Roles of NADPH Oxidase in Occurrence of Gastric Damage and Expression of Cyclooxygenase-2 During Ischemia/Reperfusion in Rat Stomachs

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Abstract. NADPH oxidase is an enzyme that converts molecular oxygen into reactive oxygen species, which cause severe damage in several organs. Cyclooxygenase (COX)-2 is an inducible enzyme that is important in gastric mucosal defense and repair processes. It is unclear whether NADPH oxidase is related to COX expression in the gastric mucosa, so we investigated the correlation. Under urethane anesthesia, a male Sprague Dawley rat stomach was mounted in an ex-vivo chamber, and ischemia/reperfusion (I/R) was performed through a cannula in the femoral vein. I/R significantly increased NADPH oxidase activity, \(\text{H}_2\text{O}_2\) production, and myeloperoxidase (MPO) activity. In contrast, ischemia alone clearly enhanced both NADPH oxidase activity and \(\text{H}_2\text{O}_2\) production but not MPO activity. Pretreatment with the NADPH oxidase inhibitor diphenylene iodonium (DPI) suppressed I/R-induced mucosal damage. On the other hand, the selective COX-2 inhibitor rofecoxib exhibited a tendency to enhance the severity of gastric damage induced by I/R, although the selective COX-1 inhibitor SC-560 and the nonselective COX inhibitor indomethacin had no effect. I/R also increased the expression of COX-2, and this increase was suppressed by pretreatment with DPI. These findings suggest that the increase in NADPH oxidase activity is involved in the occurrence of gastric mucosal damage induced by I/R and that this enzyme activity may be causally related to the upregulation of COX-2 during I/R.

Keywords: reactive oxygen species, ischemia/reperfusion (I/R), NADPH oxidase, cyclooxygenase (COX), rat gastric mucosa

Introduction

In several organs, including the heart, brain, lungs, and gastrointestinal tract, reactive oxygen species such as superoxide, hydrogen peroxide, and hydroxyl radical cause inflammatory responses and tissue damage. Reactive oxygen species have two reciprocal effects, sterilizing several types of bacteria and damaging surrounding tissues by fragmenting their cellular DNA. In the gastrointestinal tract, reactive oxygen species are generated by several types of inflammatory responses, including those induced by nonsteroidal anti-inflammatory drugs (NSAIDs), cold stress, and irritative agents, as well as \textit{Helicobacter pylori} infection in the gastric mucosa (1–5). Ischemia/reperfusion (I/R) is suggested to cause acute gastric mucosal lesions via oxidative stress, so we examined the involvement of NADPH oxidase in the oxidative stress induced by I/R in rat stomachs.

NADPH oxidase is distributed in several cell types such as phagocytic cells, vascular muscular cells, endothelial cells, fibroblasts, and adipocytes (6–10); it converts molecular oxygen into superoxide anions. NADPH oxidase consists of two membrane-integrated enzymes, including those induced by nonsteroidal anti-inflammatory drugs (NSAIDs), cold stress, and irritative agents, as well as \textit{Helicobacter pylori} infection in the gastric mucosa (1–5). Ischemia/reperfusion (I/R) is suggested to cause acute gastric mucosal lesions via oxidative stress, so we examined the involvement of NADPH oxidase in the oxidative stress induced by I/R in rat stomachs.

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components and four cytosolic components (11). We have previously reported that I/R caused hemorrhagic damage by generating reactive oxygen species via activation of NADPH oxidase, which is distributed in the gastric mucosa (12).

On the other hand, cyclooxygenase (COX) has two different isoforms, COX-1 and COX-2. COX-1 is a housekeeping enzyme that is constitutively expressed in most tissues and especially in the stomach. It forms prostaglandins (PGs) that contribute to the maintenance of mucosal integrity (13, 14), while COX-2 is an inducible enzyme that is up-regulated by proinflammatory cells, like neutrophils and macrophages, and mediates inflammatory reactions and tumor growth (15, 16). It has been reported that COX-2 is important for gastric mucosal defense and repair processes against I/R injuries (17, 18). Recently, some reports have suggested that NADPH oxidase might regulate COX-2 expression and PG synthesis in vascular smooth muscle cells, esophageal adenocarcinoma cells, and macrophages (19–22). However, it is not clear whether NADPH oxidase is related to the regulation of COX expression in the gastric mucosa damaged by I/R in rats.

In this study, we examined the role of NADPH oxidase in connection with COX-1 and COX-2 expression in rat gastric mucosa.

