Cigarette smoking delays ulcer healing: role of constitutive nitric oxide synthase in rat stomach

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Cigarette smoking delays ulcer healing: role of constitutive nitric oxide synthase in rat stomach. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G238–G248, 1999.—Epidemiological studies have shown that cigarette smoking is associated with peptic ulceration. This study aims to investigate the mechanisms by which cigarette smoking delays ulcer healing in rats. Gastric ulcers were induced by applying acetic acid to the luminal surfaces in rats. Twenty-four hours later, rats were exposed to different concentrations of cigarette smoke (0, 2, or 4%) for a 1-h period once daily for 3 or 6 days. Cigarette smoke exposure delayed ulcer healing and decreased gastric blood flow and angiogenesis at the ulcer margin. These changes were accompanied by a significant reduction of constitutive nitric oxide synthase (cNOS) activity but not PGE2 production and vascular endothelial growth factor levels. Administration of L-arginine (10 mg/kg iv) completely reversed the adverse actions on ulcer healing, gastric blood flow, and angiogenesis in the mucosa at the ulcer margin but partially restored angiogenesis in granulation tissues. In conclusion, cigarette smoke exposure delays ulcer healing through depression of gastric blood flow and angiogenesis at the ulcer margin. Reduction of cNOS expression and activity is suggested to be involved in these ulcerogenic processes.

MATERIALS AND METHODS

Animals and induction of gastric kissing ulcers. The present study was approved by the Committee on the Use of Live Animals for Teaching and Research of the University of Hong Kong. Male Sprague-Dawley rats, weighing 180–200 g, were raised under controlled temperature (22 ± 1°C), humidity (65–70%), and light-dark cycle (light from 0600 to 1800, dark from 1800 to 0600) conditions. Rats were deprived of food but had free access to tap water 24 h before ulcer induction. Gastric kissing ulcers were produced by luminal application of an acetic acid solution as described by Tsukimi and Okabe (39). Briefly, the abdomen was opened under ether anesthesia, and the stomach was exposed. The anterior and posterior walls of the stomach were clamped together with a clip that had a ring (11 mm ID) attached to the two arms of the clip. With the use of a 1-ml syringe, 0.12 ml of 60% acetic acid was injected through the forestomach into the gastric lumen between the two rings of the clipped portion. Forty-five seconds later, the acid solution was withdrawn with the same syringe and the abdomen was sutured. Thereafter, animals were fed a standard diet of laboratory chow (Ralston Purina, Chicago, IL) and given tap water ad libitum.

