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EVIDENCE FOR THE INVOLVEMENT OF NADPH OXIDASE IN ISCHEMIA/REPERFUSION-INDUCED GASTRIC DAMAGE VIA ANGIOTENSIN II

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Reactive oxygen species are known to be derived from NADPH oxidase in several tissues. Angiotensin II was suggested to be involved in the activation of NADPH oxidase; however, its role in the gastric mucosa is unclear. We examined the roles of angiotensin II receptor and NADPH oxidase in ischemia/reperfusion-induced gastric damage in rats. Under urethane anesthesia, male Sprague-Dawley rat stomachs were mounted in an *ex-vivo* chamber, had 100 mM HCl applied to them, and then a catheter was passed through the femoral vein. Ischemia/reperfusion was accompanied by blood collection and reperfusion through the catheter. Losartan, candesartan, valsartan, which are AT1 receptor blockers (ARB); PD123319, an AT2 receptor blocker; enalapril, an ACE inhibitor; or diphenylene iodonium, a NADPH oxidase inhibitor, was given i.v. 10 mins, and β -NADPH, a NADPH oxidase substrate, was given i.v. 5 mins before reperfusion. The gastric damage by ischemia/reperfusion was attenuated by treatment with any of ARB or enalapril, but was not affected by PD123319. The increase in gastric H₂O₂ production and microvascular permeability by ischemia/reperfusion was also suppressed by treatment with any of ARB or enalapril. In rat gastric mucosa, the NADPH oxidase subunit p47^{phox} was detected. Additionally, diphenylene iodonium had similar effects to ARB against ischemia/reperfusion-caused gastric damage, increased H₂O₂ production, and microvascular permeability. Ischemia/reperfusion activated NADPH oxidase in the gastric mucosa, and the activation was significantly attenuated by treatment with losartan or diphenylene iodonium. These results suggest that ischemia/reperfusion generated reactive oxygen species are derived from NADPH oxidase activation *via* AT1 receptor in rat stomachs.

Key words: *ischemia/reperfusion, gastric damage, reactive oxygen species, AT1 receptor, NADPH oxidase*

INTRODUCTION

Biological inflammatory responses play a crucial role in the immunology and pathology of most tissues including the gastrointestinal tract. In the stomach, inflammation is caused by a variety of factors including acid, stress, and *Helicobacter pylori* (*H. pylori*) and triggers the generation of reactive oxygen species such as superoxide, hydrogen peroxide (H₂O₂), and the hydroxyl radical. Reactive oxygen species have the reciprocal effects of sterilizing several bacteria but damaging the surrounding tissues, resulting in morbid inflammation. It has been reported that reactive oxygen species affect both the acute damage caused by nonsteroidal anti-inflammatory drugs (NSAID), cold stress, and irritative agents as well as the chronic damage induced by *H. pylori* infections in the gastric mucosa (1-4). Ischemia/reperfusion is suggested to cause gastric mucosal damage *via* the generation of reactive oxygen species by leukocyte migration and neutrophil infiltration. The conversion of molecular oxygen into superoxide anions by multicomponent NADPH oxidase is now recognized as the most important source of reactive oxygen species in both endothelial cells and vascular smooth muscle cells (5, 6). Some reports have suggested that NADPH oxidase is involved in the generation of reactive oxygen species in several cells (endothelial

cells, phagocytic cells, fibroblasts, and adipocytes) (7-9). NADPH oxidase is composed of two types of subunits, the membrane-binding subunit flavocytochrome b₅₅₈, which is composed of p22^{phox} and gp91^{phox}, and soluble cytosolic subunits such as p47^{phox}, p67^{phox}, and p40^{phox}. When cells possessing NADPH oxidase are stimulated by endogenous or exogenous shock, NADPH oxidase was activated through cytosolic subunit translocation and binding to the membrane subunits (10-12).

Angiotensin II, which is synthesized through the rennin-angiotensin system, is known to exert several effects: it increases the expression of adhesion molecules and chemokines and the generation of reactive oxygen species, causing vasoconstriction in the cardiovascular system (13, 14). Angiotensin AT1 receptor blockers (ARB) and angiotensin-converting enzyme (ACE) inhibitors are commonly used as antihypertensive drugs that suppress angiotensin II. As for ARB, it has been reported that blocking the AT1 receptor improved hypertension *via* the suppression of reactive oxygen species (15). NADPH oxidase is involved in the angiotensin II-regulated generation of reactive oxygen species in vascular smooth muscle cells (16). In the gastrointestinal tract, angiotensin II is involved in gastric damage *via* vasculature constriction mediated through AT1 receptor stimulation (17, 18). However, it is still unclear in

which cells of the gastric mucosa angiotensin II affects the generation of reactive oxygen species.

In the present study, we examined the relationship between angiotensin II and NADPH oxidase in ischemia/reperfusion-induced gastric damage and the role of NADPH oxidase in the pathogenesis of gastric damage.

MATERIAL AND METHODS

Animals

Male Sprague-Dawley (SD) rats (240-290 g, Nippon Charles River, Shizuoka, Japan) were used. The animals were kept in individual cages with raised mesh bottoms and were deprived of food but allowed free access to tap water for 18 hrs prior to the experiments. The studies were carried out using 3-7 rats per group. The experimental procedures employed in the present study were approved by the Experimental Animal Research Committee of the Doshisha Women's College of Liberal Arts.

Induction of gastric damage by ischemia/reperfusion and measurement of gastric mucosal blood flow (GMBF)

Acute gastric mucosal damage was produced by ischemia/reperfusion (19). Simultaneous measurement of GMBF was performed in a chambered stomach as described previously (20). Briefly, under urethane anesthesia (1.25 g/kg i.p.), each rat stomach was mounted in an *ex-vivo* chamber and had 100 mM HCl applied to it every 15 mins, and a catheter connected to a syringe was passed through the left femoral vein. After the operation, 4 ml of blood were collected using the syringe, and 30 mins later, reperfusion was achieved through the infusion of the collected blood with heparin. GMBF was measured with a laser Doppler flow-meter (model ALF 21; Advance, Tokyo, Japan), with the probe placed gently on the corpus mucosa using a balancer, and changes in GMBF were continuously monitored on a two-channel recorder (U-228; PANTOS, Kyoto, Japan). After reperfusion for 90 mins, the stomach was excised, and a lesion index was calculated using the FLOVEL Filing System (Olympus). Losartan (an ARB; 0.3-3 mg/kg), candesartan (an ARB; 3 mg/kg), valsartan (an ARB; 3 mg/kg), PD123319 (an AT2 receptor blocker; 10 mg/kg), enalapril (an angiotensin-converting enzyme (ACE) inhibitor; 0.1-1 mg/kg), or diphenylene iodonium (a NADPH oxidase inhibitor; 0.1-1 mg/kg) was given i.v. 10 mins before reperfusion or β -NADPH (a NADPH oxidase substrate; 1 mg/kg) was given i.v. 5 mins before reperfusion to precisely evaluate the hemorrhagic damage caused by reperfusion.

