by 3.5 ml of absolute ethanol to stop the reaction. The end-point absorbance of the mixture was measured at 460 nm using a spectrophotometer (Beckman DU 650, Beckman Instrument) with horseradish peroxidase (Sigma Chemical Co.) as standard. The final values were expressed as milliunits per mg of protein.

2.10. Determination of NO synthase activity

This was determined as described by Tepperman et al. (1993). The mucosal samples, 100–150 mg, were homogenized for 20 s at 0°C in a buffer (pH 7.2) containing 10 mM HEPES, 0.32 M sucrose, 0.1 mM EDTA, 1 mM dithiothreitol, 10 μg/ml of soybean trypsin inhibitor, 10 μg/ml of leupeptin, 2 μg/ml of aprotinin and 1 mg/ml of phenylmethanesulfonyl fluoride. Following centrifugation at 12,000 r.p.m. for 30 min at 4°C, the reaction mixture comprised of 100 μl supernatant and 150 μl of buffered solution (pH 7.2) containing 10 mM HEPES, 0.7 mM NADPH, 150 μM $CaCl_2$, 7 mM L-valine to inhibit any arginase and 1 μCi [^{3}H]L-arginine (1 mCi/ml; specific activity 36.1 Ci/mmol, New England Nuclear) was incubated at 37°C for 30 min. NO synthase activity was estimated from the conversion of [^{3}H]L-arginine to the NO

co-product, [^{3}H]citrulline (Knowles et al., 1990). For determining the activity of inducible NO synthase, ethylene glycol-bis N,N,N',N'-tetraacetic acid (EGTA, 1 mM) was used to inhibit the activity of calcium-dependent constitutive NO synthase. The reaction was stopped with 20% of perchloric acid and the mixture was neutralized with 160 μl of 1 M NaOH, followed by dilution with 540 μl of deionized water containing 1 mM each of L-arginine and DL-citrulline. Subsequently, the resulting mixture was applied to a column containing Dowex AG50WX-8 (Na-form, BioRad, Hercules, CA, USA) resins. Scintillation fluid (BDS, Amersham) was mixed with the eluent at a ratio of 9:1 and the mixture was counted with a scintillation counter (Beckman Instrument). The final result was expressed as picomoles of [^{3}H]citrulline formed per min per mg of protein.

2.11. Statistical analysis

The results are expressed as the means ± S.E.M. Statistical analysis was performed with an analysis of variance (ANOVA) followed by multiple comparison with Scheffé test. The time course of circulating neutrophil counts was analysed with Student's paired *t*-test. *P*-values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Effects of cigarette smoke exposure on ethanol-induced gastric mucosal damage, leukotriene B_4 concentration, myeloperoxidase and NO synthase activity

Cigarette smoke exposure for three 1 h periods concentration-dependently increased the gastric mucosal lesioning induced by 70% ethanol (Fig. 1A). A significant effect was achieved in the group exposed to 4% of cigarette smoke. Smoke exposure similarly concentration-dependently increased the mucosal leukotriene B_4 concentration, which increased further but not significantly following ethanol challenge (Table 1). Ethanol treatment did not significantly increase gastric mucosal myeloperoxidase activity (Fig. 1B). Nevertheless, there was a concentration-dependent and significant augmentation of the mucosal myeloperoxidase activity at 2 and 4% cigarette smoke. Ethanol treatment significantly reduced the constitutive NO synthase activity in the gastric mucosa (Fig. 2A); however, this treatment did not significantly increase the inducible NO synthase activity (Fig. 3B). On the other hand, smoke exposure followed by ethanol challenge for 30 min further potentiated the gastric mucosal inducible NO synthase activity. The effect reached significance at 4% but with no significant influence on constitutive NO synthase activity. Smoke exposure alone at 0, 2 or 4% did not have any significant impact on either constitutive NO synthase (3.29 ± 0.43, 3.33 ± 0.71 or 3.34 ± 0.33 pmol/min/g protein, respectively) or inducible NO synthase (0.65 ± 0.06, 0.51 ± 0.02 or 0.48 ± 0.03 pmol/min/g protein, respectively) activity.