Cigarette smoke exposure. Twenty-four hours after ulcer induction, rats were exposed to different concentrations of cigarette smoke (0, 2, or 4%, vol/vol) for a 1-h period once daily as described previously (2). Commercial cigarettes (Camel, R. J. Reynolds, Winston-Salem, NC) with or without filters were employed throughout the experiment. Initially, a balloon with a fixed concentration of smoke was prepared by emptying the residual air inside the balloon with a mechanical pump. The balloon was then connected to a 5-liter graduated bell jar. The volume of air infused into the balloon was measured by water displacement. A measured volume of cigarette smoke was delivered into either the balloon (10 liter) or a smoke chamber (20 liter) so that a known concentration of smoke and air mixture was achieved. The chamber and the balloon were then connected by silicone tubing (96400-15, Masterflex, Cole-Parmer Instrument, Vernon Hills, IL). Then, a smoke-air mixture in the balloon was delivered constantly into the chamber at a flow rate of 250 ml/min by a peristaltic pump.
smoke-air mixture for the rats inside the chamber; the
maintain a constant and fresh supply of a fixed concentration
mixture every 30 min. This procedure was necessary to
of the smoke and air in the balloon was replaced by a fresh
was made on the top of the chamber. The mixture
pump (Masterflex, Cole-Parmer Instrument). An outlet for
the smoke was made on the top of the chamber. The mixture
constant of 1.5 ms. Values for area and range of laser emission
was performed for 10 s at a frequency of 4 Hz and a time
was calibrated by placing the laser probe against a
whole glandular wall. The gastric blood flow measurements,
expressed in arbitrary units, were performed at ulcer mar-
gins by a person who was blinded from the treatments. Zero
value was calibrated by placing the laser probe against a
white board.
Assessment of ulcer severity. After gastric blood flow was
measured, rats were killed by decapitation. The stomachs
were excised, opened along their greater curvatures, and
rinsed extensively with normal saline to remove attached
debri. The stomachs were then blotted dry and spread, and
the ulcerated areas (mm²) were measured initially by tracing
the outline of the ulcer onto a glass slide that was then copied
over a square grid. After the ulcerated areas were traced, a
longitudinal section of stomach along the greater curvature,
which included the ulcer base and both sides of the ulcer
margin, was taken and fixed in 4% buffered Formalin for 24 h
at 4°C for further histological study. The remaining glandular
mucosa around the ulcer (including the ulcer margin and
adjacent normal mucosa) was scraped with a glass slide on an
ice-cold dish, or the ulcer base (mainly granulation tissues)
was cut and frozen immediately in liquid nitrogen. The
mucosal samples were stored at −70°C until assay.
Measurement of NOS activity in the gastric mucosa and
ulcer base. The tissue samples (100–150 mg) were placed in
a buffer solution containing 10 mM HEPES, 320 mM sucrose,
0.1 mM EDTA, 1 mM dithiothreitol, 10 mg/ml of soybean
trypsin inhibitor, 10 mg/ml of leupeptin, 2 mg/ml of aprotinin,
and 1 mg/ml of phenylmethylsulfonyl fluoride. Samples were
homogenized for 20 s under ice-cold condition and then
centrifuged at 20,000 × g for 20 min at 4°C. NOS activity was
estimated by the conversion of L-[3H]arginine to the NO
coproduct L-[3H]citrulline as described by Knowles et al. (17).
Briefly, the reaction mixture contained 100 µl of supernatant
and 150 µl of buffered solution (pH 7.2), consisting of 10 mM
HEPES, 0.7 mM NADPH, 150 mM CaCl₂, 7 mM L-valine to
inhibit any arginase, and 1 mCi of L-[3H]arginine (specific
activity 36.1 Ci/mmol; NEN, Boston, MA). For determination
of inducible NOS (iNOS) activity, 1 mM EGTA was used to
inhibit the activity of the Ca²⁺-dependent constitutive NOS
(cNOS). The incubation was allowed to proceed for 30 min at
37°C and then terminated by adding 50 µl of 20% perchloric
acid. The assay system was neutralized by 160 µl of 1 M
NaOH, followed by dilution with 540 µl of deionized water
containing 1 mM L-arginine and 1 mM citrulline. Subse-
dually, the resulting 1-m1 mixture was applied into a
chromatographic column containing 0.5 g of Dowex AG50WX-8
resin (sodium form; Bio-Rad, Hercules, CA) for separation from
unreacted L-[3H]arginine by cation exchange chromatog-
raphy and collected into a scintillation vial. Thereafter, the
samples were counted for radioactivity using a liquid scintil-
lation counter (LS-6500; Beckman Instruments, Fullerton,
CA). The coefficients of variation within and between assays
were 7.2 and 9.8%, respectively. The final result was ex-
pressed as picomoles of L-[3H]citrulline formed per minute
per milligram of protein. Protein concentrations of the super-
natant were measured by the method of Lowry et al. (22).
Immunostaining and quantification of cNOS expression.
Immunostaining of cNOS in the gastric mucosa was per-
formed on a Formalin-fixed, paraffin-embedded section.
Briefly, after deparaffinization, endogenous peroxidase activ-
ity in the tissue sections was quenched by immersing the
slides in 3% hydrogen peroxide-methanol solution for 10 min.
After permeabilization by trypsin for 15 min at room tempera-
ture, sections were blocked by diluted normal rabbit serum
for 1 h, which was followed by incubation with monoclonal
mouse anti-human endothelial cNOS antibody (Transduction
Laboratories, Lexington, KY) at a dilution of 1:1,000 over-
night at 4°C. After sections were washed with PBS solution,

![Fig. 1](http://ajpgi.physiology.org/)

