ELSEVIER

European Journal of Pharmacology 413 (2001) 121–129

ejp

www.elsevier.nl/locate/ejphar

Oxygen radicals mediate the final exacerbation of endothelin-1-induced gastric ulcer in rat

Spyridon Lazaratos [a], Yoko Irukayama-Tomobe [a], Takashi Miyauchi [a], Katsutoshi Goto [b,*], Akira Nakahara [a]

[a] *Department of Internal Medicine, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Japan*
[b] *Department of Pharmacology, Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan*

Received 4 September 2000; received in revised form 29 December 2000; accepted 9 January 2001

Abstract

We investigated the role of xanthine oxidase-derived oxygen radicals in the development of endothelin-1-induced gastric ulcer. Mucosal lipid peroxidation showed a peak 24 h after injection, while gastric mucosal haemodynamics were fully restored 26 h after endothelin-1 injection. Allopurinol and oxypurinol, but not superoxide dismutase or catalase, protected the gastric mucosa 24 h after endothelin-1 injection. Oxypurinol antagonized both the vasoconstrictor effect of endothelin-1 and the decrease in gastric ATP. All treatments on the second day after endothelin-1 injection significantly reduced gastric mucosal damage. Xanthine oxidase-derived oxygen radicals contributed largely to the exacerbation but they did not mediate the onset of endothelin-1-induced gastric ulcer. Pretreatment with probucol (500 mg/kg, p.o.) also protected the gastric mucosa from endothelin-1-induced mucosal injury by its antioxidant activity. Oxypurinol was gastroprotective through its vasoactive and energy saving actions. The haemodynamic background of endothelin-1-induced gastric ulcer consists of long lasting ischaemia and subsequent "reperfusion" which may be responsible for the late burst of oxygen radicals.

Keywords: Endothelin-1; Gastric ulcer experimental; Oxygen radical; Mucosal blood flow; ATP

1. Introduction

The production of oxygen-derived free radicals has been postulated as a possible pathogenetic factor in ischaemia-induced tissue injury (Perry et al., 1986; Itoh and Guth, 1985; Parks et al., 1982, 1983; Granger et al., 1981; Schoenberg and Beger, 1993; Bulkley, 1983). Granger et al. (1981) have proposed for the first time a mechanism for generation of oxygen free radicals in the ischaemic small intestine. According to their hypothesis and subsequent research, it has been shown that tissue ischaemia leads to accumulation of hypoxanthine as the result of ATP catabolism (DeWall et al., 1971) and to conversion of the nicotinamide adenine dinucleotide-reducing enzyme, xanthine dehydrogenase, to xanthine oxidase. When the tissue is subsequently reperfused, xanthine oxidase utilizes hypoxanthine and oxygen to form superoxide anion and hydrogen peroxide. Both then interact to produce the highly cytotoxic (Weiss, 1986) hydroxyl radical via the iron-catalyzed Haber–Weiss reaction (Koppenol, 1983).

The role of ischaemia in the pathogenesis of gastric mucosal injury has been the target of numerous experimental studies. These studies, with tissue ischaemia of various durations ranging from minutes (Andrews et al., 1992) to hours (Parks et al., 1982), have implicated hypoxia per se (Parks et al., 1982) and oxygen radicals in the development of gastric mucosal injury. Xanthine oxidase was shown to be the enzyme responsible for the availability of oxygen radicals during reperfusion (Smith et al., 1987). Increased iron availability during ischaemia (Baez et al., 1961; Marston, 1977; Chiney and Filnch, 1960) was indicated as the causative factor of ischaemia–reperfusion-induced gastric bleeding (Smith et al., 1987).

Reactive oxygen species can induce cellular membrane damage by peroxidation of phospholipid fatty acids (Comporti, 1985). Under conditions of increased oxidative stress, lipid peroxides breakdown produces aldehydes whose identification and measurement serve as an index of the extent of lipid peroxidation and as a tool to clarify the role

* Corresponding author. Tel.: +81-298-53-3210; fax: +81-298-53-3039.
E-mail address: kgoto@md.tsukuba.ac.jp (K. Goto).

0014-2999/01/$ - see front matter
PII: S0014-2999(01)00752-X

of aldehydes as causative agents in disease processes (Esterbauer and Cheeseman, 1990).

Nevertheless, oxygen radicals do not appear to participate in gastric mucosal epithelial damage during ischaemia (Smith et al., 1987), supporting the hypothesis that the protective action of allopurinol and its metabolite oxypurinol is due to prevention of the loss of purine bases (Parks et al., 1982; Ekman et al., 1993) or to their tissue levels (Garcia et al., 1990) and not to the inhibition of xanthine oxidase-derived oxygen radicals. Prevention of purine loss by allopurinol should preserve the nucleotide pool during hypoxic stress (Parks et al., 1982). The pathophysiology of gastric mucosal damage under prolonged periods of regional ischaemia is not yet completely understood.

We have shown that the endothelium-derived, potent vasoconstrictor peptide endothelin-1 when injected submucosally in the rat stomach, can induce, 24 h later, a gastric ulcer. This type of ulcer is characterized by long-lasting potent local vasoconstriction, and it reaches its maximum size with penetration of the muscularis mucosa 48 h after endothelin-1 injection (Lazaratos et al., 1993a,b, 1994).

The purpose of our study was to investigate whether endothelin-1-induced long-standing local tissue ischaemia will generate oxygen radicals and how these would contribute to the pathogenesis of the resulting gastric ulcer. We studied lipid peroxidation in the gastric mucosa in the course of ulcer development. We also examined the effect of xanthine oxidase inhibitors on endothelin-1-induced changes in gastric mucosal ATP level as well as gastric mucosal haemodynamics.