In some cases, the stomach was excised, and the tissue samples were then immersed in 4% paraformaldehyde, embedded in paraffin, sectioned at 5 μ m, and stained with hematoxylin and eosin (H&E).

Measurement of H₂O₂ production

H₂O₂ production in the gastric mucosa was measured after ischemia/reperfusion treatment in 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 Na₂HPO₄·12H₂O, 5.7 mM NaH₂PO₄, and 5.5 mM glucose; pH 7.35). After incubation at 4°C for 30 mins, the homogenate was centrifuged at 12000 x g for 15 mins at 4°C, and the supernatant was used as the sample. Absorbance at 540 nm was measured on a microplatereader (Multiskan BICHROMATIC; Labsystems Japan Inc., Tokyo, Japan) and expressed as μ M per mg tissue H₂O₂.

Determination of mucosal microvascular permeability

Microvascular permeability was evaluated by measuring the amount of dye (Evan's blue) extravasated into the gastric mucosa according to a method described in a previous article (21). Following ischemia/reperfusion treatment, the animal was injected with 1 ml of 1% Evan's blue (w/w) i.v. at 30 mins before sacrifice. After 90 mins of reperfusion, the stomach was removed, and the wet scraped tissues were weighed. The tissues were extracted with 1N KOH for 2 h at 37°C. The extraction of dye was performed according to the method described by Katayama *et al.* (22). The absorbance of each sample was measured at 620 nm on a spectrometer (UV mini 1240; Shimadzu, Kyoto, Japan), and the amount of dye recovered was expressed as micrograms per ml per g of wet tissue.

NADPH oxidase subunit expression according to the reverse transcription polymerase chain reaction (RT-PCR)

The stomachs were removed from chambered rats of the control, ischemia, and ischemia/reperfusion groups for the determination of p47^{phox} mRNA using RT-PCR with specific primers. Immediately after the examinations, the mucosal specimens were scraped off on ice using a slide glass. Total RNA was extracted from mucosal samples using a guanidium isothiocyanate/phenol chloroform single step extraction kit from TaKaRa Bio Inc (Shiga, Japan) based on the method described by Chomczynski P and Sacchi N (23).

Single-stranded cDNA was generated from 5 μ g of total RNA using superscript reverse transcriptase (Invitrogen, Carlsbad, CA) and random-primers (TaKaRa). The polymerase chain reaction mixture was amplified in a DNA thermal cycler (Thermo Scientific Inc, Waltham, MA). The nucleotide sequences of the primers were as follows: β -actin: sense: 5' TTG TAA CCA ACT GGG ACG ATA TGG 3'; antisense: 5' GAT CTT GAT CTT CAT GGT GCT AGG 3'; p47^{phox}: sense: 5' TCA CCG AGA TCT ACG AGT TC 3'; antisense: 5' TCC CAT GAG GCT GTT GAA GT 3'. 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, Kyoto, Japan) as a standard size marker. The gel was then photographed under UV transillumination. The signals for p47^{phox} mRNA were standardized against the β -actin signal for each sample, and the results are expressed as the ratio of p47^{phox} mRNA to β -actin mRNA.

Immunoblot analysis of NADPH oxidase subunits

Rat stomachs were lysed with lysis buffer (0.1 M Tris HCl (pH 6.8), 4% SDS, and 20% glycerol) after ischemia/reperfusion. The total protein level in each sample was determined by Bradford's method. The protein level in each lysate was adjusted to 50 μ g/20 μ l of sodium dodecyl sulfate (SDS) sample buffer, and the lysate samples were subjected to SDS-polyacrylamide gel electrophoresis and Western blotting, which was performed with anti-p47^{phox} and anti- β -actin antibodies. The blot was visualized with the ECL plus detection system (Amersham Biosciences, Piscataway, NJ). The signals for p47^{phox} protein expression were standardized against the β -actin signal for each sample, and the results are expressed as the ratio of p47^{phox} protein to β -actin protein.

Measurement of NADPH oxidase activity

NADPH oxidase activity in the gastric mucosa was measured 90 mins after the rats had been subjected to ischemia/reperfusion. 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). After incubation at 4°C for 30 mins, the homogenate was centrifuged at 12000 x g for 15 min at 4°C, and the supernatant was used as the sample solutions for a cytochrome c reductase assay. The absorbance of each sample was measured at 550 nm on a spectrometer (UV mini 1240; Shimadzu, Kyoto, Japan) and was expressed as Δ microunits per ml per g of wet tissue (one unit will reduce 1.0 μ M of oxidized cytochrome c in the presence of 100 μ M NADPH per minute at pH 7.8 and 25°C), normalized to the value of the sham group.

Preparation of drugs

The drugs used were urethane (Tokyo Kasei, Tokyo, Japan), losartan (Banyu, Tokyo, Japan), candesartan (Takeda, Osaka, Japan), valsartan (Novartis, Basle, Switzerland), PD123319 (Sigma Chemicals, St. Louis, Montana, USA), enalapril (Sigma), diphenylene iodonium (Sigma), and β -NADPH (Sigma). ARB, PD123319, enalapril, and diphenylene iodonium were dissolved in saline. Each agent was prepared immediately before use and administered intravenously in a volume of 0.1 ml per 100 g body weight. The antibodies used were mouse anti-p47^{phox} and anti- β -actin (diluted 1:200) from BD Transduction Laboratories (San Jose, CA) and peroxidase-conjugated anti mouse IgG (diluted 1:2000) from Pierce Biotechnology, Inc. (Rockford, IL).

