Mechanism of Superoxide Generation System in Indomethacin-Induced Gastric Mucosal Injury in Rats

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We studied the mechanism of the superoxide generation system in indomethacin-induced gastric mucosal injury. First, 10 mM indomethacin had no direct effect on xanthine oxidase (XOD) activity. Next, we found that NADPH oxidase activity in polymorphonuclear leukocytes (PMN) of peripheral blood was significantly increased 6h after administration of indomethacin. This phenomenon was inhibited by the injection of the NADPH oxidase inhibitor, diphenylene iodonium chloride (DIC). Activation of NADPH oxidase caused the component, p47phox, to be translocated to the plasma membrane. Since indomethacin did not directly activate NADPH oxidase, we sought another route of activation of PMN. As IL-1 and TNF α play in the inflammation, we examined these cytokines in this study. TNF α was not detected but IL-1 was increased significantly 30 min after administration of indomethacin.

Key words indomethacin; PMN; IL-1; NADPH oxidase; XOD

Indomethacin and several other nonsteroidal anti-inflammatory drugs (NSAID) are known to produce gastric mucosal injury. The pathogenesis of NSAID-induced gastric mucosal injury is reported for inhibition of prostaglandin (PG) biosynthesis and depletion of gastric mucosal blood flow. A number of studies have highlighted the importance of alterations in mucosal blood flow after NSAID administration. By this depletion of gastric mucosal blood flow, polymorphonuclear leukocytes (PMN) were infiltrated to gastric mucosal tissue. We clarified these phenomena that PMN infiltrates, generates oxygen radicals and induces direct gastric mucosal injury, and then increasing lipid peroxidation further aggravate damage in previous study. We demonstrated that neutrophil-derived free radicals are also important factors in the gastric mucosal injury induced ischemia-reperfusion in pylorus-ligated rats. But the generation system of oxygen free radicals is not clear in the indomethacin-induced-gastric mucosal injury. We examined this and report here the mechanism of pathogenesis in this type of injury.

Superoxide radicals have several generation systems, with activation of xanthine–xanthine oxidase (XOD) enzyme in vein cellular tissue and activation of NADPH oxidase enzyme in PMN being the main ones. The direct effect of indomethacin on XOD activity in vitro and NADPH oxidase in ex vivo was therefore examined.

Cytokines, IL-1 and TNF α are well known to play important roles in inflammation. The first step of PMN infiltration into an inflammation site is believed to take place in many cytokines and the growth binding factors IL-1 and TNF α from mast cells and macrophages. We measured cytokines to learn whether indomethacin acts to directly activate the oxygen radical generation system or is a second any effect.

MATERIALS AND METHODS

Animals Male Donryu rats (SPF) 8 weeks old were obtained from Charles River Co., Tokyo, and ICR mice (SPF) 8 weeks old were obtained from SLC Japan Co., Tokyo.

Materials Indomethacin, ferricytochrome C (horse heart type III), NADPH Na (type III), NBT and BCIP were obtained from Sigma, and xanthine oxidase solution from Boehringer Mannheim Yamanouchi, Tokyo. Diphenylene iodonium chloride was from ALEXIS Co., U.S.A.; Bio-Rad protein assay was from Transduction Laboratories and mouse IL-1 and the TNF α assay system were obtained from Genzyme. Other chemicals were of reagent grade and were used without purification.

Assay of Xanthine Oxidase (XOD) Activity XOD reduced cytochrome c was monitored spectrophotometrically at 550 nm. The reaction mixture consisted of 100 mM potassium phosphate buffer (pH 7.4) (1.2 ml), 0.1 mM cytochrome c (0.2 ml), 5 mM xanthine (0.2 ml) and 0.1 U/ml xanthine oxidase solution (0.2 ml). This mixture was preincubated for 5 min at 37°C and indomethacin was added to a final concentration of 0.1, 0.5, 1, 5, or 10 mM. Indomethacin was dissolved by 100% ethanol solution and finally diluted with distilled water at <1% ethanol concentration.

