Anesthesiology 2004; 101:983–90

Contribution of Interaction between Nitric Oxide and Cyclooxygenases to the Production of Prostaglandins in Carrageenan-induced Inflammation

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Background: **Nitric oxide (NO) and prostaglandins (PGs) are crucial mediators contributing to generation of inflammatory responses and pain. This study was designed to investigate the effects of peripherally released NO on cyclooxygenase (COX) expression/activation and production of PGs in carrageenan-induced inflammation.**

Methods: **A microdialysis probe was implanted subcutaneously into the skin of hind paws of rats. The concentrations of NO metabolites, PGE_2, and 6-keto-$PGF_{1\alpha}$ (metabolite of PGI_2) in the dialysate were measured. Carrageenan was injected into the plantar surface of the hind paw during perfusion of the dialysis catheter with modified Ringer's solution or N^G-monomethyl-L-arginine acetate. In addition, the effects of the selective COX-1 inhibitor SC-560 and the selective COX-2 inhibitor NS-398 on the production of NO, PGE_2, and 6-keto-$PGF_{1\alpha}$ were examined. Western blotting was performed to evaluate the expression of COX-1 and COX-2 in the skin at the site of the inflammation.**

Results: **Carrageenan injection resulted in increases in the concentrations of NO, PGE_2, and PGI_2, and these increases were completely suppressed by N^G-monomethyl-L-arginine acetate. SC-560 effectively inhibited the increase in PGE_2 and PGI_2 concentrations for the first 2 h, and NS-398 inhibited 3–6 h after carrageenan. Western blot analysis showed that the concentrations of both COX-1 and COX-2 in the skin increased after carrageenan. The up-regulation of COX-1 in the skin was observed 3 and 6 h after carrageenan and was not suppressed in the rats treated with N^G-monomethyl-L-arginine acetate. The up-regulation of COX-2 in the skin was also observed 3 and 6 h after carrageenan and was completely suppressed in the rats treated with N^G-monomethyl-L-arginine acetate.**

Conclusion: **The results of the current study suggest that NO activates COX-1 in the early phase of carrageenan and up-regulates COX-2 expression in the late phase in the skin, resulting in production of PGE_2 and PGI_2 at the site of inflammation, which would contribute to exacerbation of the inflammatory process.**

TISSUE injury and inflammation result in swelling and hyperalgesic pain, phenomena that are thought to be due, in great part, to sensitization of primary afferent nociceptors. Inflammatory mediators have been implicated in induction of the sensitization and hyperalgesia. A number of observations suggest that peripheral nitric oxide (NO) acts as a pronociceptive mediator in inflammation[1–3] in experimental models such as adjuvant arthritis[4] and carrageenan-induced inflammation in the rat.[3,5,6] Prostaglandins (PGs) are also well established as inflammatory mediators that act directly on peripheral terminals of primary afferent nociceptors[7] to sensitize nociceptors *in vitro*[8] and on spinal dorsal horn neurons[9] to produce hyperalgesia. Hyperalgesic effects of PGE_2 and PGI_2 and occasionally $PGF_{2\alpha}$ have been reported in inflammatory models.[10] Cyclooxygenase (COX) enzymes catalyze the bis-oxygenation of free arachidonic acid to PGH_2, the committed step in PG formation. It is well known that there are two isoforms of COX enzymes, referred to as COX-1 and COX-2. COX-1 is thought to produce PGs that are important for homeostasis and certain physiologic functions and is expressed constitutively in most tissues and cells.[11] In an inflammatory process, the inducible isoform of COX, COX-2, is encoded by an immediate–early gene induced by cytokines, mitogens, and endotoxins in inflammatory cells, accounting for the release of large quantities of PG at the site of inflammation.[11,12]

Several studies have suggested an interaction of NO and PG in which the production of PGE_2 is augmented further in the presence of NO. Inhibition of NO production by nitric oxide synthase (NOS) inhibitors has been found to decrease the production of PGE_2 in *in vitro* and *ex vivo* models.[5,13] It is thought that this effect of NO is due to its ability to activate the COX enzyme by an as-yet-undetermined mechanism.[6] In inflammation, the functional relation between the NO and COX pathways suggests that NO exacerbates the inflammatory process through the generation of additional PG production.[14] However, it has been reported that NO is involved in the negative regulation of the COX pathway.[15,16] Therefore, the relation between NO and PG pathways at the sites of peripheral inflammation is still controversial, and *in vivo* data are scarce.[17] Even assuming there is a relation between these pathways, the mechanisms of this interaction remain to be clarified. The current study was undertaken to determine whether NO released in carrageenan-induced inflammation would activate or up-regulate the expression of COX-1 and COX-2 in the dorsal root ganglia (DRG) and in the skin at the site of inflammation, resulting in additional production of PGE_2 and PGI_2. In addition, the effects of inhibition of COX-1 and COX-2 actions on the production of NO were examined.

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Received from the Department of Anesthesiology, Sapporo Medical University School of Medicine, Sapporo, Japan. Submitted for publication January 26, 2004. Accepted for publication June 21, 2004. Support was provided solely from institutional and/or departmental sources.

