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Lipid peroxidation induced by indomethacin with horseradish peroxidase and hydrogen peroxide: involvement of indomethacin radicals

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Abstract

Some of the side-effects of using indomethacin (IM) involve damage to the gastric mucosa and liver mitochondria. On the other hand, neutrophils infiltrate inflammatory sites to damage the tissues through the generation of reactive oxygen species by myeloperoxidase. The stomach and intestine have large amounts of peroxidase. These findings suggest that peroxidases are involved in tissue damage induced by IM. To clarify the basis for the tissue damage induced by IM in the presence of horseradish peroxidase (HRP) and H_2O_2 (HRP-H_2O_2), lipid peroxidation was investigated. When IM was incubated with liver microsomes in the presence of HRP-H_2O_2 and ADP-Fe^{3+}, lipid peroxidation was time-dependent. Catalase and desferrioxamine almost completely inhibited lipid peroxidation, indicating that H_2O_2 and iron are necessary for lipid peroxidation. Of interest, superoxide dismutase strongly inhibited lipid peroxidation, and it also inhibited the formation of bathophenanthroline-Fe^{2+}, indicating that reduction of the ferric ion was due to superoxide (O_2^-). ESR signals of IM radicals were detected during the interaction of IM with HRP-H_2O_2. However, the IM radical by itself did not reduce the ferric ion. These results suggest that O_2^- may be generated during the interaction of IM radicals with H_2O_2. Ferryl species, which are formed during the reduction of iron by O_2^-, probably are involved in lipid peroxidation.

Keywords: Horseradish peroxidase; Indomethacin; Nonsteroidal anti-inflammatory drug; Lipid peroxidation

1. Introduction

IM, one of the most effective NSAIDs, is widely used clinically to treat rheumatic and arthritic disease because it has analgesic, antipyretic, and anti-inflammatory actions; these actions are mediated by inhibiting prostaglandin synthesis by preventing the cyclooxygenase activity of prostaglandin H synthase [1,2]. However, side-effects of the administration of IM are damage to the gastric mucosa [3,4] and liver mitochondria [5,6]. Oxygen radicals and lipid peroxidation may be involved in the gastric mucosal damage induced by IM [7–9]. However, the exact role of IM in lipid peroxidation remains to be clarified.

Through its cyclooxygenase activity, prostaglandin H synthase catalyzes the bis-dioxygenation of arachidonic acid to form hydroperoxy endoperoxide prostaglandin G_2, while *via* its hydroperoxidase activity it catalyzes the reduction of the hydroperoxide groups of the endoperoxide. During this enzyme-catalyzed reduction of the endoperoxide, many xenobiotics are oxidized, *via* the hydroperoxidase activity, through a one-electron transfer [10–12]. Similarly, other peroxidases, such as lactoperoxidase, myeloperoxidase, and HRP, can also oxidize various drugs [13–17]. Neutrophils infiltrate inflammatory sites to damage the tissues through the generation of reactive oxygen species by myeloperoxidase, and they have an important role in the pathogenesis of gastric ulcers induced by NSAIDs [18]. The stomach and intestine also have large amounts of peroxidase [19]. These findings suggest that when IM acts on tissues, it may be metabolized to a free radical. The IM free radical is produced during the interaction of IM with lactoperoxidase in the presence of H_2O_2 [20]. However, whether the IM radical contributes to the tissue damage induced by IM is not clear.

In this study, we show that lipid peroxidation of microsomes is induced through the formation of IM free radicals generated during the interaction of IM with HRP and H_2O_2 (HRP-H_2O_2).

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Abbreviations: BHT, butylated hydroxytoluene; BPS, bathophenanthroline sulfonate; $HO^•$, hydroxyl radical; HRP, horseradish peroxidase; IM, indomethacin; MNP, 2-methyl-2-nitrosopropane; NSAID, nonsteroidal anti-inflammatory drug; O_2^-, superoxide; SOD, superoxide dismutase; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances.

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2. Materials and methods

2.1. Chemicals

IM and HRP were obtained from Wako Pure Chemical Industry; MNP and TBA were from the Merck Japan Co. Ltd.; and SOD (bovine erythrocyte) and catalase (bovine liver) were from the Sigma Chemical Co. Other chemicals were analytical grade products obtained from commercial suppliers.

