JPET Fast Forward. Published on February 26, 2004 as DOI: 10.1124/jpet.103.061283

JPET #61283

In Vivo Study on Cross-Talk between Inducible Nitric Oxide Synthase and Cyclooxygenase in Rat Gastric Mucosa: Effect of COX Activity on NO Production

KANAME UNO, YOSHIHITO IUCHI, JUNICHI FUJII, HIDEAKI SUGATA, KATSUNORI IIJIMA, KATSUAKI KATO, TOORU SHIMOSEGAWA, and TETSUHIKO YOSHIMURA

Laboratory of Applied Biomedicinal Chemistry, Institute for Life Support Technology, Yamagata Public Corporation for the Development of Industry, Yamagata 990-2473, Japan (K.U., T.Y.), Gastroenterology, Internal Medicine, Tohoku University Graduate School of Medicine, Sendai 980-8574, Japan (K.U., H.S., K.I., K.K., T.S.), Department of Biochemistry, Yamagata University School of Medicine, Yamagata 990-9585, Japan (Y.I., J.F.)

JPET #61283

a) Running Title: Effect of COX Activity on NO Production by iNOS

b) Corresponding author:

Tetsuhiko Yoshimura, Laboratory of Applied Biomedicinal Chemistry, Institute for Life Support Technology, Yamagata Public Corporation for the Development of Industry, Yamagata 990-2473, Japan

Tel: 023-647-3133; Fax:023-647-3138

E-mail: yoshi@yat.ymgt-techno.or.jp

c) The number of text pages: 31

Table: 0

Figures: 9

References: 35

The number of words in the Abstract: 249

Introduction: 514

Discussion: 1408

d) Abbreviations:

NO, nitric oxide; PGE2, prostaglandin E2; NOS, nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; *E.coli, Escherichia coli*; LPS, lipopolysaccharide; 1400W, N-(3-(aminomethyl)benzyl)acetamidine; IM, indomethacin; NS-398, N-(2-[cyclohexyloxy]-4-nitrophenyl)methanesulfonamide; EPR, electron paramagnetic resonance; MPO, myeloperoxidase; cAMP, cyclic adenosine monophosphate; H_4B, tetrahydrobiopterin; ANOVA, analysis of variance

e) Section assignment: Gastrointestinal, hepatic, Pulmonary & Renal

ABSTRACT

The integrity of gastric mucosa during endotoxemia is maintained by the balance of inflammatory mediators, such as prostanoids originated from cyclooxygenase-2 (COX-2) and nitric oxide (NO) from inducible nitric oxide synthase (iNOS). Thus we elucidated *in vivo* cross-talk between prostanoids and NO in gastric mucosa during endotoxemia, using an iNOS-specific inhibitor, 1400W, a non-specific COX inhibitor, indomethacin, and a COX-2 specific inhibitor, NS-398. Gastric mucosal NO and prostaglandin E2 (PGE2, a predominant product of COX), expressed as mean ± SD of 5 rats/group, were assayed by electron paramagnetic resonance spectrometry and enzyme immunoassay technique, respectively. The levels of NO and PGE2 increased gradually up to 6 hours after administration of bacterial lipopolysaccharide (LPS) (NO; control 0.35 ± 0.16, 6 hr 13.3 ± 3.3 nmol/g-tissue/30min: PGE2; control, 288 ± 16, 6 hr 806 ± 15 pg/g-tissue). Pretreatment with 1400W decreased the increase in NO level without any effect on the PGE2 level (NO; 4.0 ± 0.4 nmol/g-tissue/30min: PGE2: 788 ± 26 pg/g-tissue). In contrast, treatment with indomethacin and NS-398 inhibited not only PGE2 level but also NO level in a dose-dependent manner without any significant effect on both iNOS and COX protein and mRNA expression. These results demonstrate that in the LPS-treated rat gastric mucosa, PGE2 enhances the release of NO following activation of iNOS, although NO produced by iNOS does not stimulate the release of PGE2 by COXs. The effect of COX activity on iNOS-NO pathway can be important in the regulation of gastric mucosal integrity in inflammatory states.

The gastric mucosa is constantly exposed to various stimulants, including acid, pepsin, alcohol, *Helicobactor pylori* or drugs [e.g., nonsteroidal anti-inflammatory drugs (NSAIDs)]. Among the stimulants, NSAIDs, in particular, are well-recognized for being responsible in causing upper gastrointestinal complications, ranging from dyspeptic symptoms to life-threatening complicated ulcers (Hirschowitz, 1994). Increasing evidence suggests that endogenously produced NO maintains the gastric mucosal integrity in combination with prostanoids including prostaglandins (PGs) (Whittle et al., 1990). NO and prostanoids are synthesized by nitric oxide synthase (NOS) and cyclooxygenase (COX), respectively, both of which have constitutive and inducible isoforms (Moncada et al., 1991, Dubois et al., 1998). In the gastric mucosa, two types of constitutive NO synthase (cNOS) have been discovered by using immunohistochemical techniques: neuronal NO synthase (nNOS), localized in chief cells and mucosecretory cells of the gastric epithelium, and endothelial NO synthase (eNOS), localized in endothelia of the submucosal arterioles and muscularis mucosae (Fischer et al., 1999; Garcia-Vitoria et al., 2000). Inducible NO synthase (iNOS) is expressed in the cells involved in inflammation and immune activation through stimulation with certain cytokines and/or endotoxins (or lipopolysaccharide; LPS). COX is the rate-limiting enzyme for the production of PGs from arachidonic acid. COX-2 is the inducible form of COX enzyme, the synthesis of which is induced rapidly and transiently by various pro-inflammatory mediators and mitogenic stimuli (Clancy et al., 1998). COX-1 is a house-keeping enzyme that is constitutively expressed in the gastrointestinal tract and many other tissues (O' Neill and Ford-Hutchinson, 1993).

It has been to date demonstrated that there is a cross-talk between the products of NOS and COX enzymes. First, NOS-derived NO has been shown to interact with

COX pathways. However, contradictory results have been reported with respect to whether NO enhances, inhibits, or has little effect on COX activity (Goodwin et al., 1999; Coffey et al., 2001 and references therein). Second, it has also been reported that COX products interact with NOS pathway, although the results from various cells and tissues have been controversial (Tetsuka et al., 1994; Galea and Feinstein, 1999). Altogether, it appears that the cross-talk between NOS and COX expression and activity depend on the cell type, the isoform of the enzyme, the timing and concentration of the mediator released, and/or the specificity of selective inhibitor.

Although the gastric mucosal integrity in inflammatory states can be modulated by the concerted action of endogenous NO and PGs, little is known of the cross-talk effects between them, especially *in vivo* system. The cross-talk represents an important mechanism, by which the initial inflammatory response can be amplified or attenuated and serve as a therapeutic basis to manipulate the course of an inflammatory response. In the present study, to elucidate *in vivo* cross-talk between NO and PGs in a rat's gastric mucosa during endotoxemia, we examined the sequential changes of levels of NO, prostaglandin E2 (PGE2, a predominant product of COX), and protein and mRNA of iNOS and COXs and evaluated the effects of iNOS or COX inhibitors on these levels. NO production in the gastric mucosa was evaluated directly, using an electron paramagnetic resonance (EPR) NO trapping technique.

Materials and Methods

Animal Preparations. Male Sprague-Dawley rats, weighing 180-220 g (Charles River, Ibaraki, Japan) were used in all of the experiments. The animals were kept individual cages at a controlled temperature (23°C) on a 12-hour light-dark cycle. For 24 hours prior to sacrifice, they were deprived of food but allowed free access to tap water. To prevent coprophagy, they were placed on a raised mesh bottom. Anesthesia was induced with urethane (1.25 mg/kg, intraperitoneally; Tokyo-kasei, Tokyo, Japan). All procedures related to animal care described herein were in accordance with the criteria outlined in the Guideline for Animal Experimentation prepared by the Japanese Association for Laboratory Animal Science, 1987. Approval of the Animal Welfare Committee at the Institute for Life Support Technology was obtained for all studies.

