

Rofecoxib Produces Intestinal but not Gastric Damage in the Presence of A Low Dose of Indomethacin in Rats

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Abbreviations:

nonsteroidal antiinflammatory drug ----- NSAID

prostaglandin ----- PG

nitric oxide ----- NO

cyclooxygenase ----- COX

N^G-nitro-L-arginine methylester ----- L-NAME

constitutively expressed NO synthase ----- cNOS

inducible NO synthase ----- iNOS

reverse transcription polymerase chain reaction ----- RT-PCR

colony-forming units ----- CFU

enzyme immunoassay ----- EIA

ABSTRACT

Indomethacin in small doses is known to inhibit prostaglandin (PG) production, yet it does not damage the gastrointestinal mucosa. We examined whether a cyclooxygenase (COX)-2 inhibitor induces gastrointestinal damage in the presence of a low dose of indomethacin and investigated the ulcerogenic mechanism in relation to COX-2 expression. Rats with or without 18 hr fasting were administered rofecoxib (a selective COX-2 inhibitor: 10 or 30 mg/kg) p.o., in the absence or presence of indomethacin (3 mg/kg, p.o.), and the gastric or intestinal mucosa was examined 8 and 24 hr later, respectively. Neither indomethacin nor rofecoxib alone caused damage in the stomach or small intestine. However, indomethacin damaged the small intestine in the presence of rofecoxib, yet the same treatment did not damage the stomach. Indomethacin reduced the mucosal PGE₂ content in both tissues, while rofecoxib did not. The COX-2 mRNA was up-regulated in the intestine but not the stomach after indomethacin treatment, and the reduced PGE₂ content was significantly recovered later only in the small intestine, in a rofecoxib inhibitable manner. Indomethacin produced hypermotility in the small intestine but not the stomach, while rofecoxib had no effect. These results suggest that the PG deficiency caused by a low dose of indomethacin produces hypermotility and COX-2 expression in the small intestine but not the stomach, resulting in damage when COX-2 is inhibited. It is assumed that the hypermotility response is a key event in the expression of COX-2 and thereby important in the development of mucosal damage in the gastrointestinal tract.

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin damage the gastrointestinal tract as an adverse reaction. Although multiple factors are involved in the pathogenesis of NSAID-induced damage, a deficiency of endogenous prostaglandins (PGs) is most important as a background for such lesions (Whittle, 1981; 1995; Weissenborn et al., 1985; Takeuchi et al., 1986; 1991; 2002a; Asako et al., 1995; Wallace et al., 1995; 2000; Konaka et al., 1999). PG deficiency caused by NSAIDs is due to an inhibition of both cyclooxygenase (COX)-1 and COX-2. Recent studies including our own demonstrated that inhibition of both COX-1 and COX-2 is required for NSAID-induced gastrointestinal damage (Wallace et al., 2000; Tanaka et al., 2001; 2002a; 2002b). Furthermore, we also found the up-regulation of COX-2 expression in the gastrointestinal mucosa after administration of NSAIDs as well as the selective COX-1 inhibitor SC-560 (Tanaka et al., 2002a; 2002b). These studies suggested the prerequisite of COX-2 expression and its inhibition in the gastrointestinal ulcerogenic responses to NSAIDs.

On the other hand, indomethacin at a low dose (5 mg/kg) inhibited PG production due to COX-1 inhibition, yet did not produce damage in the gastrointestinal mucosa (Takeuchi et al., 1986). Indomethacin at lower doses reportedly inhibits COX-1 activity more potently than COX-2 activity (Warner et al., 1999). Thus, it is possible that COX-2 inhibitors damage the gastrointestinal mucosa when administered in combination with a low dose of indomethacin.

In the present study, we examined whether or not rofecoxib, a selective COX-2 inhibitor, provokes damage in the stomach and/or small intestine when a deficiency of endogenous PGs is concurrently induced by a low dose of indomethacin, in relation to the hypermotility response and COX-2 expression.

Materials and Methods

Male Sprague-Dawley rats (220-260 g; Nippon Charles River) were used. The gastric ulcerogenic response was examined in animals fasted for 18 hr prior to the experiment (Tanaka et al., 2002a), while the intestinal ulcerogenic response was examined in non-fasting animals (Tanaka et al., 2002b). In the former case, animals were kept in cages with raised mesh bottoms and deprived of food but allowed free access to tap water for 18 hr prior to the experiments. Studies were carried out using 4~6 rats per group and performed under unanesthetized conditions, unless otherwise specified. All experimental procedures described here were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University.

Evaluation of Gastric and Intestinal Ulcerogenic Responses

Various COX inhibitors such as indomethacin (a nonselective COX inhibitor: 3 mg/kg), SC-560 (a selective COX-1 inhibitor: 10 or 30 mg/kg; 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole) and rofecoxib (a selective COX-2 inhibitor: 10 or 30 mg/kg), either alone or in combination, were given p.o., and the animals were killed 8 and 24 hr later, respectively, in order to examine the gastric ulcerogenic or intestinal response to the above treatments. Upon the combined administration, SC-560 or rofecoxib was given 30 min after the administration of indomethacin. The stomach or small intestine was excised and treated with 2% formalin for fixation of the tissue walls. Then, the stomach or small intestine was opened along the greater curvature or anti-mesenteric attachment, respectively, and examined for damage under a dissecting microscope with square grids (x10). The area (mm²) of macroscopically visible lesions was measured, summed for each tissue, and used as a lesion score. The person measuring the lesions did not know the treatments given to the animals. In some cases, the effects of

atropine, ampicillin and aminoguanidine on the intestinal lesions induced by rofecoxib plus indomethacin were examined. Ampicillin (800 mg/kg) was given p.o. twice 24 hr and 30 min before the administration of indomethacin while atropine (3 mg/kg) or aminoguanidine (20 mg/kg) was given s.c. 30 min before.

Determination of Mucosal PGE₂ content

PGE₂ levels in the gastric and small intestinal mucosa were measured after p.o. administration of indomethacin (3 mg/kg), SC-560 (10 or 30 mg/kg) or rofecoxib (10 or 30mg/kg). Three, 8 or 24 hours later, the animals were killed under deep ether anesthesia, and the gastric or small intestinal tissue was isolated, weighed, and placed in a tube containing 100% ethanol plus 0.1 M indomethacin (Futaki et al., 1994). Then, the tissues were homogenized by Polytron homogenizer (IKA, Tokyo, Japan) and centrifuged at 10,000 rpm for 10 min at 4°C. After the supernatant of each sample had been evaporated with N₂ gas, the residue was resolved in assay buffer and used for determination of PGE₂. The concentration of PGE₂ was measured using a PGE₂ enzyme immunoassay (EIA) kit (Amersham Pharmacia Biotech, UK).

Analysis of COX-1, COX-2 and iNOS mRNA expression by RT-PCR

The animals, with or without 18 hr fasting, were killed under deep ether anesthesia 6 hr after p.o. administration of indomethacin (3 mg/kg), SC-560 (10 or 30 mg/kg) and rofecoxib (10 or 30 mg/kg), and the stomachs or small intestines were removed, frozen in liquid nitrogen, and stored at -80°C prior to use. In some cases, atropine (3 mg/kg) was given s.c. 30 min before indomethacin, while ampicillin (800 mg/kg) was given p.o. twice 24 hr and 30 min before administration of indomethacin. The tissue samples were pooled from 2~3 rats for extraction of total RNA, which was prepared by a single-step acid phenol-chloroform extraction procedure by use of TRIZOLE (GIBCO BRL, Gaithersburg, MD). Total RNA primed by random hexadeoxy

ribonucleotide was reverse-transcribed with the SUPERScript preamplification system (GIBCO BRL). The sequences of sense and antisense primers for the rat COX-1 were 5'-AACCGTGTGTGTGACTTGCTGAA-3' and 5'-AGAAAGAGCCCCTCAGAGCTCAGTG-3', respectively, giving rise to a 887-bp PCR product (Feng et al., 1993). For the rat COX-2, the sequences of sense and antisense primers were 5'-TGATGACTGCCCAACTCCCATG-3' and 5'-AATGTTGAAGGTGTCCGGCAGC-3', respectively, giving rise to a 702-bp PCR product (Tso et al., 1985). The sequences of sense and antisense primers for the rat iNOS were 5'-CGGTTCACAGTCTTGGTGAAAG-3' and 5'-CAGGTGTTCCCAGGTAGGTAG-3', respectively, giving rise to a 651-bp PCR product (Lyons et al., 1992). For the rat glyceraldehyde-3-phosphate dehydrogenase (G3PDH), a constitutively expressed gene, the sequence was 5'-GAACGGGAAGCTCACTGGCATGGC-3' for the sense primer and 5'-TGAGGTCCACCACCCTGTTGCTG-3' for the antisense primer, giving rise to a 310 bp PCR product (Feng et al., 1993; Tso et al., 1985). An aliquot of the RT reaction product served as a template in 35 cycles of PCR with 1 min of denaturation at 94°C, 0.5 min of annealing at 58°C and 1 min of extension at 72°C on a thermal cycler. A portion of the PCR mixture was electrophoresed in 1.8% agarose gel in TAE buffer (Tris buffer 40 mM, EDTA 2 mM and acetic acid 20 mM; pH 8.1), and the gel was stained with ethidium bromide and photographed.