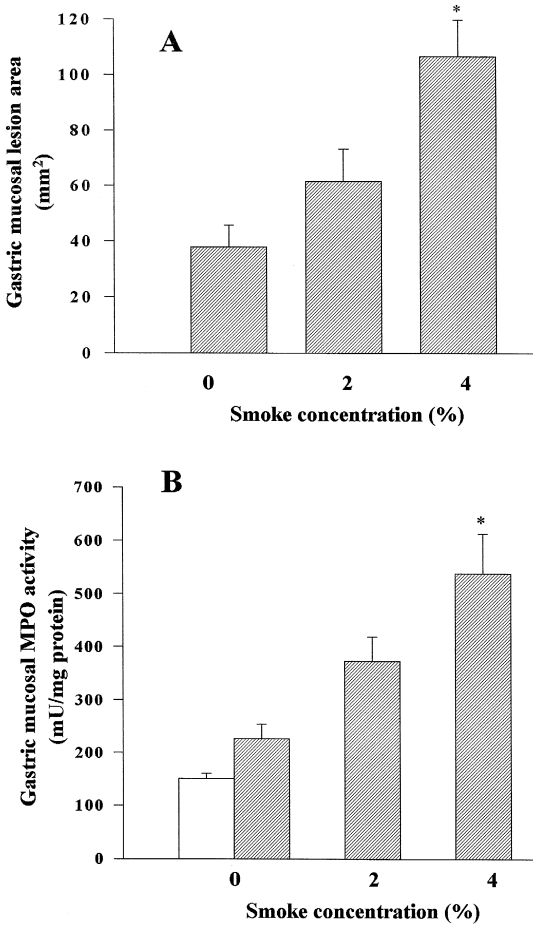

Fig. 1. Dose–response relationship of ethanol-induced gastric mucosal lesions (A) and gastric mucosal myeloperoxidase activity (B) after cigarette smoke exposure at different concentrations. Open column represents samples derived from mucosa without ethanol treatment, while hatched columns stand for samples treated with ethanol. Each column represents the mean ± S.E.M. $^*P < 0.05$ versus 0% of cigarette smoke with ethanol treatment.

Table 1
Effect of cigarette smoke exposure on gastric mucosal LTB_4 concentration (pg/mg protein)

	Smoke concentration (%)		
	0	2	4
(A) Without ethanol	100.8 ± 30.4	129.6 ± 25.5	215.2 ± 34.0 *
(B) With ethanol	158.6 ± 16.4	168.3 ± 13.6	295.4 ± 27.6 **

Values are the means ± S.E.M. for 6–10 rats.
$^*p < 0.05$ versus corresponding 0% of smoke.
$^{**}p < 0.01$ versus corresponding 0% of smoke.