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Materials and Methods

The protocol of this study was approved by the Sapporo Medical University Animal Care and Use Committee. The animals used in this study were male Sprague-Dawley rats (weight, 200–250 g; Japan SLC, Hamamatsu, Japan), which were housed individually in a temperature-controlled (21 ± 1°C) room with a 12 h light–dark cycle and were given free access to food and water. Each animal was used in only one experiment.

Construction of Microdialysis Probe and Animal Preparation

A microdialysis probe was constructed from a 2-cm length of dialysis fiber (ID of 200 μm, OD of 220 μm, and 50-kd molecular weight cutoff; DM-22; Eicom, Kyoto, Japan) that was coated with a thin layer of epoxy glue (Devcon, Danvers, MA) along the whole length, except for a 15-mm region in the middle. Each end of the fiber was attached to 10-cm polyethylene catheters (PE-10; Becton-Dickinson, Franklin Lakes, NJ), and each end of the polyethylene catheter was attached to a 50-cm Teflon tube (ID of 100 μm, OD of 400 μm; JT-10; Eicom).

The rats were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally), and additional sodium pentobarbital was administered throughout the experiment to maintain areflexia. A microdialysis probe was subcutaneously inserted into the left glabrous skin of the hind paw, as described previously.[2,3,18] The probe was perfused with modified Ringer's solution (140 mM NaCl, 4.0 mM KCl, 1.26 mM $CaCl_2$, 1.15 mM $MgCl_2$, 2.0 mM Na_2HPO_4, 0.5 mM NaH_2PO_4, and pH 7.4) at a constant flow rate of 4 μl/min for 120 min to establish a diffusion equilibrium. In another series, during general anesthesia (isoflurane in oxygen), the left sciatic nerve and L3 spinal root were exposed and transected. Seven days after neurectomy, a microdialysis probe was subcutaneously inserted.

Microdialysis Study

The microdialysis probe was perfused with modified Ringer's solution (control group) or 40 mM of nonselective NOS inhibitor, N^G-monomethyl-L-arginine acetate (L-NMMA; Dojindo Laboratories, Kumamoto, Japan) (L-NMMA group) at a constant flow rate of 4 μl/min. In the animals with denervation of the sciatic nerve and spinal root, modified Ringer's solution was perfused (denervation group). Sixty minutes after the perfusion, the sample was collected to obtain the basal concentration, and 2 mg carrageenan in a volume of 50 μl was injected into the plantar surface of the left hind paw with a 29-gauge needle. Dialysate collection was started with a 4-min delay because of the dead space of the outflow catheter to sample subcutaneous dialysate from the time of carrageenan injection. The samples were collected as 60-min fractions. Collected samples were immediately analyzed for NO or frozen at −80°C until used for analysis of PGE_2 and PGI_2. In another series of experiments, the selective COX-1 inhibitor SC-560 (10 mg/kg) or the selective COX-2 inhibitor NS-398 (10 mg/kg) was administered intraperitoneally 60 min before the injection of carrageenan to examine the effects of these inhibitors on the releases of NO, PGE_2, and PGI_2.

In four rats that had received a cannulation of right internal carotid artery, rectal temperature, respiratory rate, arterial partial pressure of oxygen, arterial partial pressure of carbon dioxide, and systemic arterial blood pressure were monitored during sodium pentobarbital anesthesia to confirm the stable preparation for the microdialysis study. The values of these parameters were stable for 8 h (data not shown).

Analysis of NO_2^-–NO_3^-

The concentrations of NO metabolites (total amount of nitrite [NO_2^-] and nitrate [NO_3^-], NO_2^-–NO_3^-) in the dialysate were analyzed using an automated NO-detecting high-performance liquid chromatography system (ENO-10; Eicom) as described previously.[19] NO_2^- and NO_3^- in the dialysate were separated by a reverse-phase separation column packed with polystyrene polymer (4.6 × 50 mm; NO-PAK; Eicom), and NO_3^- was reduced to NO_2^- in a reduction column packed with copper-plated cadmium filling (NO-RED; Eicom). NO_2^- was mixed with a Griess reagent to form a purple azo dye in a reaction coil. The separation and reduction columns and the reaction coil were placed in a column oven that was set at 35°C. The absorbance of the color of the product dye at 540 nm was measured using a flow-through spectrophotometer (NOD-10; Eicom). The mobile phase, which was delivered by a pump at a rate of 0.33 ml/min, was 10% methanol containing 0.15 M NaCl-NH_4Cl and 0.5 g/l 4Na-EDTA. The Griess reagent, which was 1.25% HCl that contained 5 g/l sulfanilamide with 0.25 g/l *N*-naphthyl ethylenediamine, was delivered at a rate of 0.1 ml/min. Contamination of NO_2^-–NO_3^- in modified Ringer's solution and reliability of the reduction column were evaluated in each experiment.

Analysis of PGE_2 and PGI_2

The concentrations of PGE_2 and 6-keto-$PGF_{1\alpha}$ (metabolite of PGI_2) in the dialysate were measured by using commercially available enzyme immunoassay kits (Cayman Chemicals, Ann Arbor, MI). Measurement was completed by using an enzyme-linked immunosorbent assay with an absorbency maximum at 405 nm.