Statistics

Data are presented as the mean \pm S.E.M. for 3~7 rats per group. Statistical analyses were performed using a two-tailed Student's *t*-test, and values of $P < 0.05$ were regarded as significant.

RESULTS

Effect of ARB on microscopic mucosal injury induced by ischemia/reperfusion

The combination of 30 mins of ischemia and 90 min of reperfusion produced hemorrhagic erosion in rat stomachs treated with 100 mM HCl. The extent of gastric erosion was reduced by pretreatment with losartan 10 mins before the reperfusion in a dose-dependent manner, and this was significant at 3 mg/kg (Fig. 1). In the case of candesartan or valsartan, which are other representative ARB, the gastric damage caused by ischemia/reperfusion was also significantly attenuated at 3 mg/kg. Whereas, pretreatment with PD123319, an AT2 receptor blocker, had no clear effect on the ischemia/reperfusion-induced damage in the rat stomach (Fig. 2). In addition, the damage was dose-dependently attenuated by pretreatment with enalapril, which was as significant at 1 mg/mg as the effects of 3 mg/kg ARB (Fig. 3). Pretreatment with either losartan or enalapril 10 mins before ischemia had no effect on the ischemia/reperfusion-induced gastric damage (not shown).

Effect of ARB on H₂O₂ production and microvascular permeability in the gastric mucosa

After ischemia/reperfusion, the mucosa was scraped, and H₂O₂ production and microvascular permeability were evaluated. The concentration of H₂O₂ in the gastric mucosa was 12.83 \pm 3.95 mM/g tissue in the sham group. H₂O₂ production was significantly promoted by ischemia/reperfusion to 34.10 \pm 3.84 mM/g tissue; whereas, pretreatment with 3 mg/kg candesartan, valsartan, or 1 mg/kg enalapril significantly decreased the amount of H₂O₂ produced by ischemia/reperfusion. Losartan at 3

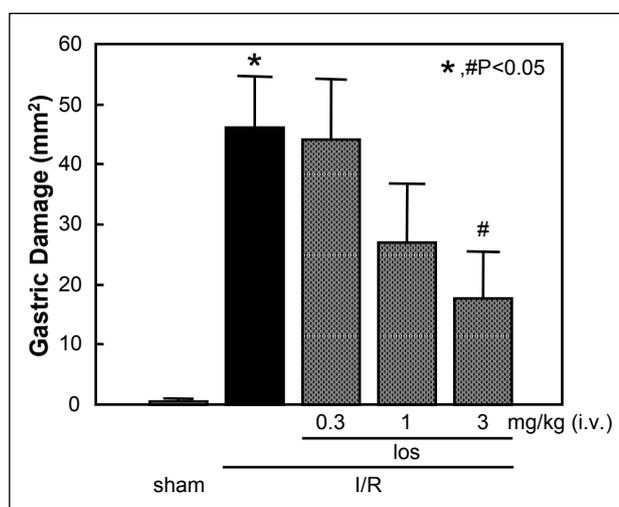


Fig. 1. The effect of losartan on an index of hemorrhagic lesions in the rat stomach. After ischemia/reperfusion (I/R), the stomach was excised, and a lesion index was calculated using microscopic visualization. Losartan (los) was given i.v. 10 mins before reperfusion. Saline was given i.v. as a placebo in the sham group. Data are presented as the mean \pm S.E.M. of 5~6 rats. Significant difference at $P < 0.05$: * from the sham value, # from the I/R value.

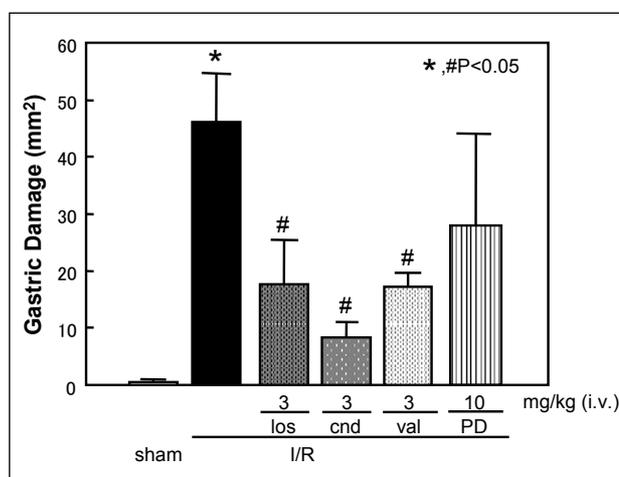


Fig. 2. The effect of ARB on an index of hemorrhagic lesions in the rat stomach. After ischemia/reperfusion (I/R), the stomach was excised, and a lesion index was calculated using microscopic visualization. Losartan (los), candesartan (cnd), valsartan (val), and PD123319 (PD) were given i.v. 10 mins before reperfusion. Saline was given i.v. as a placebo in the sham group. Data are presented as the mean \pm S.E.M. of 3~6 rats. Significant difference at $P < 0.05$: * from the sham value, # from the I/R value.

mg/kg did not have any effect ($P = 0.30$ vs. sham; $P = 0.127$ vs. ischemia/reperfusion) (Fig. 4).

Microvascular permeability was significantly increased in the ischemia/reperfusion group compared with the sham group (1.07 \pm 0.16 μ g/ml/g tissue in the sham group, 2.99 \pm 0.43 μ g/ml/g tissue in the ischemia/reperfusion group). However, the microvascular permeability after ischemia/reperfusion pretreated with 3 mg/kg losartan ($P = 0.054$ vs. ischemia/reperfusion) or valsartan ($P = 0.058$ vs. ischemia/reperfusion) tended to decrease, and that pretreated with 3 mg/kg candesartan or 1 mg/kg enalapril was significantly decreased compared with that induced by ischemia/reperfusion (Fig. 5).