Lipopolysaccharide (LPS) from *Escherichia coli* (0.3 ml, 3 mg/kg; *E. coli*, serotype 055: B55, Sigma) was administered via a tail vain of the rats. The control rats received an equal volume of saline. They were killed at 0, 2, 4, 6, 8, 10, and 12 hours after the treatments and the stomach was removed so that NO in their tissues could be measured by EPR spectrometry. An iNOS selective inhibitor, 1400W (5 mg/kg, i.v.; Cayman Chemical Co., Ann Arbor, MI, USA), a nonselective COX inhibitor, indomethacin (1, 5, 10 mg/kg, s.c.; Sigma Chemical Co., St. Louis MO, USA), and a COX-2 selective inhibitor, NS-398 (1, 5, 10 mg/kg, s.c.; Sigma Chemical Co., St. Louis MO, USA), each in saline, were administered to rats 30 minutes before LPS injection and the effect of the inhibitors on NO generation was examined at 2 and 6 hours after the LPS injection. The dependence of NO generation on COX-inhibitor doses was examined 6 hours after the LPS injection. The mucosal contents of PGE2 and both proteins and mRNA of iNOS, COX-1, and COX-2 with or without

administration of iNOS and COX inhibitors were determined at 6 hours after LPS injection. Myeloperoxidase (MPO) activity and iNOS and COX-2 expression in the gastric mucosa were also examined by an immunohistochemical procedure at 6 hours after the LPS injection.

Direct Measurement of NO by EPR Spectrometry. The NO produced in the stomach of the rats was measured by using an NO trapping technique combined with EPR spectroscopy (Nagano and Yoshimura, 2002). This technique is a method for analyzing NO production directly both *in vivo* and *in vitro*. Here, we employed an Fe-DETC complex as an NO-trapping reagent to quantify NO levels in the gastric mucosa of the LPS-treated rat. A DETC・$3H_2O$ solution (400 mg/kg; Aldrich, Milwaukee, USA) and an Fe-citrate mixture (40 mg/kg of $FeSO_4$・$7H_2O$ and 200 mg/ml of sodium citrate; Wako Chem., Osaka, Japan) were injected intraperitoneally and subcutaneously, respectively. An Fe-DETC complex thus internally formed could trap endogenously produced NO to yield an NO-Fe-DETC complex. Thirty minutes after the trapping agent was injected, the stomach was removed under deep anesthesia. The glandular mucosa at the side of the greater curvature was selectivity resected and minced. Each sample, drawn by a 1-ml plastic syringe, was collected in a glass capillary tube (75 mm in length; 46 μl inside volume), into a quartz tube (outer diameter, 5 mm). EPR spectra were recorded at ambient temperature with a spectrometer (TE-200; JEOL, Tokyo, Japan). The instrument settings were: center field, 331 mT; field scan, 4 mT; sweep time, 4 minutes; time constant, 0.3 second; modulation amplitude, 0.32 mT; modulation frequency, 100 K; microwave power, 60 mW; microwave frequency, ~ 9.5 GHz. The amplitude of the signal, which was proportional to the amount of NO, was obtained by measuring the peak-to-peak height of the lower field side signal in a three-line spectrum that is characteristic of an

NO adduct. The NO adduct concentration of the Fe-DETC complex was estimated by comparing it with the signal height of a standard solution of a chemically synthesized NO complex. The concentration in tissues, estimated at 30 minutes after the injection of NO trapping reagent, was expressed in the unit of nmol/g-tissue/30 minutes.

Prostaglandin E2 Expression Assay. The mucosal contents of PGE2 were measured in the glandular gastric mucosa that had been removed, weighed and put in a tube containing 100 % ethanol plus 0.1 M indomethacin. Then the samples were minced by scissors, homogenized, and centrifuged for 10 minutes at 12,000 x rpm at 4 °C. The supernatant of each sample was used for determination of PGE2 by EIA using PGE2-kit (Cayman Chemical Co., Ann Arbor, MI, USA).

Immunohistochemistry. Portions of the oxyntic gland area of the stomach were fixed in 10 % buffered formalin, embedded in paraffin and stained with hematoxylin-eosin. Serial sections cut from paraffin blocks (4 μm) were mounted on glass slides, dewaxed and dehydrated in xylene and washed in alcohol and phosphate-buffered saline (PBS), respectively. The sections were then incubated in 1 % hydrogen peroxide for 15 minutes to block endogenous peroxidase. Sections were then incubated overnight at 4 °C with polyclonal antibodies against for iNOS (1:1000; Santa Cruz Biotechnology, California, USA) and COX-2 (1:400; Santa Cruz Biotechnology, California, USA). After incubation with biotinylated rabbit anti-mouse and anti-goat antibody, respectively, followed by incubation with streptavidin-peroxidase complex using ABC-Kit (Amersham Pharmacia, Buckinghamshire, UK), the reaction products were detected by 0.03 % diaminobenzidine and H_2O_2 in 0.05 M Tris-HCl buffer (pH 7.6). The sections were washed in PBS three times between each step, the 3, 3-diaminobenzidine tetrahydrochloride containing 0.05 % hydrogen peroxide was applied for 3 minutes,

the sections were rinsed with tap water, then counterstained with hematoxylin. To assure the specificity of the immunohistochemical staining, the sections were processed without primary antibody.

RNA Extraction and Semi-Quantitative RT-PCR. The animals were given 3 mg/kg *E.coli* LPS i.v. and they were killed at 6 hours later. The stomach were quickly removed, frozen in liquid nitrogen and stored at -80 °C until needed. Stomach tissue samples were pooled from 5 rats for extraction of total RNA, which were prepared by a single-step acid phenol-chloroform extraction procedure, by using ISOGEN (Nippon gene, Tokyo, Japan); and the concentration of RNA was adjusted to 1 μl/μl with RNase free distilled water. Semi-quantitative RT-PCR was performed as follows: First-strand cDNA was synthesized by using Takara Best RT-PCR kit (Takara Biomed., Japan) at 65 °C 1 minute, 30 °C 5 minutes, 65 °C 5 minutes, 98 °C 5 minutes by employing a thermal cycler. PCR amplification was performed by the hot starting method, using Takara Best RT-PCR (Takara Biomed., Japan). The sequences of primers used in this study were designed according to the published study (Devaux et. al., 2001). The sequences of sense and anti-sense primers for the rat COX-2 were 5'-TTCAAAAGAAGTTCTGGAAAAGGT-3' and 5'-GATCATGTCTACCTGAGTGTCTTT-3', respectively, giving rise to 304-bp PCR product. Likewise, the sequences of sense and anti-sense primers for the rat iNOS were 5'-GATCAATAACCTGAAGCCCG-3' and 5'-GCCCTTTTTTGCTCCATAGG-3', respectively, giving rise to 578-bp PCR product. In addition, for the rat β–actin, a constitutively expressed gene, the sequences were 5'-GGACTTCGAGCAGGAGATGG-3' for sense primer and 5'-GCACCGTGTTGGCGTAGAGG-3' for anti-sense primer, giving rise to a 232-bp PCR product. After initial denaturation at 95 °C for 10 minutes, cDNA of COX-2,

iNOS, β–actin were amplified at 20-40 cycles (5-cycle interval) of amplications at 95 °C for 60 seconds, 52 °C, 60 °C, 65 °C, respectively, for 30 seconds, 72 °C for 60 seconds by using a thermal cycler. PCR products in each cycle were electrophoresed on 2 % agarose gel containing 0.1 % ethidium bromide. Detectable fluorescent bands were visualized by an ultraviolet transilluminator and the area was measured by using NIH image software. The signals for examined mRNAs were standardized against that of the β-actin mRNA from each sample and the results were expressed as PCR-product/β-actin mRNA ratio.