For that purpose, we studied: (1) the production of malondialdehyde and 4-hydroxy-2(*E*)-nonenal (4-HNE) in the gastric mucosa during endothelin-1-induced ulcer formation; (2) the effects of allopurinol (a competitive inhibitor of xanthine oxidase), oxypurinol (the product of allopurinol oxidation, an inhibitor of xanthine oxidase), superoxide dismutase (a superoxide radical scavenger), catalase (a hydrogen peroxide scavenger) and desferrioxamine (a relatively specific and potent chelator of Fe^{3+}) on the development of endothelin-1-induced gastric ulcer by quantitative histology; (3) the gastric mucosal haemodynamics on the first and second days after injection of endothelin-1; (4) the effect of oxypurinol on endothelin-1-induced changes in mucosal ATP level as well as on endothelin-1-induced gastric mucosal haemodynamic changes; and (5) the effect of oral administration of probucol on endothelin-1-induced gastric ulcer by quantitative histology.

2. Materials and methods

2.1. Animal preparation

Male Wistar rats weighing 200–250 g were fasted for 18 h prior to the experiment but allowed free access to tap water. On the day of the experiment, rats were divided in groups of 10 animals, anaesthetized with an intraperitoneal injection of urethane (1.5 g/kg) and stabilized on a surgical stand. To monitor the anaesthesia during experiments, we observed responses to twitching. Anaesthesia could be maintained over 24 h without additional injection of urethane. Blood pressure was continuously monitored with a catheter in the left carotid artery, filled with heparinized saline and connected to a unicorder through a pressure transducer. A catheter was inserted into the femoral vein to establish an intravenous line that was used for infusion of drugs. A Harvard syringe pump (Harvard Apparatus, Holliston, MS, USA) was used to infuse the drugs intravenously over 24 h at a computer-controlled speed of 0.4 ml/h. A heating lamp was used to maintain rectal temperature at 37°C during the experiment.

This study was approved by University of Tsukuba and conformed to the "Position of the American Heart Association on Research Animal Use" adopted by the Association in November 1984.

2.2. Substances, solutions and instruments

Drugs used were endothelin-1 (Peptide Institute, Osaka, Japan), allopurinol and oxypurinol (Sigma-Aldrich, St. Louis, MO, USA), superoxide dismutase (Sigma-Aldrich), catalase (Sigma-Aldrich), desferrioxamine (Sigma-Aldrich) and bovine serum albumin (Sigma-Aldrich). Probucol (Sigma-Aldrich) was suspended in olive oil for in vivo experiments. On the day of the experiment, endothelin-1 was dissolved in phosphate-buffered saline (PBS, pH 7.4) containing 0.05% bovine serum albumin.

Tissue ATP content was determined by the firefly luciferin-luciferase assay. Tissue concentrations of malondialdehyde and 4-HNE were determined by a colorimetric assay (LPO-586, Bioxytech, France).

Submucosal injection of endothelin-1 was performed with a 25-μl Hamilton microliter syringe. A laser Doppler flowmeter (PF-3, Perimed, Sweden), a tissue spectrum analyzer (TS-200, Sumitomo Electric, Japan) (Smith et al., 1987), a pressure transducer (model SCK-590, Gould, Cleveland, OH), and a unicorder (Nippon Denshi Kagaku, Japan) were used. The gastric chamber was perfused with warm Krebs' solution (pH 7.4, 37°C). We modified the gastric chamber (circle type, made of plastic) to leave the mucosal side of the stomach uncovered. The dimensions of the gastric lesions were estimated by a pathologist unaware of the treatment, by means of a planimeter (videoplan, Japan) supported by a computer (NEC, Japan). A Gilson spectrophotometer was used for the assay of malondialdehyde and 4-HNE.

2.3. Malondialdehyde and 4-HNE levels in gastric mucosa during endothelin-1-ulcer formation

Gastric mucosal samples were obtained by gently detaching the mucosa of the lesion area at 5, 15, 20, 24, 30 and 48 h after the injection of endothelin-1 (50 pmol).

Then, 1/10 tissue homogenates (g/ml) in 20 mM Tris–HCl buffer were centrifuged at 2500 × *g*. Then, 200 μl of the supernatant was added to 650 μl chromogenic reagent. We then added 150 μl methanesulfonic acid (10.4 M) and the l ml solution was left in a water bath (45°C) for 40 min. Absorbance was measured at 586 nm, and concentrations were calculated from standard curves.

2.4. Role of xanthine oxidase and superoxide radicals in onset of ulcer induced by submucosal injection of endothelin-1

2.4.1. Allopurinol and oxypurinol study

Allopurinol and oxypurinol were dissolved in normal saline by addition of the molar equivalent of 1 N NaOH to adjust the pH to 10.5. A dose of 50 mg/kg dissolved in approximately 10 ml normal saline was infused intravenously over 24 h. This is the same dose described by Itoh and Guth (1985) that is able to protect against haemorrhagic shock-induced gastric lesions.

2.4.2. Superoxide dismutase study

Superoxide dismutase was administered intravenously (20 mg/kg in approximately 10 ml normal saline) (Perry et al., 1986). The solution was kept at a low temperature (8°C) to prevent the loss of drug activity over the prolonged period of infusion. In all animals, both renovascular pedicles were ligated before administration of superoxide dismutase to prevent rapid clearance by the kidneys (Huber and Saifer, 1977).

2.4.3. Catalase study

A dose of 90,000 units/kg dissolved in approximately 10 ml normal saline was infused intravenously over 24 h (Smith et al., 1996; Ishii et al., 2000).

2.4.4. Endothelin-1 injection

In all groups, 15 min after the commencement of the continuous injection of the various drug treatments, we exposed the stomach and injected endothelin-1 (50 pmol dissolved in 20 μl 0.05% bovine serum albumin in PBS) into the submucosa of the anterior gastric wall, as we have previously described (Lazaratos et al., 1993a). The incision was sutured in layers and the rats were sacrificed 24 h later.

In all studies, the control groups received continuous intravenous infusion of normal saline, except the allopurinol and oxypurinol groups where the saline was adjusted to pH 10.5.