Western Blotting

During pentobarbital anesthesia (50 mg/kg administered intraperitoneally), the glabrous skin of the left hind paw and the left four to six lumbar DRG were rapidly removed from the rats in which modified Ringer's solution or L-NMMA was perfused through the microdialysis

probe and from the rats with denervation to analyze the expression of COX-1 and COX-2.

The removed tissue was homogenized in 0.01 M phosphate-buffered saline (PBS; pH 7.4). The crude homogenates were centrifuged at 15,000*g* for 20 min at 4°C, and protein content was determined using DC protein assay (Bio-Rad Laboratories Inc., Hercules, CA) with bovine serum albumin. The proteins were then put in a 4× sample buffer consisting of 0.2 M Tris-HCl (pH 6.8), 4% sodium dodecylsulfate, 8 M urea, 0.1 M dithiothreitol, and 0.01% bromophenol blue. Equal amounts of protein per lane were loaded onto a 7.5% polyacrylamide gel and separated by electrophoresis at 10 mA/gel for 60 min and then at 20 mA/gel for 60 min with a running buffer containing 25 mM Tris, 192 mM glycine, and 0.1% sodium dodecylsulfate. Molecular weight markers (Amersham Biosciences, Buckinghamshire, United Kingdom) were used in each gel. Electrophoresis standards of COX-1 (Cayman Chemicals) and COX-2 (Cayman Chemicals) were also used. Proteins were transferred to a polyvinylidene diflouride membrane (Immobilon-P; Millipore Corporation, Bedford, MA) at 36 V for 4 h using a transfer buffer containing 0.01 M 3-[cyclohexylamino]-1-propanesulfonic acid (pH 11)–10% methanol. The blots were incubated with 5% nonfat dry milk in PBS for 1 h at room temperature to block nonspecific binding of the antibodies. Then membrane was incubated with a mouse monoclonal anti–COX-1 antibody (1:1,000; Cayman Chemicals) or a rabbit polyclonal anti–COX-2 antibody (1:500; Cayman Chemicals) in 0.1% bovine serum albumin in PBS overnight at 4°C. The concentrations of actin, a housekeeping protein, were also measured using rabbit anti-actin antibody (1:1,000; Sigma Chemical Co., St. Louis, MO). The blot was washed for 30 min with two changes of 2% nonfat dry milk– 0.1% Tween-20 in PBS and then incubated with a goat anti-rabbit immunoglobulin G or a goat anti-mouse immunoglobulin G secondary antibody conjugated to horseradish peroxidase (1:5,000; DAKO, Glostrup, Denmark) in 0.1% bovine serum albumin in PBS for 30 min at room temperature. The membrane was washed five times for 30 min with 2% nonfat dry milk– 0.1% Tween-20 in PBS. The reaction product was visualized on x-ray film (XAR-5; Kodak, Rochester, NY) using an enhanced chemiluminescence kit (Amersham Biosciences). The amount of expressed protein was quantified and analyzed using image densitometer (NIH Image 1.63; National Institutes of Health, Bethesda, MD).

To show specificity of antibodies of COX-1 and COX-2 used in this study, Western blots were also performed on purified COX-1 and COX-2 electrophoresis proteins (Cayman Chemicals). In addition, specific antigen peptides against COX-1 and COX-2 (Cayman Chemicals) were added to primary antibody solution for preabsorption study.

Immunohistochemistry

The normal and inflamed rats were deeply anesthetized with pentobarbital and transcardially perfused with 100 ml saline followed by 300 ml paraformaldehyde (4% in 0.1 M phosphate buffer). The glabrous skins of the hind paw were dissected, postfixed in 4% paraformaldehyde, and transferred to 25% sucrose (overnight at degree). Frozen sections (30 μm) of the skins were thaw-mounted onto gelatin-coated slide glass. All of the sections were blocked with 10% normal goat serum in 0.2% Triton X-100 for 30 min at room temperature and incubated for 48 h at 4°C with primary antibodies. The sections were then incubated for 2 h at room temperature with fluorescent secondary antibodies (Alexa Fluor 488 or 594; Molecular Probes, Eugene, OR). Photographs were taken by a confocal laser scanning microscope. All analyses were performed at 20× objective magnification.

Statistical Analysis

The concentrations of NO_2^-–NO_3^-, PGE_2, and PGI_2 were expressed as mean ± SD of percentage of the basal value. Proteins were quantified using a densitometer to give COX-1:actin and COX-2:actin ratios. Relative expressions of the proteins were presented as mean ± SD. The statistical significance of the data was analyzed by a two-way analysis of variance followed by the Fisher protected least significant difference test. A P value less than 0.05 was considered to be statistically significant.

Results

Effects of L-NMMA and Denervation on NO_2^-–NO_3^- Concentration after Carrageenan Injection

A preliminary study showed that a dialysis equilibrium was obtained within 120 min after the start of modified Ringer's solution perfusion at a constant flow rate of 4 μl/min and that basal values were stable for at least 8 h (data not shown). The basal concentrations of NO_2^-–NO_3^- in the control and L-NMMA–treated rats and in the rats with denervation were shown in table 1. The basal concentrations among the three groups were comparable. The concentrations of NO_2^-–NO_3^- before and after L-NMMA were also comparable.