Western Blot Analysis. The expression of iNOS, COX-1, COX-2 and β–actin proteins was examined by Western blotting. Gastric specimens were taken from the rats and proteins were partially purified according to the method of Gierse et al. (1995). In brief, the specimens were homogenized in 25 mM Tris-HCl (pH 8.0) buffer containing 250 mM sucrose, followed by centrifugation at 10,000 x g for 20 minutes. The pellet was resuspended in a 25 mM Tris-HCl (pH 8.0) buffer containing 1 % 3-[(3-cholamidopropyl) dimethylammonio]–1-propanesulfonate (CHAPS), and the mixture was gently stirred for 2 hours at 4 °C. The supernatant was recovered after centrifugation at 30,000 x g for 30 minutes and applied onto a DEAE-Sepharose CL-4B column (Amersham Pharmacia, Buckinghamshire, UK) that had been equilibrated with a 25 mM Tris-HCl (pH 8.0) buffer containing 0.5 % CHAPS, 1 mM phenylmethylsulfonyl fluoride and 0.2 mM EDTA. After the column was washed with the same buffer supplemented with 50 mM NaCl, elution was carried out with 200 mM NaCl. After aliquot (30 μg) of the eluted proteins had been subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the separated proteins were electrophoretically transferred onto Hybound-P membranes (Amersham Pharmacia, Buckinghamshire, UK). The membranes were incubated with

the primary polyclonal antibodies (Santa Cruz Biotechnology, California, USA) against iNOS, COX-1, COX-2 and the primary monoclonal antibody (Sigma-Aldrich, St Louis MI, USA) against β–actin protein overnight at 4 °C after nonspecific binding sites had been blocked with non-fat milk. The excess primary antibody was removed by washing in buffer three times for 10 minutes. Then the membranes were incubated with the horse-radish peroxidase-conjugated secondary IgG antibody at a dilution of 1:1000 in TBST (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.05 % Triton X-100) for 1 hour. Subsequently, the membranes were washed three times at 20 min each with TBST. The proteins were detected on X-ray films (Fuji Film, Tokyo, Japan) with an enhanced chemiluminescence kit (Amersham Pharmacia, Buckinghamshire, UK). The autoradiograph was then assessed semiquantitatively utilizing computer-assisted densitometry. Comparison between different groups was made by determination of the examinated protein/β-actin protein ratio of the immunoreactive area by densitometry.

Myeloperoxidase Activity. MPO activity was determined by a modification of the method of Grisham et al. (1986). Hundred microliters of mucosal homogenates were centrifuged at 20,000 x g for 15 min at 4 °C to form the insoluble cellular debris into pellets, which were then re-homogenized in an equivalent volume of 0.05 M potassium phosphate buffer (pH 5.4), containing 0.5 % hexadecyltrimethylammonium bromide. The samples were centrifuged at 20,000 x g for 15 minutes and the supernatant was saved. MPO activity was assessed by measuring the H_2O_2-dependent oxidation of 3, 3', 5, 5'-tetramethylbenzidine. One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance of 1.0 /min at 655 nm at 25 °C.

Statistics. Data are expressed as the mean ± SD of the values from 5 rats in each

JPET Fast Forward. Published on February 26, 2004 as DOI: 10.1124/jpet.103.061283
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group. Statistical analyses were performed using a one-way analysis of variance (ANOVA) followed by the Dunnet's multiple comparison test. Values that had been associated with probability (p value) of < 0.05 were considered significant.

Results

In Vivo NO Level in Gastric Mucosa After LPS Administration. NO produced in the gastric mucosa of rats was measured at 0, 2, 4, 6, 8, and 12 hours after LPS administration. The NO levels in the unit of nmol/g-tissue/30 minutes (control, 0.35 ± 0.16, n = 5) increased gradually after administration, reached a maximum (13.3 ± 3.3, n = 5) at 6 hours and then decreased thereafter (Fig. 1).

The increase in the NO level in the unit of nmol/g-tissue/30 min at 6 hours after LPS injection was significantly suppressed (4.0 ± 0.4, n = 5) by preadministration of a selective iNOS inhibitor, 1400W, and also by that of a nonselective COX inhibitor, indomethacin, and a selective COX-2 inhibitor, NS-398 (Fig. 2). As shown in Fig. 2, pretreatment of COX inhibitors suppressed the NO level in the unit of nmol/g-tissue/30 min dose-dependently (indomethacin; 10 mg/kg, 1.97 ± 0.62; 5 mg/kg, 4.9 ± 0.8; 1 mg/kg, 9.16 ± 1.98: NS-398; 10 mg/kg, 3.36 ± 1.03; 5 mg/kg, 6.7 ± 1.0; 1 mg/kg, 11.2 ± 0.81: n = 5).

Unless otherwise stated, the various measurements shown below were performed at 6 hours after LPS administration, at that time the NO production was maximized as just described.

In Vivo PGE2 Level in Gastric Mucosa After LPS Administration. Administration of LPS caused a significant and time-dependent increase in the gastric mucosal concentration of PGE2 (control, 288 ± 16; 6hr, 806 ± 15 pg/g-tissue: n = 5) (Fig. 3). Pretreatment of the rats with an iNOS inhibitor, 1400W, had no significant effect on the concentration of PGE2 (788 ± 26 pg/g-tissue, n = 5) at 6 hours after LPS administration, while that with COX inhibitors, indomethacin and NS-398, inhibited the rise in the gastric mucosal concentration of PGE2. When compared with the selective COX-2 inhibitor, NS-398, a nonselective COX inhibitor,

indomethacin, more strongly suppressed the PGE2.

Gastric Mucosal iNOS and COX Expression after LPS Administration. Administration of LPS caused an increase in expression of iNOS in gastric mucosa when compared with that of saline-treated control rats. The increase in iNOS mRNA determined by semi-quantitative RT-PCR (Fig. 4) correlated with an increase in iNOS protein measured by Western blot analysis (Fig. 5). The up-regulation was also associated with an induction of iNOS on the gastric epithelial cells as demonstrated by immunohistochemistry (Fig. 6). On the other hand, pre-administration of neither iNOS nor COX inhibitors significantly modified iNOS mRNA and protein expression at 6 hours after LPS administration (Fig. 4, Fig. 5). Administration of LPS resulted in a time-dependent increase in COX-2 mRNA (Fig. 7) and protein (Fig. 8) expression, while it had no significant effect on the COX-1 protein (Fig. 9) expression. The up-regulation of COX-2 in the gastric mucosa after LPS administration was also evident on imunohistochemistry (Fig. 6). Alternatively, pre-administration of neither iNOS nor COX inhibitors significantly modified COX-2 mRNA (Fig. 7) and protein (Fig. 8) expression at 6 hours after LPS administration.

MPO Activity. MPO activity, an index of tissue-associated neutrophil accumulation, was assayed in gastric mucosa of rats treated with saline and LPS. No significant difference was observed in the MPO activity between the saline controls (3.747 ± 0.629 mU/mg-protein, n = 5) and the rats at 6 hours after LPS treatment (5.088 ± 0.823 mU/mg-protein, n = 5).

Discussion

In the present study, *in vivo* cross-talk between products from iNOS and COX in the rat gastric mucosa during endotoxemia was examined by evaluating the effects of iNOS or COX inhibitors on levels of NO, PGE2, and protein and mRNA of iNOS and COXs. *In vivo* NO production in the gastric mucosa was determined directly, by using an EPR NO trapping technique. We obtained following findings; 1) the administration of *E.coli* LPS caused a time-dependent increase in the levels of NO and PGE2 in the gastric mucosa and enhanced the expression of iNOS and COX-2 protein/mRNA without significant effect on MPO activity; 2) relatively specific iNOS inhibitor, 1400W suppressed the increase in NO level of the gastric mucosa after LPS administration, but did not have any effect on the increase in gastric mucosal PGE2 and expression of COX-1 and COX-2; 3) both non-specific COX inhibitor, indomethacin, and COX-2 specific inhibitor, NS-398, inhibited the increase in gastric mucosal NO and PGE2 after LPS administration without any effect on the enhancement of expression of iNOS and COX; and the inhibitory effect of indomethacin on NO and PGE2 production was greater than NS-398.

The cross-talk between NOS and COX pathway may be divided into two interactions that include NOS-derived NO with COX activity and COX-derived prostaglandins with NOS activity.