2.5. Role of xanthine oxidase and superoxide radicals in progression of ulcer induced by submucosal injection of endothelin-1

2.5.1. Animal preparation

In this study, fasted rats were lightly anaesthetized with diethyl ether, stabilized on a surgical stand and injected with endothelin-1 (50 pmol) into the submucosa of the anterior gastric wall, as described above. Then the rats were returned to cages and used 24 h after the injection of endothelin-1. The rats were anaesthetized with intraperitoneal injection of urethane (1.5 g/kg). Anaesthesia could be maintained over 24 h without additional injection of urethane. A heating lamp was used to maintain rectal temperature at 37°C during the experiment. Gastric mucosal blood flow at ulcer site was measured as the one described in Section 2.6. The doses of the drugs used were the same, as were their preparation and administration periods, with the addition of desferrioxamine.

2.5.2. Desferrioxamine study

Desferrioxamine (50 mg/kg) was dissolved in normal saline (10 ml) and administered intravenously over 24 h (Hernandez et al., 1987).

2.6. Gastric mucosal blood flow measurements at ulcer site

In this study, fasted rats were lightly anaesthetized with diethylether, stabilized on a surgical stand and injected with endothelin-1 (50 pmol) into the submucosa of the anterior gastric wall as described above. Then the rats were returned to cages and were used 15 and 26 h after the injection of endothelin-1. On the day of the experiment, rats were anaesthetized with an intraperitoneal injection of urethane (1.5 g/kg). Through a midline incision, the stomach was exposed, brought out of the peritoneal cavity, opened along the greater curvature and secured between a Lucite ring (serosa) and a modified gastric chamber over the anterior abdominal wall, as we have previously described (Lazaratos et al., 1993a). The mucosal side of the mounted stomach was flushed with warm saline (37°C) to remove any solid residue. On the area of endothelin-1 injection, the tip of the laser Doppler flowmeter and the tissue spectrum analyzer were positioned in gentle contact with and vertically to the gastric mucosa. Both analyzers were arranged a side by side on the area of endothelin-1 injection in the gastric mucosa. Both the flowmeter and the spectrum analyzer were connected to a unicorder for simultaneous recording of blood flow, mucosal oxygen content and gastric mucosal haemoglobin concentration.

In each animal ($n = 10$), we measured gastric mucosal haemodynamics both in the area of endothelin-1 injection and in the normal mucosa of the posterior gastric wall.

2.7. Effect of oxypurinol on endothelin-1-induced gastric mucosal haemodynamic changes and ATP level

Using the model preparation that was mentioned in Section 2.6, we assessed the effect of continuous intravenous infusion of oxypurinol (2 mg/kg/h) on gastric mucosal blood flow, mucosal oxygen content and gastric

mucosal haemoglobin concentration at the site of endothelin-1 injection. Briefly, under urethane anaesthesia, the rat stomach was exposed, a gastric chamber was fitted on the mucosal side, and endothelin-1 was injected submucosally under continuous blood flow measurement. Oxypurinol infusion was initiated 15 min prior to endothelin-1 (50 pmol) injection. Measurements were continued until blood flow level was restored to the baseline level or for a maximum of 20 h.

For ATP measurements, gastric tissues were weighed and mucosae were taken from gastric tissues, and homogenized in perchloric acid (1/10 of tissue weight), left at 4°C for 10 min and then processed as previously described (Stanley and Williams, 1969). After endothelin-1 injection (2 and 4 h) with and without oxypurinol infusion, the rats were killed under pentobarbital sodium (1.5 mg/kg) anesthesia. The stomachs were rapidly removed, opened along the greater curvature, rinsed with normal saline. Gastric mucosa samples were obtained by gently detaching the mucosa of the lesion. We determined the area of the superficial redness after the application of endothelin-1 as the lesion area. Baseline levels were taken from sham-operated animals.

2.8. Assessment of gastric mucosal damage

After each treatment, the rats were sacrificed and the stomach removed, opened along the greater curvature, rinsed with normal saline and pinned flat on a corkboard. They were photographed and gastric damage was evaluated by a pathologist unaware of the treatment. The specimens were subsequently fixed in formalin and embedded in paraffin. Coded slices stained with haematoxylin–eosin and Mason trichrome were microscopically evaluated. For each lesion, the maximum length (Length) as well as the percent of the full thickness of the mucosal wall (Mucosal damage: depth) that was destroyed by the lesion were estimated by computerized planimetry.

2.9. Effect of probucol on ulcer index of endothelin-1-induced gastric ulcer

Probucol was given orally in a volume of 1 ml/100 g body weight for 4 consecutive days. The rats were divided into two groups. One group was given probucol (500 mg/kg) orally, twice daily (Itoh et al., 1998). The other group was given vehicle instead of test drug as control. On the second day, fasted rats were lightly anaesthetized with diethylether, stabilized on a surgical stand and injected with endothelin-1 (50 pmol) into the submucosa of the anterior gastric wall as described above. Then the rats were returned to cages and used 48 h after the injection of endothelin-1, as described above.

2.10. Statistics

All data are presented as mean ± S.D. One factor analysis of variance (ANOVA) was used for multiple comparisons between groups. Probability values of less than 0.05 were regarded as significant.

3. Results

3.1. Malondialdehyde and 4-HNE levels in gastric mucosa during endothelin-1-induced ulcer formation

The total concentration of malondialdehyde and 4-HNE showed a significant increase at 24 h after endothelin-1 injection (26.55 ± 9.87 nmol/mg tissue compared to 13.32 ± 6.06 nmol/mg tissue at 20 h, $n = 5$, $P < 0.01$) (Fig. 1). Tissue homogenates at 5 and 15 h included a significant amount of pigments, most probably due to thrombus and haemorrhage, which gave a strong pink color after incubation and therefore were excluded from the study.