Determination of Gastric and Intestinal motility

Gastric and intestinal motility was determined using a miniature balloon according to the previously published method (Takeuchi et al., 1986; 2002a). For determination of gastric motility, the balloon and the support catheter were placed into the stomach through an incision of the forestomach under ether anesthesia. The animals were then kept in Bollman cages, and gastric motility was monitored on a Hitachi recorder (Model 056, Mito, Japan) using a pressure transducer (Narco Telecare, Model 151-T,

Houston, Tx., U.S.A.) and a polygraph device (San-ei, Model 6M-72, Tokyo, Japan) after complete recovery from anesthesia. On the other hand, intestinal motility was determined under urethane anesthesia (1.25 g/kg, i.p.). The trachea was cannulated to ensure respiration. A midline incision was then made to expose the small intestine, and a thin, saline-filled balloon, made from silicone rubber and attached to a polyethylene catheter, was introduced into the jejunum via a small incision and tied in place avoiding large blood vessels. The volume in the balloon was adjusted to give an initial resting pressure of 5 mmHg, which was not sufficient to cause active distension of the intestinal wall, and after allowing the preparation to rest for 30 min, intestinal motility was monitored on a recorder (U-228, Tokai-Irika, Tokyo, Japan) as intraluminal pressure changes, through a pressure transducer and polygraph device (Nihon Kodan, Ibaragi, Japan). Indomethacin (3 mg/kg), SC-560 (10 or 30 mg/kg) or rofecoxib (10 or 30 mg/kg) was given i.d. after basal gastric or intestinal motility had well stabilized, and the motility was measured for 3 hr thereafter. In some cases, atropine (3 mg/kg) or aminoguanidine (20 mg/kg) was given s.c. 2 hr after the administration of indomethacin, while ampicillin (800 mg/kg) was given p.o. 24 hr before. Quantitation of gastric or intestinal motility was performed by measuring the area of motility changes in a recording sheet using NIH image 1.61 (NIH, Bethesda, MD), and the data were expressed as the motility index (arbitrary units).

Determination of enterobacterial counts

The enterobacteria were enumerated according to a modified version of the method originally described by Reuter et al. (1997). Six hours after administration of indomethacin (3 mg/kg), the animals were killed under deep ether anesthesia, and their small intestines were removed. After the rinsing of each intestine with sterile saline, the mucosa was scraped with glass slides, weighed, and homogenized in 1 ml of sterile phosphate-buffered saline (PBS) per 100 mg of wet tissue. Aliquots of the homogenate

were placed on blood agar and GAM agar (Nissui, Osaka, Japan). Blood agar plates were incubated at 37°C for 24 hr under aerobic conditions, while GAM agar plates were incubated for 48 hr under standard anaerobic conditions (BBL Gas Pack Pouch Anaerobic System, Becton Dickinson, Maryland, MD). Plates containing 10~200 colony-forming units (CFU) were examined for numbers of enterobacteria, and the data were expressed as log CFU/g tissue. In some rats, ampicillin (800 mg/kg) was given p.o. twice 24 hr and 30 min before the administration of indomethacin while atropine (3 mg/kg) was given s.c. 30 min before.

Determination of iNOS activity

The animals were killed under deep ether anesthesia 24 hr after the administration of indomethacin (3 mg/kg) and rofecoxib (10 mg/kg), either alone or in combination, and their small intestines were removed. After the rinsing of the intestine with cold saline, the mucosa was scraped with glass slides, weighed, and used for the determination of iNOS activity. The iNOS activity was measured by determining the conversion of radiolabeled L-arginine to citrulline, according to the method described by Brown et al. (1992). The tissue was homogenized in ice-cold buffer (Tris-HCl 50 mM, sucrose 32 mM, dithiothreitol 1 mM, leupeptin 10 µg/ml and aprotinin 2 µg/ml), adjusted to pH 7.4 with NaOH, and centrifuged for 20 min at 10,000 rpm at 4°C. The supernatant was incubated for 60 min at 37 °C in a reaction buffer containing [³H]-L-arginine at 0.5 µCi/ml. The level of activity of constitutively expressed NOS (cNOS) was determined from the difference in the presence and absence of 1 mM EGTA; the activity of iNOS was evaluated in the presence of 1 mM EGTA. Sample protein content was estimated by a Hitachi spectrophotometer (U-2000, Hitachi, Ibaraki, Japan), and the NOS activity was expressed as pmol citrulline/min/mg protein.

Preparation of drugs

The drugs used were indomethacin, ampicillin (Sigma Chemicals, St. Louis, MO), SC-560 (Cayman Chemical, Ann Arbor, MI), rofecoxib (synthesized in our laboratory), ampicillin, aminoguanidine, atropine (Nakarai Tesque, Kyoto, Japan) and urethane (Tokyo Kasei, Tokyo, Japan). All COX inhibitors were suspended in a hydroxy propyl cellulose (HPC) solution (Wako, Osaka, Japan) while other drugs were dissolved in saline. All drugs were prepared immediately before use and administered p.o., i.d., i.p. or s.c. in a volume of 0.5 ml/100 g body weight.

Statistics

Data are presented as the mean \pm SE of 4 to 6 rats per group. Statistical analyses were performed using the two-tailed Dunnett's multiple comparison test, and values of $P<0.05$ were considered significant.

Results

Gastric and Intestinal Ulcerogenic Response to Indomethacin plus

Rofecoxib

Neither SC-560 nor rofecoxib at 30 mg/kg alone produced any damage in the stomach (**Figure 1A**). Similarly, these agents alone at 10 mg/kg did not damage the small intestine (**Figure 1B**). However, when these agents were given together, severe damage was induced in both the stomach and intestine at an incidence of 100%. Likewise, indomethacin at 3 mg/kg did not cause any damage in the stomach or intestine. However, when administered together with rofecoxib, the selective COX-2 inhibitor, it produced damage in the small intestine but not the stomach.

The intestinal lesions caused by indomethacin (3 mg/kg) plus rofecoxib (10 mg/kg) were significantly attenuated by the antibiotic ampicillin (800 mg/kg) and the anticholinergic drug atropine (3 mg/kg) as well as the selective iNOS inhibitor aminoguanidine (20 mg/kg), at doses that reportedly prevent intestinal lesions induced by a high dose of indomethacin (10 mg/kg) (Takeuchi et al., 2002a; Tanaka et al., 2002b) (**Figure 2**).

Effects of Various COX Inhibitors on Mucosal PGE₂ Production

Levels of PGE₂ in the normal rat gastric mucosa and small intestine were 15.3±4.1 ng/g tissue and 31.1±6.0 ng/g tissue, respectively (**Figures 3A and 3B**). Indomethacin at 3 mg/kg significantly decreased the mucosal PGE₂ content in the stomach and small intestine when determined 3 hr after the administration. Similarly, SC-560 at the doses used significantly inhibited PGE₂ production in these tissues, while rofecoxib did not. This inhibitory effect of indomethacin on PGE₂ production was not affected by co-

administration of rofecoxib (not shown).

The PGE₂ content remained reduced in the stomach 8 hr after the administration of indomethacin, and this effect was not influenced by additional treatment with rofecoxib. However, when the PGE₂ content was measured in the small intestine 24 hr after indomethacin treatment, the PGE₂ content was significantly recovered, reaching approximately 70% of control levels, in a rofecoxib-inhibitable manner. In the animals treated with SC-560, the PGE₂ content was significantly recovered in the stomach 8 hr later and in the small intestine 24 hr later, and these changes were significantly inhibited by the combined administration of rofecoxib, consistent with our previous studies (not shown)(Tanaka et al., 2002a; 2002b).

Effect of Indomethacin on COX-2 mRNA Expression in The Stomach and Small Intestine

We previously reported that NSAID up-regulates the expression of COX-2 in the gastrointestinal mucosa and that inhibition of this enzyme activity is a key to NSAID-induced damage in these tissues (Tanaka et al., 2002a; 2002b). Since indomethacin at 3 mg/kg decreased PGE₂ contents in both the stomach and small intestine, as effectively as SC-560, we examined whether the expression of COX-2 is also up-regulated in these tissues by indomethacin at the non-ulcerogenic dose of 3 mg/kg.

The gene expression of COX-2 was negligible in the normal rat stomach and small intestine, although the expression of G3PDH, the house keeping gene was clearly observed in these tissues (**Figure 4A and 4B**). Indomethacin at 3 mg/kg induced COX-2 mRNA expression in the small intestine but not the stomach. The up-regulation of COX-2 expression in the small intestine of the indomethacin-treated rats was attenuated by prior administration of ampicillin (800 mg/kg) or atropine (3 mg/kg).