Subcutaneous injection of carrageenan significantly ($P < 0.05$) increased the NO_2^-–NO_3^- concentrations in the dialysate under the condition of perfusion with modified Ringer's solution (fig. 1). The increases were observed for more than 6 h. The perfusion of L-NMMA through the microdialysis probe significantly ($P < 0.05$), and almost completely, suppressed the carrageenan-induced increase in NO_2^-–NO_3^- concentration (fig. 1, *left*). In the animals with denervated sensory nerves in the hind paw, the increase in the concentration of NO_2^-–NO_3^- after carrageenan was completely suppressed until 2 h after carrageenan injection and was significantly ($P < 0.05$) lower than that of the control

Table 1. Baseline Values of NO_2^-–NO_3^-, PGE_2, and 6-Keto-$PGF_{1\alpha}$

	Control	L-NMMA		Denervated
		Before	After	
NO_2^-–NO_3^-, pmol/10 μl	24.4 ± 8.0	22.0 ± 6.5	24.1 ± 6.2	20.0 ± 4.5
PGE_2, pg/ml	23.0 ± 7.6	28.9 ± 6.1	29.3 ± 8.3	32.4 ± 9.9
6-Keto-$PGF_{1\alpha}$, pg/ml	14.9 ± 6.9	12.0 ± 4.2	11.1 ± 4.4	19.0 ± 7.0

Data are presented as mean ± SD.

L-NMMA = N^G-monomethyl-L-arginine acetate; NO_2^- = nitrite; NO_3^- = nitrate; PG = prostaglandin.

group during the study period but significantly ($P < 0.05$) higher than that of the L-NMMA group 3 h after carrageenan injection and thereafter.

Effects of L-NMMA and Denervation on PGE_2 and 6-Keto-$PGF_{1\alpha}$ Concentrations after Carrageenan Injection

A dialysis equilibrium of PGE_2 and 6-keto-$PGF_{1\alpha}$ was obtained within 60 min after the start of modified Ringer's solution perfusion, and basal values were stable for more than 8 h (data not shown). The basal values of PGE_2 and 6-keto-$PGF_{1\alpha}$ concentrations in control and L-NMMA–treated rats and in the rats with denervation are shown in table 1. The concentrations among the three groups were comparable.

Prostaglandin E_2 and 6-keto-$PGF_{1\alpha}$ concentrations in the dialysate significantly ($P < 0.05$) increased after carrageenan injection under the condition of perfusion with modified Ringer's solution (control group), as shown in figure 2. These increased concentrations were observed for up to 6 h in this study. The perfusion with L-NMMA through the microdialysis probe significantly ($P < 0.05$) suppressed the carrageenan-induced increase in the concentrations of PGE_2 and 6-keto-$PGF_{1\alpha}$. In the rats with denervation, the concentrations of PGE_2 and 6-keto-$PGF_{1\alpha}$ after carrageenan injection were significantly ($P < 0.05$) lower than those of the control group but significantly ($P < 0.05$) higher than those of the L-NMMA group.

Effects of SC-560 and NS-398 on NO_2^-–NO_3^-, PGE_2, and 6-Keto-$PGF_{1\alpha}$ Concentrations after Carrageenan Injection

The concentrations of NO_2^-–NO_3^- after carrageenan in the animals that had received pretreatment with the selective COX-1 inhibitor SC-560 were comparable with those in control animals (fig. 1, *right*). Selective COX-2 inhibitor NS-398 also did not show any effects on the increased concentrations of NO_2^-–NO_3^- seen in control animals.

Figure 3 shows the effects of SC-560 and NS-398 on the concentrations of PGE_2 and 6-keto-$PGF_{1\alpha}$ after carrageenan injection. Pretreatment with SC-560 significantly ($P < 0.05$) suppressed the increases in PGE_2 and 6-keto-$PGF_{1\alpha}$ concentrations for the first 2 h after carrageenan but not at 3 h and thereafter. In contrast, NS-398 significantly ($P < 0.05$) suppressed the increasing in PGE_2 and 6-keto-$PGF_{1\alpha}$ concentrations 3 h after carrageenan and thereafter but not for the first 2 h.

Expressions of COX-1 and COX-2

Figure 4A shows the specificity of COX-1 and COX-2 antibodies to purified COX-1 and COX-2 proteins, respectively. Preabsorption of the COX-1 and COX-2 antibodies abolished the immunoreactivity of COX-1 and COX-2 isoforms, respectively (fig. 4B).

Figure 5 shows the expressions of COX-1 and COX-2 in the DRG. In normal condition, the DRG expressed detectable concentrations of COX-1 and COX-2. Injection

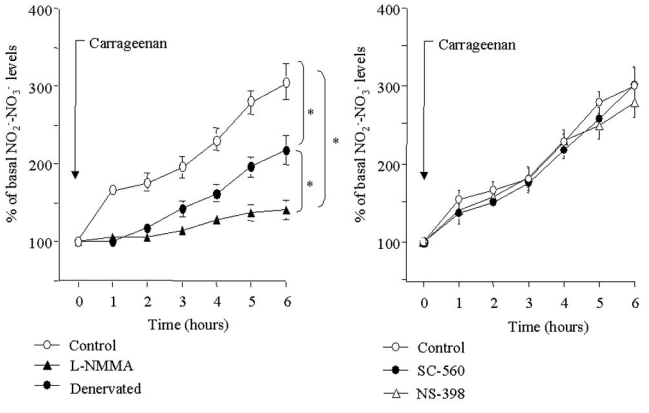

Fig. 1. (*Left*) Time courses of the subcutaneous concentration of nitrite (NO_2^-)–nitrate (NO_3^-) after injection of carrageenan. (*Right*) Effects of SC-560 and NS-398 on subcutaneous concentrations of NO_2^-–NO_3^-. Data are presented as mean ± SD from six rats in each group. * $P < 0.05$. L-NMMA = N^G-monomethyl-L-arginine acetate.