On the interaction of NO with COX activity, contradictory results have been reported with respect to whether NO enhances, inhibits, or has little effect on the COX activity (Goodwin et al., 1999; Coffey et al., 2001 and references therein). A variety of chemical reactivities of the NO radical would affect both conformation and activity of COX enzyme. NO can attack COX enzyme directly through the coordination to heme iron in the prosthetic group of the enzyme or the reaction with

tyrosyl radical located in the active site (Salvemini et al., 1993; Gunther et al., 1997). In addition, NO influences the supply of COX substrate (Ma et al., 1996) and also COX-2 gene transcription (Schmedjte et al., 1997; Dela Torre et al., 1997). These *in vitro* experiments suggest that NO has the ability to lead the COX enzyme to two opposed states; activation or inactivation, depending on cell types employed or experimental conditions.

Several studies performed *in vivo* are also controversial with respect to the interaction of NO with COX activity (Paya et al., 1997; Hamilton et al., 1998; Devaux et al., 2001). In these studies, the iNOS inhibitors with different selectivity were employed and NO production was evaluated indirectly by measuring plasma or urinary levels of NOx (nitrite plus nitrate) which can be derived not only from endogenously produced NO but also from nitrate in a diet. On the one hand, an iNOS inhibitor, 1400W, has been shown to have markedly high selectivity for iNOS among the inhibitors reported and to be a useful tool in discriminating the roles of iNOS and cNOS *in vivo* (Hamilton et al., 1998). On the other hand, at present, direct NO assay method applicable to *in vivo* measurement is limited to the EPR NO trapping technique with Fe-DETC complex as an NO trapping agent (Nagano and Yoshimura, 2002). In the present study, accordingly, we employed 1400W as a selective iNOS inhibitor and the EPR NO trapping technique as a direct NO assay method. Here, we found that 1400W prevented iNOS activation but not its induction in the gastric mucosa caused by LPS treatment, although this compound did not affect the accompanying rise in gastric mucosal PGE2 contents as a consequence of COX-2 activation. These results negate the concept that iNOS-derived NO in inflammatory states *in vivo* may contribute to either induction or activation of COX-2.

Another consideration to the cross-talk between iNOS and COX-2 would be

required from the angle that COX-2 and its products including PGE2 may modulate iNOS pathway. Several studies focused on this point have been performed by the application of either COX inhibitors or PGE2 to *in vivo* or *in vitro* investigations (Bulut et al., 1993; Amin et al., 1995; Chen et al., 1999; Galea and Feinstein, 1999; Boje et al., 2003; Dobashi et al., 2003). These studies showed that the COX enzyme system widely contributes to iNOS gene induction and resultant NO production. In this study, performed *in vivo*, we showed that both non-selective and selective COX inhibitors, indomethacin and NS-398, respectively, suppressed the up-regulation in the gastric mucosal NO and PGE2 production that had been caused by systemic administration of LPS with their dose-dependent fashions, although the both inhibitors exerted neither reduction on iNOS nor COX-2 at their protein and mRNA levels. These results were essentially consistent with those observed in the cerebral tissues of restraint-stressed rat pretreated with NS-398 (Madrigal et al., 2003). Both indomethacin and NS-398 had been confirmed to have no direct inhibitory effect on enzymatic activity of iNOS in a cell free system (Posadas et. al., 2000). Altogether, these findings suggest that COX-2 activity may be closely related to the functional activation of iNOS but not to the expression of iNOS protein and mRNA in the gastric mucosa of LPS-treated rats.

The production of NO derived from iNOS is dependent on the intracellular availability of either 1) co-substrates for NO generation; L-arginine, NADPH and oxygen, or 2) co-factors cooperating with the enzyme; tetrahydrobiopterin (H_4B), heme, flavin mononucleotide, and flavin adenine dinucleotide. These factors may play critical roles in NO production from iNOS especially in *in vivo* conditions, even though the enzyme expression is sufficient to product NO in the gastric mucosa. On the other hand, two catalytic steps of COX-2, cyclooxygenase and peroxidase, are

associated with the production of not only prostanoids but also reactive oxygen species (Vane et al., 1998). The peroxidase action of COX-2 may affect intracellular oxygen status and effect on the NO-production derived from iNOS (Madrigal et al., 2003). In addition, hydrogen peroxide, one of the reactive oxygen species, is known to stimulate H_4B synthesis in vascular endothelial cells (Shimizu et al., 2003). Moreover, PGE2 may also play important roles in functional activation of iNOS enzyme through its receptor-ligated signaling mediated by a cyclic adenosine monophosphate (cAMP) (Bulut et al., 1993; Chen et al., 1999). PGE2 has been shown to increase intracellular cAMP in gastric mucosal cells (Hiraishi et al., 1986). The cAMP is known to participate in functional activation of iNOS enzyme through 1) accelerating a transmembrane intake of L-arginine from the outside and 2) suppressing intracellular metabolic conversion to L-citrulline in the cells (Ferro et al., 1999). Another way of the contribution of cAMP to iNOS activity is achieved by the induction of GTP cyclohydrolase I, a rate-limiting enzyme in H_4B biosynthesis (Pluss et al., 1996). These suggest that COX-2 enzyme activity and/or its products, such as PGE2, may be closely implicated in functional activation of iNOS enzyme through regulating its co-substrates and co-factors in addition to the expression of iNOS mRNA/protein. Alternatively, the post-translational modification –including protein stabilization, dimerization, phosphorylation, and subcellular localization– might explain the discrepancy between effects of PGE2 on NO production and iNOS protein/mRNA level. Consequently, these possibilities might lead to the discrepancy between the intracellular effects of COX activity on NO production and iNOS protein/mRNA expression. Regardless of the precise mechanisms, this *in vivo* study demonstrates that endogenous COX activity can modulate LPS-stimulated iNOS activity in the post-translational way. However, we analyzed only the production of

PGE2 as the representative of COX activity, thus it is difficult to rule out the possibility that the NO production was influenced not only by PGE2 but also by another COX products, such as thromboxane A2 or PGI2. Further study will be required to clarify the mechanisms of the post-translational modification of COX activity on NO production and, moreover, the regulatory mechanism of NO production by PGE2. The possible stimulatory role played by COX activity in NO production, in response to intracellular pathogens, is interesting in the light of a possible pharmacological regulation of the beneficial or detrimental effects of NO.

In summary, our data shows that PGE2 in the LPS-treated rat gastric mucosa increases the release of NO following activation of iNOS, although NO produced by iNOS does not stimulate the release of PGE2 by COXs in the rat gastric mucosa. During the pathogenesis of endotoxin-induced gastric injury, COX activity may be an important factor of NO production, including a co-factor and co-stimulator of iNOS activity. The effect of COX activity on iNOS pathway may be important in fine-tuning and regulation of gastric mucosal integrity in inflammatory states.

Acknowledgements

The authors thank Dr. Hidekazu Suzuki (Keio University School of Medicine) for his kind direction on myeloperoxidase activity assay.

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Footnotes

This work was supported in part by a Grant-in-Aid to T. Y. for Scientific Research from Japan Society for the Promotion of Science (15590087) and for Scientific Research on Priority Areas (Area No. 769) from the Ministry of Education, Culture, Sports, Science and Technology (15087212).

Figure legends

Fig. 1. Time course of NO production of the gastric mucosa after LPS injection. NO produced in the gastric mucosa of rat was measured at 0, 2, 4, 6, 8, and 12 hours after LPS administration (3 mg/kg), using an EPR NO trapping technique. The NO trapping agent (Fe-DETC complex) was injected 30 minutes before taking measurement. The amplitude of the signal, which was proportional to the amount of NO, was obtained by measuring the peak-to-peak height of the lower field side signal in a three-line spectrum which is characteristic of NO adduct. The NO adduct concentration of Fe-DETC complex was estimated by comparing it with the signal height of a standard solution of a chemically synthesized NO complex. The concentration in tissues estimated at 30 minutes after the injection of NO trapping reagent is expressed in the unit of nmol/g-tissue/30 min. The instrument settings were: center field, 331 mT; field scan, 4 mT; sweep time, 4 minutes; time constant, 0.3 seconds; modulation amplitude, 0.32 mT; modulation frequency, 100 K; microwave power, 60 mW; microwave frequency, ~ 9.5 GHz. Each plot represents mean ± S.D. (n = 5). (**p< 0.01 vs. LPS 0 hr; ***p < 0.001 vs. LPS 0 hr)