Effects of Indomethacin, SC-560 and Rofecoxib on Gastric and Intestinal Motility

The normal rat stomachs contracted at a frequency of 16~20/10 min with an amplitude of 18.6 ± 3.2 cmH₂O. The gastric motility was markedly enhanced by intragastric administration of SC-560 (30 mg/kg), while neither indomethacin (3 mg/kg) nor rofecoxib (30 mg/kg) had any effect (**Figure 5A**). By contrast, the intestinal motility was increased by both indomethacin (3 mg/kg) and SC-560 (10 mg/kg) but not rofecoxib (10 mg/kg) given i.d. (**Figure 5B**). The enhanced intestinal motility following the indomethacin treatment was completely inhibited by atropine (3 mg/kg, s.c.) (**Figure 6**). Neither ampicilline (800 mg/kg, i.d.) nor aminoguanidine (20 mg/kg, s.c.) had any influence on the enhanced intestinal motility caused by indomethacin.

Effect of Indomethacin on Enterobacterial Numbers in the Intestinal Mucosa

The aerobic and anaerobic bacterial numbers in the normal intestinal mucosa were 6.89 ± 0.19 log CFU/g tissue and 7.03 ± 0.13 log CFU/g tissue, respectively (**Table 1**). Six hours after the administration of indomethacin (3 mg/kg, p.o.), bacterial numbers in both aerobic and anaerobic conditions increased significantly, the values being 8.35 ± 0.30 log CFU/g tissue and 7.89 ± 0.31 log CFU/g tissue, respectively. The antibiotic ampicillin (800 mg/kg, p.o.) or atropine (3 mg/kg, s.c.) significantly prevented the bacterial invasion following indomethacin treatment, and in the former case the numbers of both aerobic and anaerobic bacteria decreasing even below control levels seen in the normal mucosa.

Effect of Indomethacin on iNOS mRNA Expression in Intestinal Mucosa

It is known that NSAIDs up-regulate iNOS expression in the intestinal mucosa

(Tanaka et al., 1999; 2002c; Takeuchi et al., 2003) and that NO derived from iNOS plays a pathogenic role in the development of intestinal lesions induced by NSAIDs (Whittle et al., 1995; Konaka et al., 1999; Takeuchi et al., 2002a; Tanaka et al., 2002c). To examine the involvement of iNOS/NO in the pathogenesis of intestinal lesions induced by a low dose of indomethacin plus rofecoxib, we investigated whether indomethacin at 3 mg/kg causes the expression of iNOS mRNA in the small intestine.

As shown in **Figure 7A**, RT-PCR analysis revealed that iNOS mRNA was expressed in the intestinal mucosa 6 hr after administration of indomethacin (3 mg/kg, p.o.), although it was not detected in the normal rat intestine with vehicle treatment. Up-regulation of the iNOS mRNA expression caused by indomethacin was clearly prevented by prior administration of ampicillin (800 mg/kg, p.o.) as well as atropine (3 mg/kg, s.c.).

Neither indomethacin (3 mg/kg, p.o.) nor rofecoxib (10 mg/kg, p.o.) alone had any effect on the iNOS activity in the small intestinal mucosa when examined 24 hr after the administration (**Figure 7B**). However, the combined administration of indomethacin plus rofecoxib markedly increased the iNOS activity, the values reaching about 8 times greater than control levels.

Discussion

The present study demonstrated that rofecoxib provoked damage in the small intestine but not the stomach when administered with a low dose of indomethacin (non-ulcerogenic dose). The PG deficiency caused by a low dose of indomethacin induces hypermotility and COX-2 expression in the intestine but not the stomach, resulting in damage when COX-2 is inhibited, again supporting the idea that COX-2 expression is a key to induction of damage in the gastrointestinal mucosa.

First, we confirmed our previous findings (Tanaka et al., 2002b; 2000c) that neither SC-560 nor rofecoxib at 10 mg/kg alone caused intestinal damage in non-fasted rats, yet these agents given in combination produced gross lesions. Similarly, it was confirmed that the combined administration of SC-560 and rofecoxib at 30 mg/kg produced gastric lesions in fasted rat stomachs, although either agent alone did not (Wallace et al., 2000; Tanaka et al., 2001; 2002a). These data strongly support the contention that inhibition of both COX-1 and COX-2 is required for induction of gastrointestinal damage (Wallace et al., 2000; Tanaka et al., 2002b). We found in the present study that indomethacin at 3 mg/kg alone did not cause damage in the stomach or intestine, yet this agent when administered together with the selective COX-2 inhibitor rofecoxib damaged the small intestine but not the stomach. This dose of indomethacin significantly decreased mucosal PGE₂ content in both the stomach and small intestine when determined 3 hr after the treatment. Certainly, SC-560 at the doses used significantly inhibited PG production in these tissues, while rofecoxib did not. Thus, the effects of indomethacin at a low dose were very much similar to those of SC-560, the selective COX-1 inhibitor. It was previously reported that SC-560 up-regulates the expression of COX-2 in the stomach and intestine, and the PGs derived from COX-2 may play a compensatory role in maintaining

the mucosal integrity under PG deficiency caused by COX-1 inhibition (Tanaka et al., 2002a; 2002b). Since indomethacin even at 3 mg/kg decreased the PGE₂ contents, as effectively as SC-560, it is expected that COX-2 expression might also be up-regulated in these tissues by this dose of indomethacin. However, indomethacin at 3 mg/kg induced COX-2 expression in the small intestine but not the stomach. These findings are interesting when considering that indomethacin at this dose produced damage in the small intestine but not the stomach, in the presence of rofecoxib. Indeed, the PGE₂ content was reduced by 3 mg/kg of indomethacin in both the stomach and intestine, but recovered significantly 24 hr later only in the small intestine. Certainly, this recovery of PGE₂ was prevented when rofecoxib was administered together with indomethacin. The same has previously been shown in the animals after administration of SC-560 with or without rofecoxib (Tanaka et al., 2002a; 2002b). As shown in our previous study, the reduced PGE₂ content in response to SC-560 was significantly recovered in either the stomach and small intestine, at 8 hr or 12 hr after the administration, respectively (Tanaka et al., 2002a; 2002b).

We previously reported that the up-regulation of COX-2 expression after administration of an ulcerogenic dose of indomethacin (10 mg/kg) is prevented by the antibiotic ampicillin, suggesting a role for enterobacteria in this phenomenon [Tanaka et al., 2005]. As expected, the COX-2 expression observed following administration of a low dose of indomethacin was also attenuated by prior administration of ampicillin at the dose that prevented intestinal lesions induced by a low dose of indomethacin plus rofecoxib. These results confirmed a causal relationship between bacterial invasion and COX-2 expression and suggested that this event is critical for development of intestinal lesions induced by a low dose of indomethacin plus rofecoxib. We also examined the enterobacterial counts after administration of 3 mg/kg of indomethacin. As expected, the number of bacteria in the mucosa was significantly increased after administration of a low

dose of indomethacin. Since intestinal damage induced by a low dose of indomethacin plus rofecoxib was significantly prevented by the antibiotic ampicilline as well as the selective iNOS inhibitor aminoguanidine, it is assumed that the pathogenic mechanism of intestinal damage induced by a low dose of indomethacin plus rofecoxib is very much similar to that of NSAID-induced intestinal damage, involving enterobacteria and iNOS/NO as the causative factor.

The expression of COX-2 in the stomach and intestine following administration of NSAIDs was curtailed by atropine at the dose that inhibited gastrointestinal hypermotility in response to NSAIDs, suggesting a causal relationship between the COX-2 expression and hypermotility (Takeuchi et al., 1986; 2002a; 2002b; 2004, Tanaka et al., 2005). This idea was further supported by the findings of Ohno et al (2004), who showed that the nonselective NOS inhibitor N^G-nitro L-arginine methyl ester (L-NAME) caused intestinal hypermotility as well as up-regulation of COX-2 expression, and resulted in intestinal damage when rofecoxib was co-administered with L-NAME. Abnormal contraction of the intestinal wall results in disruption of the unstirred mucus layer over the epithelium, leading to increased mucosal susceptibility to pathogens and irritants (Anthony et al., 1993). Indeed, atropine potently inhibited intestinal hypermotility as well as bacterial invasion and other inflammatory changes in response to indomethacin, resulting in prevention of intestinal damage (Takeuchi et al., 2002a). However, we found in the present study that indomethacin at 3 mg/kg did not affect gastric motility but produced a marked increase in intestinal motility, in an atropine-inhibitable manner. Certainly, the intestinal lesions induced by indomethacin plus rofecoxib were significantly prevented by pretreatment with atropine, similar to ampicillin and aminoguanidine. These results may provide an explanation as to why a low dose of indomethacin did not up-regulate COX-2 expression in the stomach and produced no damage even in the presence of rofecoxib.

The question remains why indomethacin at 3 mg/kg caused hypermotility in the small intestine but not in the stomach, although this agent decreased PGE₂ content in these two tissues, similar to SC-560. The latter agent increased motility in both the stomach and small intestine, suggesting that the hypermotility response is associated with a decrease in PG production due to COX-1 inhibition. At present, the reason why a low dose of indomethacin induced the different motility responses in the stomach and small intestine remains unknown, though it may be related to the mechanism for the hypermotility response induced in these tissues by NSAIDs, including indomethacin. The gastric hypermotility response induced by indomethacin at the ulcerogenic dose (10 mg/kg) occurs via vagal-cholinergic mechanisms, since this event is completely inhibited by bilateral vagotomy or prior administration of atropine (Takeuchi et al., 1986; 1990; 1991). By contrast, the intestinal hypermotility response in small intestine was inhibited by atropine but not vagotomy or hexamethonium (unpublished data). It is assumed that the intestinal hypermotility response is due to a deficiency of PGs in the peripheral tissue, while the hypermotility response in the stomach may require PG deficiency not only in the peripheral but also in the central nervous system, involving vagal-cholinergic nerves. Further study is certainly required to clarify the mechanism of gastrointestinal hypermotility responses to NSAIDs.