3.2. Role of xanthine oxidase and superoxide radicals in onset of ulcer induced by submucosal injection of endothelin-1

Continuous intravenous administration of allopurinol resulted in a significant decrease in the overall endothelin-1-induced gastric mucosal damage. Macroscopically, the damage was attenuated compared to the endothelin-1 only group (data not shown). Histological examination revealed

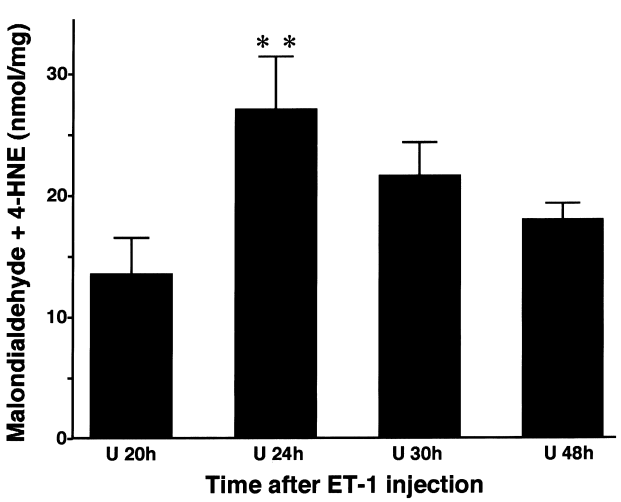

Fig. 1. Concentrations of Malondialdehyde and 4-HNE in gastric mucosa at different times after endothelin-1 (ET-1) injection. $^{**}P < 0.01$ vs. 20 h group. U20, U24, U30, U48 stand for ulcers at 20, 24, 30 and 48 h after endothelin-1 injection, respectively. Each column and bar represent mean and S.D. of six animals, respectively.

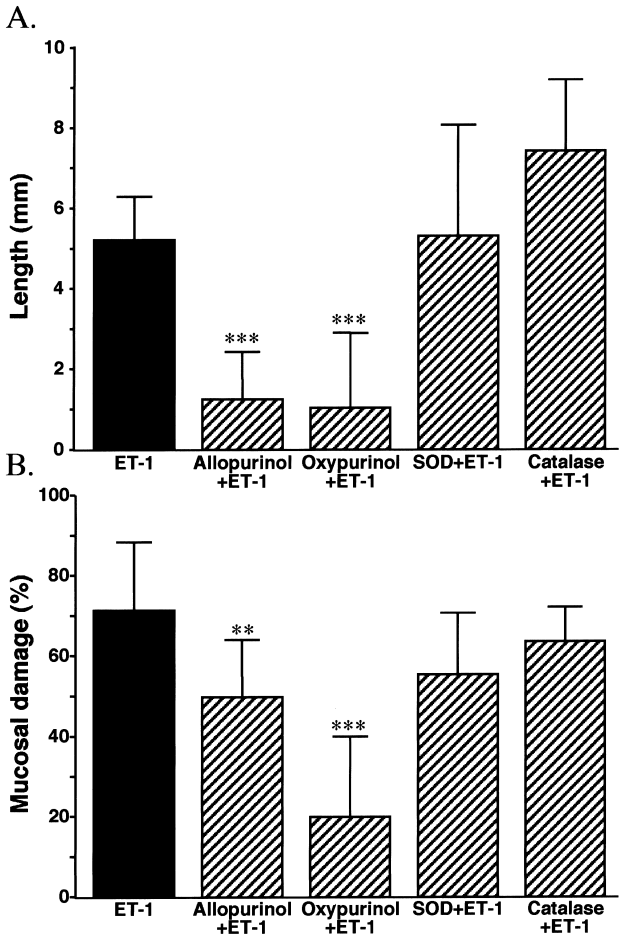

Fig. 2. Effects of allopurinol, oxypurinol, superoxide dismutase and catalase on endothelin-1 (ET-1)-induced gastric mucosal damage 24 h after injection of endothelin-1. (A) Length of gastric mucosal damage. (B) Percentage of mucosal wall damage. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. endothelin-1 only group. Each column and bar represent mean and S.D. of 10 animals, respectively.

that both the length (Fig. 2A) (Length) and depth (Fig. 2B) of the lesion (Mucosal damage) were significantly reduced (1.17 ± 1.09 mm and $49.80 \pm 13.22\%$ vs. 5.23 ± 0.87 mm and $72.15 \pm 14.87\%$ of the endothelin-1 only group, $P < 0.001$, $n = 10$). Superoxide dismutase and catalase both failed to exert any gastroprotective action both macroscopically (data not shown) and microscopically (Fig. 2). When we intravenously infused these pharmacological agents alone to rats, there was no effect on gastric mucosa macroscopically (data not shown). The submucosal injection of the vehicle did not induce any detectable mucosal damage macroscopically and microscopically (data not shown).

3.3. Role of oxygen radicals in progression of ulcer induced by submucosal injection of endothelin-1

When allopurinol was infused continuously during the second day after the injection of endothelin-1, the gastric mucosal damage was significantly attenuated both macroscopically (data not shown) and microscopically (0.62 ± 0.85 mm and $39.99 \pm 35.99\%$ vs. 4.99 ± 1.72 mm and $88.07 \pm 15.47\%$ of the endothelin-1 only group, $P < 0.001$, $n = 10$) (Fig. 3). In contrast to the results of the previous protocol, oxypurinol was not more effective than allopurinol in reducing gastric mucosal damage (Fig. 3). Superoxide dismutase and catalase effectively reduced endothelin-1-induced gastric mucosal damage, and desferrioxamine was also effective in reducing the size and severity of gastric mucosal damage (% mucosal damage: $36.34 \pm 33.34\%$ vs. $88.07 \pm 15.47\%$ of the endothelin-1 only group, $P < 0.001$, $n = 10$).

