Lipid Peroxidation in Gastric Mucosal Lesions Induced by Indomethacin in Rat

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The peroxidation of lipids and changes in the activities of related enzymes in the gastric mucosa were studied in a rat model of gastric mucosal injury induced by the nonsteroidal anti-inflammatory drug indomethacin. The area of gastric erosion and the amount of thiobarbituric acid reactive substances (TBARS) in gastric mucosa were significantly increased beginning 4 h after administration of indomethacin. Xanthine oxidase (XOD) activity in the gastric mucosa also increased immediately after administration of the drug. Although XOD activity was significantly suppressed by allopurinol treatment, the induction of gastric mucosal injury and the increase of TBARS in the gastric mucosa were not. Myeloperoxidase (MPO), a marker enzyme of leukocytes, was unaffected by indomethacin administration. But the depletion of polymorphonuclear leukocyte (PMN) counts induced by an injection of anti-rat PMN antibody inhibited both the injury and the increase in TBARS. Indomethacin activated PMN in peripheral blood at 30 mg/kg per os and enhanced release of oxygen radicals from PMN in peripheral blood. As compared with the XOD system, the generation of oxygen free radicals may derived mainly from activated PMN. On the other hand, superoxide dismutase (SOD) and glutathione peroxidase (GSH-px) were reduced by the administration of indomethacin. Decreases in SOD and GSH-px activity in gastric mucosa may aggravate mucosal injury by free radicals and lipid peroxidation.

Key words: indomethacin; gastric mucosal injury; oxygen free radical; lipid peroxidation; polymorphonuclear leukocyte; xanthine oxidase

The gastrointestinal irritant properties of indomethacin and several other nonsteroidal anti-inflammatory drugs (NSAIDs) continue to be the major impediment to the use of NSAIDs in the treatment of inflammatory diseases such as rheumatoid arthritis. These drugs are well known to produce gastric mucosal injury in the gastrointestinal tract. They have also been shown to inhibit the cyclo-oxygenase enzyme system that is responsible for prostaglandin (PG) biosynthesis from arachidonic acid in vitro and in vivo. It has been suggested that the production of gastric lesions by indomethacin and other NSAIDs is due to inhibition of PG biosynthesis. Mengu and Desbaillets and Kauffman et al. reported that NSAIDs induced gastric mucosal injury, caused inhibition of gastric mucosal secretion and reduced basal gastric mucosal blood flow. In recent years, a number of studies have highlighted the importance of alterations in mucosal blood flow after NSAID administration in the pathogenesis of ulceration in several experimental models. Despite extensive research, the pathogenesis of NSAID-induced damage to the gastric mucosa is still not fully understood.

It has been suggested that oxygen free radicals may play an important role in the pathogenesis of tissue injury induced by ischemia-reperfusion and hemorrhagic shock. The possible involvement of oxygen free radicals in the development of NSAID-induced mucosal damage has not received much attention, yet this could theoretically be one of the most important events in the initiation of mucosal injury. It was for this reason that we studied the relationship between the pathogenesis of gastric mucosal injury and lipid peroxidation. We measured thiobarbituric acid reactive substances (TBARS), xanthine oxidase (XOD) and myeloperoxidase (MPO) activities and oxygen radical release from polymorphonuclear leukocytes (PMN) in peripheral blood, and investigated the effects of the XOD inhibitor allopurinol and anti-rat PMN antibody. We also measured the levels of superoxide dismutase (SOD) (superoxide radical scavenger), and glutathione peroxidase (GSH-px) (an enzyme that protects animal cells against peroxidative damage) in the gastric mucosa in order to elucidate the systems that protect tissues against peroxidative damage, and resolve the lipid peroxidation products.

MATERIALS AND METHODS

Animals Male Donyru rats (SPF), 8 weeks old and weighing 200—230 g, were used. The animals were obtained from Charles River Co., Tokyo.

Materials Thiobarbituric acid was obtained from BHD Chemicals, Ltd., England; phosphotungstic acid was from Merck, West Germany; and allopurinol was from Fukuyu Pharmaceuticals, Ltd., Tokyo. Glutathione reductase, glutathione peroxidase standard solution and xanthine oxidase solution were obtained from Boehringer Mannheim Yamanouchi, Tokyo. Hydroxylamine hydrochloride, sulfonic acid, and uric acid test kit were obtained from Wako Pure Chemical Industries Ltd., Tokyo. N-(1-Naphthyl)ethylenediamine dihydrochloride was from Tokyo Kasei, Tokyo, 1-nicotinamide adenine dinucleotide phosphate was from Kojin Co., Ltd., Tokyo, and glutathione and o-dianisidine dihydrochloride were from Sigma, U.S.A. Other chemicals were of reagent grade and were used without purification.