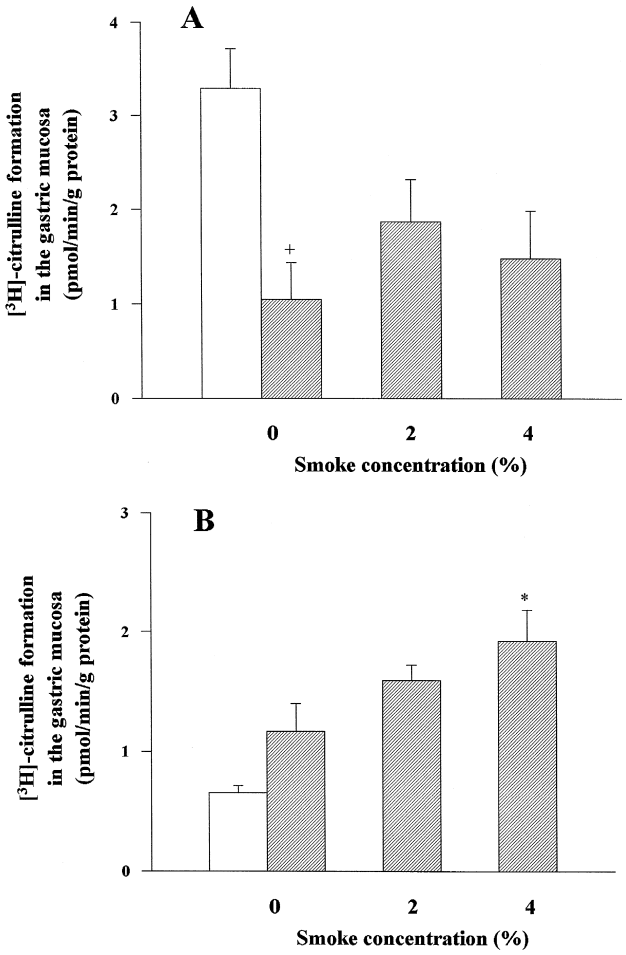

Fig. 2. Dose–response relationship of the gastric mucosal constitutive nitric oxide synthase activity (A) and inducible nitric oxide synthase activity (B) after smoke exposure, based on the amount of [^{3}H]L-citrulline formed during the 30 min incubation-period. Open column represents samples derived from mucosal samples without ethanol treatment and hatched columns stand for samples treated with ethanol. Each column represents the mean ± S.E.M. $^{+}P < 0.05$ versus 0% without ethanol treatment; whereas $^{*}P < 0.05$ versus the corresponding 0% of cigarette smoke.

3.2. Effects of antineutrophil serum treatment on gastric lesions, myeloperoxidase and inducible NO synthase activity

One hour after antineutrophil serum treatment, there was a significant reduction in circulatory neutrophil counts when compared with those of the normal serum-treated rats (Fig. 3). There was a more than 90% reduction in circulating neutrophils 24 h after antineutrophil serum treatment. On the other hand, the number of circulating neutrophils in rats treated with normal serum increased initially in the first 8 h but returned to normal after 24 h. Neutropenia induced by antineutrophil serum completely abolished the potentiation of gastric mucosal lesioning in

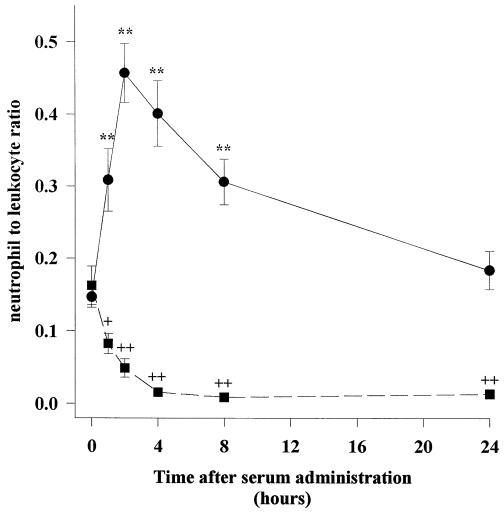

Fig. 3. Time course of circulating PMNs to leukocytes ratio in rats before and after treatment with either normal serum (●) or anti-neutrophil serum (■). One hour after normal serum administration, PMNs were significantly increased with a peak at 2 h. The ratio dropped eventually and returned to its basal level 24 h after normal serum administration. One h after antineutrophil serum treatment the ratio was significantly reduced, reaching a minimum at 4 h. The ratio did not return to the basal level 24 h later. There was a more than 90% reduction in the ratio 4 h after administration. Each column represents the mean ± S.E.M. of 8 rats. $^{**}P < 0.01$ versus corresponding 0 h; $^{+}P < 0.05$, $^{++}P < 0.01$ versus corresponding normal serum-treated group.

the 4% smoking group (Fig. 4). This treatment also markedly reduced the potentiating action of cigarette smoke on myeloperoxidase activity (Fig. 5). In addition, neutropenia reduced the inducible NO synthase activity back to the level of the ethanol-treated control (Fig. 6).