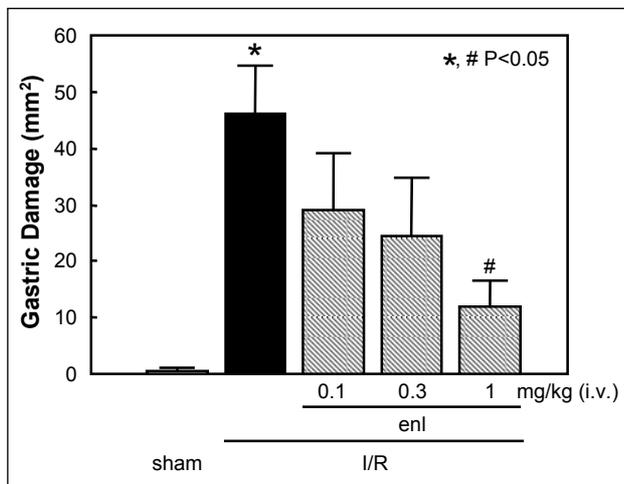


Fig. 3. The effect of enalapril on an index of hemorrhagic lesions in the rat stomach. After ischemia/reperfusion (I/R), the stomach was excised, and a lesion index was calculated using microscopic visualization. Enalapril (enl) was given i.v. 10 mins before reperfusion. Saline was given i.v. as a placebo in the sham group. Data are presented as the mean±S.E.M. for 4-6 rats. Significant difference at P<0.05: * from the sham value, # from the I/R value.

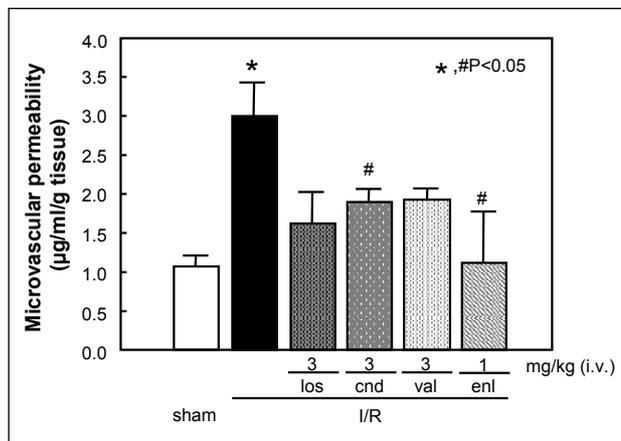


Fig. 5. The effect of ARB on the microvascular permeability induced by ischemia/reperfusion (I/R) in the rat stomach. The chambered stomach was scraped, the dye was extracted, and the amount of dye recovered was expressed as micrograms per ml per g of wet tissue. Losartan (los), candesartan (cnd), valsartan (val), or enalapril (enl) were given i.v. 10 mins before reperfusion. Saline was given i.v. as a placebo in the sham group. Data are presented as the mean±S.E.M. of 4-6 rats. Significant difference at P<0.05: * from the sham value, # from the I/R value.

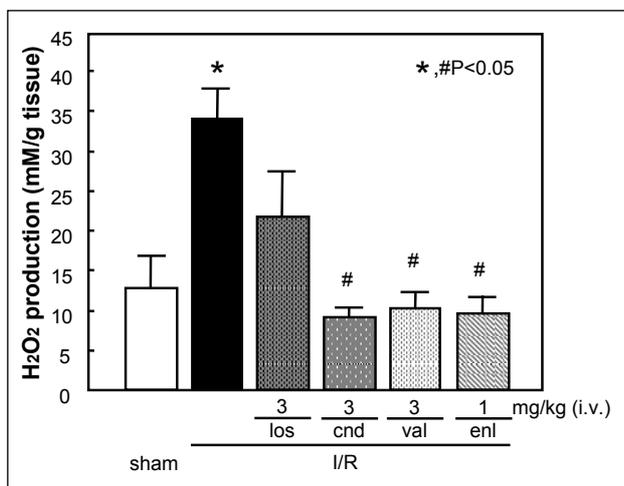


Fig. 4. The effect of ARB on H₂O₂ production induced by ischemia/reperfusion (I/R) in the rat stomach. The mounted stomach was excised, and the tissue was homogenized in Krebs-Ringer phosphate buffer (pH 7.4). After centrifugation, the supernatant was measured at 540 nm, and the absorbance was expressed as mM H₂O₂. Losartan (los), candesartan (cnd), valsartan (val), or enalapril (enl) were given i.v. 10 mins before reperfusion. Saline was given i.v. as a placebo in the sham group. Data are presented as the mean±S.E.M. of 4-6 rats. Significant difference at P<0.05: * from the sham value, # from the I/R value.

Distribution of NADPH oxidase in the rat gastric mucosa

We studied the expression of p47^{phox}, one of the cytosol components of NADPH oxidase in the rat stomach by RT-PCR and western blotting to demonstrate the existence of NADPH oxidase in the gastric mucosa. The expression of p47^{phox} mRNA was detected in the gastric mucosa both in the sham and ischemia/reperfusion groups, and no significant difference in the level of mRNA expression was found between the two groups (Fig.

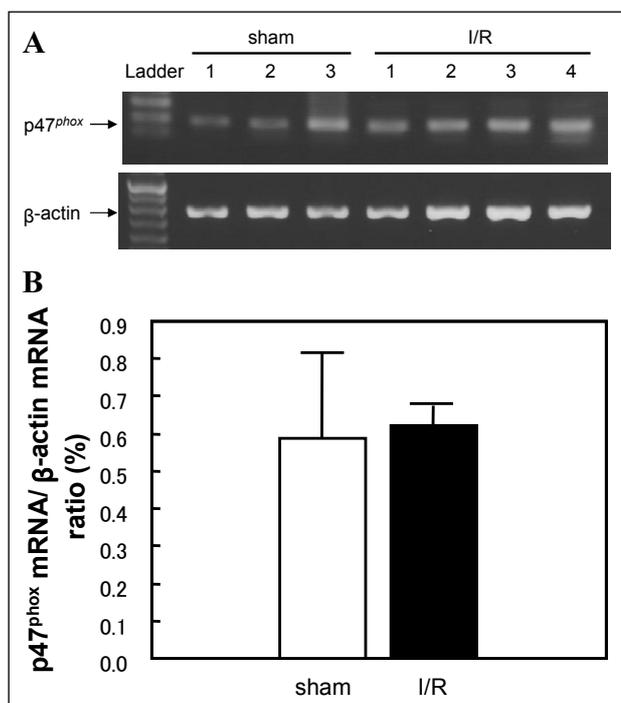


Fig. 6. Expression of the NADPH oxidase subunit p47^{phox} mRNA in the rat stomach. The chambered stomach was excised, and the homogenate was used for generating single standard cDNA by RT-PCR. (A) The PCR products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide. The gel was photographed under UV transillumination. (B) The figure shows the ratio of p47^{phox} mRNA to β-actin mRNA. Mean±S.E.M. (N=3-4).