**Fig. 1.** Effect of cigarette smoking on ulcer healing. A: rats were exposed to different concentrations of smoke (0, 2, or 4%) 1 day after ulcer induction for a 1-h period once daily for 3 or 6 days. B: L-arginine (Arg) was given 5 min before each cigarette smoke exposure in another 4% treatment group for 3 or 6 days. Columns and vertical bars represent means ± SE of 10–12 rats. *P < 0.05, **P < 0.01 vs. corresponding 0% group; †P < 0.05 vs. corresponding 4% group.
biotinylated anti-mouse IgG was applied for 1 h at room temperature. The sections were further washed and incubated with avidin-biotin complex (Vector Stain ABC kit, Vector Laboratories, Burlingame, CA) for another 40 min and were detected after a diaminobenzidine reaction. Negative control was performed by removing either the primary or secondary antibody. Quantification of cNOS expression was performed by a person who was blinded from the treatments, at ×100 magnification field by field (0.46 × 0.46 mm) consecutively starting from the edge of ulcer margin under an image analyzer (Q500W, Leica Immaging Systems, Cambridge, UK). The areas of cells demonstrating positive staining for cNOS (dark brown) were measured [given as µm²/field (0.46 mm × 0.46 mm)]. The threshold for determination of positive staining cells in the section was controlled by a computer throughout the measuring procedure.

Measurement of PGE2 levels in the gastric mucosa and ulcer base. The tissue samples (100–150 mg) were homogenized for 20 s in an ice-cold Krebs solution containing (in mM) 128 NaCl, 4.7 KCl, 3.3 CaCl₂, 1.2 KH₂PO₄, 14.3 NaHCO₃, 11.1 glucose, and 28 indomethacin, to prevent any neoformation of PG during the extraction process. The homogenates were centrifuged at 20,000 g for 20 min at 4°C. PGE₂ was measured by an RIA kit (Amersham, Arlington Heights, IL). The coefficients of variation within and between assays were 5.3 and 7.6%, respectively. The final values obtained are given in picograms per milligram of protein. In addition, protein concentration of the supernatant was again determined (22).

Measurement of VEGF levels in the gastric mucosa and ulcer base. The tissue samples (100–150 mg) were homogenized for 20 s in ice-cold 10 mM PBS (pH 7.4) and centrifuged at 20,000 g for 20 min at 4°C. VEGF levels in the supernatant were measured by a commercial ELISA kit (Chemicon International, Temecula, CA). The coefficients of variation within and between assays were 8.0 and 11.9%, respectively. The final values are given in picograms per milligram of protein.

Determination of angiogenesis in the granulation tissue and mucosa at ulcer margin. Angiogenesis was assessed by counting the number of neomicrovessels on 5-µm-thick sections using the immunohistochemical method for von Willebrand factor expression (42). The procedure was similar to that for the detection of cNOS expression. Sections were incubated overnight at 4°C with rabbit anti-human von Willebrand factor antibody (DAKO, Copenhagen, Denmark) at a dilution of 1:1,000. After sections were washed with PBS solution, a secondary antibody was applied for 1 h at room temperature. The sections were further washed and incubated with avidin-biotin complex (Vector Laboratories) for another 1 h. Thereafter, the neomicrovessels could be visualized after a diaminobenzidine reaction. These were counted under an image analyzer (Q500W, Leica Immaging Systems) at ×200 (for granulation tissue) or ×400 (for mucosa) magnification by a person who was blinded from the treatments. Each slide was counted twice. Any brown-stained endothelial cell or endothelial cell cluster that was clearly separate from adjacent microvessels was considered a single, countable microvessel. The whole procedure was confirmed by an independent pathologist. The final values are given in number of microvessels per square millimeter of granulation tissue and mucosa at the ulcer margin.