3.4. Gastric mucosal blood flow measurements in area of endothelin-1 injection

Evaluation of the changes in gastric mucosal haemodynamics 15 h after endothelin-1 injection showed a signifi-

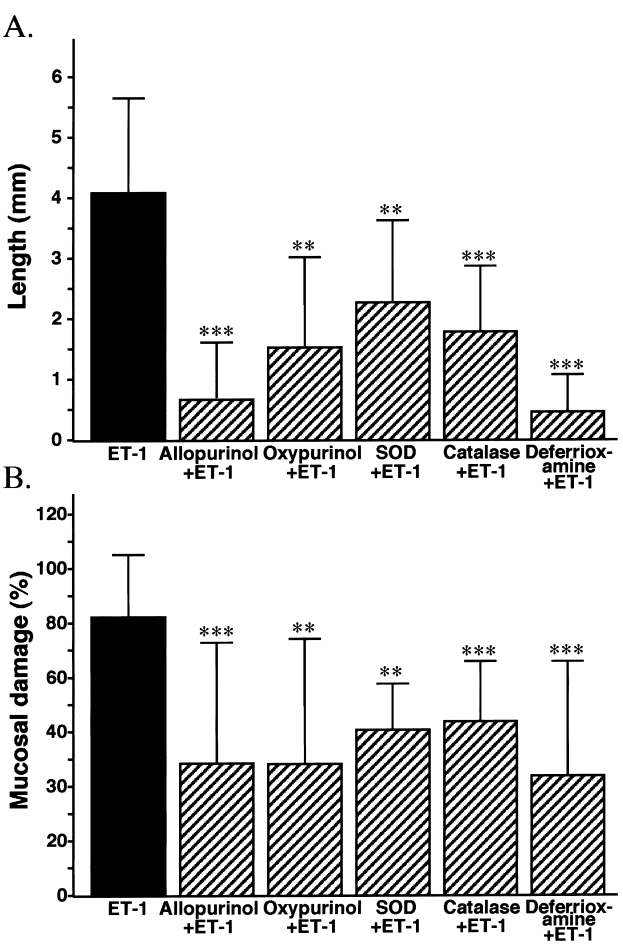

Fig. 3. Effects of allopurinol, oxypurinol, superoxide dismutase, catalase and desferrioxamine on endothelin-1 (ET-1)-induced gastric mucosal damage 48 h after injection of endothelin-1. (A) Length of gastric mucosal damage. (B) Percentage of mucosal wall damage. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. endothelin-1 only group. Each column and bar represent mean and S.D. of 10 animals, respectively.

cant marked decrease of blood flow in the area of the ulcer, as we have already reported (Lazaratos et al., 1993a). No significant difference between the ulcerated area and the normal mucosa of the opposite gastric wall was seen 26 h later (Fig. 4). Both laser Doppler flowmetry and tissue reflectance spectrophotometry showed gastric blood flow, haemoglobin concentration and tissue oxygen content to have been completely restored.

3.5. Effect of oxypurinol on endothelin-1-induced gastric mucosal haemodynamic changes and ATP level

Submucosal injection of endothelin-1 resulted in prolonged local vasoconstriction verified by the simultaneous decrease of blood flow, mucosal oxygen content and gastric mucosal haemoglobin concentration. Continuous intravenous infusion of oxypurinol restored blood flow to the levels before endothelin-1 injection by approximately 2.75 $\pm$ 0.12 h after endothelin-1 injection (Fig. 5). And the other haemodynamic indexes (mucosal oxygen content and gastric mucosal haemoglobin concentration) also restored to the levels before endothelin-1 injection (data not shown). There was no evident effect of the drug on systemic blood pressure or blood flow of the posterior gastric corpus that was simultaneously measured.

Gastric ATP level was significantly reduced by endothelin-1 injection. Oxypurinol infusion did not affect the endothelin-1-induced reduction in ATP content of the gastric mucosa at 2 h, but resulted in an increase of the mucosal content of ATP 4.5 h after endothelin-1 injection (Fig. 6).

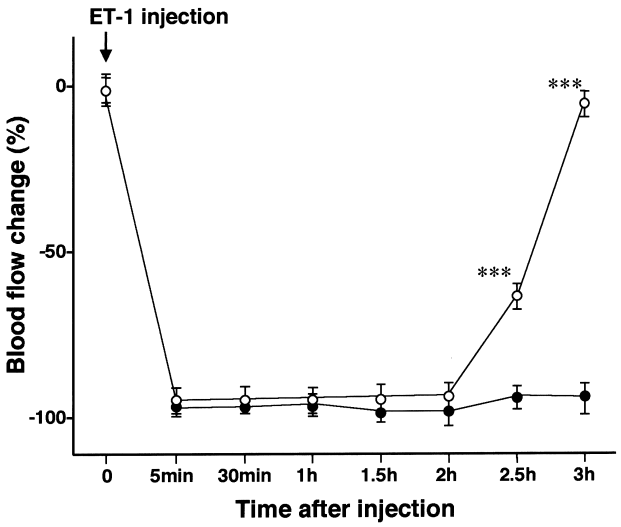

Fig. 5. Effect of oxypurinol infusion (2 mg/kg/h) on endothelin-1(ET-1)-induced gastric mucosal blood flow changes determined by laser Doppler flowmetry. Filled circles: without oxypurinol; open circles: with oxypurinol infusion. $^{***}P < 0.001$ vs. endothelin-1 only group. Each circle and bar represent mean and S.D. of four animals, respectively.

3.6. Effect of probucol on ulcer index of endothelin-1-induced gastric ulcers

Oral administration of probucol (500 mg/kg twice daily, p.o.) resulted in a significant decrease in the overall endothelin-1-induced gastric mucosal damage. Macroscopically, the damage was attenuated compared to the endothelin-1 only group, and histological examination re-

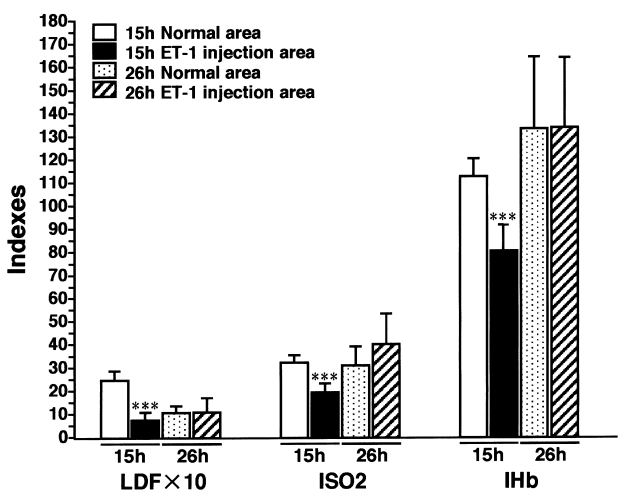

Fig. 4. Gastric mucosal blood flow determined by laser Doppler flowmetry (LDF), tissue oxygen content (ISO_2) and haemoglobin concentration (IHb) 15 and 26 h after injection of endothelin-1 (ET-1). Open columns: normal mucosa of posterior gastric wall 15 h after endothelin-1 injection; filled columns: endothelin-1 injection site at 15 h; dotted columns: normal mucosa of posterior gastric wall 26 h after endothelin-1 injection; and hatched columns: endothelin-1 injection site at 26 h. Significantly different from respective controls (normal area), $^{***}P < 0.001$. Each column and bar represent mean and S.D. of 10 animals, respectively.