2.2. Lipid peroxidation

Microsomes were prepared from the livers of Wistar strain rats weighing 200–250 g as follows [21]: livers were minced and then homogenized in a buffer consisting of 0.25 M sucrose, 1.0 mM EDTA, and 10 mM HEPES at pH 7.4. The homogenate was filtered through double-layer gauze and then was centrifuged at 1,600 *g* for 10 min at 4°. The supernatant was centrifuged at 9,000 *g* for 20 min at 4°, and the mitochondrial pellets were discarded. Microsomal pellets were obtained by ultra-centrifugation at 105,000 *g* for 1 hr at 4°. To remove the sucrose, which interferes with TBARS formation, the microsomal pellets were washed three times with HEPES buffer at pH 7.4, and they were stored at −80°. Protein was measured using the bicinchoninic acid assay with bovine serum albumin as a standard [22]. The reaction mixture contained microsomes (0.1 mg protein/mL), 0.12 μM HRP, 100 μM H_2O_2, 10.0 μM ADP-Fe^{3+} (0.17 mM ADP, 10 μM $FeCl_3$), and 0.15 M NaCl in 3.0 mL of 10 mM HEPES buffer at pH 7.4. Various concentrations of IM were added to the reaction mixture. The formation of TBARS was determined as reported by Buege and Aust [23] with minor modification. The peroxidation reaction was stopped by adding 0.1 mL of 30% trichloroacetic acid. The precipitate from the reaction mixture was discarded after centrifugation at 1,500 *g* for 10 min at room temperature. TBARS formation was assayed by measuring the absorption at 535 nm after heating for 30 min at 100°, and the absorbance was expressed as nmol TBARS/mg protein, using $\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$ [24].

2.3. Reduction of Fe^{3+}

The reduction of Fe^{3+} induced by IM was monitored spectrophotometrically at 530 nm as BPS-Fe^{2+} formation ($\varepsilon = 22.14\ mM^{-1}\ cm^{-1}$) [25].

2.4. ESR measurement

ESR signals of IM radicals were measured using the JEOL model JES-RE1X with MNP as a spin trapper. The ESR setting was microwave power, 10 mW; modulation frequency, 100 kHz; modulation field, 0.5 G; receiver gain, 1000; and time constant, 0.3 sec. The relative signal intensity was measured using Mn^{2+} as a standard.

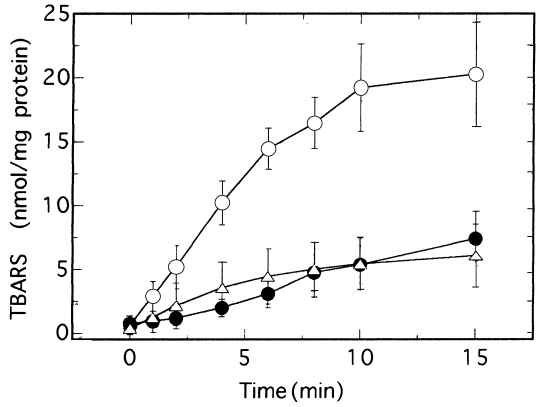

Fig. 1. Lipid peroxidation induced by IM with HRP-H_2O_2 in the presence of ADP-Fe^{3+}. The complete reaction mixture contained: microsomes (0.1 mg protein/mL), –IM (10 μM), H_2O_2 (100 μM), HRP (0.12 μM), –ADP-Fe^{3+} (10 μM), and 0.15 M NaCl in 10 mM HEPES buffer at pH 7.4. After the mixture was incubated at 37°, TBARS formation was measured as described in Section 2. Each point represents the mean ± SD of five experiments. Key: (○) complete reaction mixture; (●) IM, and (△) ADP-Fe^{3+}.

3. Results

3.1. Lipid peroxidation induced by IM

Fig. 1 shows that when microsomes were incubated with IM and HRP-H_2O_2 in the presence of ADP-Fe^{3+}, lipid peroxidation, which was measured in terms of the formation of TBARS, was induced with time. Only low lipid peroxidation was observed in the reaction lacking IM or ADP-Fe^{3+}. Fig. 2 shows that the lipid peroxidation was dependent upon the IM concentration. At 20 μM IM, TBARS formation reached about 20 nmol/mg protein. These results suggest that IM induced the lipid