6A, 6B). Correspondingly, both in the sham and ischemia/reperfusion groups, we recognized the protein expression of p47^{phox}, and the level of protein expression of the sham group was similar to that of the ischemia/reperfusion group (Fig. 7A, 7B).

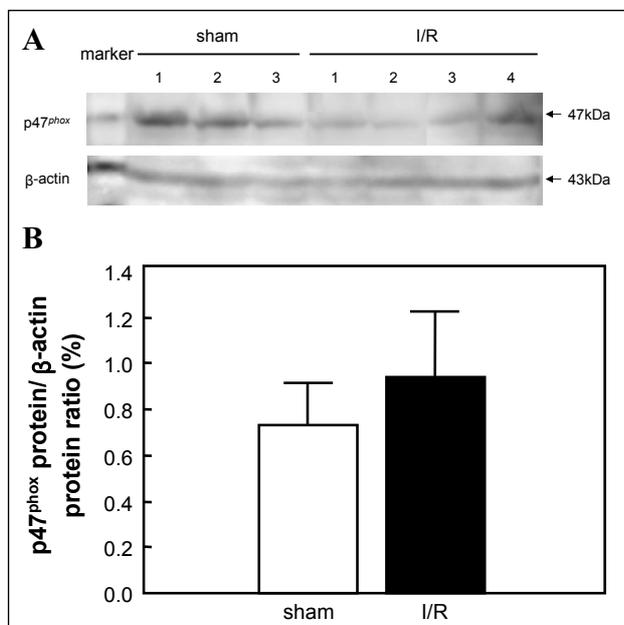


Fig. 7. Expression of the NADPH oxidase subunit p47^{phox} protein in the rat stomach. The chambered stomach was excised, and the homogenate was used for Western blotting. The scraped tissues were lysed with lysis buffer. After the total protein level in each sample had been determined by Bradford's method, the sample was subjected to SDS-PAGE and western blotting. (A) The membrane was photographed under a luminoimage analyzer. (B) The figure shows the ratio of p47^{phox} protein to β-actin protein. Mean±S.E.M. (N=3~4).

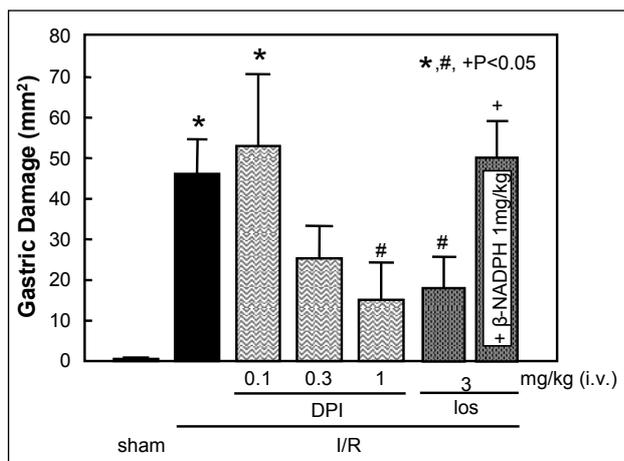


Fig. 8. The effect of diphenylene iodonium and β-NADPH on an index of hemorrhagic lesions in the rat stomach. After ischemia/reperfusion (I/R), the stomach was excised, and a lesion index was calculated. β-NADPH was given i.v. 5 mins before reperfusion, in addition, diphenylene iodonium (DPI) or losartan (los) was given i.v. 10 mins before reperfusion. Saline was given i.v. as a placebo in the sham group. Data are presented as the mean±S.E.M. of 4~7 rats. Significant difference at P<0.05: * from the sham value, # from the I/R value, + from the value for I/R pretreated with 3 mg/kg losartan.

Effect of diphenylene iodonium on gastric erosion and damage parameters induced by ischemia/reperfusion

The damage caused by ischemia/reperfusion was dose-dependently suppressed by pretreatment with diphenylene

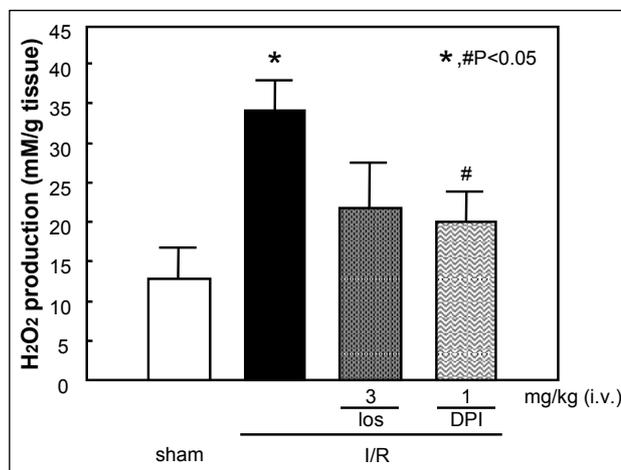


Fig. 9. The effect of diphenylene iodonium on H₂O₂ production induced by ischemia/reperfusion (I/R) in the rat stomach. The mounted stomach was excised, and the tissue was homogenized in Krebs-Ringer phosphate buffer (pH 7.4). After centrifugation, the supernatant was measured at 540 nm, and the absorbance was expressed as mM H₂O₂. Losartan (los) or diphenylene iodonium (DPI) was given i.v. 10 mins before reperfusion. Saline was given i.v. as a placebo in the sham group. Data are presented as the mean±S.E.M. of 4~6 rats. Significant difference at P<0.05: * from the sham value, # from the I/R value.