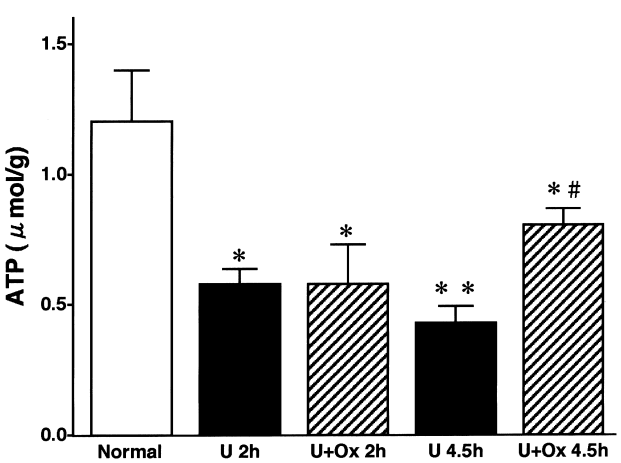

Fig. 6. Effect of oxypurinol infusion on gastric ATP level. From left to right are normal group, ulcer 2 h after endothelin-1 injection, ulcer with oxypurinol after 2 h, ulcer 4.5 h after endothelin-1 injection, and ulcer with oxypurinol after 4.5 h. ATP is expressed as μmol/g dry weight. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. normal group. $\#P < 0.01$. vs. U4.5 h. Each column and bar represent mean and S.D. of four animals, respectively.

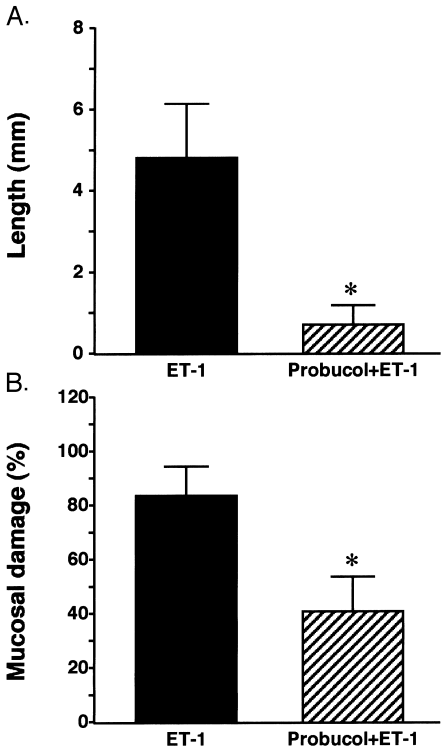

Fig. 7. Effect of probucol (500 mg/kg twice daily, p.o.) on endothelin-1-induced gastric mucosal damage 48 h after injection of endothelin-1 (ET-1). (A) Length of gastric mucosal damage. (B) Percentage of mucosal wall damage. * $P < 0.05$ vs. endothelin-1 only group. Each column and bar represent mean and S.D. of six animals, respectively.

vealed both the length (Fig. 7A) (Length) and the depth (Fig. 7B) of the lesion (Mucosal damage) to have been significantly reduced (0.75 ± 0.56 mm and $40.5 \pm 12.0\%$ vs. 4.85 ± 1.35 mm and $83.0 \pm 10.50\%$ of the endothelin-1 only group, $P < 0.05$, $n = 6$).

4. Discussion

In the present study, we investigated whether prolonged endothelin-1-induced local tissue ischaemia generates oxygen radicals through the xanthine oxidase reaction and their concomitant pathogenic effect on the onset and development of this type of ulcer.

We found evidence that (1) endothelin-1-induced gastric ulcer has a prolonged ischaemic component which is followed by a "reperfusion" phase characterized by a burst of oxygen radical generation, (2) xanthine oxidase-derived oxygen radicals were not generated during the first 24 h after the injection of endothelin-1 and therefore could not be incriminated in the onset of this type of ulcer, (3) the gastroprotective effect of oxypurinol was not only due to inhibition of xanthine oxidase-induced oxygen radical production but also to vasoactive and energy-saving actions, (4) oxyradicals generated by xanthine oxidase were responsible mainly for the exacerbation of endothelin-1-induced gastric mucosal damage, and (5) oral administration of probucol (a lipid-lowering agent with antioxidant properties) is effective in preventing endothelin-1-induced ulcer formation and ulcer healing.

Parks et al. (1982) have noticed that although oxygen radicals were the initial mediators of ischaemic tissue injury produced by 3 h of regional ischaemia, under conditions different to those they have employed (e.g. longer duration of ischaemia, haemorrhagic shock or total vascular occlusion) other mediators may negate the protective effect of superoxide dismutase or catalase. In our study, both of the enzymes were incapable of any gastroprotective effect during the onset of endothelin-1-induced mucosal damage, supporting the aforementioned hypothesis and suggesting the existence of other mechanisms mediating the initialization of gastric mucosal damage. Moreover, the present data support our previous findings of extremely long-lasting local ischaemia at the site of endothelin-1 injection (Lazaratos et al., 1993a).