Fig. 2. Effect of inhibitors of iNOS and COX on NO production in the gastric mucosa. An iNOS selective inhibitor, 1400W (5 mg/kg, i.v.), a nonselective COX inhibitor, indomethacin (IM; 1, 5, 10 mg/kg, s.c.), and a COX-2 selective inhibitor, NS-398 (1, 5, 10 mg/kg, s.c.) in saline administered into rats 30 minutes before LPS injection; and the effect of the inhibitors on NO generation was examined at 2 and 6 hours after the LPS injection. The dependence of NO generation on COX-inhibitor doses was examined at 6 hr after LPS injection. Data are mean ± S.D. (n = 5). (*p < 0.05 IM 1

mg/kg + LPS vs. IM 5 mg/kg + LPS; **p < 0.01 LPS 6 hr vs. 1400W + LPS, IM 1 mg/kg + LPS vs. IM 10 mg/kg + LPS, NS398 1 mg/kg + LPS vs. NS398 5 mg/kg + LPS; ***p<0.001 LPS 6 hr vs. IM 10 mg/kg + LPS, NS398 10 mg/kg + LPS, NS398 1 mg/kg + LPS vs. NS398 10 mg/kg + LPS)

Fig. 3. Effect of inhibitors of iNOS and COX on prostaglandin E2 production in the gastric mucosa. An iNOS selective inhibitor, 1400W (5 mg/kg, i.v.), a nonselective COX inhibitor, indomethacin (IM; 10 mg/kg, s.c.), and a COX-2 selective inhibitor, NS-398 (10 mg/kg, s.c.) in saline were administered into rats 30 minutes before LPS injection; and the effect of the inhibitors on PGE2 generation in the glandular gastric mucosa was examined at 6 hours after the LPS injection. Data are mean ± S.D. (n = 5). (***p< 0.001 vs. LPS 6 hr)

Fig. 4 Effect of inhibitors of iNOS and COX on iNOS mRNA induction. An iNOS selective inhibitor, 1400W (5 mg/kg, i.v.), a nonselective COX inhibitor, indomethacin (IM; 10 mg/kg, s.c.), and a COX-2 selective inhibitor, NS-398 (10 mg/kg, s.c.), in saline administered into rats 30 minutes before LPS injection; and the effect of the inhibitors on iNOS mRNA in the glandular gastric mucosa was examined at 6 hours after the LPS injection. (A) Representative RT-PCR profile of rat stomach mRNA expression. The PCR products were detected as 578- and 232-bp bands for iNOS and β-actin mRNA, respectively. (B) Semi-quantitative analysis of iNOS mRNA using densitometric scanning of the amplified PCR products. Each iNOS signal was standardized against the corresponding β-actin signal, and results are expressed as an iNOS/β-actin ratio. Data are mean ± S.D. (n = 5). (*p<0.05 vs. LPS 6 hr)

Fig. 5 Effect of inhibitors of iNOS and COX on iNOS protein expression. An iNOS selective inhibitor, 1400W (5 mg/kg, i.v.), a nonselective COX inhibitor, indomethacin (IM; 10 mg/kg, s.c.), and a COX-2 selective inhibitor, NS-398 (10 mg/kg, s.c.), in saline administered into rats 30 minutes before LPS injection; and the effect of the inhibitors on iNOS protein in the glandular gastric mucosa was examined at 6 hours after the LPS injection. (A) Representative Western blot profile of rat stomach protein expression. The proteins of iNOS and β-actin were detected as 131- and 42-kDa bands, respectively. (B) Quantitative analysis of iNOS protein expression using densitometric scanning of Western blots. Each iNOS signal was standardized against the corresponding β-actin signal and results are expressed as an iNOS/β-actin ratio. Data are mean ± S.D. (n = 5). (*p< 0.05 vs. LPS 6 hr; **p < 0.01 vs. LPS 6 hr)

Fig. 6. A study of immunohistchemistry for iNOS and COX-2 protein. Immunostaining for iNOS in the intact gastric mucosa (A) and 6 hours after exposure to LPS (B). Immunostaining for COX-2 in the intact gastric mucosa (C) and 6 hours after exposure to LPS (D). (Original magnification 400 x) iNOS and COX-2 staining was absent in the intact gastric epithelium but there was iNOS and COX-2 staining of the gastric epithelium in 6 hours after exposure to LPS.

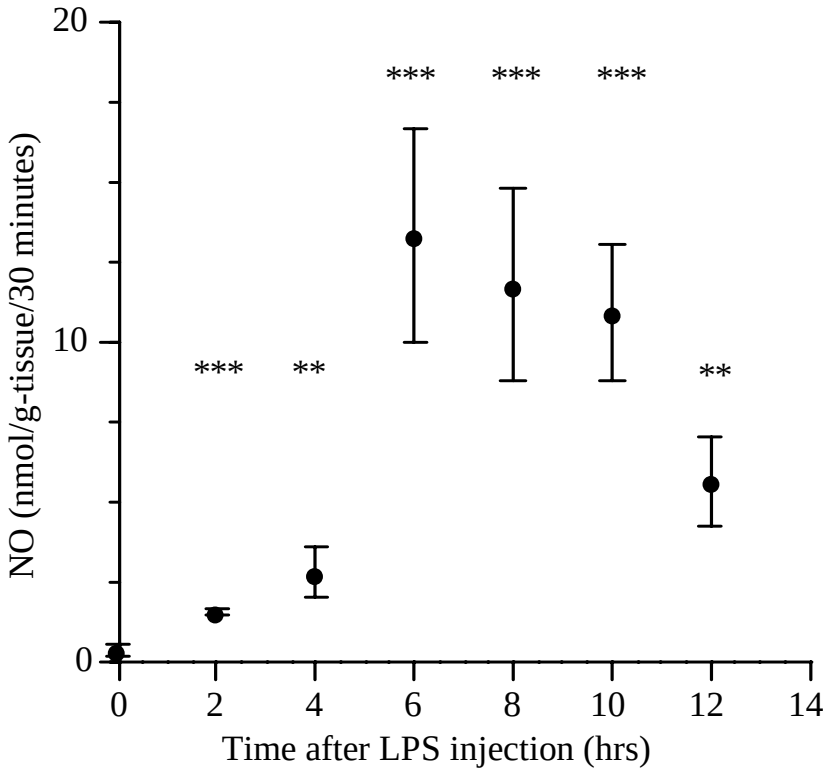
Fig. 7. Effect of inhibitors of iNOS and COX on COX-2 mRNA induction. An iNOS selective inhibitor, 1400W (5 mg/kg, i.v.), a nonselective COX inhibitor, indomethacin (IM; 10 mg/kg, s.c.), and a COX-2 selective inhibitor, NS-398 (10 mg/kg, s.c.), in saline administered to rats 30 minutes before LPS injection; and the effect of the inhibitors on COX-2 mRNA in the glandular gastric mucosa was examined at 6 hours after the LPS injection. (A) Representative RT-PCR profile of rat stomach mRNA