Statistical analysis. Results are expressed as means ± SE. Statistical analysis was performed with ANOVA and un-
Table 1. Effect of L-arginine on angiogenesis in gastric mucosa at ulcer margin in rats with cigarette smoke exposure 4 and 7 days after ulcer induction

<table>
<thead>
<tr>
<th>Groups</th>
<th>Fields (×400) Starting From Edge of Ulcer Margin</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>4 Day</td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>163.4 ± 4.8</td>
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<tr>
<td>4%</td>
<td>139.2 ± 7.5a</td>
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<tr>
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<td>192.3 ± 8.5a</td>
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<tr>
<td>0%</td>
<td>167.3 ± 9.6</td>
</tr>
<tr>
<td>4%</td>
<td>126.8 ± 8.6b</td>
</tr>
<tr>
<td>4% + Arg</td>
<td>168.8 ± 13.7</td>
</tr>
</tbody>
</table>

Values are means ± SE of 8–10 rats given in number of microvessels/field. Rats were divided into 3 treatment groups: 0% and 4% smoke exposure (Arg, 10 mg/ml iv). Numbers of microvessels in the gastric mucosa at the ulcer margin were counted consecutively field by field from the edge of the ulcer margin. *P < 0.05, bP < 0.01 vs. corresponding 0% group; dP < 0.05, eP < 0.01, *P < 0.001 vs. corresponding 4% group.

RESULTS

Effects of cigarette smoke exposure on body weight gain and food and water consumption. Cigarette smoke exposure for a 1-h period once daily for 6 days did not significantly affect the normal body weight gain in rats with gastric ulcers. The average body weight gains (g·rat⁻¹·day⁻¹) during this experimental period in the 0, 2, and 4% smoking groups were 8.1 ± 0.7, 1.3, and 1.75, respectively (n = 7–8 per group). Neither food consumption nor water intake was affected during the 6-day cigarette smoke exposure. Food consumptions (g·rat⁻¹·day⁻¹) of the 0, 2, and 4% smoking groups were 29.7 ± 1.6, 27.2 ± 1.5, and 26.6 ± 1.4, respectively, whereas water intakes (ml·rat⁻¹·day⁻¹) were 37.2 ± 1.3, 37.7 ± 1.5, and 38.4 ± 1.0, respectively (n = 7–8 per group).

Effect of cigarette smoke exposure on ulcer healing. Two circular ulcers at the anterior and posterior walls were observed 24 h after acetic acid application. They were similar in shape and size. No adhesion to the adjacent organs was observed at the surface of serosa. Histological study showed that the damage penetrated through the muscularis mucosae to the muscle layer. The damage induced by the method used in this study was validated as a true ulcer in the same type of animal (39). Rats were then exposed to 0, 2, or 4% concentrations of cigarette smoke for a 1-h period once daily at a fixed time for 3 or 6 days. One day after ulcer induction, the ulcer size in all three groups was similar (111.8 ± 5.6, 111.3 ± 6.1, and 114.4 ± 5.9 mm², respectively). Ulcers healed rapidly in the control group from day 1 to day 7 and almost healed on day 10. However, the healing rate was delayed by cigarette smoke exposure because the ulcer sizes were significantly larger in the smoke-exposed groups, at a concentration-dependent manner, both on the 4th and 7th day after ulcer induction (Fig. 1A). This finding indicated that cigarette smoke exposure indeed delayed ulcer healing in the acetic acid ulcer model. However, smoke from cigarettes with filters did not significantly delay ulcer healing. The ulcer sizes on day 7 were 36.4 ± 1.75, 39.8 ± 4.8, and 44.4 ± 4.4 mm² in the 0, 2, and 4% smoking groups, respectively.

Effect of cigarette smoke exposure on gastric blood flow. The positions of ulcer base and normal area could be easily distinguished by color differences at the serosal surface after ulcer induction. The ulcer margin was defined as the interface between the ulcer base and normal area. Because the range of laser emission of the laser was validated as a true ulcer in the same type of animal (39). Rats were then exposed to 0, 2, or 4% concentrations of cigarette smoke for a 1-h period once daily at a fixed time for 3 or 6 days. One day after ulcer induction, the ulcer size in all three groups was similar (111.8 ± 5.6, 111.3 ± 6.1, and 114.4 ± 5.9 mm², respectively). Ulcers healed rapidly in the control group from day 1 to day 7 and almost healed on day 10. However, the healing rate was delayed by cigarette smoke exposure because the ulcer sizes were significantly larger in the smoke-exposed groups, at a concentration-dependent manner, both on the 4th and 7th day after ulcer induction (Fig. 1A). This finding indicated that cigarette smoke exposure indeed delayed ulcer healing in the acetic acid ulcer model. However, smoke from cigarettes with filters did not significantly delay ulcer healing. The ulcer sizes on day 7 were 36.4 ± 1.75, 39.8 ± 4.8, and 44.4 ± 4.4 mm² in the 0, 2, and 4% smoking groups, respectively.