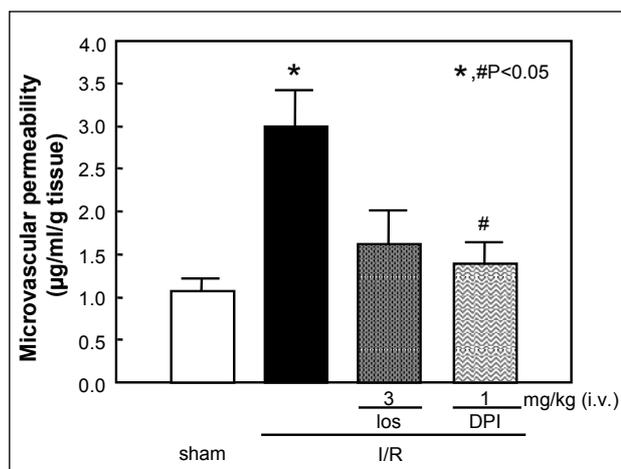


Fig. 10. The effect of diphenylene iodonium on the microvascular permeability induced by ischemia/reperfusion (I/R) in the rat stomach. The chambered stomach was scraped, the dye was extracted, and the amount of dye recovered was expressed as micrograms per ml per g of wet tissue. Losartan (los) or diphenylene iodonium (DPI) were given i.v. 10 mins before reperfusion. Saline was given i.v. as a placebo in the sham group. Data are presented as the mean±S.E.M. from 4~6 rats. Significant difference at P<0.05: * from the sham value, # from the I/R value.

iodonium, as NADPH oxidase inhibitor, and this was significant at 1 mg/kg. The inhibitory effect of losartan on the ischemia/reperfusion-related damage was significantly reversed by pretreatment with 1 mg/kg β-NADPH, an NADPH oxidase substrate, at 5 mins before reperfusion (Fig. 8).

In addition, we also examined the effect of 1 mg/kg diphenylene iodonium on H₂O₂ production and microvascular

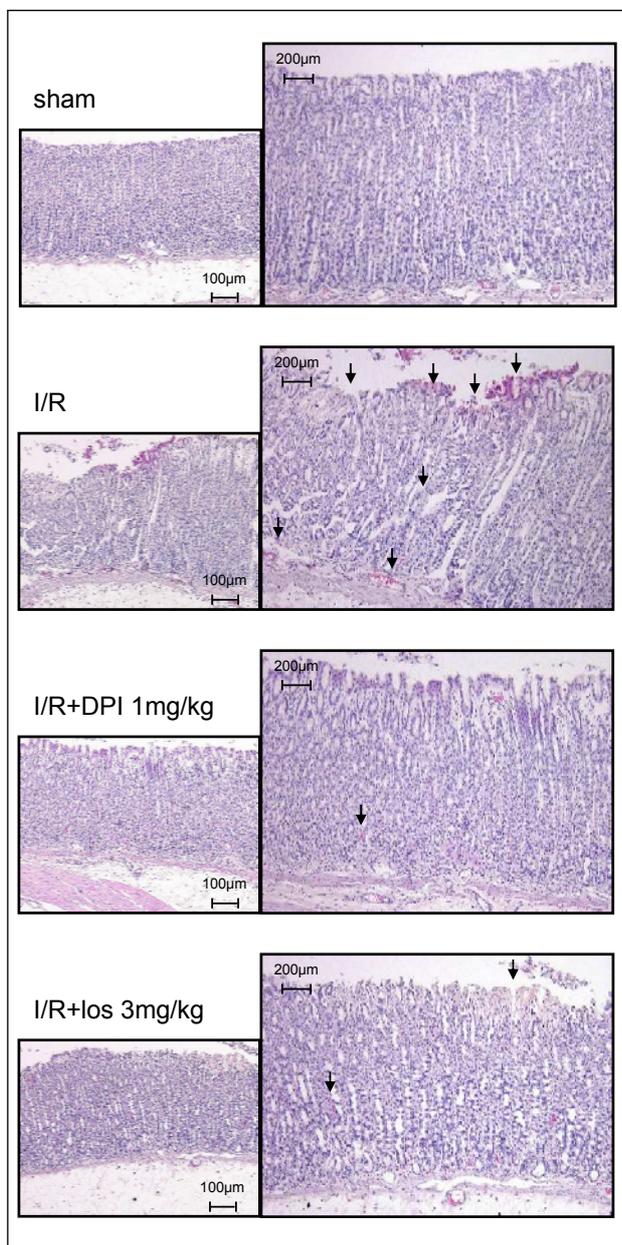


Fig. 11. Representative histological observations of the effect of losartan and diphenylene iodonium on the rat gastric mucosa. The mounted stomach was excised after ischemia/reperfusion (I/R), fixed with 4% paraform aldehyde, embedded in paraffin, and stained by H&E. The black arrows show marked migration of leukocytes. The pictures show sham, I/R, I/R pretreated with diphenylene iodonium (DPI) 1 mg/kg (i.v.), and I/R pretreated with losartan (los) 3 mg/kg (i.v.).

permeability after ischemia/reperfusion. The increased H_2O_2 production induced by ischemia/reperfusion was significantly suppressed by pretreatment with 1 mg/kg diphenylene iodonium (19.99 ± 3.78 mM/g tissue), having a similar effect to 3 mg/kg losartan (21.69 ± 5.88 mM/g tissue) (Fig. 9). As for the result for H_2O_2 production, the facilitation of microvascular permeability induced by ischemia/reperfusion was significantly attenuated by 1 mg/kg diphenylene iodonium pretreatment (1.39 ± 0.25 μ g/ml/g tissue), having a similar effect to 3 mg/kg losartan (1.62 ± 0.40 μ g/ml/g tissue) (Fig. 10).