It should be noted that a low dose of indomethacin did not increase the iNOS activity in the intestinal mucosa, despite causing the up-regulation of iNOS mRNA expression. The combined administration of indomethacin plus rofecoxib, however, markedly increased the iNOS activity. Similar results were previously observed using SC-560 with or without rofecoxib in rat small intestines, indicating that the up-regulation of iNOS is associated with the inhibition of COX-1 (Tanaka et al., 2002c). This is understandable, because iNOS expression is triggered by endotoxin released from

enterobacteria (Boughton-Smith et al., 1993), and because bacterial invasion is causally related with intestinal hypermotility due to COX-1 inhibition (Takeuchi et al., 2002a, 2004b). SC-560 alone did not increase iNOS activity, although it caused an up-regulation of iNOS expression through enterobacterial invasion, and significantly increased iNOS activity only in the presence of rofecoxib. Both a low dose of indomethacin and SC-560 induced COX-2 expression in addition to the up-regulation of iNOS. Thus, it is assumed that PGs derived from COX-2 may inhibit the iNOS activity, and the additional treatment with rofecoxib unmasks the increase in iNOS activity by inhibiting PG production through COX-2 inhibition. Indeed, Kobayashi et al. (2001) recently reported that COX-2 downregulated iNOS expression in rat intestinal epithelial cells.

Given the findings in the present study, we concluded that a low dose of indomethacin produced damage in the small intestine but not in the stomach when administered together with rofecoxib. The pathogenic mechanism of intestinal lesions induced by a low dose of indomethacin plus rofecoxib was very much similar to that caused by the ulcerogenic dose of NSAIDs, involving PG deficiency, enterobacteria, iNOS/NO and hypermotility. PG deficiency caused by a low dose of indomethacin produces hypermotility and COX-2 expression in the small intestine, resulting in damage when COX-2 is inhibited. In the stomach, however, PG deficiency alone causes neither gastric hypermotility nor COX-2 expression, resulting in no damage even in the presence of rofecoxib. It is assumed that the hypermotility response is a key event in the expression of COX-2 and thereby important in the development of mucosal damage in the gastrointestinal tract.

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Footnotes

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Figure Legends

Figure 1. The gastric (A) and intestinal (B) ulcerogenic response to indomethacin, SC-560 and rofecoxib, either alone or in combination, in rats. In Fig A, the animals fasted for 18 hr were administered indomethacin (3 mg/kg), SC-560 (30 mg/kg) or rofecoxib (30 mg/kg) p.o., and killed 8 hr later. In Fig. B, the animals without fasting were administered indomethacin (3 mg/kg), SC-560 (10 mg/kg) or rofecoxib (10 mg/kg) p.o., and killed 24 hr later. Upon the combined administration in both cases, rofecoxib was given p.o. 30 min after indomethacin or SC-560. Data are presented as the mean \pm SE for 4-5 rats. * Significant difference from control.

Figure 2. Effects of aminoguanidine, atropine or ampicillin on the intestinal ulcerogenic response caused by indomethacin plus rofecoxib in rats. The animals were first administered indomethacin (3 mg/kg) and 30 min later rofecoxib (10 mg/kg) p.o., and then killed 24 hr later. Aminoguanidine (20 mg/kg) or atropine (3 mg/kg) was given s.c. 30 min before indomethacin, while ampicillin (800 mg/kg) was given twice 24 hr before and 30 min after indomethacin. Data are presented as the mean \pm SE for 5-6 rats. * Significant difference from vehicle.

Figure 3. Effects of indomethacin, SC-560 and rofecoxib on mucosal PGE₂ levels in the rat stomach (A) and small intestine (B). The animals were administered indomethacin (3 mg/kg), SC-560 (10 or 30 mg/kg) or rofecoxib (10 or 30 mg/kg) p.o., and killed 3 hr, 8 hr or 24 hr later, then the PGE₂ content was measured by EIA. In some cases, the animals were given rofecoxib together with indomethacin. Values are presented as the mean \pm SE for 4-6 rats. Significant difference at P<0.05; * from control; # from indomethacin alone.

Figure 4. Gene expression of COX-2 and G3PDH in the rat stomach **(A)** or small intestine **(B)** after administration of indomethacin. Indomethacin (IM: 3 mg/kg) was given p.o., and the COX-2 expression was examined 6 hr later in both the stomach and small intestine. In some rats, atropine (3 mg/kg) was given s.c. 30 min before indomethacin, while ampicillin (Amp: 800 mg/kg) was given p.o. twice 24 hr and 30 min before indomethacin. M: Marker

Figure 5. Representative recordings of gastric **(A)** and intestinal **(B)** motility changes after administration of various COX inhibitors in rats. In Fig. A, indomethacin (3 mg/kg), SC-560 (30 mg/kg) or rofecoxib (30 mg/kg) was given i.d., and gastric motility was recorded for 2 hr thereafter. In Fig. B, indomethacin (3 mg/kg), SC-560 (10 mg/kg) or rofecoxib (10 mg/kg) was given i.d., and intestinal motility was recorded for 2 hr thereafter.

Figure 6. Representative recordings showing the effects of atropine, ampicillin and aminoguanidine on intestinal hypermotility after administration of indomethacin in rats. Indomethacin (3 mg/kg) was given i.d., and gastric motility was recorded for 2 hr thereafter. Atropine (3 mg/kg) or aminoguanidine (20 mg/kg) was given s.c. as a single injection 2 hr after indomethacin while ampicillin (800 mg/kg) was given i.d. 24 hr.

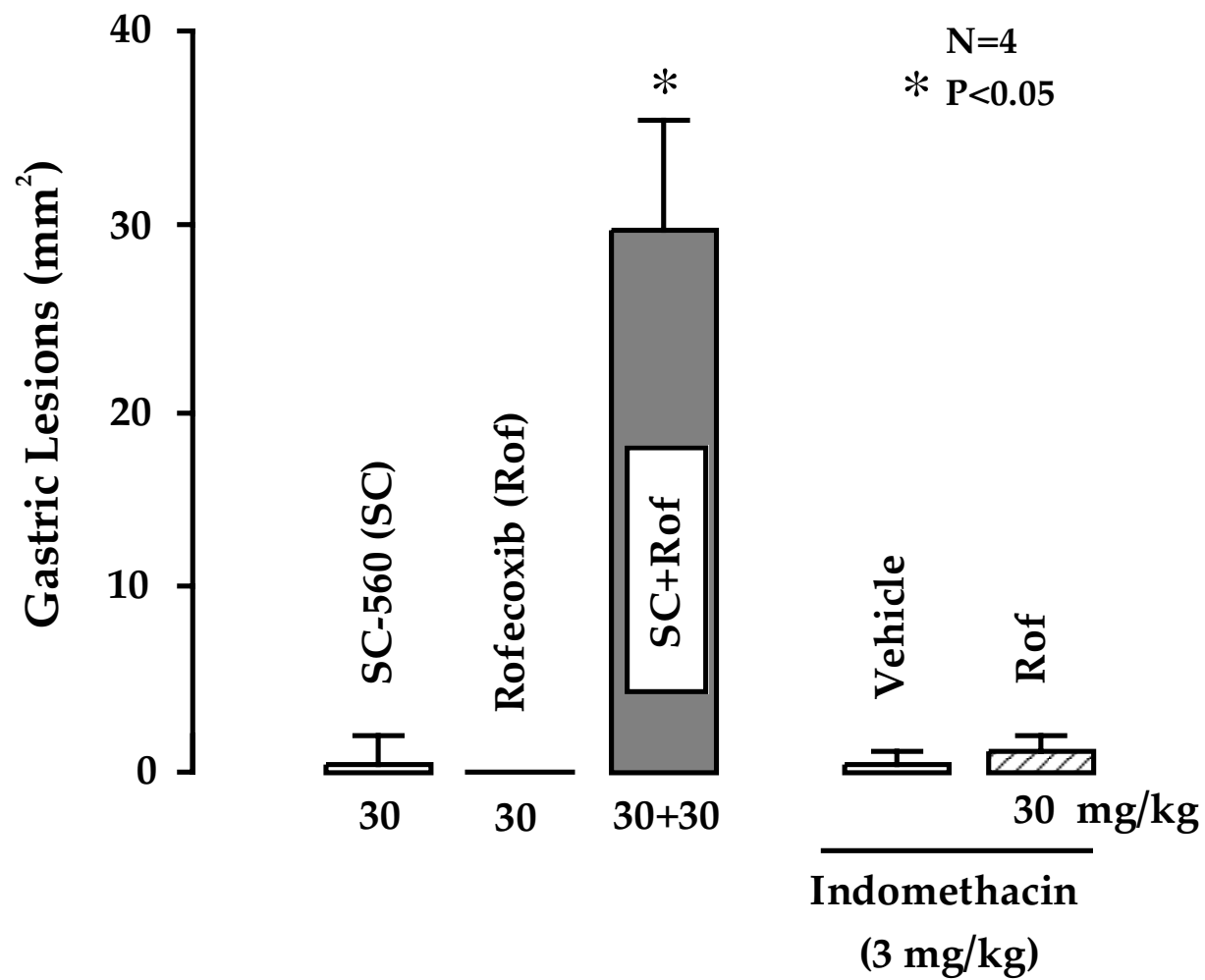
Figure 7. Gene expression of iNOS and G3PDH **(A)** and iNOS activity **(B)** in the rat small intestine after administration of indomethacin. In Fig. A, indomethacin (3 mg/kg) was given p.o., and the iNOS expression was examined 6 hr later in the small intestinal mucosa. In some rats, atropine (Atr: 3 mg/kg) was given s.c. 30 min before indomethacin, while ampicillin (Amp: 800 mg/kg) was given p.o. twice 24 hr and 30 min before indomethacin.