Experimental Ulcers Rats were deprived of food, but given water, for 18—24 h before an experiment. Gastric mucosal damage was induced in five groups by oral administration of indomethacin at a dose of 30 mg/kg. The groups were then killed at one of the following times after administration: 1, 3, 4, 5 or 7 h. Non-treated

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rats were killed 5 h after administration of a vehicle. Allopurinol was administered orally to rats once a day for three days before indomethacin treatment. Gastric damage was scored, the stomach of each rat was removed, and the collected gastric mucosal membranes were frozen at −90 °C until use.

Preparation of Anti-rat PMN Antibody Following the technique of Ward et al., a suspension of PMN isolated from the rat abdominal cavity and Freund's complete adjuvant (Difco Laboratories, Detroit, U.S.A.) was subcutaneously injected into domestic rabbits. This immunizing procedure was repeated weekly a total of 4 times in each rabbit, and one week after the final immunization, blood was taken. The serum was frozen at −80 °C until used.

PMN-Depletion To make PMN-depleted rats, anti-rat PMN antibody was intraperitoneally injected at a dose of 10 ml/kg 15 h before indomethacin treatment. The same amount of normal domestic rabbit serum was administered to the control group.

Determination of Lipid Peroxidation The level of TBARS in the gastric mucosa was measured according to the method of Okawa et al. The gastric mucosa was homogenized with 1.15% KCl solution to make a 10% homogenate solution. After adding sodium dodecyl sulfate and TBA solution, the reaction product was assayed spectrophotometrically (532 nm).

Measurement of Xanthine Oxidase (XOD) XOD-catalyzed uric acid formation in the gastric mucosa was monitored spectrophotometrically at 550 nm. The reaction mixture consisted of 100 mm phosphate-boric buffer (pH 8.2) (0.3 ml), xanthine (0.2 ml), distilled water (0.3 ml) and sample solution (0.2 ml). A sample of supernatant fluid with 20% homogenate solution and 1.15° KCl was made by centrifugation at 4000 × g for 10 min at 4 °C. The level of uric acid in the reaction mixture was measured by using a Wako uric acid test kit after incubation for 3 h at 37 °C.

Measurement of Myeloperoxidase (MPO) MPO activity was determined according to the modified method of Bradley and his coworkers. The reaction mixture consisted of 50 mm phosphate buffer (pH 6), 1.03 mm o-dianisidine and 1% H₂O₂ in a total volume of 180 μl. Samples (20 μl) were added to the above mixture, and the absorbance at 450 nm was immediately recorded for 5 min with a Micropile reader. A sample of supernatant fluid with 10% homogenate solution and 1.15° KCl was obtained by centrifugation at 4000 × g for 10 min at 4 °C. Activity was calculated from OD per minute.

Measurement of Superoxide Radical from PMN in Peripheral Blood PMN in peripheral blood were isolated following the technique of Boyum from Donryu rats. The cells were separated by using 2% dextran. The PMN were freed of erythrocytes by hypotonic lysis and washed once in Krebs-Ringer phosphate buffer (KRb), pH 7.4. The proportion of PMN obtained in this manner averaged 60%.

Measurements of O₂⁻ Generation The generation of O₂⁻ was measured in terms of the reduction of ferricytochrome c (horse heart type III) as described by Goldstein et al. Leucocytes (1 × 10⁷) and 0.1 mm ferricytochrome c were incubated in the presence of opsonised zymosan for 30 min at 37 °C. The final volume of the reaction mixture was adjusted to 2.0 ml. Opsonated zymosan was prepared by incubating 50 mg of zymosan in 1 ml of freshly prepared normal rat serum for 40 min at 37 °C. The opsonized particles were collected by centrifugation at 1700 × g for 15 min, then suspended again at a concentration of 50 mg/ml in phosphate buffered saline. This suspension was stored at −80 °C before use.