Materials and Methods

Animals

Male Sprague Dawley rats (220–300 g; Nippon Charles River, Shizuoka) were used. The animals were kept in individual cages with raised mesh bottoms and deprived of food but allowed free access to tap water for 18 h prior to the experiments. Studies were carried out using 3–10 rats per group. The experimental procedures employed in the present study were approved by the Experimental Animal Research Committee of Doshisha Women’s College of Liberal Arts.

Induction of gastric injuries by I/R

Acute gastric mucosal damage was produced by I/R (23, 24). Briefly, under urethane anesthesia (1.25 g/kg, i.p.), a rat stomach was mounted in an ex-vivo chamber, 100 mM HCl was applied every 15 min, and a catheter connected to a syringe was passed through the left femoral vein. After the operation, 4 ml of blood was collected in a syringe, and 30 min later, reperfusion was achieved through the infusion of the collected heparinized blood. After reperfusion for 90 min, the stomach was excised, and the index of gastric corpus damage was calculated using the FLOVEL Filing System (Olympus, Tokyo). Diphenylene iodonium (DPI, a NADPH oxidase inhibitor) was given i.v. 10 min before ischemia; and indomethacin (a nonselective COX inhibitor), SC-560 (a selective COX-1 inhibitor), or rofecoxib (a selective COX-2 inhibitor) was given i.v. 30 min before ischemia.

Measurement of NADPH oxidase activity

NADPH oxidase activity in the gastric mucosa was measured after the rats had been subjected to ischemia or I/R. The chambered stomach was removed, and the gastric mucosa was scraped with glass slides, weighed, and homogenized in 5 ml/g tissue of an isotonic extraction buffer [10 mM HEPES (pH 7.8), 250 mM sucrose, 25 mM potassium chloride, and 1 mM EGTA] at 4°C. They were incubated at 4°C for 30 min and then centrifuged at 12,000 × g for 15 min at 4°C, and the supernatant was used as the sample solution for the cytochrome c reductase (NADPH) assay kit. The absorbance of each sample was measured at 549 nm in a spectrometer (UV mini 1240; Shimadzu, Kyoto) and was expressed as units per mg protein (one unit will reduce 1.0 μM of oxidized cytochrome c in the presence of 100 μM NADPH per min at pH 7.8 and 25°C).

Measurement of H2O2 production

H2O2 production in the gastric mucosa was measured after ischemia or I/R treatment in the rats. The chambered stomach was removed, and the gastric mucosa was scraped with glass slides, weighed, and homogenized in 5 ml/g tissue of a Krebs-Ringer phosphate buffer (145 mM NaCl, 4.86 mM KCl, 0.54 mM CaCl2, 1.22 mM MgSO4·7H2O, 5.7 mM NaH2PO4·12H2O, 5.7 mM NaH2PO4, and 5.5 mM glucose; pH 7.35) at 4°C. They were incubated at 4°C for 30 min and then centrifuged at 12,000 × g for 15 min at 4°C, and the supernatant was used as a sample solution for the hydrogen peroxide/peroxidase assay kit (Molecular Probes, Inc., Eugene, OR, USA). Absorbance at 540 nm was measured by a microplate reader (Multiskan BICHROMATIC; Labsystems Japan, Inc., Tokyo) and expressed as μmol per g protein.

Measurement of myeloperoxidase (MPO) activity

As an index of neutrophil activation and the reactive oxygen species generated by ischemia or I/R, MPO activity was measured after the experiments according to a modified version of the method of Krawisz et al. (25). The chambered stomach was removed, and the gastric mucosa was scraped with glass slides, weighed, and homogenized in 50 mM phosphate buffer containing 0.5% hexadecyltrimethyl-ammonium bromide (pH 6.0; Sigma-Aldrich, St. Louis, MO, USA). The homogenized samples were subjected to freezing and thawing three times.
times and centrifuged at 600 × g for 10 min at 4°C. MPO activity in the supernatant was determined by adding 100 μl of supernatant to 1.9 ml of 10 mM phosphate buffer (pH 6.0) and 1 ml of 1.5 M α-dianisidine hydrochloride (Sigma-Aldrich) containing 0.0005% (w/v) hydrogen peroxide. The changes in absorbance at 450 nm of each sample were recorded in a spectrometer. Sample protein content was estimated using a protein assay (Nacalai Tesque, Kyoto). MPO activity was obtained from the slope of the reaction curve and expressed as units per g protein.