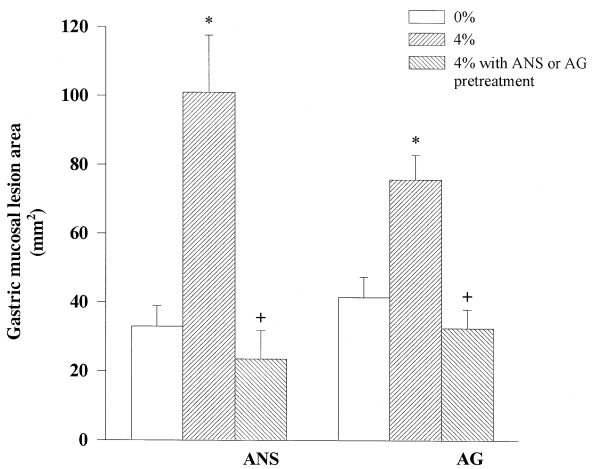

Fig. 4. Effects of antineutrophil serum (ANS) or aminoguanidine (AG) on the potentiation of smoke exposure on ethanol-induced gastric mucosal lesion formation. Normal serum or ANS (0.4 ml/rat, i.v.) was given 24 h prior to ethanol or normal saline or AG (15 mg/kg, i.v.), 90 min prior to ethanol administration. ANS and AG (inducible nitric oxide synthase inhibitor) pretreatment significantly reduced the potentiating action of smoke on lesion formation. Each column represents the mean ± S.E.M. $^{*}P < 0.05$ versus 0% of cigarette smoke; $^{+}P < 0.05$ versus 4% of cigarette smoke alone.

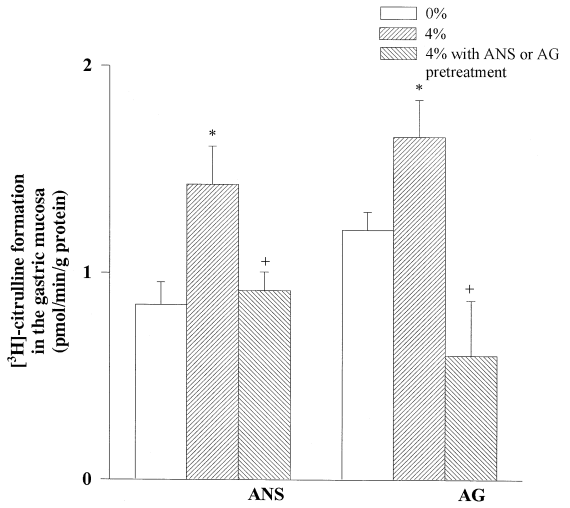

Fig. 5. Effects of antineutrophil serum (ANS) or aminoguanidine (AG) on the potentiation of gastric mucosal myeloperoxidase activity. Normal serum or ANS (0.4 ml/rat, i.v.) was given 24 h before ethanol or normal saline or AG (15 mg/kg, i.v.), 90 min before ethanol administration. ANS but not AG pretreatment significantly abolished the rise in enzyme activity. Each column represents the mean ± S.E.M. $^{*}P < 0.05$ versus 0% of cigarette smoke; $^{+}P < 0.05$ versus 4% of cigarette smoke alone.

3.3. Effects of aminoguanidine treatment on gastric lesion, myeloperoxidase and inducible NO synthase activity

Similar to the effects of antineutrophil serum, aminoguanidine pretreatment at a dose of 15 mg/kg significantly attenuated the potentiating effect of cigarette smoke on ethanol-induced gastric mucosal damage (Fig. 4) and significantly reduced inducible NO synthase activity (Fig. 6). Pretreatment with aminoguanidine, however, did not reverse the augmentation of mucosal myeloperoxidase activity by cigarette smoke 30 min after ethanol challenge (Fig. 5).