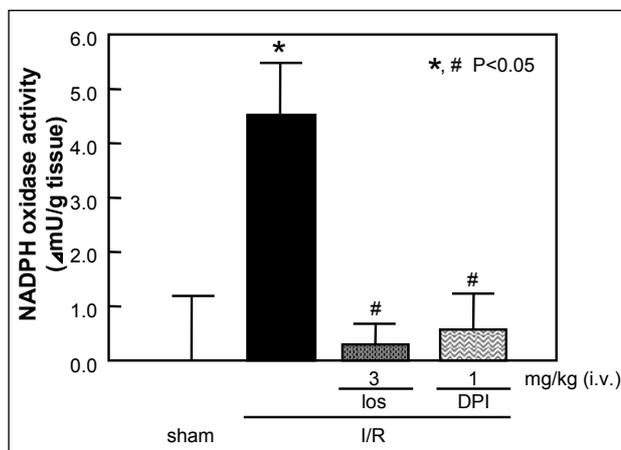


Fig. 12. The effect of losartan and diphenylene iodonium on NADPH oxidase activity induced by ischemia/reperfusion (I/R) in the rat stomach. The chambered stomach was excised, and the homogenate was used to measure NADPH oxidase activity with a cytochrome c reductase assay kit. The homogenate was measured at 550 nm, and the absorbance was expressed as Δ microunits per g tissue. Losartan (los) or diphenylene iodonium (DPI) was given i.v. 10 mins before reperfusion. Saline was given i.v. as a placebo in the sham group. Data are presented as the mean \pm S.E.M. of 4–6 rats. Significant difference at $P < 0.05$: * from the sham value, # from the I/R value.

Histological observation of damage induced by ischemia/reperfusion in the gastric mucosa

After ischemia/reperfusion, we histologically examined the gastric mucosa using H&E staining (Fig. 11). Ischemia/reperfusion induced the migration of leukocytes, and this was accompanied by marked hemorrhaging and erosion not only around the epithelial area but also throughout the gastric submucosa. However, pretreatment with losartan (3 mg/kg) caused less migration than ischemia/reperfusion alone. Diphenylene iodonium (1 mg/kg) pretreatment also caused less migration than ischemia/reperfusion.

NADPH oxidase activity in the rat gastric mucosa

We examined NADPH oxidase activity in the rat gastric mucosa. After ischemia/reperfusion, the scraped mucosa was used to measure NADPH oxidase activity with a cytochrome c reductase assay. Ischemia/reperfusion significantly activated NADPH oxidase in the gastric mucosa compared with the sham group. The increase in NADPH oxidase activity induced by ischemia/reperfusion was significantly attenuated by pretreatment with 3 mg/kg losartan or 1 mg/kg diphenylene iodonium (Fig. 12).

Effect of losartan and diphenylene iodonium on the change in GMBF during ischemia/reperfusion

GMBF was measured during the ischemia/reperfusion examination. Ischemia significantly reduced GMBF to 50–66 % of the value in sham group. After ischemia, GMBF increased quickly at the baseline and was significantly increased in the early stage (10 and 20 mins) of reperfusion compared with that of the sham group. As well as ischemia/reperfusion, pretreatment with 3 mg/kg losartan or 1 mg/kg diphenylene iodonium significantly reduced GMBF during ischemia and

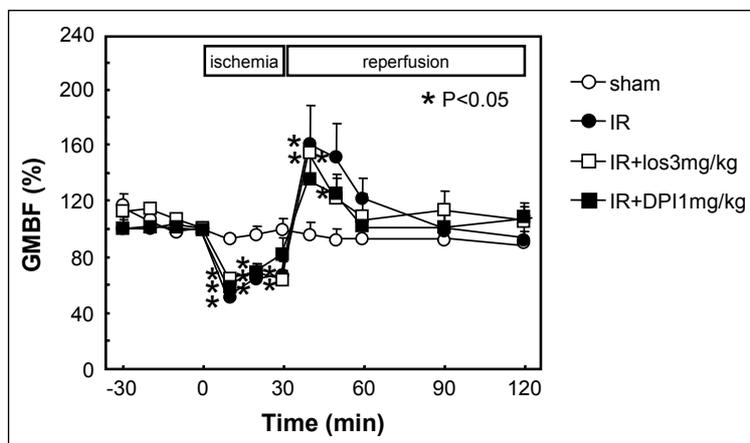


Fig. 13. The effect of losartan and diphenylene iodonium on changes in GMBF during ischemia/reperfusion (I/R). GMBF was measured in a chambered stomach with a laser Doppler flow-meter, and changes in GMBF were continuously monitored on a recorder. Losartan (los) or diphenylene iodonium (DPI) was given i.v. 10 mins before reperfusion. Saline was given i.v. as a placebo in the sham group. The values of GMBF are shown as a percentage of the value at the baseline. Data are presented as the mean±S.E.M. of 4–6 rats. *Significant difference from the sham value at $P<0.05$.

reversed it during reperfusion (Fig. 13). Pretreatment with 1 mg/kg enalapril showed similar effects to losartan and enalapril (data not shown).

DISCUSSION

Reactive oxygen species play a role in preventing bacterial infections, but can cause various illnesses by injuring surrounding cells. Reactive oxygen species are derived from reduced oxygen and are produced by several cells including neutrophils, macrophages, endothelial cells, surface epithelial cells, and vascular smooth muscle cells, resulting in cellular damage and inflammation. In the gastrointestinal tract, it has been demonstrated that reactive oxygen species are involved in ethanol-, NSAID-, and stress-related acute gastritis, as well as in ischemia/reperfusion-induced gastric injury (1–3). Likewise, *H. pylori* infection also causes chronic gastritis resulting from the generation of reactive oxygen species in gastric epithelial cells (4). In addition, it was reported that activation of the renin-angiotensin system was accompanied by functional effects in the stomach (24), intestine (25), and colon (26) because angiotensin II is a powerful inducer of reactive oxygen species in the brain, heart, and liver (15, 27). In particular, the stomach has been reported to exhibit angiotensin I, angiotensin II, and ACE activity in rats (28). These reports support the assertion that the generation of reactive oxygen species plays an important role in gastrointestinal damage. Initially, we focused on the ability of angiotensin II to induce the generation of reactive oxygen species. To investigate the relationship between NADPH oxidase and angiotensin II, we used three types of angiotensin-related drugs at doses that did not affect blood pressure: ARB, a selective AT2 receptor blocker, and an ACE inhibitor, which are all involved in renin-angiotensin-mediated vasoconstriction and inflammatory responses. Although the ARB had a marked effect on the parameters of the reactive oxygen species, we used other types of drugs to ensure that the suppressive effects of the ARB against ischemia/reperfusion-induced generation of reactive oxygen species were mediated *via* the AT1 receptor, which was reported to suppress the generation of reactive oxygen species (15). Pretreatment with ARB attenuated ischemia/reperfusion-induced gastric damage and increased microvascular permeability and H_2O_2 production, which is a relatively stable and membrane-permeable reactive oxygen species. Pretreatment with ARB 10 mins before ischemia did not have significant effects on ischemia/reperfusion-induced gastric damage unlike that before reperfusion (data not shown). In this study, we examined the effect of ARB pretreatment administered 10 mins before reperfusion because ischemia/reperfusion induces the expression of

proinflammatory genes and inflammatory cytokines including TNF- α and IL-1 β , and we suggest that reactive oxygen species are generated by inflammatory reactions. This study clarified that angiotensin II is involved in gastric damage and the generation of reactive oxygen species induced by ischemia/reperfusion *via* the AT1 receptor by examining the effects of pretreatment with ARB and enalapril. It was shown that angiotensin II acted as an inducer of reactive oxygen species in the rat stomach as well as in the above-mentioned organs.

