JPET Fast Forward. Published on December 2, 2004 as DOI: 10.1124/jpet.104.078717

Significance of cyclooxygenase-2 induced via p38 mitogen-activated protein kinase in mechanical stimulus-induced peritoneal adhesion in mice

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Running title: Significance of cyclooxygenase-2 in peritoneal adhesion

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Text pages : 24

Tables : 0

Figures : 8

References : 36

Abstract word count : 249

Introduction word count : 473

Discussion word count : 993

Non-standard abbreviations :

COX, cyclooxygenase; PG, prostaglandin; MAP kinase, mitogen-activated protein kinase; DMSO, dimethylsulfoxide; PBS, phosphate-buffered saline; PPAR; peroxisome proliferator-activated receptor

Section assignment : Inflammation & Immunopharmacology

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Abstract

Postoperative peritoneal adhesion represents a major complication of surgery, but the molecular mechanism underlying pathogenesis of adhesion is not fully understood. The present study investigated the roles of cyclooxygenase (COX)-1 and COX-2 in peritoneal adhesion induced by scraping the surface of the cecum and abdominal wall in mice. Slight, but macroscopically observable, peritoneal adhesion was induced even on day 1, and the extent of adhesion reached a maximum on day 7 and beyond. COX-1 mRNA was constitutively expressed in the intact cecum, and its expression level was not altered after the mechanical stimulus. In contrast, expression of the COX-2 gene was markedly increased after the stimulus, and maximum expression was observed on days 3 - 7. Mofezolac, a specific COX-1 inhibitor, had no effect on peritoneal adhesion at 30 mg/kg, and had only marginal effects on PGE_2 levels in the cecum or peritoneal fluid. On the other hand, two highly selective inhibitors for COX-2, NS-398 and CAY10404, dose-dependently inhibited both adhesion formation and the increase in PGE_2 levels (3 - 30 mg/kg). The effects of NS-398 were eliminated when PGE_2 or (R)-butaprost was administered exogenously. A COX-2 antisense oligonucleotide also attenuated adhesion formation. Activation of p38 mitogen-activated protein (MAP) kinase was observed in the traumatized cecum, and a MAP kinase inhibitor, SB202190, inhibited adhesion formation (54% inhibition at 15 μM) and also reduced the COX-2 mRNA level and PGE_2 levels. In

conclusion, COX-2, but not COX-1, plays a significant role in mechanical stimulus-induced peritoneal formation in the mouse cecum.

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Introduction

Postoperative abdominal adhesion and fibrosis represent major complications of surgery and often result in infertility, abdominopelvic pain and small bowel obstruction. Adhesions are generally thought to be the result of inflammatory responses to surgery-derived tissue trauma, bacterial infection, hemorrhage or foreign substances in the peritoneal cavity. However, the molecular mechanisms underlying adhesion formation still await elucidation.

Cyclooxygenase (COX) is a key enzyme in inflammation. COX catalyzes the conversion of arachidonic acid to eicosanoids, including prostaglandins (PGs) and thromboxanes, and these COX-derived eicosanoids (especially PGE_2) are heavily involved in inflammatory diseases. Prostaglandins have also been implicated in adhesion formation, because tissue PGE_2 levels are elevated in bowels traumatized by mechanical stimuli (Celebioglu et al., 1999). There are two COX isozymes: COX-1 is constitutively expressed in most tissues and plays various basic "house-keeping" roles; in contrast, COX-2 is induced in response to various stimuli and is involved in the pathogenesis of various diseases, including inflammation and tumors. Recent reports suggest a significant role for COX-2 in adhesion formation in the uterine horn (Guvenal et al., 2001; Saed et al., 2003), whereas the contribution of COX-1 to abdominal adhesion is not fully understood. A role for COX-1 in inflammation was confirmed by studies using mice in which the Ptgs1 gene encoding COX-1 was

disrupted (Langenbach et al., 1995). Involvement of COX-1 in the development of inflammation has been also demonstrated in a rat model of collagen-induced arthritis (Ochi and Goto, 2002). Because COX-1 is constitutively expressed throughout the gastrointestinal tract in several species, including humans (Kargman et al., 1996), the roles of COX-1 in abdominal adhesion formation require closer investigation.