Fig. 4. Effect of cigarette smoking on constitutive nitric oxide synthase (cNOS) activities in the gastric mucosa (A) and ulcer base (B). Rats were exposed to different concentrations of smoke (0, 2, or 4%) 1 day after ulcer induction for a 1-h period once daily for 3 or 6 days. cNOS activities in the gastric mucosa and ulcer base were determined. Columns and vertical bars represent means ± SE of 8–10 rats. *P < 0.05 vs. corresponding 0% group.
probe was 5 mm, the gastric blood flow measured by this method represented that of the whole glandular wall. The data obtained from the serosal surface were similar to those from the mucosal surface. Four and seven days after ulcer induction, the blood flow at the ulcer margin was higher than that at the adjacent normal mucosa (ulcer margin hyperemia). Cigarette smoke exposure for a 1-h period once daily induced a concentration-dependent reduction in ulcer margin hyperemia 24 h after exposure, which was significant at the higher concentration on the 7th day after ulcer induction (Fig. 2A). However, it had no significant effect on the gastric blood flow at the adjacent normal area, although a single exposure to smoke decreased the basal gastric blood flow within the first 40–50 min after exposure (unpublished observations).

Effects of cigarette smoke exposure on angiogenesis in granulation tissues and mucosa at the ulcer margin. After completion of immunostaining for the von Willebrand factor expressed in the endothelium, the newly formed blood vessels were counted under a microscope. The granulation tissue at the ulcer base did not develop even 1 day after ulcer induction. Four days later, however, the granulation tissue formed, and many microvessels were found to grow at the ulcer margin. The numbers of microvessels in granulation tissues and mucosa at the ulcer margin were counted, and results are shown in Fig. 3 and Table 1, respectively. Cigarette smoke exposure at 4% significantly reduced the vascular growth both in granulation tissues (Fig. 3A) and in mucosa at the margin areas (Table 1).

Effects of cigarette smoke exposure on cNOS activity and PGE2 and VEGF levels in the gastric mucosa and ulcer base. As shown in Fig. 4, cigarette smoke at 4% for 3 or 6 days markedly reduced the cNOS activities in both the gastric mucosa (Fig. 4A) and ulcer base (Fig. 4B). However, iNOS activities in these two areas were not significantly affected by cigarette smoke exposure. In the gastric mucosa, iNOS activities were 4.03 ± 0.61, 5.05 ± 0.61, and 5.42 ± 0.47 pmol·min⁻¹·g protein⁻¹ in the 0, 2, and 4% smoking groups on day 4 and 4.54 ± 0.37, 4.21 ± 0.26, and 3.66 ± 0.47 pmol·min⁻¹·g protein⁻¹ on day 7, respectively. In the ulcer base, iNOS activities were 0.27 ± 0.06, 0.32 ± 0.06, and 0.32 ± 0.03 pmol·min⁻¹·g protein⁻¹ in the 0, 2, and 4% smoking groups on day 4 and 0.11 ± 0.05, 0.19 ± 0.05, and 0.13 ± 0.04 pmol·min⁻¹·g protein⁻¹ in the 0, 2, and 4% smoking groups on day 7, respectively (n = 8–10 per group). Also, cigarette smoke exposure during the observation period resulted in no significant changes in PGE2 (Fig. 5) and VEGF (see Fig. 8) contents in either gastric mucosa or ulcer base.

Effect of cigarette smoke exposure on cNOS expression in the gastric mucosa. With the use of the immunohistochemical method, endothelial cNOS was detected not only in blood vessels (Fig. 6, A–C) but also in cells of gastric glands, including parietal cells (Fig. 6D) existing in the region starting ~0.5 mm from the edges of the ulcer margin. These regions were not stained in the absence of mouse anti-endothelial cNOS antibody or anti-mouse IgG antibody. Quantification of cNOS expression was performed using an image analyzer at ×100 magnification field by field starting from the edge of the ulcer margin. The results are shown in Table 2. Cigarette smoke exposure at 4% markedly reduced the activity and expression of endothelial cNOS in the gastric mucosa on both the 4th and 7th day after ulcer induction (Fig. 7 and Table 2).