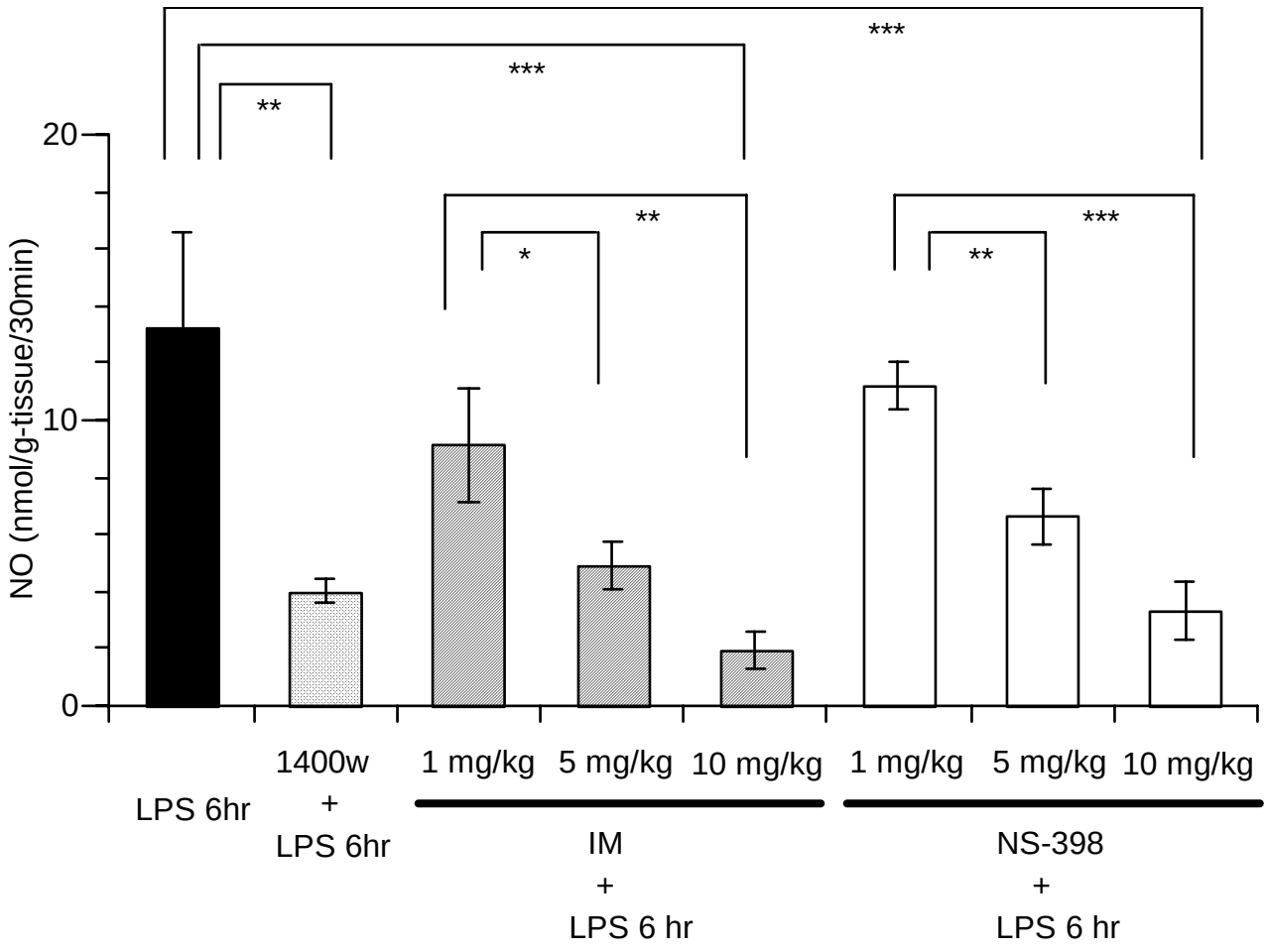
expression. The PCR products were detected as 304- and 232-bp bands for COX-2 and β-actin mRNA, respectively. (B) Semi-quantitative analysis of COX-2 mRNA using densitometric scanning of amplified PCR products. Each COX-2 signal was standardized against the corresponding β-actin signal and results are expressed as a COX-2/β-actin ratio. Data are mean ± S.D. (n = 5). (**p< 0.01 vs. LPS 6 hr)

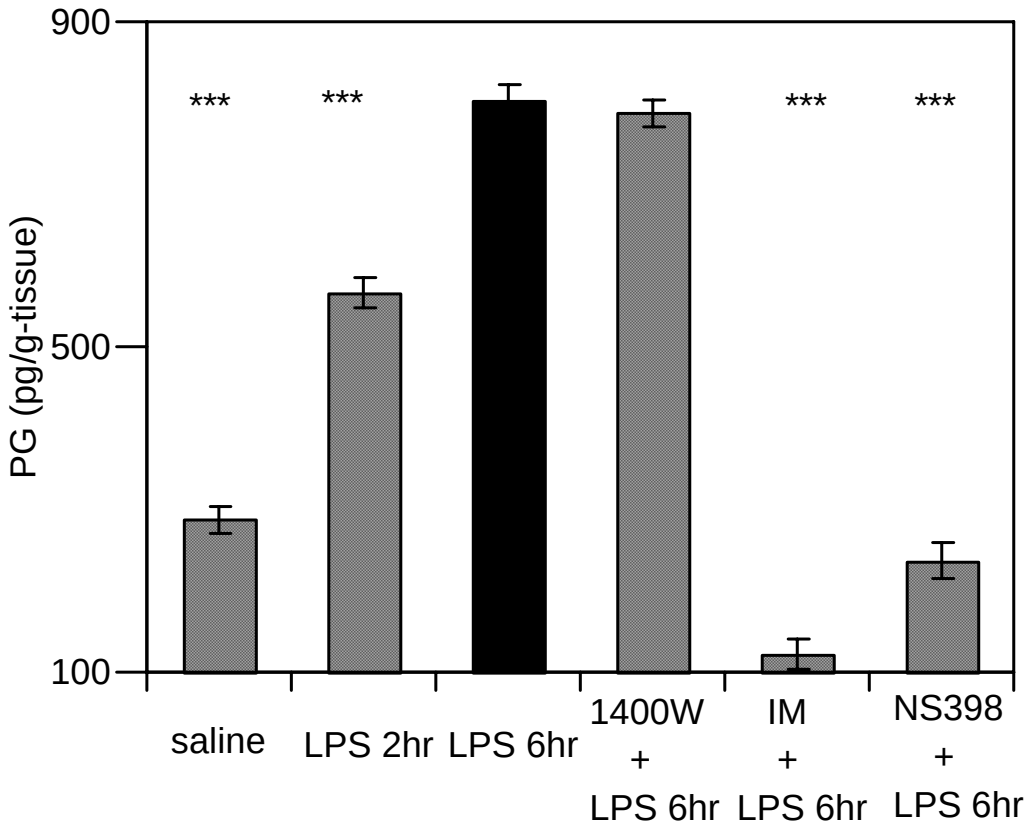
Fig. 8. Effect of inhibitors of iNOS and COX on COX-2 protein expression. An iNOS selective inhibitor, 1400W (5 mg/kg, i.v.), a nonselective COX inhibitor, indomethacin (IM; 10 mg/kg, s.c.), and a COX-2 selective inhibitor, NS-398 (10 mg/kg, s.c.), in saline administered to rats 30 minutes before LPS injection; and the effect of the inhibitors on COX-2 protein in the glandular gastric mucosa was examined at 6 hours after the LPS injection. (A) Representative Western blot profile of rat stomach protein expression. The proteins of COX-2 and β-actin were detected as 81- and 42-kDa bands, respectively. (B) Quantitative analysis of COX-2 protein expression using densitometric scanning of Western blots. Each COX-2 signal was standardized against the corresponding β-actin signal, and results are expressed as a COX-2/β-actin ratio. Data are mean ± S.D. (n = 5). (*p< 0.05 vs. LPS 6 hr; **p < 0.01 vs. LPS 6 hr)

Fig. 9. Effect of inhibitors of iNOS and COX on COX-1 protein expression. An iNOS selective inhibitor, 1400W (5 mg/kg, i.v.), a nonselective COX inhibitor, indomethacin (IM; 10 mg/kg, s.c.), and a COX-2 selective inhibitor, NS-398 (10 mg/kg, s.c.), in saline administered to rats 30 minutes before LPS injection; and the effect of the inhibitors on COX-1 protein in the glandular gastric mucosa was examined at 6 hours after the LPS injection. (A) Representative Western blot profile of rat stomach protein

expression. The proteins of COX-1 and β-actin were detected as 69- and 42-kDa bands, respectively. (B) Quantitative analysis of COX-1 protein expression using densitometric scanning of Western blots. Each COX-1 signal was standardized against the corresponding β-actin signal and results are expressed as a COX-1/β-actin ratio. Data are mean ± S.D. (n = 5).