M: marker; V: vehicle. In Fig. B, animals were given indomethacin (3 mg/kg) p.o., and intestinal iNOS activity was measured 24 hr later. Rofecoxib (10 mg) was given 30 min before indomethacin. Data are presented as the mean \pm SE for 4 rats. * Significant difference from control, at $P < 0.05$.

Table 1 Changes in Enterobacterial Numbers in The Rat Intestinal Mucosa After Administration of Various COX Inhibitors

Treatment	No. of Rats	No. of Enterobacteria (log CFU/g tissue)	
		Aerobic	Anaerobic
Normal	5	6.83 ± 0.19	7.09 ± 0.13
Indomethacin 3 mg/kg	5	8.35 ± 0.30 *	7.82 ± 0.31 *
+Ampicillin 800 mg/kg	5	4.97 ± 0.38 #	4.82 ± 0.35 #
+Atropine 3 mg/kg	5	6.97 ± 0.28 #	6.82 ± 0.21 #

All values are presented as the mean±SE for 5 rats per group. Indomethacin (3mg/kg) was administered p.o., and the animals were killed 6 hr later. Ampicillin (800 mg/kg) was given p.o. twice 24 hr and 30 min before the administration of indomethacin while atropine (3 mg/kg) was given s.c. 30 min before. Significant difference at P<0.05; * from normal; # from indomethacin alone.