Effects of L-arginine on ulcer size, gastric blood flow, and angiogenesis. One day after ulcer induction, rats were divided into three groups: 0% and 4% smoke exposure and 4% smoke exposure with L-arginine treatment. Again, it was confirmed that the ulcer areas in the 4% smoking group were significantly larger than those in the 0% control group on both day 4 and day 7. Intravenous administration of L-arginine (10 mg/kg) 5 min before each cigarette smoke exposure completely reversed the delay in ulcer healing induced by cigarette smoke exposure (Fig. 1B). However, the same dose of L-arginine did not significantly affect the ulcer sizes in nonsmoking animals (69.8 ± 2.1 and 35.5 ± 4.4 mm² on day 4 and day 7, respectively). Similarly, L-arginine also completely restored the reduction of gastric blood flow at the ulcer margin induced by cigarette smoke exposure (Fig. 2B). Furthermore, L-arginine significantly reversed the inhibitory action of cigarette smoke exposure.
exposure on angiogenesis in both the granulation tissue (Fig. 3B) and mucosa at the ulcer margin (Table 1) on day 4 and day 7.

**DISCUSSION**

Smoking has been recognized as one of the significant risk factors of increased incidence, delayed healing, and activated relapse of peptic ulcers. Gastric blood flow is important for maintenance of the integrity of gastric mucosa and protection against mucosal injury. Cigarette smoke exposure reduces basal gastric blood flow and potentiates ulcer formation (23). Adequate gastric blood flow is also essential for ulcer healing (12). Endoscopic studies have shown that hyperemia at the margin of an established gastric ulcer (10, 14) is essential for ulcer healing in patients. In the present study, the laser Doppler technique showed that gastric blood flow at the ulcer margin was higher than that in the normal area. This finding is consistent with other reports that used the hydrogen gas clearance method to measure mucosal blood flow (13). Similar to Iwata and Leung’s experiment conducted in unconscious animals, our study also showed that cigarette smoke exposure in conscious rats for a 1-h period once daily for 3 or 6 days...
Table 2. Effect of cigarette smoke exposure on cNOS expression in gastric mucosa in rats with ulcer induction

<table>
<thead>
<tr>
<th>Groups</th>
<th>Fields (0.46 × 0.46 mm) Starting From Edge of Ulcer Margin</th>
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<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>4 Day</td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>1.76 ± 0.41</td>
</tr>
<tr>
<td>4%</td>
<td>0.29 ± 0.10</td>
</tr>
<tr>
<td>7 Day</td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>3.41 ± 0.12</td>
</tr>
<tr>
<td>4%</td>
<td>1.27 ± 0.34</td>
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</table>

Values for areas of cell demonstrating positive staining (dark brown) for constitutive nitric oxide synthase (cNOS) expression are means ± SE of 6–8 rats, given in 10³ µm²/field. With the use of immunohistochemical staining, cNOS expression was quantified field by field consecutively from the edge of the ulcer margin under an image analyzer. *P < 0.05 vs. corresponding 0% group.

Fig. 7. Immunostaining for endothelial cNOS in the gastric mucosa 4 days (A and B) and 7 days (C and D) after ulcer induction. Cigarette smoke exposure at 4% (B or D) significantly reduced cNOS expression vs. corresponding 0% group (A or C). Magnification = ×100.
significantly delayed the healing of acetic acid-induced gastric ulcer in a concentration-dependent manner. This was accompanied by a reduction of gastric blood flow at the ulcer margin (Figs. 1 and 2). It is envisaged that the poor local circulation could partly explain why cigarette smoke exposure delays gastric ulcer healing in rats. However, the fact that smoke from cigarettes with filters did not significantly affect the healing rate indicated some ulcerogenic substances might have been retained in the filters. Identification of these substances should be of great interest and needs further study.