In general, the conversion of oxygen into reactive oxygen species is catalyzed by NADPH oxidase, which is present in several cells such as vascular muscle cells and endothelial cells (16, 29), which suggests a relationship between the generation of reactive oxygen species and the activation of NADPH oxidase in cardiovascular events. In the gastrointestinal tract, reactive oxygen species generated from gastrointestinal cells *via* NADPH oxidase have been suggested to be involved in mucosal inflammation and tissue damage (30). However, it is not clear whether NADPH oxidase is a key enzyme in the inflammation of the gastric mucosa. Moreover, the relationship between the activation of NADPH oxidase and proinflammatory substances in the stomach has not been elucidated either. In this study, we determined the role of NADPH oxidase in the gastric damage induced by ischemia/reperfusion *via* angiotensin II in rats.

In this study, we demonstrated the distribution of NADPH oxidase in gastric mucosal cells by detecting mRNA and protein of its subunit p47^{phox} (Fig. 6 and 7). In both examinations, p47^{phox} was expressed to a similar extent between the sham and ischemia/reperfusion groups. Thus, we suggest that NADPH oxidase is not expressed but rather is activated by ischemia/reperfusion. In the gastrointestinal tract, Nox1 and Duox2 (subtypes of NADPH oxidase) are mainly expressed (31, 32). Additionally, it has been reported that guinea pig gastric mucosal cells constitutively express Nox1 and possess potent NADPH oxidase-like activity (33). In future, we will study which type of NADPH oxidase is present in gastric cells.

Diphenylene iodonium, which inhibits NADPH oxidase by suppressing flavocytochrome, suppressed ischemia/reperfusion-induced gastric damage, H_2O_2 production, and increases in microvascular permeability; *i.e.*, showed similar results to ARB pretreatment. We already reported the effect of diphenylene iodonium on gastric damage induced by ischemia/reperfusion, and suggested that the activation of NADPH oxidase was involved in the generation of reactive oxygen species (34). However, we did not examine and discuss the relation between angiotensin II and NADPH oxidase unlike this study. Interestingly, the effects of ARB were almost abolished by β -NADPH, a reduced form NADPH oxidase substrate. β -NADPH

is a pyridine nucleotide and is used as an electron donor for NADPH oxidase (35). Thus, β -NADPH activates NADPH oxidase and promotes the generation of reactive oxygen species in the rat gastric mucosa (36). From the result that β -NADPH counteracted the effects of losartan, NADPH oxidase is believed to be activated *via* the angiotensin AT1 receptor. Incidentally, we administered β -NADPH at 5 mins before reperfusion unlike the other agents (ARB and diphenylene iodonium) to avoid a rapid increase in body fluid volume in this case.

In addition, we have to consider the effect of ARB and diphenylene iodonium on GMBF, a gastroprotective factor. Generally, hypovolemia involving ischemia leads to the activation of vasopressor systems such as the renin/angiotensin system in order to maintain arterial pressure, but this endangers microcirculation. GMBF is an important gastroprotective factor and is affected by vasodilators like nitric oxide, prostaglandins, and bradykinin (37). However, the doses of losartan and diphenylene iodonium used in this study were considered to have no significant influence on GMBF. So in this study, we considered the effect of pretreatment with losartan or diphenylene iodonium to be a significant reduction in the negative effects of ischemia/reperfusion induced *via* the AT1 receptor/NADPH oxidase system but not changes in GMBF.

It is important to identify in which cells NADPH oxidase is activated by ischemia/reperfusion. Mugge *et al.* (38) reported that the p22^{phox} expressed in vascular smooth muscle had >90% homology with neutrophil p22^{phox}. However, vascular NADPH oxidase produces continuously low levels of superoxide in contrast to the burst-like activity of neutrophils. Some reports have provided evidence that neutrophils are recruited to inflamed tissue during ischemia/reperfusion (39). On the other hand, NADPH oxidase is also abundant in mitochondria. It has been reported that acutely infused angiotensin II activated mitochondria derived reactive oxygen species through NADPH oxidase (40). The role of NADPH oxidases in mitochondrial electron transport is well known. The opening of mitochondrial adenosine 5'-triphosphate-sensitive potassium (mitoK_{ATP}) channels was suggested to be important in mitochondria derived reactive oxygen species production *via* NADPH oxidase (41). Some reports have shown a relationship between mitochondrial NADPH oxidase and ischemia/reperfusion heart injuries (42) or the development of oral carcinoma (43). The data in this study cannot demonstrate the existence of NADPH oxidase in the rat stomach; however, we have demonstrated the possible involvement of NADPH oxidase in gastric mucosal cells.

This study demonstrated that pretreatment with diphenylene iodonium suppressed ischemia/reperfusion -induced gastric injury with a concomitant suppression of H₂O₂ production and a facilitation of vascular permeability similar to that observed after pretreatment with ARB or enalapril, suggesting that NADPH oxidase is closely involved in the pathogenesis of ischemia/reperfusion-induced gastric damage through a reactive oxygen species-generating system induced *via* angiotensin II. Investigation of the AT1 receptor/NADPH oxidase system is valuable for the elucidation of the pathogenesis of gastric mucosal inflammation and injury, and the inhibition of NADPH oxidase may represent a new target for anti-inflammatory drugs.

Conflict of interests: None declared.

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