**COX expression by reverse transcription polymerase chain reaction (RT-PCR)**

The stomachs were removed from chambered rats of the control, ischemia, and I/R groups for the determination of COX-1 and COX-2 mRNA using RT-PCR with specific primers. Immediately after the examinations, the mucosal specimens (about 200 mg) were scraped off on ice using a glass slide, immediately snap frozen in liquid nitrogen, and stored at −80°C until analysis. Total RNA was extracted from mucosal samples using a guanidium isothiocyanate/phenol chloroform single-step extraction kit from TaKaRa Bio, Inc. (Shiga) based on the method described by Chomczynski and Sacchi (26).

Single-stranded cDNA was generated from 5 μg of total RNA using superscript reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random-primers (TaKaRa Bio). The polymerase chain reaction mixture was amplified in a DNA thermal cycler (Thermo Scientific, Inc., Waltham, MA, USA). The nucleotide sequences of the primers were as follows: β-actin: sense, 5’ TTG TAA CCA ACT GGG ACG ATA TGG 3’ and antisense, 5’ GAT CTT GAT CTT CAT GGT GCT AGG 3’; COX-1: sense, 5’ CTT CAT GTG GCT GTG GAT GTC ATC 3’ and antisense, 5’ GGT CTT GGT GAG GCA GAC CAG 3’; COX-2: sense, 5’ TGA TGA CTG CCC AAC TCC CAT G 3’ and anti-sense, 5’ AAT GGT TGA GGT TGC CGG CAG C 3’. The primer sequences for β-actin, COX-1, and COX-2 were based on the sequences of their published cDNA (17, 27). The polymerase chain reaction products were detected by electrophoresis on a 1.5% agarose gel in Tris–EDTA–acetic acid buffer containing ethidium bromide. The location of the predicted products was confirmed using a 100-bp ladder (Nacalai Tesque) as a standard size marker. The gel was then photographed under UV transillumination. The signals for COX mRNA were standardized against the β-actin signal for each sample, and the results were expressed as the ratio of COX-1 or COX-2 mRNA to β-actin mRNA.

**Preparation of drugs**

The drugs used were urethane (Tokyo Kasei, Tokyo), SC-560 (Cayman Chemical, Ann Arbor, MI, USA), rofecoxib (Toronto Research Chemicals, Inc., North York, Canada), and DPI (Sigma Chemicals, St. Louis, MO, USA). DPI was dissolved in saline, and indomethacin, SC-560, and rofecoxib were suspended in saline with Tween80 (Wako, Osaka). Each agent was prepared immediately before use. DPI was administered intravenously in a volume of 0.1 ml, while indomethacin, SC-560, and rofecoxib were administered subcutaneously in a volume of 0.5 ml per 100 g body weight.

**Statistics**

Data are presented as the mean ± S.E.M. of 3–10 rats per group. Statistical analyses were performed by the two-tailed Student’s t-test, and values of P<0.05 were regarded as significant.

**Results**

**Changes in NADPH oxidase activity and H2O2 production induced by ischemia and I/R in the gastric mucosa**

The level of NADPH oxidase activity was 0.26 ± 0.07 U/mg protein in the sham group, and it was significantly activated immediately after 30-min ischemia treatment (0.50 ± 0.04 U/mg protein) as well as after the combination of treatment of 30 min I/R for 90 min (0.59 ± 0.05 U/mg protein) (Fig. 1). Gastric H2O2 production was also significantly increased after ischemia (5.14 ± 0.98 μmol/g protein) as well as after I/R (4.11 ± 1.02 μmol/g protein) compared with the sham treatment (1.44 ± 0.27 μmol/g protein) (Fig. 2).

![Fig. 1. Change in NADPH oxidase activity induced by ischemia or ischemia/reperfusion (I/R) in rat gastric mucosa. After 30-min ischemia or I/R, the gastric mucosa was scraped, and the homogenate was used for measuring NADPH oxidase activity. Samples were measured at 549 nm, and the absorbance was expressed as units per mg protein. Mean ± S.E.M. (n = 4 – 6). *P<0.05, compared to sham.](image-url)
Change in MPO activity induced by ischemia and I/R in the gastric mucosa

To exclude the possibility that NADPH oxidase activity in the gastric mucosa might be dependent on neutrophil migration, we examined MPO activity in the gastric mucosa immediately after ischemia alone or I/R. MPO activity was significantly increased by I/R (16.39 ± 2.78 U/g protein) compared with sham treatment (6.57 ± 0.27 U/g protein), but was not increased by ischemia alone for 30 min (6.42 ± 0.07 U/g protein) (Fig. 3). Furthermore, ischemia alone for 120 min did not have any effect on MPO activity (8.897 ± 3.11 U/g protein).