**Figure 1A**

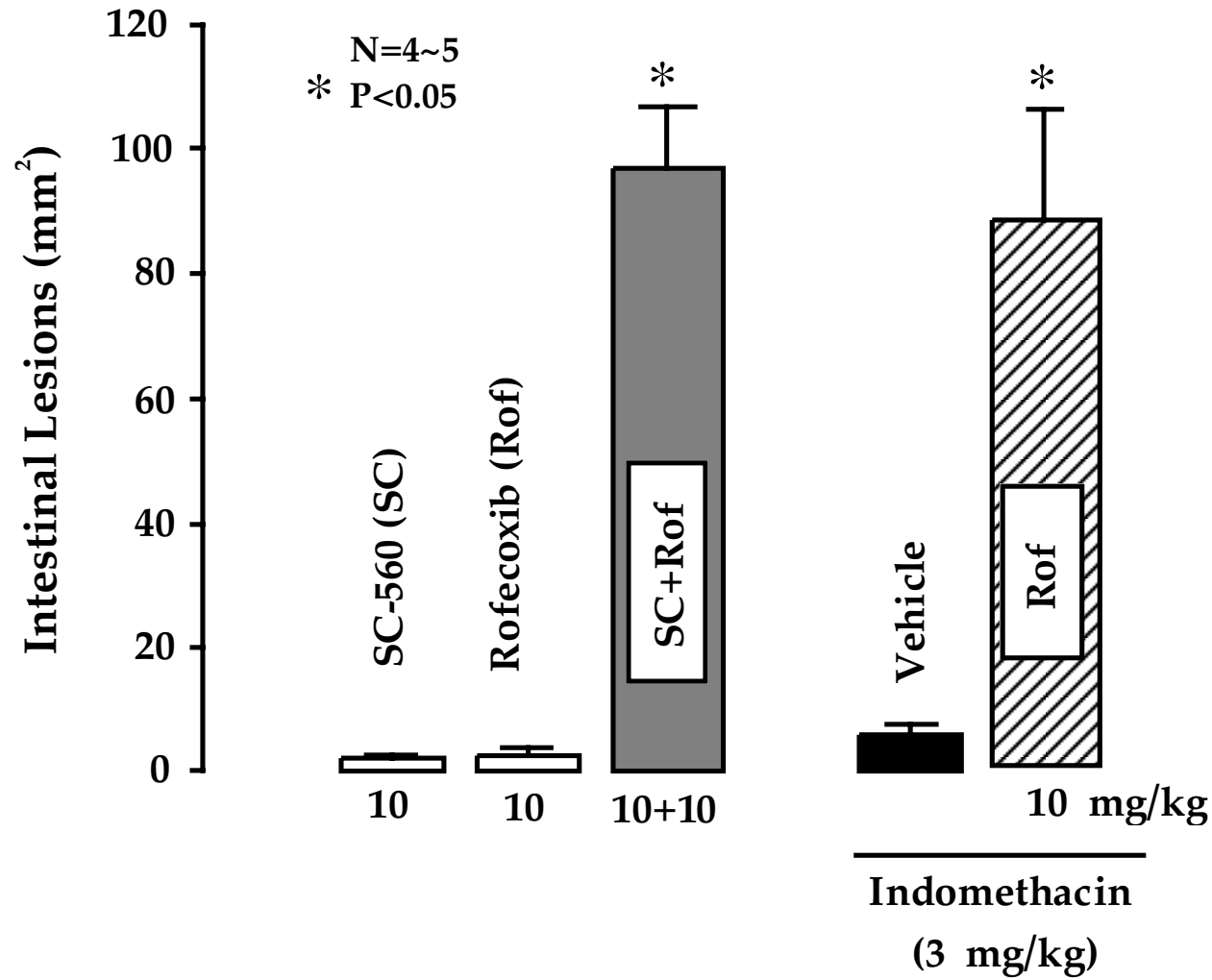


Figure 1B

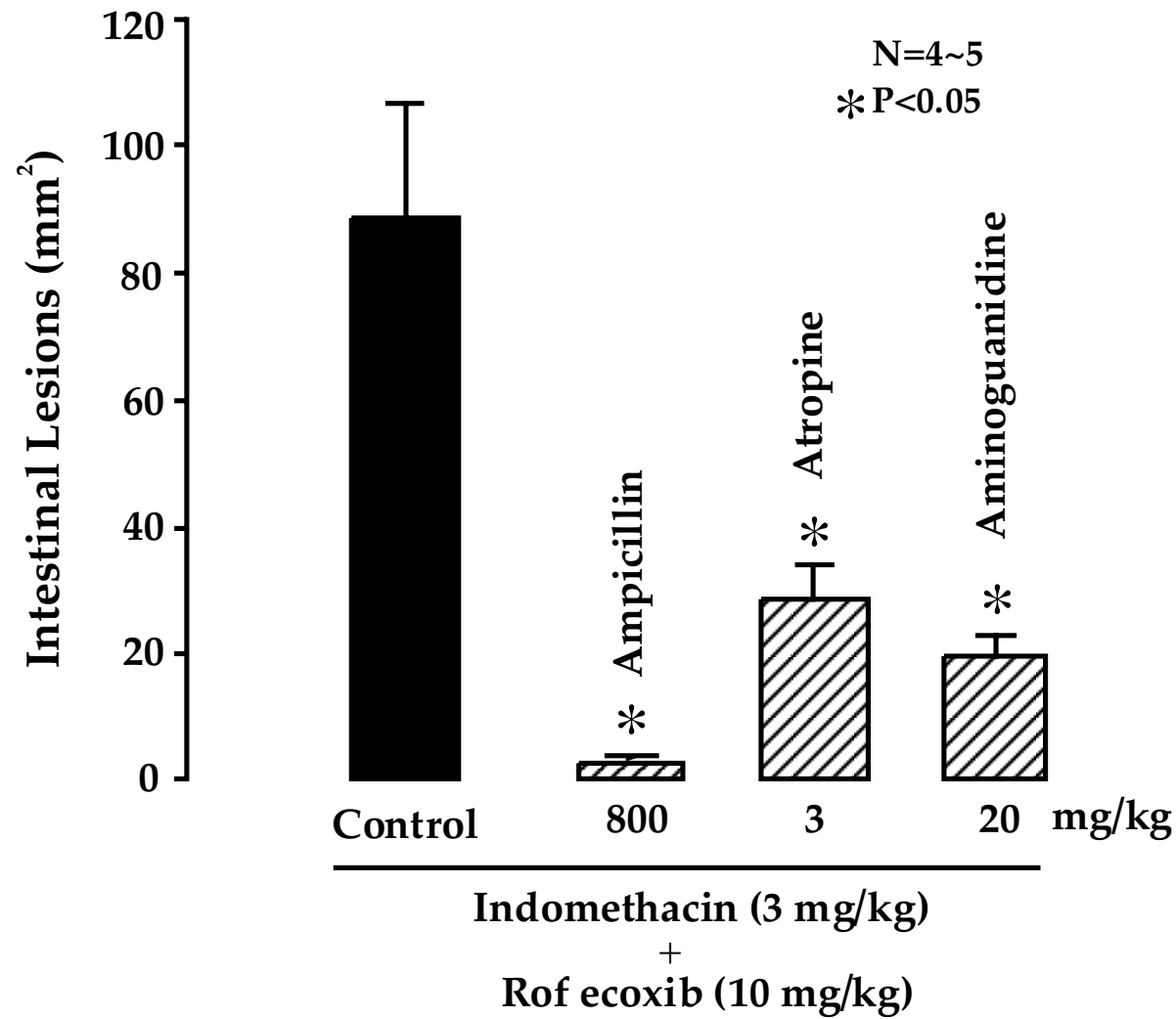


Figure 2

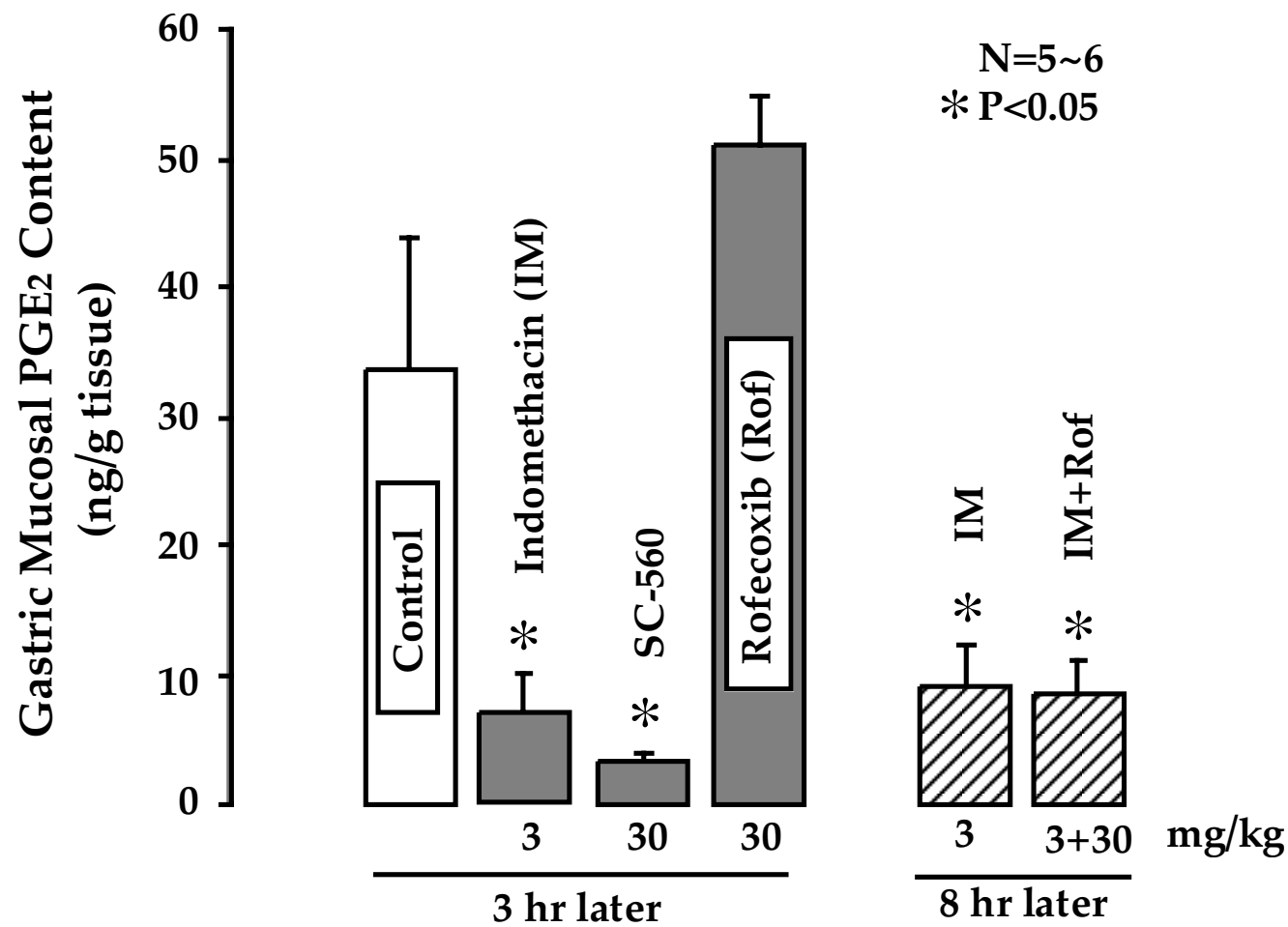


Figure 3A

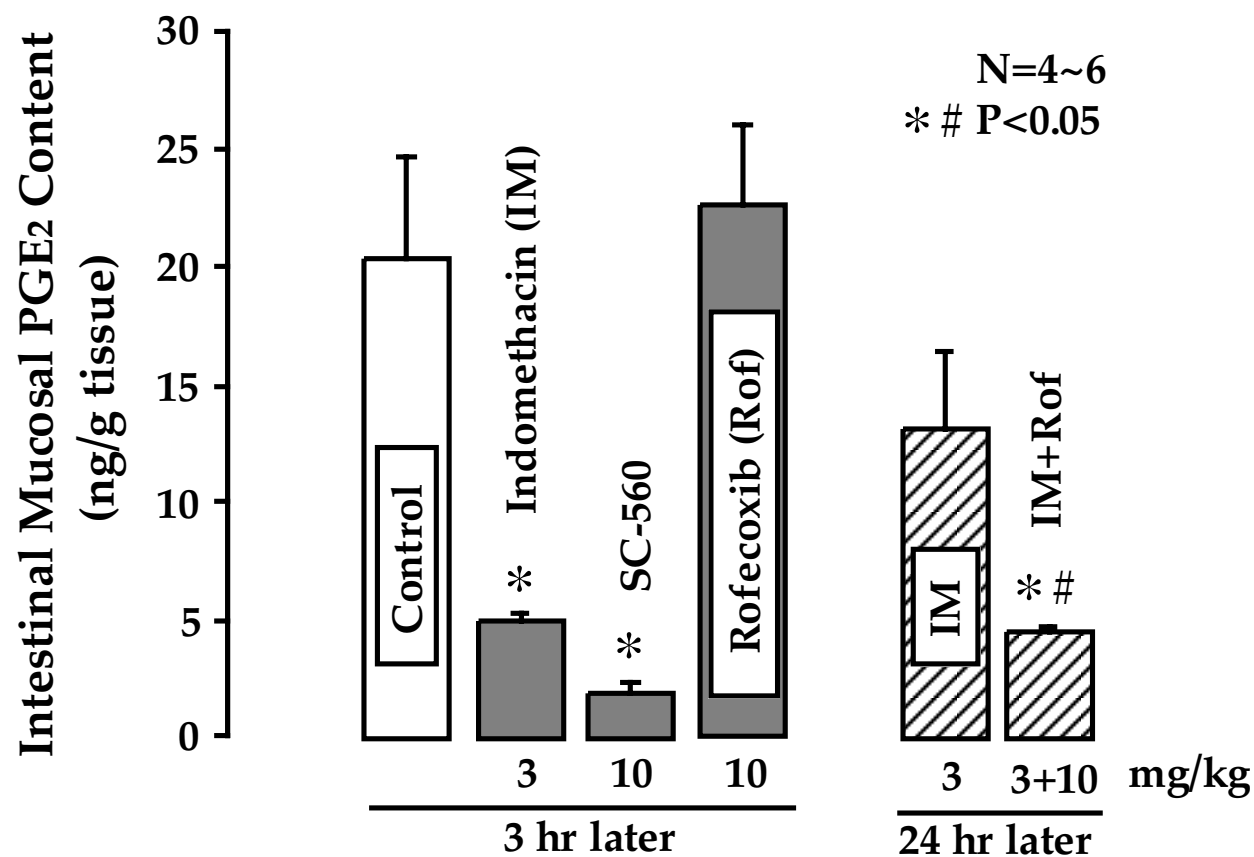
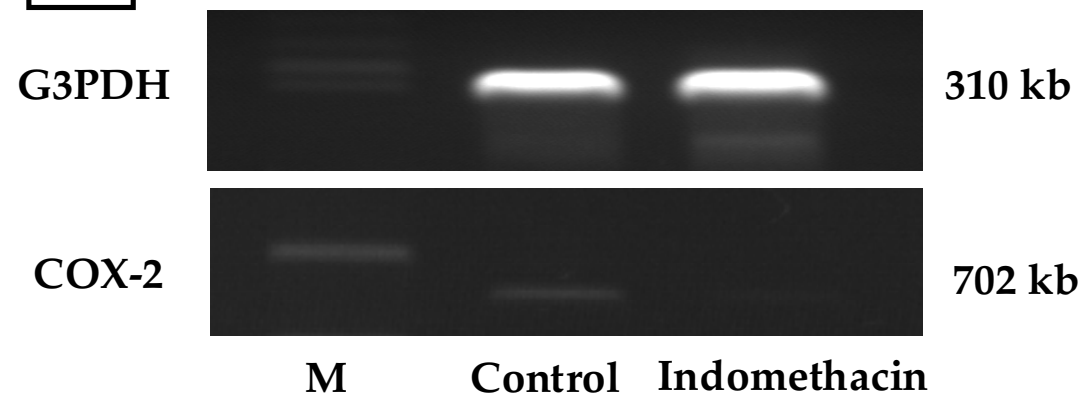