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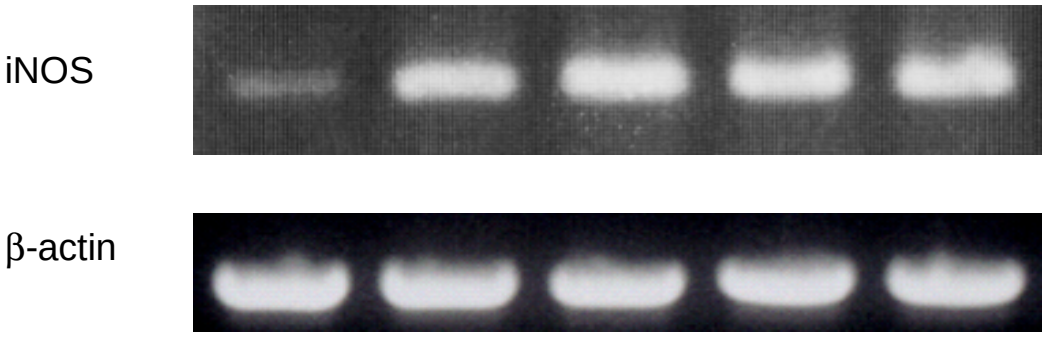

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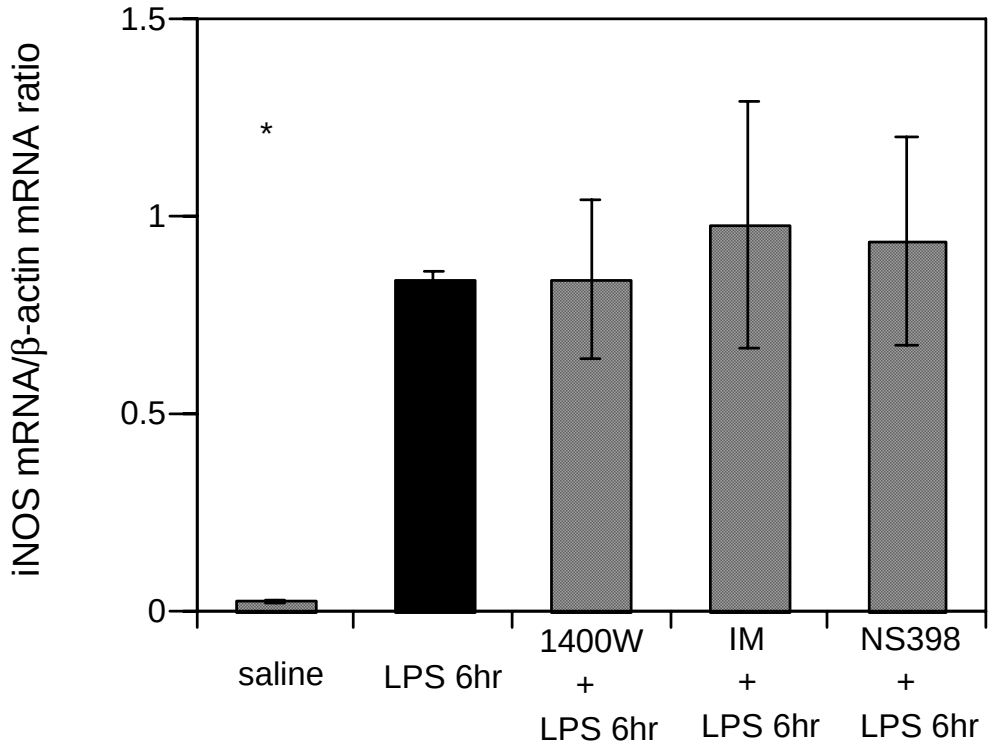

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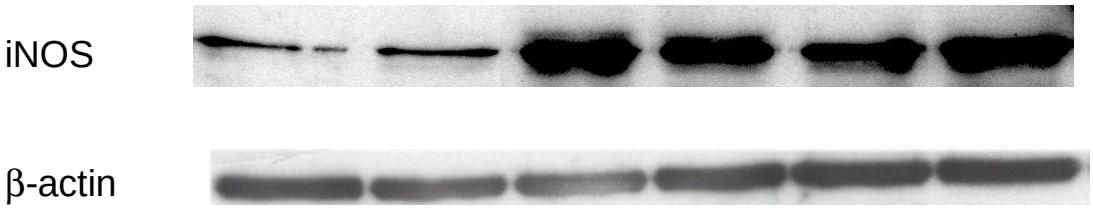

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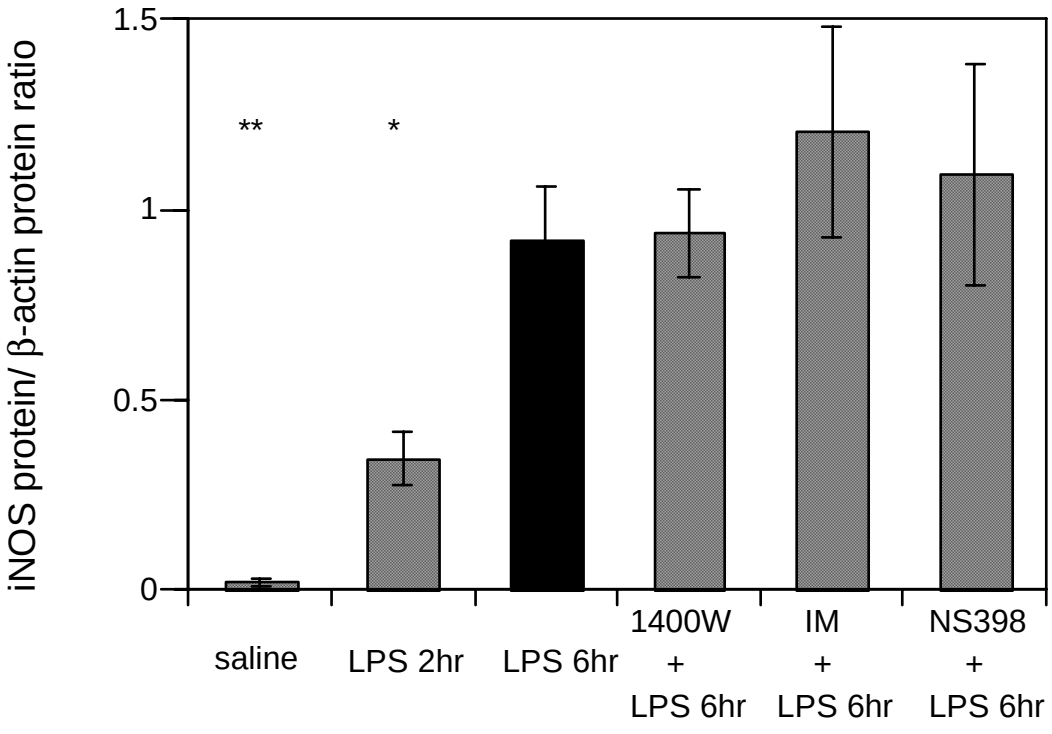
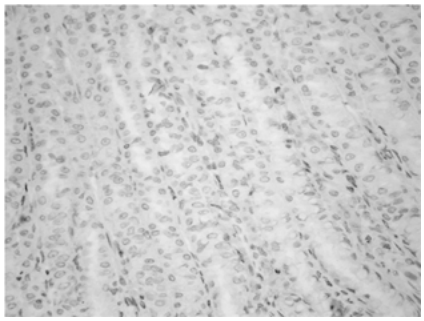
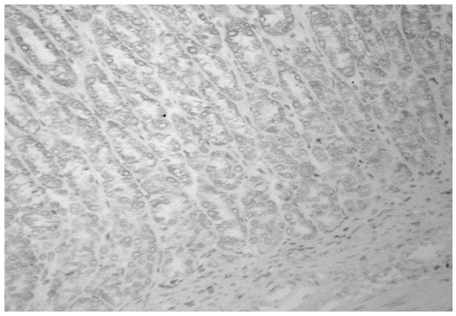


A. iNOS (control)



C. COX-2 (control)



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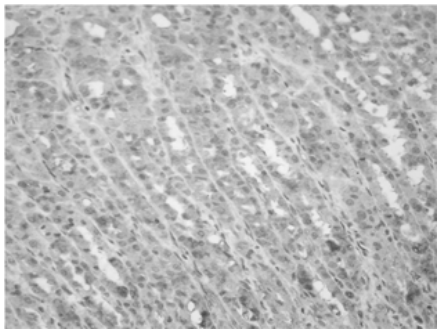