Gastric Mucosal Lesions Induced by Indomethacin Rats were deprived of food, but given water, for 24h before an experiment. Gastric mucosal damage was induced by oral administration of indomethacin at a dose of 30 mg/kg. The groups were then killed at 6h after administration. The NADPH oxidase inhibitor, diphenylene iodonium chloride (DIC) at a dose of 125 mg/kg was administered i.p. at 30 min before administration of indomethacin.

In another experiment, mice were deprived of food, but given water for 9h before it began. Gastric mucosal damage was induced in six 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: 0.5, 1, 3, 6h. Non-treated rats were killed 6h after administration of a vehicle. Serum was collected each time.

Preparation of NADPH Oxidase from Polymorphonuclear Leukocyte (PMN) in Peripheral Blood Rats were treated previously by 30 mg/kg indomethacin. Blood was collected with heparin from the celiac artery. Blood was centrifuged with 2% dextran (blood : dextran = 20 : 13).

The conjugation was allowed to stand 20 min at room temperature. The upper layer was removed and freed of erythrocytes by hypotonic lysis. The proportion of PMN obtained in...
this manner averaged 95%.

PMN was resuspended in 1 ml of ice-cold 0.34 M sucrose containing 0.05 M PMSF and the cells were disrupted at 0 °C. After disrupting the cells, the preparation was centrifuged at 250×g for 30 min at 4 °C to remove nuclei and unbroken cells. The supernatant was then centrifuged at 30000×g at 30 min at 4 °C. The pellet, which contains the O$_2^-$ forming activity, was resuspended in 1 ml of sucrose.

**Measurement of NADPH Oxidase** The oxidase activity was measured by determining the rate of O$_2^-$ formation in the presence of NADPH. The method was based on the spectrophotometric determination of superoxide-mediated ferricytochrome c reduction as originally described by Asada et al. To a tube containing 0.5 ml of 0.1 M potassium phosphate buffer (pH 7.4) was added 0.2 mM flavin-adenine dinucleotide (FDA) (0.1 ml), 1.0 mM NADPH (0.1 ml), 0.2% Triton X-100 solution (0.1 ml), 0.1 mM cytochrome c (0.1 ml) and 0.1 ml D.W. This mixture was incubated for 5 min at 37 °C.

To this solution 0.2 ml of O$_2^-$ forming particles was added, and this was incubated for 30 min at 37 °C. The reaction was stopped by adding 10 μl of SOD solution and centrifugation followed at 3000 rpm 4 °C for 10 min. The amount of reduced cytochrome c was measured spectrophotometrically (550 nm).

The NADPH oxidase inhibitor DIC was used in the same way on gastric mucosal lesions induced by indomethacin.

**Measurement of Cytokine of Mouse Serum** IL-1 and TNF α were measured by a kit made by the Genzyme Co., Ltd. ICR male mice were administered indomethacin at dose of 30 mg/kg p.o., and 0.5, 1, 3, 6 h later blood was collected from a vein. Serum was separated and IL-1 and TNF α were measured according to the kit method.

**Detection of p47phox Using Immunoblotting** Membrane fractions and cytoplasmic fractions were separated from PMN in the peripheral blood. The fraction mixture was added in the same volume as the sample buffer (10% SDS solution: Tris–HCl buffer pH 6.8: glycerol: 2-mercaptoethanol=6:1:2:1) and boiled for 5 min at 100 °C. Fifteen microliters of the sample was applied to 5—20% SDS page (Marisol Co., Ltd.). The low molecular weight marker was used which was made by Pharmacia Co., Ltd.). The low molecular weight marker was added which was made by Pharmacia Co., Ltd.. After electrophoresis, band the sample was applied to 5—20% SDS page (Marisol Co., Ltd.). The low molecular weight marker was used which was made by Pharmacia Co., Ltd.. After electrophoresis, band

**Statistical Method** The results were calculated as mean values±standard error (mean±S.E.). Differences between treatment groups were assessed using an analysis of variance followed by Dunnett’s multiple range test or Student’s t test. Probability values <0.05 were considered statistically significant.