Angiogenesis, the formation of new microvessels from preexisting blood vessels, plays a significant role in wound healing and tissue regeneration (38). The importance of angiogenesis in gastroduodenal ulcer healing has also been confirmed in a number of studies. Folkman et al. (7) demonstrated that stimulation of angiogenesis in granulation tissues with basic fibroblast growth factor dramatically accelerates the healing of experimental duodenal ulcers in rats. In another study, Schmassmann et al. (31) showed that chronic indomethacin administration inhibits angiogenesis in granulation tissues and delays healing of experimental gastric ulcers in the same type of animals. The present study shows that cigarette smoke exposure for a 1-h period once daily for 3 or 6 days also depresses angiogenesis not only in granulation tissues but also in the mucosa of the ulcer margin (Fig. 3A and Table 1). Therefore, it is suggested that depression of angiogenesis at the ulcer margin is one of the pathogenic mechanisms involved in the delay of ulcer healing in rats. We therefore further studied the contributory factors leading to reduction of both ulcer margin hyperemia and angiogenesis by cigarette smoke exposure.

Both NO (28, 47) and PGE₂ (8, 24, 30) are believed to have angiogenic and vasodilation functions. Several isoforms of NOS have been identified. The most important ones are the cNOS present mainly in the endothelium (endothelial cNOS) and neural tissue (neural cNOS) and the iNOS (1). NO constantly formed through cNOS in the endothelium of the gastric tissue dilates blood vessels and increases blood flow in the gastric mucosa (28, 44). On the other hand, iNOS, another isozyme for NO biosynthesis, existing in vascular smooth muscle cells and activating immunocytes such as macrophages, neutrophils, and mast cells, can be largely induced under certain pathological conditions (1). NO from these activated immune cells acts as a killer molecule and is involved in inflammation. Therefore, it is plausible that the adverse effect of cigarette smoke exposure on ulcer healing is mediated through reduction of cNOS and/or increase of iNOS activity in the gastric mucosa. However, there has been no report regarding the effect of smoking on the synthesis of endogenous NO. Of most interest, our results indicate that cigarette smoke exposure for a 1-h period once daily for 3 or 6 days significantly reduced cNOS activities not only in the gastric mucosa around the ulcer area (Fig. 4A) but also in the ulcer base (mainly granulation tissues) (Fig. 4B). However, neither the mucosal nor the granulation tissue iNOS activity was affected in this study. These results implied that inhibition of cNOS could account for the adverse action of cigarette smoking on ulcer healing.

To confirm further whether reduction of NO synthesis followed by depression of gastric blood flow and angiogenesis would contribute largely to the adverse effect of cigarette smoke exposure on ulcer healing, we used L-arginine, a substrate for NO synthesis. Intravenous administration of L-arginine (10 mg/kg) 5 min before each cigarette smoke exposure once daily for 3 or 6 days completely reversed the adverse action of cigarette smoke exposure on ulcer healing (Fig. 1B) and gastric blood flow (Fig. 2B). It also significantly restored angiogenesis in the granulation tissue and mucosa at ulcer margin (Fig. 4B and Table 1). The complete recovery of gastric blood flow by L-arginine probably depended on both the direct vasodilating action (27) and angiogenic effect (47) of NO produced in the gastric mucosa. This phenomenon was supported by the fact that inhibition of NOS with N⁵-G-monomethyl-L-arginine delayed healing of chronic gastric ulcers, whereas supplement of L-arginine, a substrate for NO production, or exogenous NO by an NO donor, such as glyceryl trinitrate, reversed this effect (18). The present
study not only confirmed but also further elaborated that reduction of NO synthesis, mainly from cNOS, contributed significantly to the delay of ulcer healing. In addition, immunostaining of endothelial cNOS was detected in blood vessels located in the region of the gastric glands and submucosal (Figs. 6, A–C), consistent with the finding of Price et al. (29) and also compatible with the involvement of NO in regulation of gastric blood flow (44).