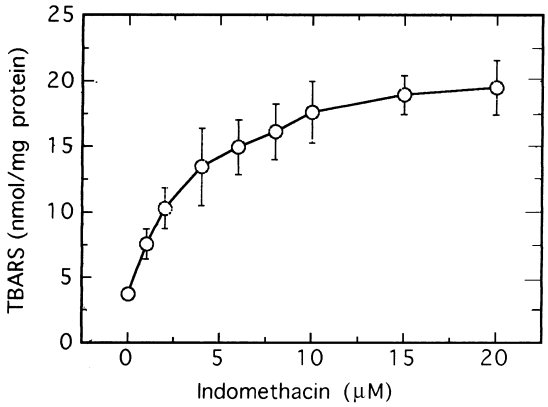

Fig. 2. Effect of IM concentration on lipid peroxidation. After the mixture was incubated for 10 min, TBARS formation was measured. Other conditions were the same as described in the legend of Fig. 1. Each point represents the mean ± SD of five experiments.

Table 1
Effects of radical scavengers and desferrioxamine on lipid peroxidation

Additions	Concentration (μM)	TBARS (nmol/mg protein)	Inhibition (%)
None	–	18.6 ± 0.9	–
Catalase	0.4	0.9 ± 0.2	95.2
SOD	0.3	12.1 ± 2.8	34.9
Dimethyl sulfoxide	1×10^4	17.2 ± 0.9*	7.5
Mannitol	1×10^4	17.7 ± 0.8*	5.1
BHT	10	1.3 ± 0.4	92.9
Desferrioxamine	100	3.0 ± 0.3	84.1
GSH	1×10^3	9.6 ± 0.2	48.2

Conditions were the same as described in the legend of Fig. 1 except for adding radical scavengers and desferrioxamine. IM (10 μM) was added to the reaction mixture. After the mixture was incubated for 10 min, TBARS formation was measured. Each value represents the mean ± SD of five experiments. Data were analyzed using the *t*-test.

* $P > 0.05$, versus no addition.

peroxidation of microsomes through interaction with HRP-H_2O_2 in the presence of ADP-Fe^{3+}.

3.2. Effect of radical scavengers and iron chelators

The ability of radical scavengers and desferrioxamine to inhibit lipid peroxidation induced by IM with HRP-H_2O_2 was tested, and the results are summarized in Table 1. Catalase completely inhibited lipid peroxidation, indicating that H_2O_2 was necessary for lipid peroxidation induced by IM with HRP. Gastric mucosal damage and membrane lipid peroxidation induced by IM has been shown to be partially inhibited by SOD [26]. In this study, SOD inhibited lipid peroxidation, indicating that lipid peroxidation was induced O_2^--dependently by IM with HRP-H_2O_2. BHT, a typical antioxidant, and the iron chelator desferrioxamine sharply inhibited lipid peroxidation. These results suggest that iron is necessary for lipid peroxidation. However, lipid peroxidation was independent of the hydroxyl radical (HO•), because mannitol and dimethyl sulfoxide, typical HO• scavengers, had no effect. GSH strongly inhibited lipid peroxidation activity. It has been reported that GSH can react with HRP compounds I and II [13]. Presumably, the reaction of IM with HRP compound I or II is depressed by GSH.

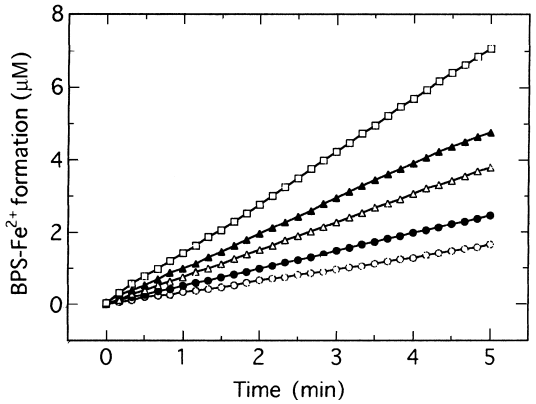

Fig. 3. Ferrous ion formation caused by IM with HRP-H_2O_2. The reaction mixture contained BPS (1.0 mM), ADP-Fe^{3+} (10.0 μM), various concentrations of IM, H_2O_2 (100 μM), HRP (0.12 μM), and 0.15 M NaCl in 10 mM HEPES buffer at pH 7.4. Ferrous ion formation was monitored by measurement of absorption at 530 nm. Each point represents the mean of three experiments. The variation was less than 10%. Key: (○) no IM; (●) 1.0 μM IM; (△) 3.0 μM IM; (▲) 5.0 μM IM, and (□) 10.0 μM IM.