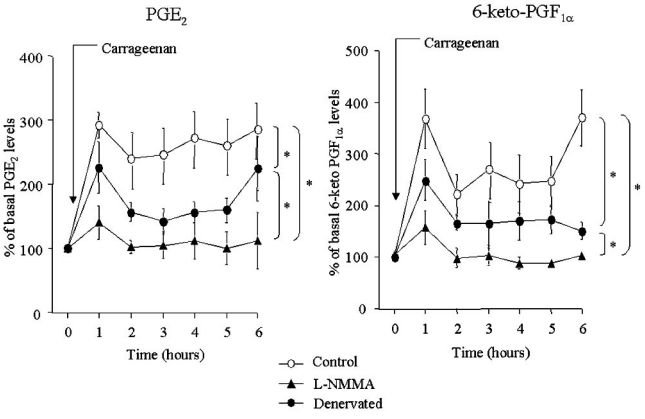

Fig. 2. Time courses of the subcutaneous concentration of prostaglandin (PG) E_2 (*left*) and 6-keto-$PGF_{1\alpha}$ (*right*) after injection of carrageenan. Data are presented as mean ± SD from six rats in each group. * $P < 0.05$. L-NMMA = N^G-monomethyl-L-arginine acetate.

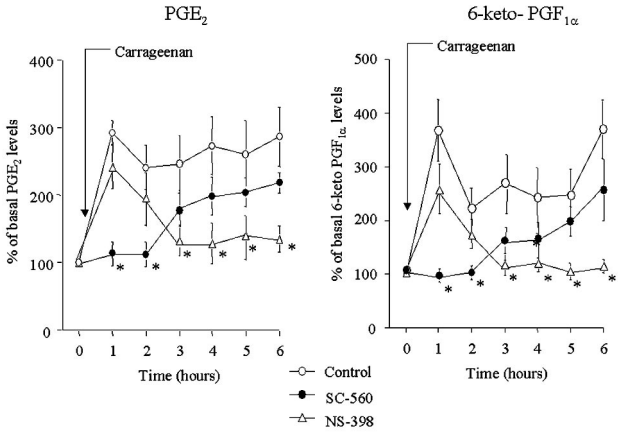

Fig. 3. Effects of SC-560 and NS-398 on subcutaneous concentrations of prostaglandin (PG) E_2 (*left*) and 6-keto-$PGF_{1\alpha}$ (*right*). Data are presented as mean ± SD from six rats in each group. * $P < 0.05$ *versus* control.

of carrageenan did not affect the basal expression of either COX-1 or COX-2 at each time point (3 and 6 h after carrageenan injection).

Figure 6 shows the expressions of COX-1 and COX-2 in the skin of the hind paw. The expression level of COX-1 protein after injection of carrageenan showed significant ($P < 0.05$) increases at 3 and 6 h, but not 1 h, after carrageenan injection (figs. 6A, *left*, and 6B, *left*). The expression levels of COX-1 after carrageenan under the condition of perfusion of L-NMMA were comparable with those under the condition of modified Ringer's solution perfusion (fig. 6B, *left*). In the animals with denervation, the carrageenan-induced increase in the COX-1 protein expression level was significantly ($P < 0.05$) suppressed.

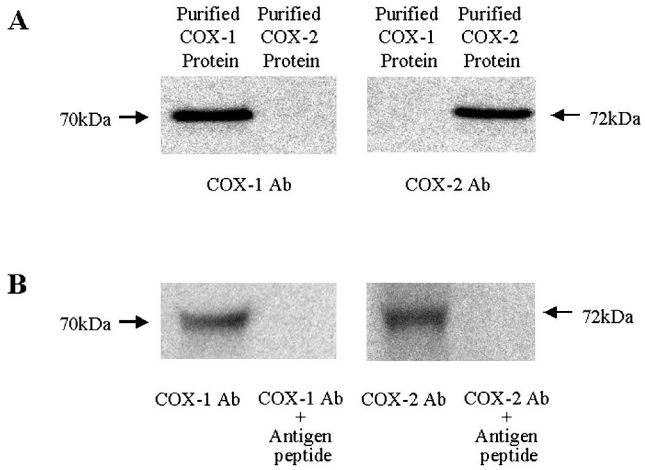

Fig. 4. (*A*) Western blots for cyclooxygenase (COX)-1 (*left*) and COX-2 (*right*) using 100 ng purified COX-1 protein and 30 ng purified COX-2 protein. (*B*) Western blots for COX-1 (*left*) and COX-2 (*right*) proteins extracted from skin of the rat that was injected with carrageenan 6 h before with use of COX-1 and COX-2 antibodies and the preabsorbed antibodies with each specific antigen peptide. Ab = antibody.