Measurement of Superoxide Dismutase (SOD) SOD activity in the gastric mucosa was assayed by a modification of the nitrite kit method utilized for the xanthine oxidase–naphthyl ethylenediamine system. Optical absorption was measured at 550 nm. The reaction mixture consisted of 100 mm phosphate-boric buffer (pH 8.2) (0.2 ml), 5 mm xanthine (0.2 ml), 1 mm hydroxylamine HCl (0.1 ml), distilled water (0.1 ml), xanthine oxidase solution (0.1 U/ml), and sample solution (0.1 ml). A sample of supernatant fluid with 10% homogenate solution and 1.15° KCl was obtained by centrifugation at 4000 × g for 10 min at 4 °C.

Measurement of Glutathione Peroxidase (GSH-px) GSH-px activity was determined according to the method of Lawrence and Burk. The reaction mixture consisted of 50 mm potassium phosphate buffer (pH 7), 1 mm EDTA, 1 mm Na₂S₂O₃, 0.2 mm reduced nicotinamide adenine dinucleotide phosphate (NAPDH), 1 E.U./ml oxidized glutathione (GSSG) reductase, 1 mm GSH, and 0.25 mm H₂O₂ in a total volume of 1 ml. The ingredients, except for the enzyme source and peroxide, were combined at the beginning of each assay. Samples (0.1 ml) were added to 0.8 ml of the above mixture and incubated for 5 min at 25 °C before initiating the reaction with the addition of 0.1 ml of peroxide solution. A sample of supernatant fluid with 10% homogenate solution and 1.15° KCl was made by centrifugation at 4000 × g for 10 min at 4 °C. The absorbance at 340 nm was recorded for 5 min. The activity was then calculated from the slope of the plot as μmol of NADPH oxidized per minute. The blank datum (the enzyme was replaced with distilled water) was subtracted from each value.

Measurement of Protein The amount of protein was determined by the method of Lowry and his coworkers.

Statistical Analysis All data are expressed as mean ± S.D. Differences between treatment groups were compared by using an analysis of variance followed by Dunnett’s multiple range test, or the Wilcoxon test. Probability values <0.05 were considered statistically significant. Correlation coefficients were calculated by Pearson’s method (Program ID: IFEK 320)

RESULTS

The time course of indomethacin-induced gastric mucosal damage is shown in Fig. 1. An index of the gastric mucosal injury was determined using the product of length. Total area of gastric mucosal injury increased from 4 h after the oral administration of indomethacin.

The time course of TBARS in the gastric mucosa (an index of lipid peroxidation) increased from 4 h after oral administration of indomethacin. The results are shown in
Fig. 1. Time Course of Ulceration Induced by Indomethacin
Time course curve of indomethacin induced gastric lesions. Indomethacin was given orally at a dose of 30 mg/kg. At different time points, animals were killed, the stomach was removed and the lesions measured. Each value is the mean ± S.D. from 4–5 determinations.

Fig. 2. Time Course of TBARS Level in Gastric Mucosa
TBA reactant substance level in gastric mucosa induced by indomethacin administration (30 mg/kg p.o.). Each value is mean ± S.D. from 4–16 determinations. Untreated rats were used as time 0. **p < 0.01; * p < 0.05 significantly different from time 0.

Fig. 2. The level of TBARS in the stomach followed the time course curve of the gastric mucosal injury index. The change of TBARS in the gastric mucosa was highly correlated with the gastric mucosal injury index (correlation coefficient, r = 0.955 until 5 h, r = 0.702 until 7 h).

Time course of XOD activity in the gastric mucosa is shown in Fig. 3. This activity increased soon after oral administration of indomethacin (p < 0.001), that the time course was not correlated with that of TBARS in the gastric mucosa (r = 0.451).

Changes in MPO activity are shown in Fig. 4. MPO activity decreased significantly after indomethacin administration. But the depletion of PMN counts induced by treatment with anti-rat PMN antibody significantly inhibited aggravation of the gastric mucosal lesions or the increase in TBARS in gastric mucosa induced by indomethacin. Furthermore, the aggravation of gastric mucosal lesions and the increase in TBARS were not inhibited by treatment with allopurinol. The results are summarized in Table 1.

Superoxide generation from PMN in peripheral blood stimulated with zymosan for 30 min is shown in Table 2.

Fig. 3. Time Course of XOD Activity in Gastric Mucosa
XOD activity in gastric mucosa induced by indomethacin administration (30 mg/kg p.o.). Each value is the mean ± S.D. from 4–16 determinations. Untreated rats were used as time 0. ***p < 0.001; **p < 0.01 significantly different from time 0.