Figure 3B

A



B

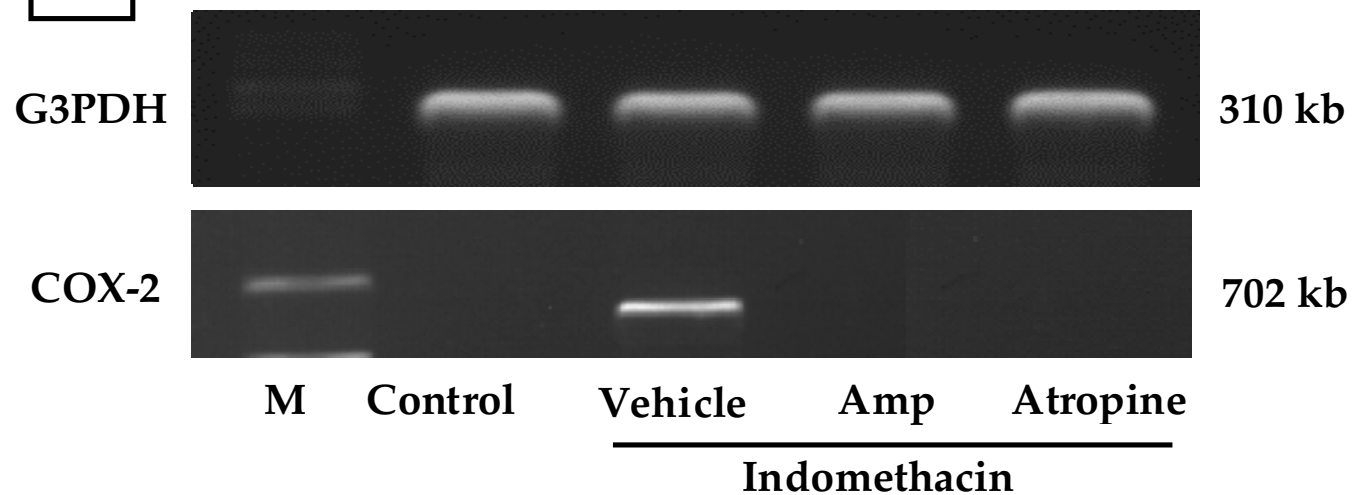
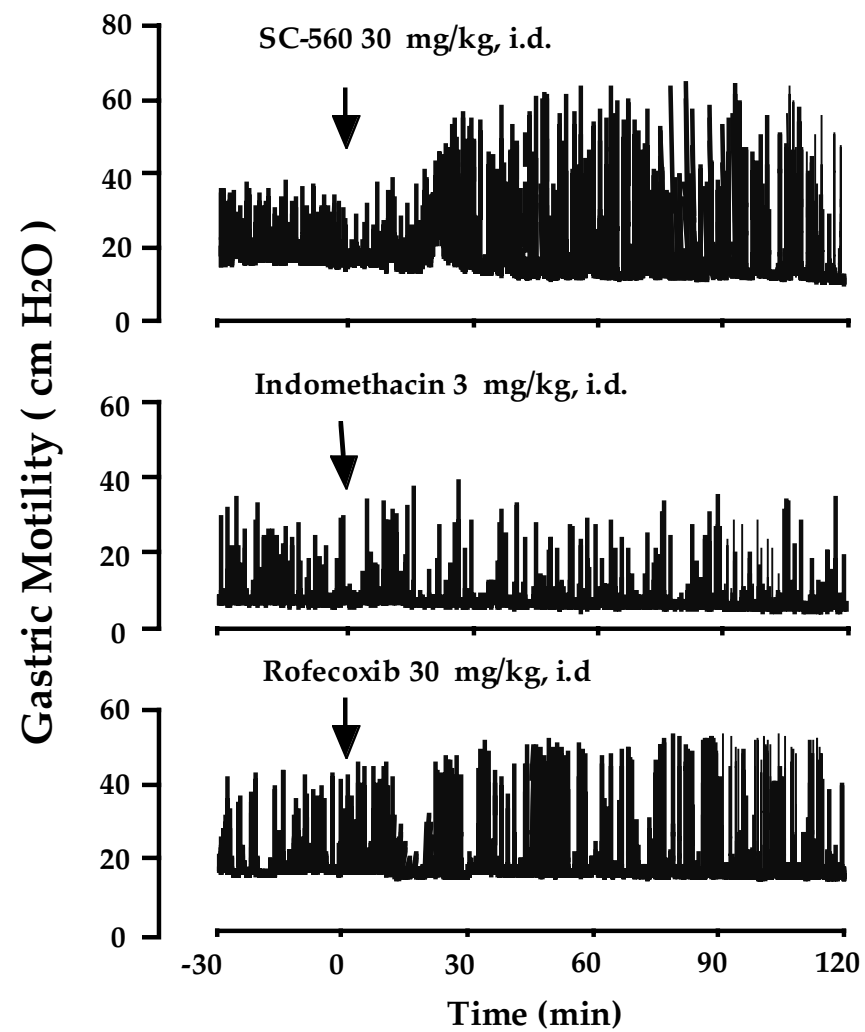
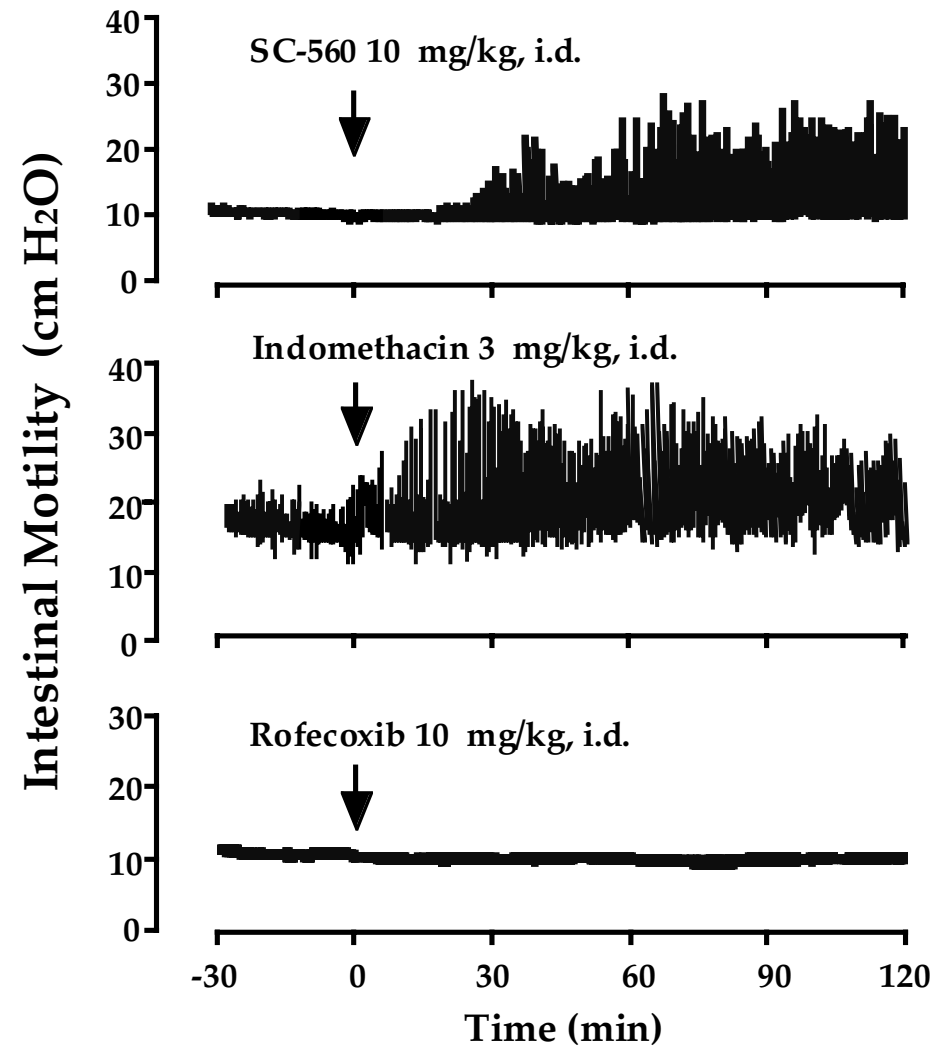
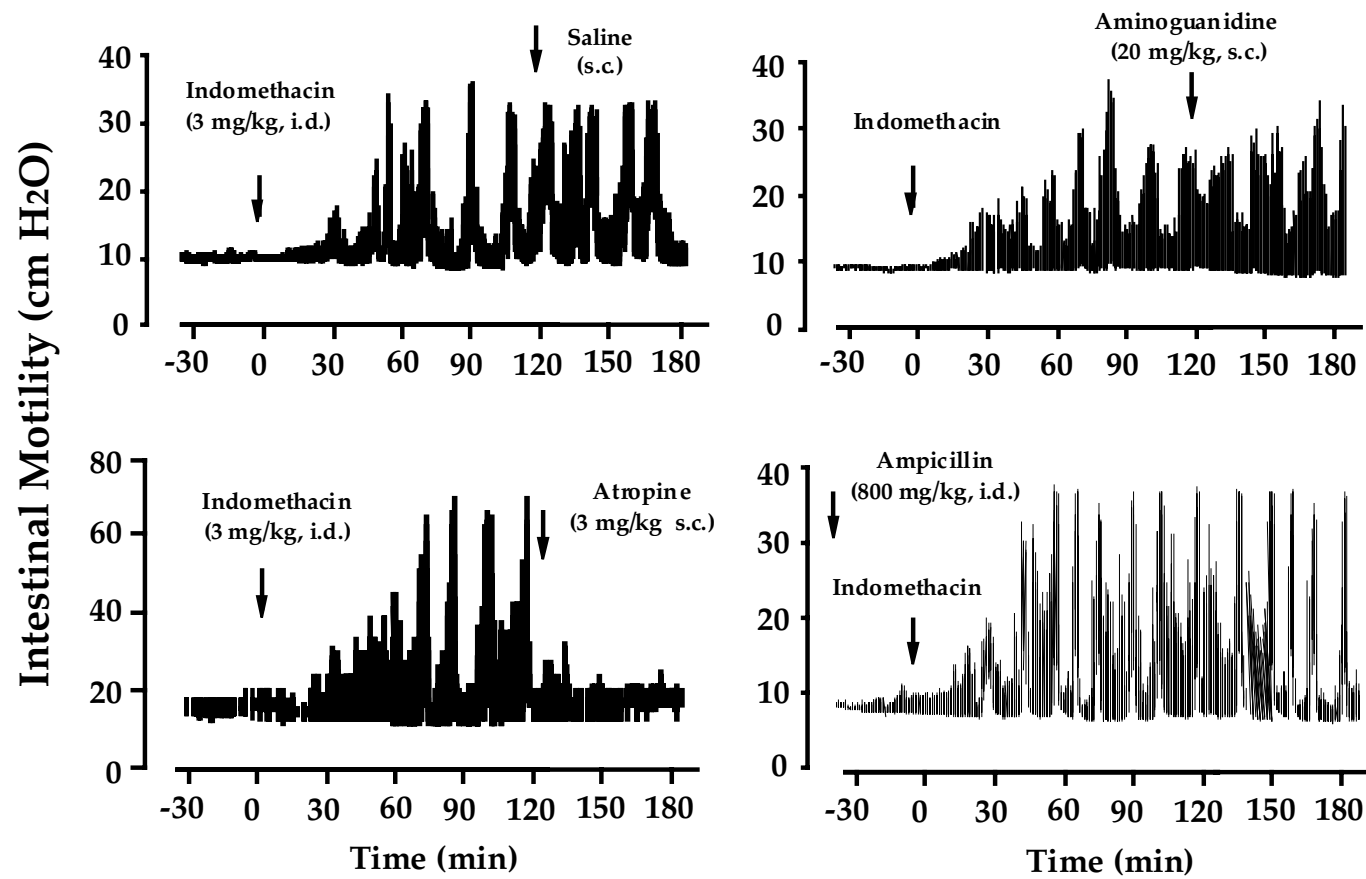