Ischaemia-induced gastric lesions have been shown to be significantly reduced by allopurinol pretreatment (Itoh and Guth, 1985). One possible explanation is that allopurinol, by inhibiting xanthine oxidase-induced oxygen radical generation, protected the gastric mucosa. The same authors also speculated a link between the protective effect of allopurinol and preservation of the pool of purine bases by its inhibition of their irreversible conversion to uric acid under oxygen deficiency. Garcia et al. have shown that the protective effect of allopurinol in intestinal ischaemia was not related to the blockade of xanthine oxidase but rather to the allopurinol levels in the intestinal wall (Garcia et al., 1990). Further, in water immersion stress ulcer, marked decomposition of adenine nucleotides and an increase in hypoxanthine, xanthine and xanthine oxidase concentrations were found in the gastric mucosa after only 4 h of stress, suggesting the involvement of energy metabolism in the formation of gastric mucosal damage (Itoh et al., 1991).

In the present study, given the inability of superoxide dismutase and catalase to protect the mucosa for the first 24 h, the effect of allopurinol and oxypurinol cannot be attributed to the inhibition of xanthine oxidase-derived oxygen radicals. In this situation, the significant effects of oxypurinol and allopurinol in preventing endothelin-1-induced mucosal lesions even during the first 24 h might be accounted for by the fact that oxypurinol effectively preserved mucosal ATP 4.5 h after endothelin-1 injection and might thereby antagonize the vasoconstrictor effect of endothelin-1 in the gastric microcirculation, although the precise mechanisms have yet to be investigated. The relatively stronger gastroprotective action of oxypurinol than allopurinol may be due to the elevated concentrations of the substrates under ischaemia and to the fact that oxidation of allopurinol itself can generate superoxide radicals (Spector, 1988).

The endogenous antioxidant enzymes superoxide dismutase and catalase are highly specific and have been extensively used to prove the involvement of free radicals in various pathophysiologic conditions (Granger et al., 1981; Schoenberg and Beger, 1993; McCord, 1983). Since their half-lives are only 6–9 min, we chose to infuse them continuously. Both drugs effectively protected the gastric mucosa only when they were administered during the second day of endothelin-1 injection. This finding suggests an accumulation of oxyradical substrates during the first 24 h and a subsequent burst of free radical generation most probably due to the return of gastric mucosal blood flow at the site of injection. Since allopurinol was able to efficiently reduce gastric mucosal damage, it is feasible to think that xanthine oxidase was mainly responsible for the free radical generation.

The deleterious effect of xanthine oxidase-derived oxygen anions and hydrogen peroxide is attributed to the formation of hydroxyl radicals by the iron catalyzed Haber–Weiss reaction. During ischaemia it is known that extracellular iron level rises, although its origin is still unclear (Baez et al., 1961; Chiney and Filnch, 1960). The effect of desferrioxamine showed the significant contribution of hydroxyl radicals to the late phase of the development of this type of ulcer.

In their model of ischaemia–reperfusion, Dale and Granger (1986) conceived the mechanism of tissue injury in terms of a division into two temporally consequent systems i.e., the so-called 'hypoxia counter-current exchange', characterized by energy metabolism deficiency and increase in hypoxanthine, xanthine and xanthine oxidase concentrations and the 'oxygen free radicals' created during reperfusion from the accumulated substrates and the resupplied oxygen. In the model of endothelin-1-induced gastric ulcer, the prolonged reduction of gastric mucosal blood flow may account not only for tissue hypoxia but also for enhancement of the irreversible loss of purine bases, whose relative significance in the ensuing gastric mucosal injury remains to be clarified. The finding that gastric mucosal blood flow was restored at the site of injection, 26 h after the application of endothelin-1, coupled with that of the lipid peroxidation peak at 24 h, suggests that a resumption of blood flow after prolonged ischaemia may lead to a late burst of oxygen free radicals and their subsequent contribution to the exacerbation of mucosal injury.

In the present experiment, probucol also exhibited gastroprotective properties. Ito et al. reported that oral administration of probucol (250–1000 mg/kg) dose-dependently prevented HCl plus ethanol-induced gastric mucosal injury and the increase in thiobarbituric acid-reactive substances, an index of lipid peroxidation, in injured mucosa (Itoh et al., 1998). They also reported that repeated oral administration of probucol (250–1000 mg/kg twice daily) dose-dependently accelerated the healing of acetic acid-induced gastric ulcers, by inhibiting the increase in content of thiobarbituric acid-reactive substances in the ulcerated region. In this study, probucol may have partly protected the gastric mucosa from endothelin-1-induced mucosal injury by its antioxidant activity, although further experiments are necessary to examine other actions than the free radical-scavenging action of this drug.

In summary, we have shown that in endothelin-1-induced gastric ulcer, oxygen radicals are generated from xanthine oxidase at least 24 h after endothelin-1 injection and contribute to the deterioration of the inflicted mucosal damage, but they do not mediate the early mucosal injury. The gastroprotective role of oxypurinol encompasses effects on the gastric microcirculation, gastric ATP level and inhibition of oxyradical production. Finally, marked long-lasting local ischaemia followed by reperfusion may be the haemodynamic mechanism responsible for the formation of this type of ulcer. Probucol may exhibit gastroprotective actions in the endothelin-1-induced ulcer model, at least in part, as a result of its antioxidant activity.

Acknowledgements

This study was supported by grants-in-aid for scientific research from the Ministry of Education, Science, Sports, and Culture of Japan (11357019, 11557047, 12470147, 12680012), and by a grant from the Miyauchi Project of Center for Tsukuba Advanced Research Alliance in the University of Tsukuba. We thank Dr. Wendy Gray for editing the English language of our manuscript.

References

Andrews, F., Malcontenti, C., Obrien, P., 1992. Sequence of gastric mucosal injury following ischaemia and reperfusion. Dig. Dis. Sci. 37 (9), 1356–1361.

Baez, S., Herschel, S.G., Robenstein, E.A., 1961. Vasotrophic substances in intestinal ischaemic shock. Am. J. Physiol. 200, 1245–1253.

Bulkley, G., 1983. The role of oxygen free radicals in human disease processes. Surgery 94 (3), 407–411.