3.3. Involvement of iron

Generally, lipid peroxidation proceeds through the reduction of iron [27]. We therefore examined the mechanism by which iron is reduced. Fig. 3 shows that BPS-Fe^{2+} formation was dependent upon the IM concentration. At 10 μM, IM formed 1.5 μM Fe^{2+}/min in the presence of HRP-H_2O_2. Only a low amount of BPS-Fe^{2+} was formed in the reaction lacking IM. Evidently, ferric ion was reduced to ferrous ion during the interaction of IM with HRP-H_2O_2. Both SOD and catalase strongly inhibited the formation of BPS-Fe^{2+} (Fig. 4a), indicating that reduction of the ferric ion depended predominantly upon O_2^- and

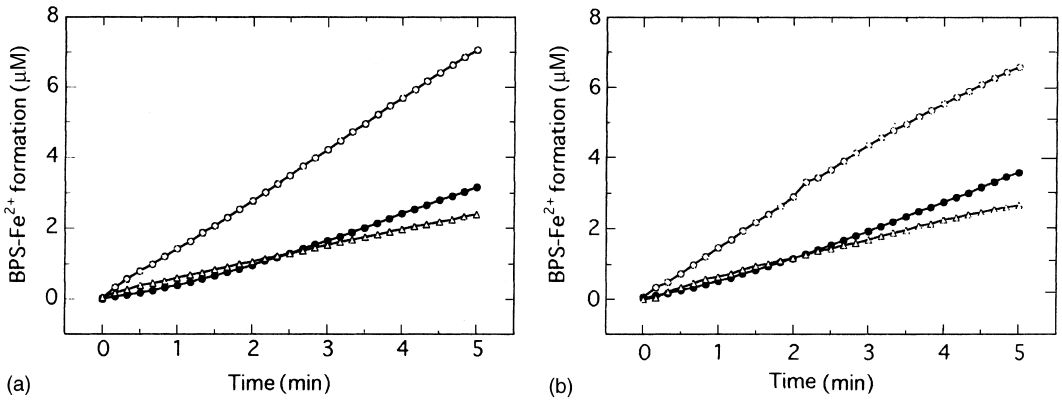

Fig. 4. Inhibitory effect of SOD and catalase on ferrous ion formation under aerobic (a) or anaerobic (b) conditions. The conditions were the same as described in the legend of Fig. 3 except for the presence of 10 μM IM. SOD and catalase were added to the reaction mixture. For anaerobic experiments, the reaction mixture was purged with argon for 15 min before the reactions were carried out. Key: (○) no addition; (●) SOD (0.3 μM); and (△) catalase (0.4 μM). Each point represents the mean of three experiments. The variation was less than 10%.

H_2O_2. Even under anaerobic conditions, BPS-Fe^{2+} was formed by the interaction of IM with HRP-H_2O_2, while, interestingly, SOD and catalase efficiently inhibited its formation (Fig. 4b).

Peroxidase oxidizes various organic compounds by a one-electron transfer [13–16], suggesting that IM radicals are formed by IM with HRP-H_2O_2 and are involved in lipid peroxidation. Fig. 5 shows that ESR signals of IM radicals ($a_N = 15.6$) were detected from an aerobic reaction mixture consisting of IM, HRP-H_2O_2, MNP, and 5% ethanol, which was used to dissolve IM and MNP. No ESR signals of IM radicals were observed in the reaction mixture lacking IM (Fig. 5b), H_2O_2 (Fig. 5c), or HRP (Fig. 5d), indicating that radicals of IM were formed through the interaction of IM with HRP-H_2O_2. Adding 0.1 mM ferric ion did not affect the ESR signals of IM radicals (Fig. 5e),