Figure 4

**Figure 5A**

**Figure 5B**

**Figure 6**

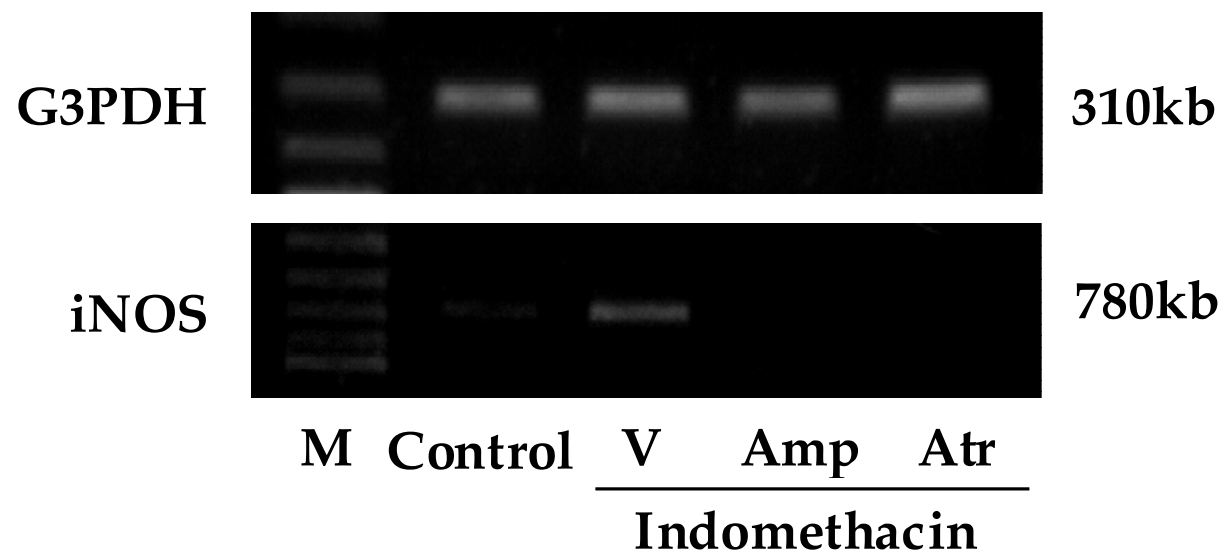
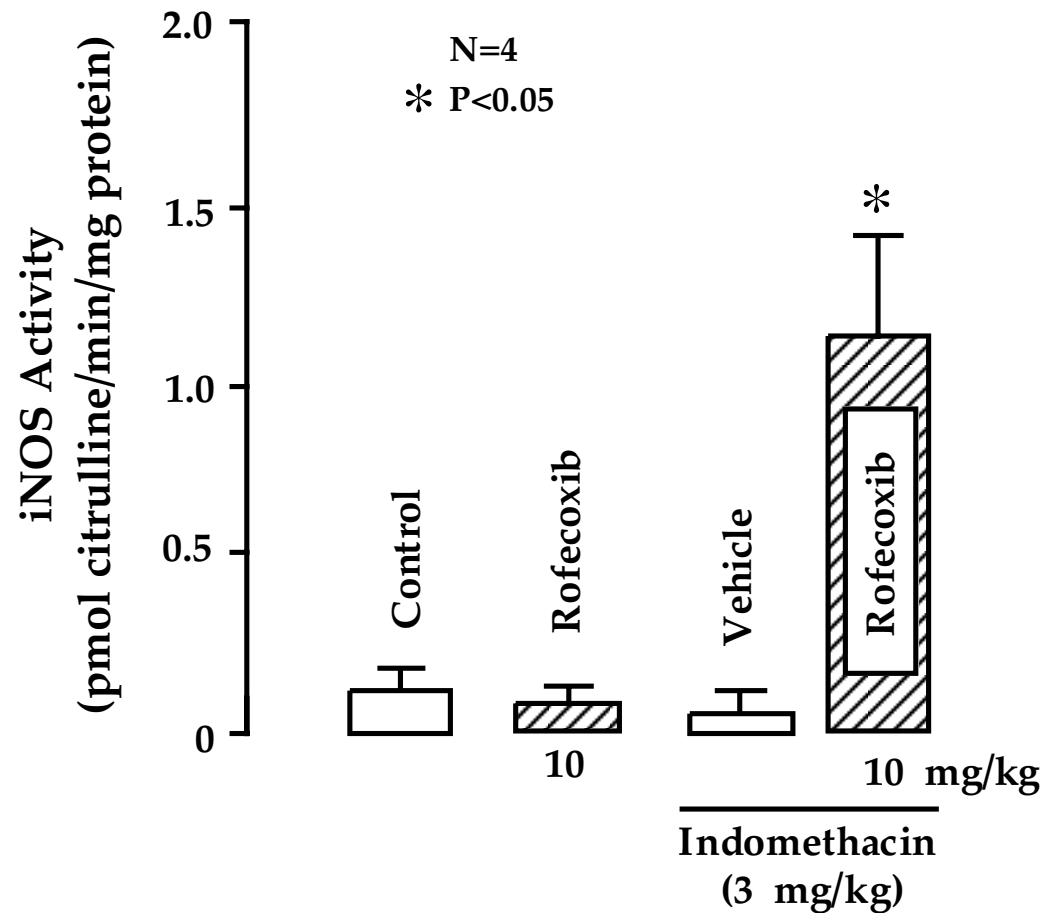


Figure 7A

**Figure 7B**