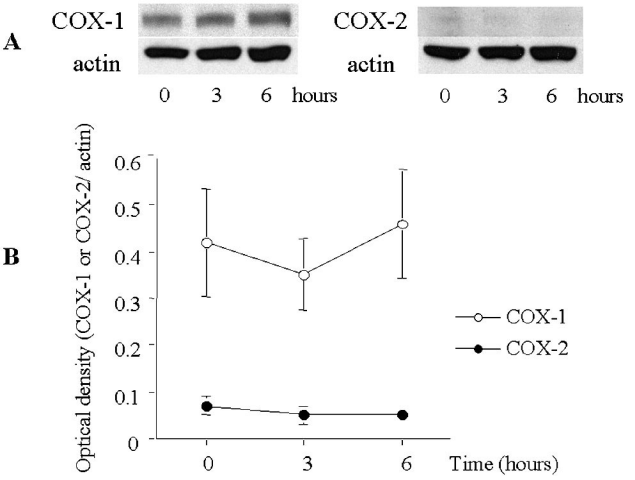

Fig. 5. Cyclooxygenase (COX)-1 and COX-2 expressions in dorsal root ganglia. (*A*) Western blots for COX-1 (*left*) and COX-2 (*right*) protein extracted from dorsal root ganglia after the injection of carrageenan. COX-1, COX-2, and actin were immunodetected as shown in representative blots. (*B*) Optical density analyses of COX-1:actin and COX-2:actin ratios. Data are presented as mean ± SD from six rats at each time point.

The expression level of COX-2 protein in the skin increased significantly ($P < 0.05$) at 3 and 6 h, but not 1 h, after carrageenan injection (figs. 6A, *right*, and 6B, *right*). L-NMMA completely suppressed the carrageenan-induced increase in the expression level of COX-2 (fig. 6B, *right*). COX-2 expression levels in the animals with denervation also increased after carrageenan, but the magnitude of increase was significantly ($P < 0.05$) lower than that in the control group. There was a significant ($P < 0.05$) difference of COX-2 concentrations among the three groups.

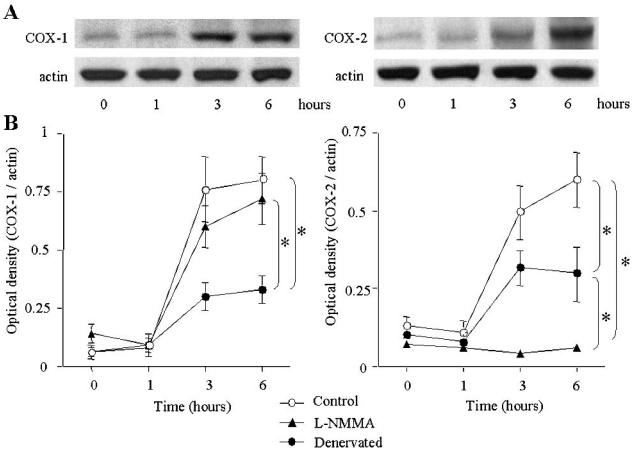

Fig. 6. Cyclooxygenase (COX)-1 and COX-2 expressions in skin and the effects of N^G-monomethyl-L-arginine acetate (L-NMMA) and denervation. (*A*) Western blots for COX-1 (*left*) and COX-2 (*right*) proteins extracted from skin after the injection of carrageenan. COX-1, COX-2, and actin were immunodetected as shown in representative blots. (*B*) Optical density analyses of COX-1:actin (*left*) and COX-2:actin (*right*) ratios. Data are presented as mean ± SD from six rats at each time point. * $P < 0.05$.