Fig. 4. Time Course of MPO Activity in Gastric Mucosa
MPO activity in gastric mucosa induced by indomethacin administration (30 mg/kg p.o.). Each value is the mean ± S.D. from 4–16 determinations. Untreated rats were used as time 0. **p < 0.01; * p < 0.05 significantly different from time 0.

Table 1. Effect of PMN-Depletion and Allopurinol on Total Area of Gastric Mucosal Injury and Level of TBARS in Gastric Mucosa after Treatment of Indomethacin

<table>
<thead>
<tr>
<th>Condition</th>
<th>Ulcer index (mm)</th>
<th>TBARS in gastric mucosa (nmol/10 mg protein)</th>
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<tr>
<td>0.1% CMC p.o.</td>
<td>60.89 ± 4.96</td>
<td>6.03 ± 1.48</td>
</tr>
<tr>
<td>Anti-rat PMN antibody s.c.</td>
<td>6.41 ± 2.55***</td>
<td>4.30 ± 0.72***</td>
</tr>
<tr>
<td>Normal rabbit serum s.c.</td>
<td>72.21 ± 14.43</td>
<td>6.64 ± 1.05</td>
</tr>
<tr>
<td>Allopurinol 100 mg/kg p.o.</td>
<td>62.00 ± 28.59</td>
<td>6.68 ± 1.22</td>
</tr>
<tr>
<td>No treatment</td>
<td>—</td>
<td>4.41 ± 0.59**</td>
</tr>
</tbody>
</table>

Gastric mucosal injury index was measured at 5 h after indomethacin administration in rat. Each value is the mean ± S.D. from 5–9 determinations are presented. ***p < 0.001; **p < 0.01 significantly different from 0.1% CMC.

Six hours after indomethacin administration, superoxide generation from PMN in peripheral blood was increased compared with that in non-treated rats (p < 0.01).

The SOD activity in the gastric mucosa (Fig. 5)
Table 2. Effect of Indomethacin on O$_2^-$ Generation from PMN of Peripheral Blood

<table>
<thead>
<tr>
<th></th>
<th>O$_2^-$ generation (nmol/min/1 × 10$^6$ cells)</th>
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<tbody>
<tr>
<td>Non-treated rat</td>
<td>288.31 ± 90.91</td>
</tr>
<tr>
<td>Indomethacin-treated rat</td>
<td>498.30 ± 97.44**</td>
</tr>
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O$_2^-$ generation from PMN stimulated with opsonized zymosan for 30 min. PMN were prepared from peripheral blood. Each value is the mean ± S.D. from 5 determinations are presented. ** p<0.01 significantly different from non-treated rat.

![Fig. 5. Time Course of SOD Activity in Gastric Mucosa](image)

SOD activity in gastric mucosa induced by indomethacin administration (30mg/kg p.o.). Each value is the mean ± S.D. from 4–16 determinations. Untreated rats were used as time 0. ***p<0.001; **p<0.01; *p<0.05 significantly different from time 0.

decreased soon after oral administration of indomethacin (p<0.001). The correlation between SOD activity and TBARS in the gastric mucosa was negative (r = –0.711).

GSH-px activity in the gastric mucosa likewise decreased soon after administration of indomethacin (p<0.01). Though we could not find a correlation between the time course of GSH-px and that of TBARS in the gastric mucosa, GSH-px activity showed a negative correlation with XOD activity (r = –0.118, r = –0.837). The results are shown Fig. 6.

**DISCUSSION**

The gastrointestinal irritant properties of nonsteroidal anti-inflammatory drugs (NSAIDs) continue to be the major impediment to their use in the treatment of inflammatory diseases such as rheumatoid arthritis.1 In recent years, a number of studies have highlighted the importance of alterations in mucosal blood flow after NSAID administration in the pathogenesis of ulceration in several experimental models.7,8 These observations suggest a role for circulating leukocytes in the pathogenesis of NSAID-induced ulceration. It is well known that the activation of leukocytes is accompanied by the release of oxygen-derived free radicals, which can attack and initiate a free radical chain reaction known as lipid peroxidation.19 The role of free radicals and lipid peroxidation in NSAID-induced gastrointestinal injury has not been extensively investigated.