Effect of COX inhibitors on NADPH oxidase activation induced by I/R in the gastric mucosa

The NADPH oxidase activation induced by I/R was significantly decreased by the injection of DPI. SC-560 caused a significant enhancement of NADPH oxidase activation induced by I/R, and indomethacin and rofecoxib also exhibited a tendency to enhance the NADPH oxidase activation induced by I/R (P = 0.123 and P = 0.226, respectively) (Fig. 5).
The expression of COX-2 mRNA was significantly detected in the gastric mucosa after it had been damaged by I/R, but COX-2 mRNA expression in ischemia alone for 30 min was as little as that in sham. The increase in COX-2 mRNA expression was almost totally suppressed by DPI (Fig. 6: A and B). In contrast, the expression of COX-1 mRNA was observed in the normal gastric mucosa (sham), and this expression was not affected by any treatment (Fig. 6: A and C). On the other hand, the expression of COX-2 mRNA was clearly enhanced by ischemia alone 120 min later, and this enhancement was also significantly prevented by DPI (Fig. 7: A and B). The expression of COX-1 mRNA was not affected by these treatments (Fig. 7: A and C).

Discussion

The generation of reactive oxygen species plays an important role in gastrointestinal damage. In the stomach, it has been demonstrated that reactive oxygen species are involved in ethanol-, NSAIDs-, stress-, and Helicobacter pylori–related gastritis, as well as in I/R-induced gastric injuries (2 – 4, 28). I/R is used as a model of the gastric mucosal hemorrhagic damage through the generation of reactive oxygen species originating from leukocyte and neutrophil filtration (29, 30) or the xanthine/xanthine oxidase system (31).

Ischemia in the early stage of I/R is an especially
important factor for building up resistance to the deleterious effects of prolonged I/R in the heart (32). The preconditioning of organs remote from the stomach exerts a potent protective influence on gastric mucosa subjected to prolonged I/R (33). Therefore, it is conceivable that the understanding of the effects of brief ischemia is greatly significant to the gastric mucosa.

NADPH oxidase was discovered as an enzyme that produces reactive oxygen species. It is composed of a membrane-integrated cytochrome b$_{558}$ (a heterodimer formed by gp91$^{phox}$ and p22$^{phox}$) and cytosolic components (p47$^{phox}$, p67$^{phox}$, and the small GTPase Rac) in phagocytes, in which the oxidase was first identified (11). Monocyte differentiation into macrophages, possibly triggered by unquenched reactive oxygen species, may contribute to the increased inflammatory responses associated with atheromata (34). Recently, several homologs of gp91$^{phox}$ have been identified and were named the NADPH oxidase/dual oxidase family (35, 36).

It has been reported that guinea-pig gastric mucosal cells in primary culture express Nox1 and spontaneously produce reactive oxygen species (37, 38) and that the human gastric mucosa expresses Nox2, Nox5, and NOXA1 in normal mucosa (39, 40). The Nox1 and NOX01 present in gastric glands and cancer cells are known to be associated with chronic inflammation such as Helicobacter pylori–associated diseases (37, 41 – 43). Recently, it was suggested that Nox1-derived reactive oxygen species regulate local innate immunity, inflammation, cell growth, and vascular tone and contribute to carcinogenesis (44).

In this study, NADPH oxidase activity was measured using the cytochrome c reductase assay, which monitors the cytochrome b$_{558}$ of NADPH oxidase, and it was found to be enhanced during ischemia alone as well as I/R. Significant facilitation of MPO activity in the gastric mucosa occurred after I/R, but not after ischemia, resulting in a reduced possibility of the involvement of the NADPH oxidase present in neutrophils in the early stage of I/R. From the results shown in Figs. 1 – 3, it was suggested that the development of gastric damage induced by I/R was involved in the activation of NADPH oxidase, which was localized in the gastric mucosa, but not in migrated neutrophils (45 – 47). These results are in agreement with our previous study showing that gastric NADPH oxidase is involved in the development of I/R-induced gastric damage in rats (12). Yet, the physiological and pathological roles of Nox1 in I/R are not clear in rat gastric mucosa.