COX-2 is an inducible isozyme and several pro-inflammatory stimuli increase the expression level of COX-2 at inflammatory sites. The molecular mechanisms that regulate COX-2 expression have been extensively studied, and involvement of several factors, including mitogen-activated protein (MAP) kinases (Guan et al., 1998; Ridley et al., 1998; Scherle et al., 1998), peroxisome proliferator-activated receptors (PPARs), and nuclear factor (NF)-κB (Plummer et al., 1999; Charalambous et al., 2003), has been suggested (Pang et al., 2003). Accumulating evidence suggests that the major signaling pathway responsible for COX-2 induction differs, depending on the stimulus and the cell type. Hence, it has been shown that p38 MAP kinase can be activated by mechanical stimuli (Dieckgraefe et al., 1997; Pyles et al., 1997), and mechanical stimuli-induced COX-2 expression is mediated by activation of p38 MAPK in podocytes (Martineau et al., 2004). Based on these reports, we were interested in determining whether the p38 MAPK pathway plays a significant role in COX-2 expression in the mechanically-stimulated cecum.

The aims of the present study were to elucidate the contribution of COX-1 and COX-2 in adhesion formation and to determine the signaling pathways implicated in the induction of COX-2 in the traumatized cecum, with a particular focus on the involvement of p38 MAP kinase.

METHODS

Animals and Drugs

Six-week-old male ICR mice (purchased from SLC, Shizuoka, Japan) were used for the experiments. Mice that were homozygous null (COX-2 KO) for targeted disruption of the COX-2 gene (B6;129S7-Ptgs2^{tm1Jed}, The Jackson Laboratory, Bar Harbor, ME) and littermates with the same genetic background (B6129SF2 wild-type mice, WT) were also used in some experiments. We confirmed that our protocol induced similar peritoneal adhesion in ICR and B6129SF2 WT mice. The animals were fed standard rodent chow and tap water *ad libitum*, housed in communal cages and maintained at controlled temperature and humidity. All experimental protocols conformed to international guidelines and were approved by the institutional review board.

Mofezolac (Mitsubishi Pharma, Tokyo, Japan) is a highly selective COX-1 inhibitor (Goto et al., 1998), and NS-398 (N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide) and CAY10404 (3-(4-methylsulphonylphenyl)-4-phenyl-5-trifluoromethylisoxazole), purchased from Cayman Chemical (Ann Arbor, MI), were used as specific COX-2 inhibitors (Habeeb et al., 2000). PGE_2 and (R)-butaprost, an analog of PGE_2 with good selectivity for the EP2 receptor subtype (Kiriyama et al., 1997), and AH6809 (6-isopropoxy-9-oxoxanthene-2-carboxylic acid) and SC-19220

(8-chloro-dibenz[b,f][1,4]oxazepine-10(11H)-carboxy-(2-acetyl)hydrazide) were also obtained from Cayman Chemical (Ann Arbor, MI). SB202190 (4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole), which is a widely-used pyridinyl imidazole inhibitor of p38α and p38β, but not p38γ and p38δ (Jiang *et al.*, 1996; Jiang *et al.*, 1997; Kumar *et al.*, 1997; Stein *et al.*, 1997; Wang *et al.*, 1997), was obtained from Calbiochem (San Diego, CA). Antibodies for p38 and phosphorylated p38 (Thy180 / Tyr182) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for COX-1 or COX-2 for use in Western blotting were also obtained from Santa Cruz Biotechnology.