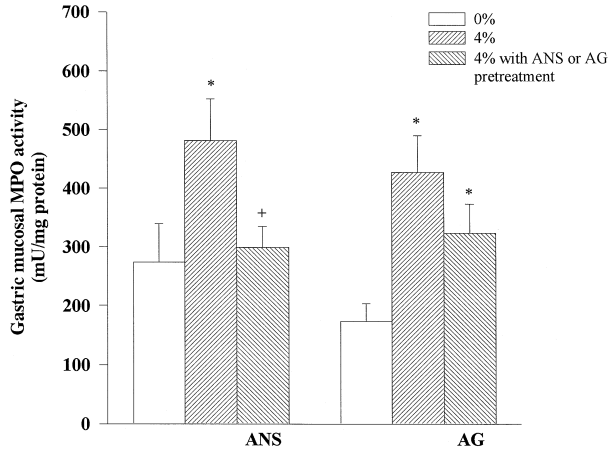

Fig. 6. Effects of antineutrophil serum (ANS) or aminoguanidine (AG) on the potentiation of gastric mucosal inducible nitric oxide synthase activity. Normal serum or ANS (0.4 ml/rat, i.v.) was given 24 h or normal saline or AG (15 mg/kg, i.v.), 90 min before ethanol administration. ANS and AG pretreatment significantly reduced the increased inducible nitric oxide synthase activity. Each column represents the mean ± S.E.M. $^{*}P < 0.05$ versus 0% of cigarette smoke; $^{+}P < 0.05$ versus 4% of cigarette smoke alone.

4. Discussion

In peptic ulcer disease, cigarette smoking is a risk factor for ulcer development and is an even greater risk factor for ulcer relapse, delay in ulcer healing and resistance to drug therapy (Kikendall et al., 1984). Indeed, using the same animal model as reported upon in the present study, Chow et al. (1996) have demonstrated that cigarette smoke exposure potentiates ethanol-induced gastric mucosal lesions. The serum nicotine levels were found to be similar to those of smokers, which were as high as 0.1–0.2 ng/μl in the arterial circulation (Benowitz, 1991). These levels were not significantly affected by ethanol administration (Chow et al., 1996). This smoking model, however, did not disturb the normal physiological functions and also did not impose any stress on the animals during the experimental period, as indicated by the absence of any significant change in heart rate and blood pressure and also of the acid–base balance in the systemic circulation. These findings suggest that there are some underlying mechanisms which act specifically on the gastric mucosa and thereby aggravate gastric ulceration. However, which factors are involved in such an adverse reaction are still undefined.

Cigarette smoking has been shown to increase the burden of neutrophils in the lower respiratory tract. Ludwig and his coworkers (Ludwig and Hoidal, 1982; Ludwig et al., 1985) demonstrated that smokers have more neutrophils in their lavage fluid than nonsmokers. It is therefore plausible that cigarette smoking could induce neutrophil infiltration in the gastroduodenal mucosa. However, this phenomenon has not been investigated in depth. In the present study, it was found that smoke exposure for three 1-h periods significantly potentiated the appearance of ethanol-induced gastric mucosal lesions. This effect was accompanied by a concentration-dependent increase in myeloperoxidase activity and possibly neutrophil aggregation (Bradley et al., 1982). Coincidentally, the leukotriene B_4 concentration in gastric mucosa was also increased in a parallel fashion both before and after ethanol challenge. Leukotriene B_4 is a potent chemoattractant for granulocytes released from various cell types including eosinophils, neutrophils and mononuclear phagocytes (Rigaud et al., 1979; Kurihara et al., 1984; Frigas and Gleich, 1986). It is likely that neutrophil aggregation could be activated further by over-expression of leukotriene B_4 in the gastric mucosa after ethanol challenge. It was also noted that a short-term increase of leukotriene B_4 after smoke exposure without ethanol did not produce any injury to the gastric mucosa, suggesting that an acute rise in leukotriene B_4 alone would not produce any observable lesions in the organ. Indeed, it has been evidenced that leukotriene B_4

itself, although it can induce penetration of neutrophils between endothelial cells of post-capillary venules, does not produce any degranulation of neutrophils or local injury to endothelial cells (Thureson-Klein et al., 1984). However, in the presence of noxious agents like ethanol, such an increase in mucosal leukotriene could lead to ulceration in the gastric mucosa. Therefore, it is envisaged that activation of leukotriene B_4 by cigarette smoke, followed by more neutrophil aggregation in the gastric mucosa, could explain in part how cigarette smoke aggravates ethanol-induced gastric damage in rats.