![Fig. 6. Time Course of GSH-px Activity in Gastric Mucosa](image)

GSH-px activity in gastric mucosa induced by indomethacin administration (30mg/kg p.o.). Each value is the mean ± S.D. from 4–16 determinations. Untreated rats were used as time 0. ***p<0.001; **p<0.01; *p<0.05 significantly different from time 0.

In the present study, we used a rat model to study the relationship between the pathogenesis of gastric mucosal injury and lipid peroxidation. First, we found that the production of gastric mucosal injury and TBARS levels in the gastric mucosa increased significantly after oral administration of indomethacin (Figs. 1 and 2). This change of TBARS in the gastric mucosa was shown to be highly correlated with the gastric mucosal injury index (r = 0.955 up to 5h). These observations suggested that lipid peroxidation plays an important role in the pathogenesis of indomethacin-induced ulceration, although the source of the oxygen free radicals involved in this ulceration is not yet clear. It was in an attempt to identify the source that we undertook this study.

Based on the report that NSAID reduce gastric mucosal blood flow, we measured XOD activity in the gastric mucosa. Depression of ATP concentration following reduced blood flow is accompanied by a rise in adenosine monophosphate levels. This converts xanthine dehydrogenase to the oxidative form of the enzyme, which is capable of producing tissue-destructive oxygen radicals. Though XOD activity increased soon after oral administration of indomethacin, the time course of XOD activity was not correlated with the time course of TBARS in the gastric mucosa (Fig. 3). Although XOD activity in the gastric mucosa was inhibited by treatment with the XOD inhibitor allopurinol, gastric mucosal injury was not ameliorated, and the TBARS level did not change (Table 1, Fig. 3). These data indicate that XOD is not important in the injury induced by indomethacin. Vaananen et al. reported that allopurinol did not significantly affect the extent of macroscopically visible mucosal damage induced by indomethacin or acid.20 We conclude that oxygen free radicals induced by indomethacin were produced by a mechanism other than the XOD system.

The activation of neutrophils is known to be accompanied by the release of oxygen free radicals (through the NADPH oxidase system).21 In recent years, it has become increasingly clear that neutrophil-derived free radicals play an important role in the gastrointestinal damage associated
with hemorrhagic shock and ischemia-reperfusion.\textsuperscript{22,23} We demonstrated that these neutrophil-derived free radicals are also important factors in the gastric injury induced by ischemia-reperfusion in pylorus-ligated rats.\textsuperscript{53} MPO activity was therefore measured in the gastric mucosa. But MPO, which is a marker enzyme of leukocytes,\textsuperscript{13} decreased significantly after administration of indomethacin, contrary to our expectation. Shaeter et al.\textsuperscript{24} and Kettle and Winterbourn\textsuperscript{25} demonstrated that NSAIDs inhibit MPO, so we investigated the use of anti-rat PMN antibody. By depression of circulating PMN numbers with the antibody, indomethacin-induced gastric damage was reduced significantly, as were TBARS levels in gastric mucosa (Table 1). Wallance et al. also reported that the gastric damage induced by indomethacin can be significantly reduced by prior depletion of circulatory neutrophils.\textsuperscript{26} We previously reported that a high concentration of indomethacin did not inhibit $O_2^-$ production by NADPH oxidase.\textsuperscript{27} In fact, PMN in peripheral blood showed enhanced $O_2^-$ release when stimulated by zymosan after administration of indomethacin (Table 2). We considered that indomethacin-induced gastric mucosal injury occurred as a result of oxygen free radical generation from PMN.

It is known that several enzymes protect animal cells against lipid peroxidation. In this study, we examined the effects of two protective enzymes, SOD and GSH-px, against tissue peroxidative damage to the gastric mucosa. SOD is a scavenger of superoxide radicals and GSH-px resolves lipid peroxide. Our studies revealed that SOD and GSH-px activities were significantly decreased in gastric mucosa treated with indomethacin. It seemed that these decreases in activity aggravated mucosal injury by free radicals and lipid peroxidation. The factors responsible for triggering PMN activation after indomethacin administration are not yet clear, but together with our previous studies,\textsuperscript{23,27} the present results suggest that a high dose of indomethacin induced a reduction of blood flow, activation of PMN, superoxide generation from PMN and mucosal injury by free radicals and lipid peroxidation. Further, depression of the protective enzymes, SOD and GSH-px aggravated the gastric mucosal injury.

REFERENCES