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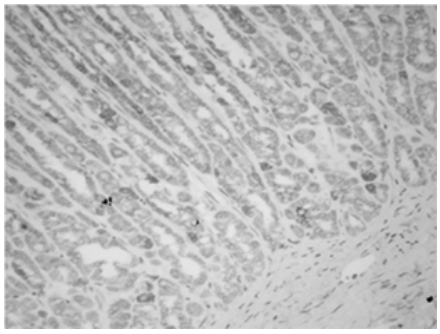

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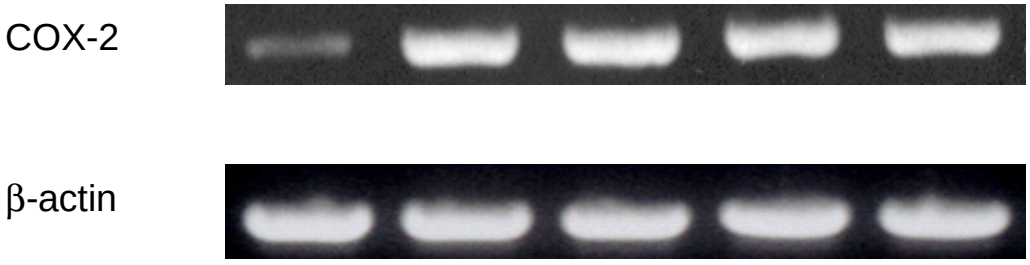

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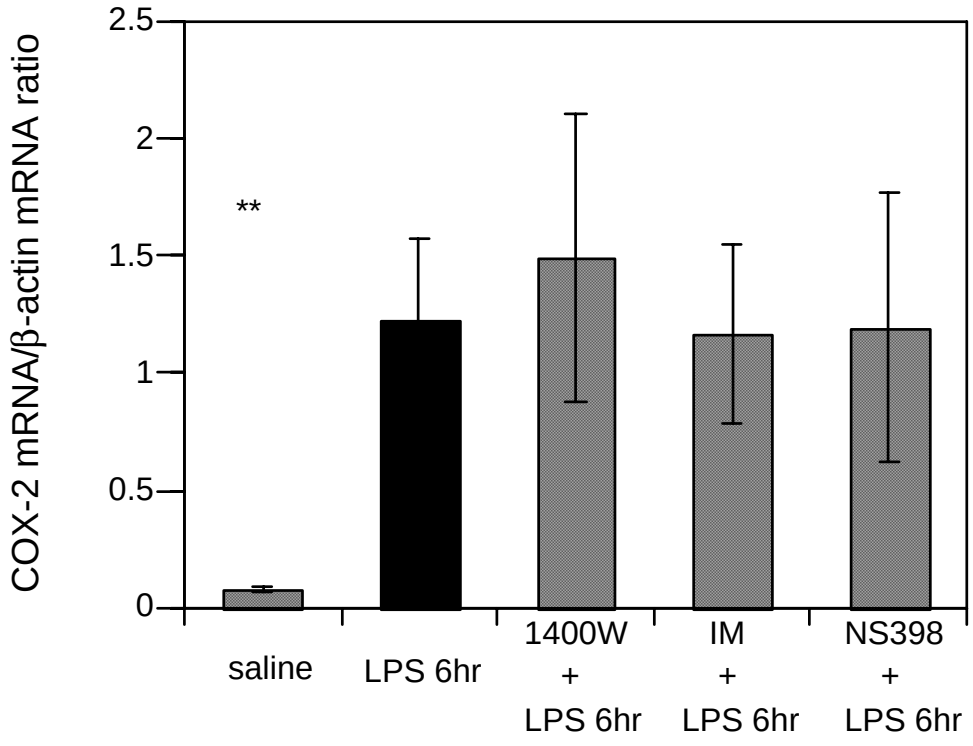

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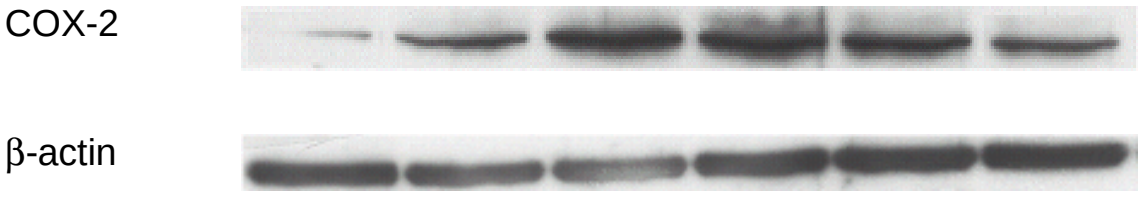

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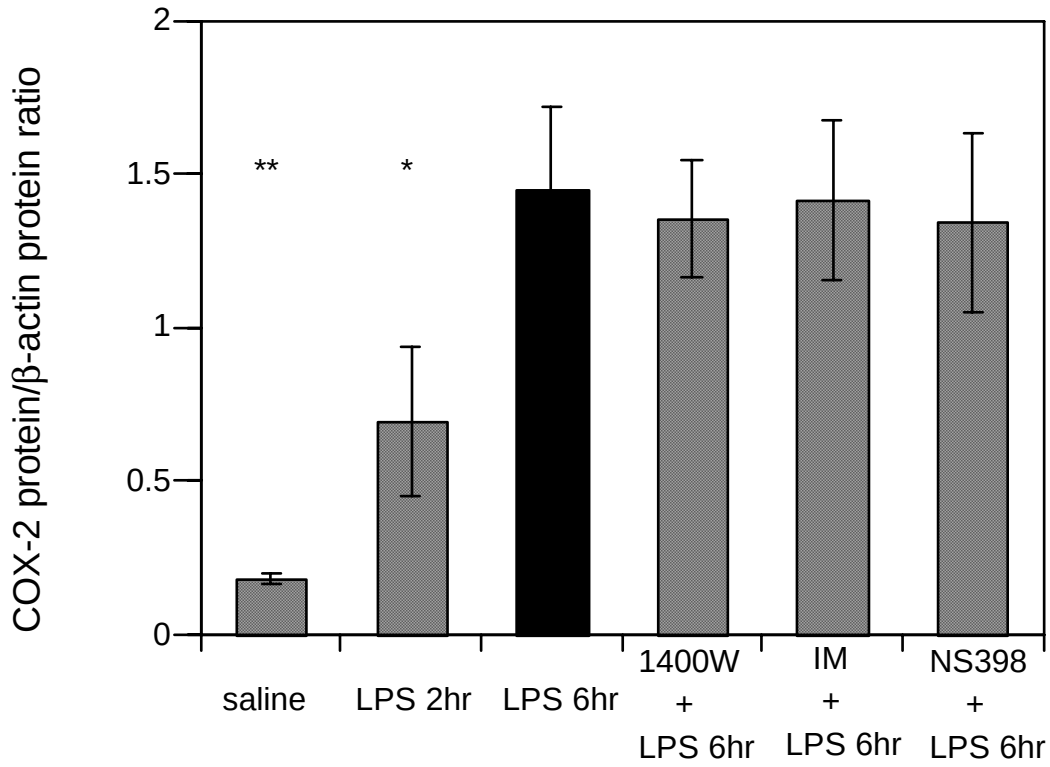

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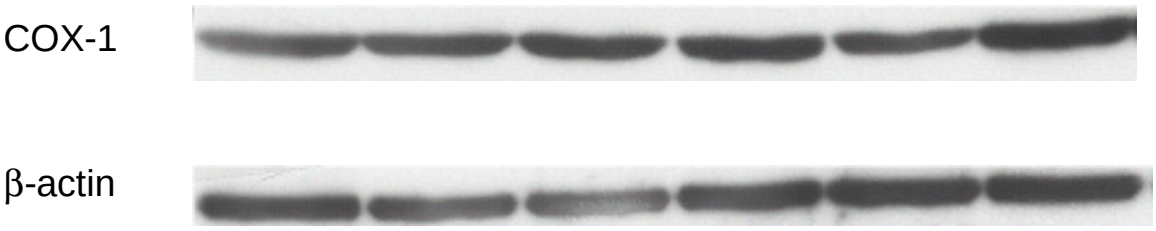

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