The involvement of neutrophils in lesion formation was further confirmed by the anti-lesion action of antineutrophil serum which also markedly reduced the number of circulating neutrophils. In addition, the mucosal myeloperoxidase activity was also dramatically attenuated by antineutrophil serum after smoke exposure followed by ethanol challenge. Indeed, neutrophil mobilization and recruitment are the critical steps leading to inflammation and release of damaging substances, such as superoxide, hydrogen peroxide, myeloperoxidase, proteases and hypochlorous acid (Harlan, 1985). Furthermore, experimental evidence has indicated that neutrophil aggregation is responsible for gastric injury and is associated with an increase in the extent of ethanol-induced hemorrhagic damage in the gastric mucosa both in vitro and in vivo (Kvietys et al., 1990; Tepperman et al., 1993). Neutrophil activation was also found to be capable of damaging the gastric mucosal surface cells (Kozol et al., 1994).

NO released from the inducible isozyme is another pro-inflammatory mediator which can be induced in various cells, especially macrophages, by cytokines or endotoxin (Knowles et al., 1990). The inducible NO synthase activity was shown to be dose dependently up-regulated by smoke exposure in the ethanol-treated animals. It is plausible that this increase in inducible NO synthase activity could play a significant role in the potentiating action of cigarette smoking on ethanol ulceration. We studied further the role of inducible NO synthase in such an adverse interaction by using a potent inducible NO synthase inhibitor, aminoguanidine (Wu et al., 1995). This agent has been shown to be less potent as an inhibitor of constitutive NO synthase in cultured cells and isolated blood vessels in vitro (Corbett et al., 1992; Griffiths et al., 1993). The 15-mg/kg dose of aminoguanidine was chosen because it did not cause any increase in blood pressure in control rats, but significantly inhibited inducible NO synthase activity (Wu et al., 1995). Treatment with aminoguanidine indeed completely abolished the potentiating effect of cigarette smoke on gastric lesion formation and on inducible NO synthase activity. These results indicated that both neutrophil aggregation and inducible NO synthase activation in the gastric mucosa are involved in the potentiating effect of cigarette smoking on gastric damage.

We also investigated the temporal relationship between these two pathways during the ulcerogenic processes in the gastric mucosa. Both antineutrophil serum and aminoguanidine pretreatment completely reversed the increase of inducible NO synthase activity back to the normal level. However, only antineutrophil serum, but not aminoguanidine, significantly prevented the potentiating effect of cigarette smoking on myeloperoxidase activity. It is anticipated that adherence of neutrophils to the endothelium could activate the inflammatory cascade including stimulation of inducible NO synthase activity in the gastric mucosa. Antineutrophil serum pretreatment neutralized the circulating neutrophils and thereby prevented these leukocytes from adhering to the endothelium and subsequently decreased inducible NO synthase activation. On the other hand, aminoguanidine inhibited inducible NO synthase activity after neutrophil adherence, therefore myeloperoxidase activity (a marker for neutrophils) was not significantly affected. All these findings suggest that neutrophil adherence is an initiator for the potentiating action of cigarette smoke on ethanol-induced gastric damage. Subsequently, NO derived from inducible NO synthase is the critical step in the propagation of the whole inflammatory cascade. Ethanol administration dramatically reduced constitutive NO synthase activity. This finding is consistent with other results (Tepperman et al., 1993). This low level of constitutive NO synthase was, however, not significantly affected by cigarette smoking, implying that the involvement of this isozyme in the potentiation of ethanol ulceration by smoke exposure might be negligible.

Together, the results showed that short-term cigarette smoking dose dependently potentiated ethanol-induced gastric mucosal damage. The adverse action was shown to be mediated through neutrophil aggregation and NO production derived from inducible NO synthase. We also conclude that neutrophil infiltration preceded the activation of inducible NO synthase during the inflammatory processes which were indeed aggravated by cigarette smoking.

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