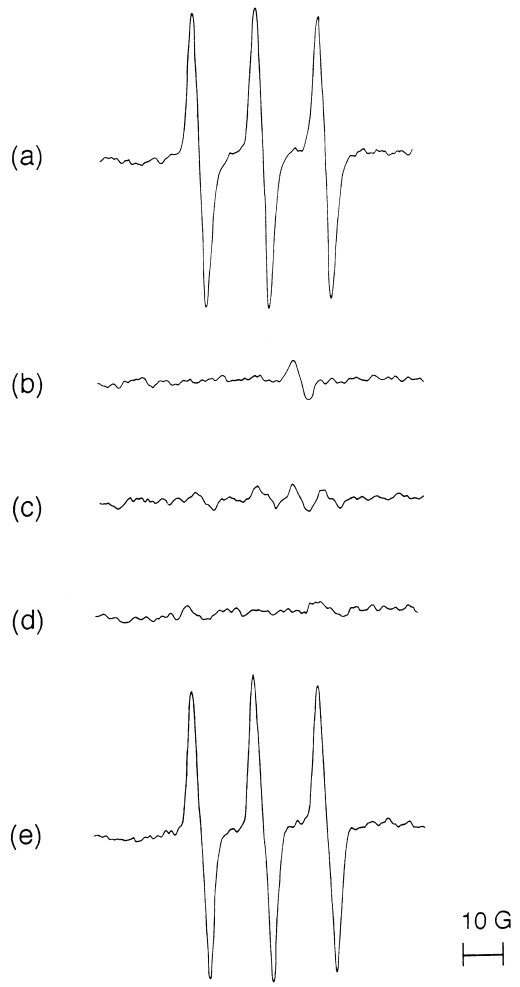

Fig. 5. ESR spectra of IM radicals formed by IM with HRP-H_2O_2. The reaction mixture contained IM (5.0 mM), HRP (2.5 μM), H_2O_2 (100 μM), MNP (2.9 mM), and 5% ethanol in 100 mM HEPES buffer at pH 7.4. The reaction was started by adding IM. The signals were recorded under anaerobic conditions. For anaerobic experiments, the reaction mixture was purged with argon for 15 min before the reactions were carried out. Other conditions were as described in Section 2. Key: (a) IM added; (b) without IM; (c) without H_2O_2; (d) without HRP, and (e) IM with ADP-Fe^{3+} (0.1 mM).

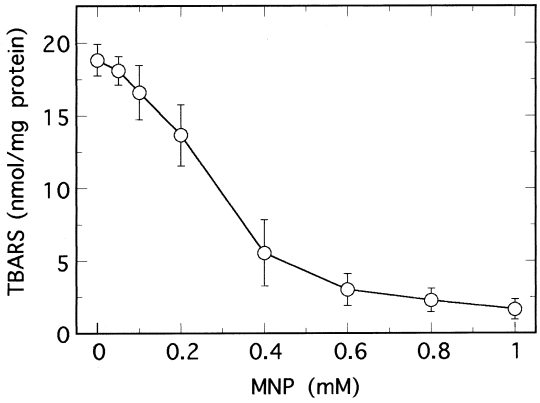

Fig. 6. Inhibition of lipid peroxidation by MNP. The conditions were the same as described in the legend of Fig. 1 except for MNP. After the mixture was incubated for 10 min, TBARS formation was measured. Each point represents the mean ± SD of five experiments.

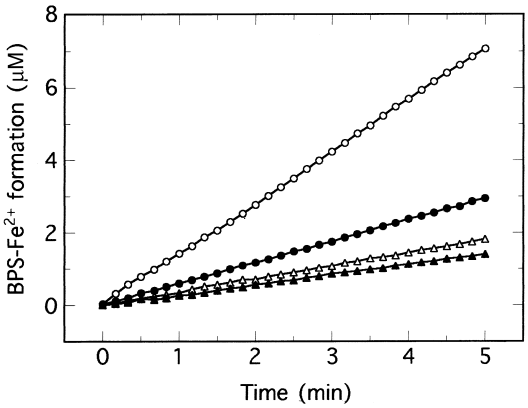

Fig. 7. Inhibition of Fe^{2+} formation by MNP. The conditions were the same as described in the legend of Fig. 3 except for MNP. Each point represents the mean of three experiments. The variation was less than 10%. Key: (○) no MNP; (●) 1.0 mM MNP; (△) 3.0 mM MNP; and (▲) 5.0 mM MNP.

indicating that the IM radical by itself did not reduce the ferric ion. However, MNP, a spin-trapping agent, strongly inhibited both lipid peroxidation (Fig. 6) and the formation of BPS-Fe^{2+} in a concentration-dependent manner (Fig. 7). These results suggest that IM radicals are involved in lipid peroxidation through the reduction of the ferric ion.