Chiney, B., Filnch, Ca., 1960. Effect of inosine on iron absorption in rat. Proc. Soc. Exp. Biol. Med. 103, 37–38.

Comporti, M., 1985. Lipid peroxidation and cellular damage in toxic liver injury. Lab. Invest. 53 (6), 599–623.

Dale, A.P., Granger, N., 1986. Contributions of ischaemia and reperfusion to mucosal lesion formation. Am. J. Physiol. 250, G749–G753.

DeWall, R.A., Vasko, K.A., Stanley, E.L., 1971. Responses of the ischaemic myocardium to allopurinol. Am. Heart J. 82, 362.

Ekman, T., Risberg, B., Soussi, B., Bagge, U., 1993. Gastric mucosal microcirculation and purine nucleotide metabolism after retransfusion of rats in haemorrhagic shock. Scand. J. Gastroenterol. 28, 173–178.

Esterbauer, H., Cheeseman, K., 1990. Determination of aldehyde lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. Methods Enzymol. 186, 407–421.

Garcia, J.C., Rollan, M., Enrinquez, R., Madruga, M.H., Hernandez, E.M., Nunez, M., Alonso, G., 1990. Improved survival in intestinal ischaemia by allopurinol not related to xanthine-oxidase inhibition. J. Surg. Res. 48, 144–146.

Granger, D., Rutili, G., McCord, J., 1981. Superoxide radicals and feline intestinal ischaemia. Gastroenterology 81, 22–29.

Hernandez, L.A., Grisham, M.B., Granger, D.N., 1987. A role for iron in

oxidant-mediated ischemic injury to intestinal microvasculature. Am. J. Physiol. 253, G49–G53.

Huber, W., Saifer, M., 1977. Orgotein, a drug version of bovine Cu–Zn superoxide dismutase. A summary account of safety and pharmacology in laboratory animals. Superoxide and Superoxide Dismutase. Academic Press, 517.

Ishii, M., Shimizu, S., Nawata, S., Kiuchi, Y., Yamamoto, T., 2000. Involvement of reactive oxygen species and nitric oxide in gastric ischemia–reperfusion injury in rats. Dig. Dis. Sci. 45 (1), 93–98.

Itoh, M., Guth, P., 1985. Role of oxygen-derived free radicals in haemorrhagic shock-induced gastric lesions in rats. Gastroenterology 88, 1162–1167.

Itoh, Y., Suzuki, K., Matsubara, M., Takeyama, N., Kitazawa, Y., Tanaka, T., 1991. Changes in gastric mucosal content of adenosine, xanthine and hypoxanthine induced by restrained water immersion stress: antiulcer effects of tetraprenylacetone. Digestion 48, 25–33.

Itoh, M., Suzuki, Y., Ishihara, M., Suzuki, Y., 1998. Anti-ulcer effects of antioxidants: effect of probucol. Eur. J. Pharmacol. 354, 189–196.

Koppenol, W., 1983. Thermodynamics of the Fenton-driven Haber–Weiss and related reactions. Oxyradicals and Their Scavenger Systems, vol. 1. Elsevier, pp. 84–88.

Lazaratos, S., Kashimura, H., Nakahara, A., Fukutomi, H., Osuga, T., Urushidani, T., Miyauchi, T., Goto, K., 1993a. Gastric ulcer induced by submucosal injection of ET-1: role of potent vasoconstriction and intraluminal acid. Am. J. Physiol. 265, G491–G498.

Lazaratos, S., Kashimura, H., Nakahara, A., Fukutomi, H., Osuga, T., Miyauchi, T., Goto, K., 1993b. Endothelin-1-induced gastric ulcer is attenuated by cetraxate. Life Sci. 53 (7), 123–128.

Lazaratos, S., Nakahara, A., Kashimura, H., Goto, K., Mogi, T., Ono, Y., Saginoya, H., Fukutomi, H., 1994. The development of the endothelin-1-induced gastric ulcer: time sequence analysis of morphologic changes. Int. J. Exp. Pathol. 75, 345–355.

Marston, A., 1977. Laboratory studies of intestinal ischaemia. Intestinal Ischaemia. Yearbook Medical Publishers, pp. 43–69.

McCord, J., 1983. The superoxide free radical: its biochemistry and pathophysiology. Surgery 94 (3), 412–414.

Parks, D., Buckley, G., Granger, D., Hamilton, S., McCord, J., 1982. Ischaemic injury in the cat small intestine: role of superoxide radicals. Gastroenterology 82, 9–15.

Parks, D., Bulkley, G., Granger, D., 1983. Role of oxygen-derived free radicals in digestive tract diseases. Surgery 94 (3), 415–422.

Perry, M., Wadhwa, S., Parks, D., Pickard, W., Granger, D., 1986. Role of oxygen radicals in ischaemia-induced lesions in the cat stomach. Gastroenterology 90, 62–367.

Schoenberg, M., Beger, H., 1993. Reperfusion injury after intestinal ischaemia. Crit. Care Med. 21 (9), 1376–1386.

Smith, M., Grisham, M., Manci, E., Granger, D., Kvietys, P., 1987. Gastric mucosal injury in the rat. Gastroenterology 92, 950–956.

Smith, G.S., Mercer, D.W., Cross, J.M., Barreto, J.C., Miller, T.A., 1996. Gastric injury induced by ethanol and ischemia–reperfusion in the rat. Differing roles for lipid peroxidation and oxygen radicals. Dig. Dis. Sci. 41 (6), 1157–1164.

Spector, T., 1988. Oxypurinol as an inhibitor of xanthine oxidase-catalyzed production of superoxide radical. Biochem. Pharmacol. 37 (2), 349–352.

Stanley, P.E., Williams, S.G., 1969. Use of liquid sintillation spectrometer for determining adenosine triphosphate by the luciferase enzyme. Anal. Biochem. 29, 381–392.

Weiss, S.J., 1986. Oxygen, ischaemia and inflammation. Acta Physiol. Scand. 126 (Suppl. 548), 9–37.