NSAIDs, inhibitors of PGs, which regulate mucosal integrity and inflammatory responses, are highly effective drugs, but their use is frequently associated with

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**Fig. 7.** Effect of a NADPH oxidase inhibitor on the COX expression induced by ischemia for 120 min in rat gastric mucosa. After ischemia for 120 (Ischemia120), the gastric mucosa was scraped, and the homogenate was used for generating single standard cDNA by RT-PCR. Diphenylene iodonium (DPI: 1 mg/kg) was given i.v. 10 min before ischemia. A) The PCR products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide. The gel was photographed under UV transillumination. B) The figure shows the ratio of COX-2 mRNA to β-actin mRNA. Mean ± S.E.M. (n = 3 – 5). *P<0.05, compared to sham; *P<0.05, compared to ischemia120. C) The figure shows the ratio of COX-1 mRNA to β-actin mRNA. Mean ± S.E.M. (n = 3 – 5).
side effects that induce gastrointestinal damage and impair the ability of the mucosa to defend itself against luminal challenges. This impairment is, in part, caused by the reduction of mucosal blood flow, mucus secretion, bicarbonate production, and epithelial cell turnover that occurs with PG suppression (48–50). The selective inhibition of COX-2 does not affect gastric PG synthesis derived from COX-1 and is associated with a greatly reduced incidence of gastric erosion compared with that observed with NSAIDs (51). As for the role of COX-1 in the stomach, some reports have demonstrated that selective inhibition or deletion of COX-1 resulted in substantial suppression of gastric PGs synthesis and decreased mucosal injury (52–56). On the other hand, as for the effects of selective COX-2 inhibitors on the adaptive response of the gastric mucosa to a topical irritant, several reports have suggested that the presence of COX-2 in inflammatory gastric mucosa contributes to mucosal defense and healing (18, 57, 58).

Under the experimental conditions, the increased COX-2 expressed in the endothelial cells played a protective role against gastric injury induced by I/R (17, 59). However, COX-1 has demonstrated inconsistent findings with regard to its role in gastric mucosa damaged by I/R with some studies assigning it a protective or neutral role (33, 60) and another giving it an aggressive role (59). The discrepancy between these results could be attributed to differences among animal species, methods of I/R induction, doses of SC-560 chosen, durations of the ischemia and reperfusion, and experimental conditions employed. Normally, animals that have suffered gastric damage would be chosen and used as clamping models, but in this study, the rats were used as cannulated models that physiologically approximated clinical ischemia pathology.

In this study, we showed that the expression of COX-2 was significantly upregulated by I/R, and this upregulation was significantly prevented by DPI. Interestingly, we further observed that ischemia alone for 120 min induced upregulation of COX-2 expression in the stomach, and this response was also significantly suppressed by DPI. However, ischemia alone did not cause any macroscopic gastric damage and change in MPO activity even 120 min later. Barbieri et al. (61) demonstrated that attenuation of NADPH oxidase occurred to prevent NF-κB activation and COX-2 expression. Thus, it is likely that the activation of NADPH oxidase may induce the upregulation of COX-2 in the gastric mucosa during I/R and ischemia. Further studies are needed to elucidate this point in more detail.

In this study, we found that several COX inhibitors showed a tendency to enhance the activation of NADPH oxidase during I/R. Likewise, Li et al. (20) reported that COX inhibition by NSAIDs enhanced the expression of vascular and cardiac NADPH oxidase in spontaneous hypertensive rats and human endothelial cells. These findings suggest that PGs derived from either COX-1 or COX-2 may affect negatively NADPH oxidase. In contrast, we observed that the severity of damage induced by I/R was aggravated, although not significantly, by rofecoxib, while slightly suppressed by SC-560. The aggravation of damage by rofecoxib was also significantly attenuated by DPI. Several reports demonstrated the upregulation of COX-2 accompanied with an ischemic period in gastric mucosa resulted in mucosal protection (62, 63). Thus, it is assumed that the upregulation of COX-2/PGs during I/R may play a protective role in the gastric mucosa, at least in part, through inhibition of NADPH oxidase. It is well known that COX-1 is coupled with the generation of thromboxanes, which are a potent vasoconstrictor of gastric vasculature (64) and are involved in the pathogenesis of gastric mucosal injury (65). Indeed, it has been reported that inhibition of thromboxane production suppressed the severity of gastric damage induced by water-immersion stress in rats (66). Thus, it is possible that the suppressive effect of SC-560 on I/R-induced gastric damage observed in this study may be due to inhibition of thromboxane production. Further studies are needed to elucidate the role of COX isozymes in the gastric mucosa during I/R.

In conclusion, the increase in NADPH oxidase activity is involved in the occurrence of gastric mucosal damage induced by I/R. Furthermore, this enzyme activity may be causally related to the upregulation of COX-2 expression in the gastric mucosa during I/R.

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References