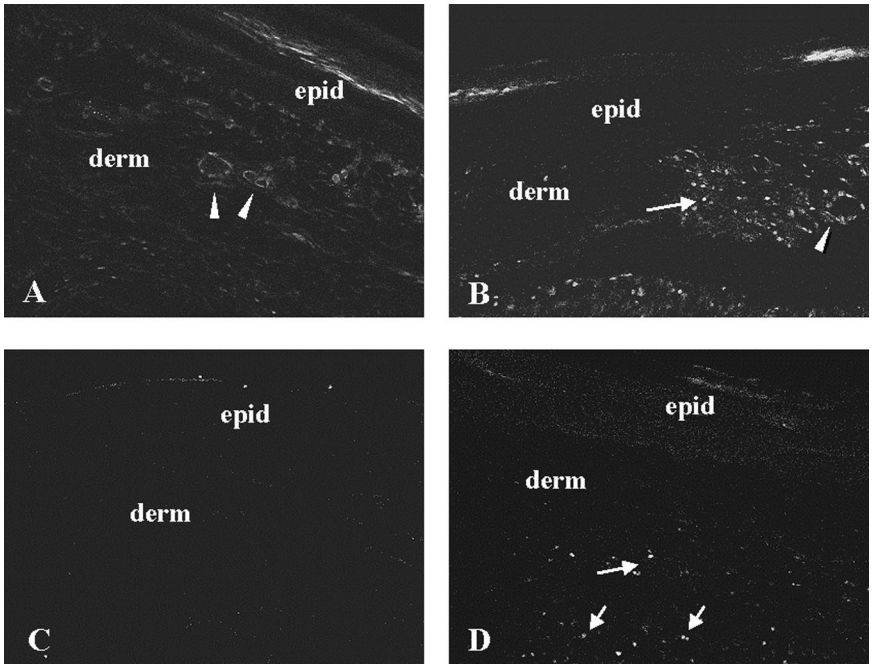

Fig. 7. Photographs of immunofluorescent staining of cyclooxygenase (COX)-1 (*A* and *B*) and COX-2 (*C* and *D*). (*A* and *C*) COX-1 and COX-2 immunoreactivity in the normal glabrous skin of the rat hind paw, respectively. (*B* and *D*) COX-1 and COX-2 immunoreactivity in the inflamed glabrous skin of the hind paw. *Arrow* = COX-1 or COX-2 immunoreactive cells; *arrowhead* = blood vessel; derm = dermis; epid = epidermis.

Cyclooxygenase-1 and COX-2 immunoreactivities in the glabrous skin of the rat hind paw are shown in figure 7. The COX-1 immunoreactivity was observed in the blood vessels in the normal skin. Six hours after carrageenan injection, many COX-1 immunoreactive cells were observed in the dermis and in the blood vessels. The COX-2 immunoreactivity was very limited in normal glabrous skin. However, 6 h after carrageenan injection, COX-2 immunoreactive cells were markedly increased in the dermis.

Discussion

The current study demonstrated that carrageenan-induced inflammation increased peripheral concentrations of NO, PGE_2, and PGI_2, and the increases were completely inhibited by NOS inhibitor L-NMMA. In the animals that had undergone neurectomy, the increases in NO and PGs after carrageenan were partially inhibited. The COX-1 inhibitor suppressed the increases in the concentrations of PGs for the first 2 h, and the COX-2 inhibitor suppressed the increasing 3 h after carrageenan and thereafter. Neither the COX-1 nor the COX-2 inhibitor suppressed the increasing in NO concentrations after carrageenan. It was also found that injection of carrageenan increased the COX-1 and COX-2 expression levels in the skin of the hind paw but not in the lumbar DRG. L-NMMA inhibited the up-regulation of COX-2 but not that of COX-1. The carrageenan-induced up-regulation of COX-1 and COX-2 expressions in the skin was partially inhibited in the animals with denervated sensory nerves.

Peripheral NO, PGE_2, and PGI_2 in Inflammation

We previously reported that carrageenan-induced production of NO was mediated by neuronal NOS (nNOS) in the early phase (~3 h) and by both nNOS and inducible NOS (iNOS) in the late phase (~3.5 hours) of carrageenan-induced inflammation.[3] This finding is consistent with the results of the current study, in which carrageenan-induced inflammation increased peripheral concentrations of NO, which were completely inhibited by L-NMMA, and in which increases in NO concentrations in the animals that had undergone neurectomy were completely inhibited until 2 h after carrageenan and partially inhibited thereafter. The nNOS is primarily found within the nervous system, and iNOS is present in various cell types, including macrophages, chondrocytes, and neutrophils, and is also found in central nervous system glial cells.[20] Therefore, it seems that nNOS in peripheral nerves and iNOS in inflammation-related cells contribute to the production of peripherally released NO in inflammation.

Hyperalgesic effects of PGs have been reported in several inflammatory models of nociception. Although PGE_2 has attained wide recognition as a mediator of hyperalgesia,[7,8] PGI_2 has also been shown to produce hyperalgesia as potently as PGE_2.[21] When the effects of PGE_2 and PGI_2 on sensory neurons are compared directly, PGI_2 is equally effective or even more effective as a hyperalgesic or sensitizing agent both *in vivo*[22,23] and *in vitro*.[24,25] This study has shown that not only PGE_2 but also PGI_2 concentrations increased in carrageenan-induced inflammation, and these increases should contribute to the induction and maintenance of inflammatory responses and pain.

NO-induced Prostaglandin Production

We demonstrated in this study that the inhibition of NO production by NOS inhibitor perfusion was associ-

ated with complete inhibition of not only NO but also PGE_2 and PGI_2 release; therefore, it is possible that the antiinflammatory and antihyperalgesic potencies of the NOS inhibitors are correlated with their ability to block both NO and PG releases. This is consistent with the previously reported results showing that an inhibition of edema with nonselective NOS inhibitor was associated with inhibition of NO release and clear inhibition of PGE_2 release in carrageenan-induced inflammation.[6] These indicate that PG production is in part mediated by NO. Because NO release is mediated by nNOS in the early phase and by both nNOS and iNOS in the late phase, it seems that NO derived from not only iNOS but also nNOS contributes to the enhancement of production of PGs in inflammation.