4. Discussion

In gastric mucosal injury induced by IM, lipid peroxidation mediated by oxygen radicals destroys and damages the cell membranes, leading to mucosal injury [7–9,26]. However, the mechanism of lipid peroxidation induced by IM is not clear. In this study, we demonstrated that IM radicals, produced from the interaction of IM with HRP-H_2O_2, were involved in lipid peroxidation. In the absence of ADP-Fe^{3+}, lipid peroxidation was not induced by IM with HRP-H_2O_2, indicating that the IM radical by itself did

not cause lipid peroxidation. Evidently, IM is involved in lipid peroxidation through the reduction of iron. Furthermore, catalase and desferrioxamine strongly inhibited lipid peroxidation, indicating that iron and H_2O_2 are necessary for lipid peroxidation. SOD also strongly inhibited both lipid peroxidation and the formation of BPS-Fe^{2+}. Superoxide by itself does not initiate lipid peroxidation. Therefore, lipid peroxidation should be caused through the reduction of the ferric ion by O_2^-. However, IM radicals seem unlikely to participate in the reduction of the ferric ion because adding ferric ion did not diminish the ESR signals. MNP, a spin-trap agent, strongly inhibited both lipid peroxidation and the formation of BPS-Fe^{2+}, indicating that IM radicals are involved in lipid peroxidation through the reduction of the ferric ion by O_2^-. SOD and catalase inhibited BPS-Fe^{2+} formation not only under aerobic conditions, but also under anaerobic conditions. From these results, we speculated that O_2^- should be formed by the interaction of the IM radical ($IM^\bullet$) with H_2O_2 as follows:

$$IM^\bullet + H_2O_2 \rightarrow IM + O_2^- + 2H^+ \quad (1)$$

Participation of $HO^\bullet$ in iron-catalyzed lipid peroxidation is assessed by using SOD, catalase, or $HO^\bullet$ scavengers. In this study, SOD and catalase, but not $HO^\bullet$ scavengers, strongly inhibited the lipid peroxidation induced by IM with HRP-H_2O_2, suggesting that $HO^\bullet$ is not involved in the lipid peroxidation induced by IM with HRP-H_2O_2. $HO^\bullet$ formation can be detected by its ability to degrade deoxyribose to fragments that generate pink chromogen upon heating with TBA at a low pH [28]. However, no marked deoxyribose degradation was induced during the interaction of IM with HRP-H_2O_2 in the presence of ADP-Fe^{3+} (data not shown). These results indicate that $HO^\bullet$ was not formed during the interaction of IM with HRP-H_2O_2 in the presence of ADP-Fe^{3+}. Intermediates formed in the Fenton reaction consist of $HO^\bullet$ and iron species [29]. The efficiency of $HO^\bullet$ generation varied with the nature of the iron chelators used. The oxidizing species of iron, ferryl, FeO_2^+, or Fe(IV)=O, are generated effectively in the presence of ADP. Hypervalent iron, such as ferryl or perferryl ion Fe(V)=O_3^+, are formed by the reaction of the ferrous ion with H_2O_2.

$$Fe^{2+} + H_2O_2 \rightarrow FeO_2^+ + H_2O \quad (2)$$

The ferryl or perferryl ion can extract a hydrogen atom from polyunsaturated fatty acids of membranes to initiate lipid peroxidation [30–32].

Many studies have shown that gastric mucosal damage, including ulcers and lipid peroxidation induced by IM, is inhibited by SOD [7,8,26]. From the present study, we believe that SOD blocks gastric mucosal damage by preventing the reduction of iron by O_2^-.

Prostaglandin H synthase has both cyclooxygenase and hydroperoxidase activities, and the gastric toxicity of IM is believed to be due to its ability to inhibit cyclooxygenase. However, data in this study suggest that it is the peroxidase, and not the cyclooxygenase, activity of prostaglandin H synthase that is involved in gastric toxicity. Harvison *et al.* [33] showed that the oxidation of acetaminophen by prostaglandin H synthase in the presence of H_2O_2 (but not arachidonic acid) is not inhibited by IM, indicating that the peroxidase activity of prostaglandin H synthase is not inhibited by IM. These findings suggest that IM acts as a stimulator of lipid peroxidation in the presence of H_2O_2.

Xanthine oxidase induces lipid peroxidation depending on the substrate. SOD effectively inhibits lipid peroxidation [34,35]. The digestive tract provides a large amount of xanthine oxidase. However, IM did not affect the lipid peroxidation induced by xanthine oxidase with hypoxanthine and ADP-Fe^{3+} (data not shown). Xanthine oxidase probably does not contribute to lipid peroxidation induced by IM in the digestive tract. Gastric peroxidase, but not xanthine oxidase, may have an important role in gastric mucosal damage induced by IM.

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