Besides the blood vessels, our study demonstrated that cNOS was also expressed in mucosal cells in the more mature mucosa starting ~0.5 mm from the two sides of the ulcer margin (Fig. 7). This is consistent with the finding that cNOS activity in the gastric mucosa is much higher than that in the ulcer base (Fig. 4). Moreover, unlike other intracellular messenger, NO generated within a cell also has the potential to modify the biological activities in adjacent cells because of the ease of this gaseous molecule to pass through cell membranes (1). Mathematical evidence suggests that NO generated from a point source may be active over distances up to and beyond 0.2 mm within a few seconds (45). All these findings imply that expression of cNOS in blood vessels and in the proximity of the ulcer margin may be beneficial for ulcer healing by augmenting gastric blood flow and angiogenesis at the surrounding areas of ulcer margin. Therefore, replenishment of NO by L-arginine would completely restore the angiogenesis, especially at the ulcer margin (Table 1), and accelerate ulcer healing in animals exposed to cigarette smoke (Fig. 1A).

Both in vitro and in vivo studies showed that VEGF acts as a mitogen for endothelial cell proliferation (19, 34) and migration (30). A large body of evidence demonstrated that VEGF could be markedly induced by hypoxia (25), which is the characteristic feature of healing wounds (16). Indeed, this was reflected not only by the marked elevation of VEGF levels in the gastric mucosa after acute ulcer formation (unpublished observations) but also by the fact that the VEGF level in the ulcer base was ~14 times higher than that in the gastric mucosa around the ulcer area (Fig. 8) because of a more severe hypoxic condition in the granulation tissues at the ulcer base. Furthermore, it has been reported that NO lies downstream from VEGF-induced angiogenesis (46), and there is a reciprocal relation between VEGF and NO in regulation of endothelial integrity (40). However, in the present study, VEGF levels in both the gastric mucosa and the granulation tissues were not significantly affected by cigarette smoke exposure, although the cNOS activity was markedly reduced. These findings indicated that cigarette smoking did not attenuate VEGF production during ulcer healing but impaired its downstream NO production for angiogenesis, suggesting that VEGF may not contribute to the depression of angiogenesis in the gastric mucosa and granulation tissues after cigarette smoking.

The involvement of PGE2 in angiogenesis was also examined. Indomethacin, a PG inhibitor, delays ulcer healing, whereas synthetic PG reverses this action (11, 41), suggesting that endogenous PGs are involved in the process of ulcer healing. Experimental evidence demonstrates that impairment of epithelial renewal and angiogenesis by indomethacin during ulcer healing may also be due to its PG-depleting action (41). In fact, PGE1 and PGE2 in the extravascular space also stimulate capillary sprouting directly from veins (4). Recently, it was reported that PGE1 and PGE2 markedly stimulated the expression of VEGF, both by human gastric fibroblasts and epithelial cells (4). However, experimental and clinical studies on the effects of smoking on mucosal PGE2 concentration are still in conflict (6). Our data showed that cigarette smoke exposure had no significant effect on PGE2 levels in either gastric mucosae or granulation tissues (Fig. 5). This was in accord with our previous finding with a different regimen of smoking (3). Therefore, it is likely that reduction of gastric blood flow and angiogenesis at the ulcer margin induced by cigarette smoke exposure is also not mediated through the depression of PGE2 production in the gastric mucosa.

It is interesting to note that cigarette smoke exposure during the experimental period neither affected the body weight gain nor food and water consumption, suggesting that the detrimental effects of cigarette smoke exposure on ulcer healing did not result from the reduction of dietary intake accompanied by the general decrease of protein synthesis and adverse physical conditions imposed on the animals. Although the present experimental conditions may not fully reflect the situation of smokers with gastric ulcers, since the duration of smoking and also the ulcer induction method are not the same as those experienced by humans, we do, however, demonstrate for the first time that cigarette smoke exposure for a relatively short period of time indeed can delay ulcer healing in conscious animals. The mechanisms for this action are likely to be due to the depression of gastric blood flow and angiogenesis at the ulcer margin. Also, deficiency of cNOS rather than VEGF and PGE2 in the gastric mucosa around the ulcer area and at the ulcer base is responsible for these adverse reactions to cigarette smoking. All these findings suggest that cigarette smoking even for a short term can adversely affect the stomach. Supplementation of NO could be beneficial to smokers with peptic ulcer disease.

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