Several *in vitro* studies have suggested that NO activates COX activity, but the molecular mechanism by which NO activates COX remains to be identified, and moreover, *in vivo* data are scarce.[17] Some possible mechanisms have been proposed: antioxidant effect of NO by removal of O_2^-, which is involved in the autoinactivation of the COX enzyme[26,27]; NO-induced formation of nitrosothiols from COX enzyme, leading to changes in the structure of the COX[28]; and NO-induced generation of oxidant peroxynitrite, which acts as an activator of COX activity.[29] Also, the study of Posadas *et al.*[30] showed that administration of an iNOS inhibitor reduced COX-2 activity and the content of PGE_2 in exudates and did not affect the expression of COX-2 in a model of experimental inflammation in mice that were injected with zymosan. These suggest that NO enhances COX activity in inflammation without affecting the expression of COX. In the current study, however, we found the up-regulation of COX expression in the late phase in the skin at the site of inflammation. The fact that L-NMMA perfusion suppressed the expression of COX-2 but not that of COX-1 suggests the up-regulation of COX-2 expression by peripheral NO. In addition, in the animals that had been denervated, the up-regulation of the COX-2 expression in the skin was partially suppressed. These findings indicate that NO produced by nNOS in peripheral nerves partially contributes to this up-regulation. A large variety of agents such as proinflammatory cytokines and growth factors are thought to induce expression of the COX-2 gene[31,32] and iNOS.[33] Therefore, it is possible that these mediators induce NO release mediated by iNOS, leading to up-regulation of the expression of COX-2. Therefore, it seems that the up-regulation of COX-2 is due to NO produced by both iNOS and nNOS and that the NO-induced PG production in inflammation is due to enhancement of the expression of COX-2 and probably due to activation of COX-2 in the late phase. Although it has been uncertain whether NO interacts directly or indirectly with COX-2, a recent study by Chun *et al.*[34] suggested that NO induces COX-2 up-regulation in mouse skin through an activation of enkaryotic transcription factor NF-κB, which is a critical regulator of COX-2 expression.

The current study showed that the selective COX-1 inhibitor SC-560 inhibited the production of PGE_2 and PGI_2 in the early phase of carrageenan-induced inflammation, and the selective COX-2 inhibitor NS-398 inhibited the production in the late phase. In addition, neither COX-1 nor COX-2 expression was up-regulated in the early phase. These facts suggest that the activation of COX-1 contributes to production of PGs in the early phase. In contrast to COX-2, the up-regulation of COX-1 in the skin observed in the late phase was not mediated by NO, because L-NMMA did not suppress the up-regulation of COX-1 expression. It seems that the NO-independent up-regulation of COX-1 in the late phase does not contribute to the production of PGs, because the COX-1 inhibitor did not suppress the production of PGs in this phase. Furthermore, the expression was inhibited in the animals with denervation. These suggest that up-regulation of COX-1 in inflammation is independent on the action of NO but is mediated by the actions of other mediators derived from nerves. Although there are abundant data indicating that COX-2 is important in inflammation and pain, COX-1 has also been suggested to play a role in inflammatory processes.[35] Some studies have demonstrated that selective inhibition of COX-2 only partially reduced the concentrations of PGs at sites of inflammation in comparison with nonsteroidal antiinflammatory drugs, which reduced PGs to undetectable concentrations.[36,37] Therefore, COX-1 may contribute to the total pool of PG at a site of inflammation. Consistent with these results, activation and expression of both COX-1 and COX-2 contribute to the production of PGs, as shown in this study. Smith *et al.*[38] reported that either COX-1 or COX-2 inhibition prevented the increases in concentrations of PGs above basal concentrations in carrageenan-induced inflammation in the rat hind paw and that COX-2 but not COX-1 inhibition prevented the development of edema and hyperalgesia. A therapeutic dose of a COX-1 inhibitor did not reverse either hyperalgesia or edema but reduced the concentration of PGE_2 in the inflamed foot pad.[38] Therefore, it is possible that COX-1–derived PGs do not mediate inflammatory responses in a model of carrageenan-induced inflammation. This suggests that COX-2–derived PGs might be distinguishable from those synthesized by COX-1. Further study is needed to clarify the role of COX-1 in inflammation.

The current study demonstrated that although COX-1 and COX-2 exist in the DRG in normal state, up-regulation of expressions of these enzymes was not observed for up to 6 h after carrageenan injection. This indicates that the up-regulation of COX-1 and COX-2 expressions observed in the late phase occurs at peripheral sites other than peripheral nerves (fig. 7).

Prostaglandins and NO Production

The effect of PGs on the NOS pathway has not been fully explored. In the current study, we demonstrated that neither a selective COX-1 inhibitor nor a selective COX-2 inhibitor, at the dose that effectively inhibited PGs production, inhibited the increasing in NO concentrations after carrageenan. This suggests that PGs do not modify the NOS pathway. Tetsuka *et al.*[39] reported that endogenously released PGE_2 down-regulates iNOS induction and exogenously administered PGI_2 up-regulates iNOS induction in rat mesangial cells. These opposite effects of PGE_2 and PGI_2 might offset each other to induction of NOS after inflammation.

In conclusion, subcutaneous carrageenan injection induces peripheral release of PGE_2 and PGI_2 due to activation of COX-1 in early phase of carrageenan-induced inflammation and up-regulation of COX-2 expressions in the late phase in the skin. The activation of COX-1 and the up-regulation of the expression of COX-2 are facilitated by NO. Therefore, NO may exacerbate the inflammatory process through the generation of additional PG production. In addition, an NOS inhibitor may have the advantage of alleviating inflammatory symptoms through dual inhibition of NO and NO-driven COX activation and expression.

The authors thank Hitoshi Sohma, Ph.D. (Associate Professor, Department of Biochemistry, Sapporo Medical University School of Medicine, Sapporo, Japan), for suggestions on Western blot analysis.

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