**RESULTS**

As shown in Fig. 1, 8.7 nmol of O$_2^-$ was released in 30 min by xanthine-XOD activity. Treatment with 0.1 and 0.5 mM indomethacin caused about 24.4 and 21.4% inhibition but not significant. O$_2^-$ release were increased by treatment with 1, 5 and 10 mM indomethacin, but treatment with 10 mM indomethacin was not even significant.

The total area of gastric mucosal injury was measured 6 h after indomethacin administration, and length of the injury was determined by vernier micrometer. The results are shown in Table 1. The change was significantly inhibited by treatment with NADPH oxidase inhibitor, diphenylene iodonium chloride (DIC) (p<0.001).

As shown in Fig. 2, NADPH oxidase activity was measured in rat polymorphonuclear leukocyte (PMN) of the celiac artery. A significant increase was recognized found 4, 6 h after indomethacin administration. Administration of DIC

![Fig. 1. The Effect of Indomethacin on XOD Activity in Vitro](image)

**Table 1. Effect of Diphenylene Iodonium Chloride (DIC) on Indomethacin Induced Gastric Ulcer Index**

<table>
<thead>
<tr>
<th>Indomethacin 30 mg/kg p.o. + saline i.p.</th>
<th>Ulcer index (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indomethacin 30 mg/kg p.o. + DIC 125 mg/kg i.p.</td>
<td>61.72±9.29 ***</td>
</tr>
</tbody>
</table>

Gastric mucosal injury index was measured at 6h after indomethacin administration in rats. Each value is the mean±S.E. from 6 determinations. ***p<0.001 significantly different from saline treated rat.

![Fig. 2. Time Course of NADPH Oxidase Activity in Rat Polymorphonuclear Leucocytes of Peripheral Blood after Indomethacin Administration](image)
We previously reported that a number of studies have highlighted the importance of alterations in mucosal blood flow after nonsteroidal anti-inflammatory drug (NSAID) administration in the pathogenesis of ulceration in several experimental methods.3,5 These observations suggest a role for circulating leukocytes in the pathogenesis of NSAID-induced ulceration. We have reported a previous study7 on this, but in this report, XOD activity in gastric mucosa was increased immediately after indomethacin administration. We therefore examined the direct effect of indomethacin on XOD and found that XOD was not affected by indomethacin at a dose of 0.1—10 μM in vitro (Fig. 1). The rise in XOD activity appeared to be a secondary effect.

The activation of neutrophils is known to be accompanied by the release of oxygen free radicals (through the NADPH oxidase system).10 In the previous study,7 PMN in peripheral blood showed enhanced O₂⁻ release when stimulated by zymosan after administration of indomethacin. And then PMN-depleted rats were not induced gastric damage by administration of indomethacin. We thus believed that indomethacin induced gastric mucosal injury occurred as a result of oxygen free radical generation from PMN. In fact, in this report, indomethacin induced gastric mucosal injury was inhibited by treatment of the NADPH oxidase inhibitor, 125 mg/kg DIC (Table 1). We also previously reported that a high concentration of indomethacin (1 mM) did not inhibit O₂⁻ production by NADPH oxidase.11 Here, we examined the direct effect of indomethacin on XOD oxidase once more and found that 10 mM indomethacin did not affect XOD activity in gastric mucosa. Six hours after the administration of indomethacin, NADPH oxidase activity was significantly increased in rat PMN of peripheral blood. This phenomenon was correlated with the gastric mucosal injury index (correlation coefficient, r=0.985); this increase was inhibited by 125 mg/kg DIC i.p. Activation of NADPH oxidase caused the component, p47phox to be translocated to the plasma membrane (Fig. 5). DIC is NADPH-dependent flavoprotein inhibitor. DIC directly inhibits the activity of a variety of flavoprotein.13 Exactly p47phox activity was inhibited by DIC administration. Since indomethacin did not directly activate NADPH oxidase (data not shown), we sought another route of activation of PMN. Again, the rise in NADPH oxidase activity seemed second to be any effect. These observations suggested that another NADPH oxidase and XOD activating factor was related to the indomethacin induced ulceration.

Cytokines such as IL-1 and TNFα are known to play an important role in inflammation.9 Ding et al.14 and Appleyard et al.15 reported that plasma TNFα level was increased significantly after indomethacin administration, and suggested that TNFα plays a critical role in indomethacin-induced gastric mucosal damage. Welborn et al.16 and Garcia-

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**DISCUSSION**

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**Fig. 3. NADPH Oxidase Activity in Rat Polymorphonuclear Leukocytes of Peripheral Blood after Indomethacin Administration**

Each value is the mean±S.E. from 6 determinations. NADPH oxidase activity was measured 6 h after indomethacin administration. *p<0.01 significant difference from non-treated rat. Differences between treatment groups were compared by using an analysis of Dunnett's, multiple range test.

**Fig. 4. IL-1 and TNFα Concentration in Mouse Serum after Indomethacin Administration**

Each value is the mean±S.E. from 6 determinations. Differences between treatment groups were compared using an analysis of student's t test. **p<0.01 significantly different from time 0.

**Table 2. The Effect of DIC on IL-1 Concentration in Mouse Serum at 30 min after Indomethacin Administration**

<table>
<thead>
<tr>
<th>Condition</th>
<th>IL-1 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indomethacin 30 mg/kg p.o.+ saline i.p.</td>
<td>91.80±17.20 N.S.</td>
</tr>
<tr>
<td>Indomethacin 30 mg/kg p.o.+ DIC 125 mg/kg i.p.</td>
<td>97.80±6.68 N.S.</td>
</tr>
</tbody>
</table>

IL-1 concentration was measured at 30 min after indomethacin administration. Each value is the mean±S.E. from 6 determinations. Difference between DIC administration group was compared using an analysis of student's t test. N.S. = not significant.

**Fig. 5. Translocation of NADPH Oxidase Components in Neutrophils Stimulated with Indomethacin**

At 6 h after 30 mg/kg indomethacin administration, neutrophil collected from peripheral blood. p47phox in neutrophil was detected by immunoblot method. I, indomethacin; D, diphenylene iodonium chloride; C, non-treated; M, membrane; Cy, cytosol

inhibited this activity and returned it to the control level (Fig. 3). As shown Fig. 4, IL-1 activity in serum was significantly increased 0.5 h after indomethacin administration. DIC was no effect to this change (Table 2). After 3 h, the IL-1 level returned to 0, while, TNFα activity in serum remained stationary.

We then investigated the effect of indomethacin on translocation of NADPH oxidase components from cytosole to the plasma membrane in neutrophils. As shown in Fig. 5, indomethacin aggravated the translocation of these components. Thirty minutes prior, 125 mg/kg DIC was injected intraperitoneally inhibited translocation of NADPH oxidase components to the plasma membrane. Control animals received the vehicle.

**Fig. 6. Concentration of IL-1 in Serum at 30 min after Indomethacin Administration**

Each value is the mean±S.E. from 6 determinations. Difference between DIC administration group was compared using an analysis of student's t test. N.S., not significant.
Criado et al.\textsuperscript{17) reported that plasma TNF \(\alpha\) and IL-1 level were significantly increased on the ischemia-reperfusion model. So we examined whether they have an influence on indomethacin induced gastric mucosal injury. TNF was not detected in serum until 6h after administration of indomethacin \textit{p.o.}, although IL-1 was increased immediately after this administration. After rising, the IL-1 concentration decreased immediately. And IL-1 activation was unaffected by DIC. It seemed that rising IL-1 level have an influence on PMN activation. Exactly, Leuckocyte adhesion molecule (ICAM-1) was induced 2—4 h after IL-1 treatment.\textsuperscript{18,19) NADPH oxidase activity in rat PMN of peripheral blood was significantly increased from 4 h after indomethacin administration. So we considered that NADPH oxidase activation from 4 h after indomethacin administration depend on IL-1 activation.

In conclusion, a high concentration of indomethacin seemed to reduce blood flow and activated mast cell, macrophage and monocyte release of IL-1 in this study. The rise in IL-1 level in plasma seemed to have an influence on XOD activity in blood vessel endothelium and NADPH oxidase in PMN. This activation releases oxygen free radicals immediately, so that oxygen free radical generation from PMN and blood vessel endothelium appeared to induce gastric mucosal injury.

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\textbf{REFERENCES}