Formation of experimental adhesion

Mice were anesthetized with a subcutaneous injection of sodium pentobarbital (40 mg/kg). After treatment of the abdomen with a povidone iodine solution, a vertical midline incision was made and the cecum was exteriorized. Both surfaces of the cecum were scraped 40 times with a swab, without injuring the mesenteric arteries and veins. The cecum was returned to its original position and the abdominal wall facing the treated cecum was also scraped with a swab. The abdomen was closed in two layers with a silk suture. All procedures were conducted under sterile conditions. Sham-operated mice were prepared in the same manner, but no scraping was performed.

Mofezolac, NS-398 and CAY10404 were orally administered to the mice once daily for 7 days. Vehicle control mice were given 0.5% methylcellulose solution orally. AH-6809, SC19220 and SB202190 were dissolved in dimethyl sulfoxide (DMSO) and diluted 1000-fold with sterile phosphate-buffered saline (PBS) to obtain the desired concentrations. 0.5 mL of each solution was administered to the mice by intraperitoneal (i.p.) injections. Although SB202190 is known to be toxic, no signs of toxicity (including no decrease in body weight) were observed in SB202190-treated mice at the doses used, compared with vehicle-treated mice. Administration of 0.5 mL of a 15 μM SB202190 solution results in a systemic dose of 82.5 μg/kg, which gives little or no toxicity in mice in a 7-day experiment (Zhang et al., 2003).

Antisense treatment

An antisense phosphorothioate oligonucleotide for murine COX-2 (5'-GCAGAGCAGCACCGA-3') and a randomized control oligonucleotide were synthesized as previously described (Kage et al., 1999). An aqueous solution of each oligonucleotide was intraperitoneally administered daily at a dose of 3 mg/kg, starting 2 hr before mechanical stimulation (Khan et al., 2002).

Assessment of adhesion

One week after the surgery, the mice were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and intraperitoneal adhesion formation was assessed using three different methods. Assessment was performed by a single research worker throughout the study, and this person was blinded to the treatment. Firstly, the severity of adhesion was evaluated by macroscopic observation, using a modified Mazuji classification system (Mazuji et al., 1964). Scores of 0-4 (with 4 being the most severe) were assigned based on the severity of adhesion, as follows: 0, no adhesion; 1, mild adhesion (localized and easy to separate); 2, moderate adhesion; 3, severe adhesion (very widespread and difficult to separate). Cecum-cecum adhesion, cecum-abdominal wall adhesion and cecum adhesion to other tissues, including the small intestine and liver, were evaluated independently. Therefore, the severest adhesion formation could receive a score of 12. Secondly, the maximum force required to detach the adhered cecum completely was determined using a piezo-electrical device, as described in a previous report (Wiseman et al., 2004). Finally, the cecum and intestine were opened along the mesenteric attachment for performance of planimetry (Muller et al., 2003). Tension-free specimens were placed on a glass plate and photographed, and the adhesion area was computed by analyzing the images using NIH Image software.

RT-PCR study

After evaluation of adhesion formation, the cecum was excised, washed thoroughly with ice-cold PBS, and quickly frozen with liquid nitrogen. Total RNA was extracted with ISOGEN reagent (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. Real-time PCR was conducted as previously described (Paliege et al., 2004). Oligonucleotides for primers and probes were designed using Primer Express 1.0 (Applied Biosystems, Foster City, CA). COX-1 sense, 5'-GCTGAAGGAGGAGC ATCCC-3', antisense, 5'-AGGCGAGTGGTCTGGAAGAG-3', and probe, 5'-CGTGGGATGATGAGC-3'; COX-2 sense, 5'-CCCTGAAGCCGTACACATCA-3', antisense, 5'-TGTCACTGTAGAGGGCTTTCAATT-3', and probe, 5'-TGCAGCCATTTCCTTCTCTCCTGTAAGTTCT-3'. Real-time PCR amplification was performed using TaqMan Universal PCR Master Mix and the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Relative amounts of mRNA, normalized against β-actin mRNA, were calculated from the threshold cycle numbers.

Determination of PGE_2 levels in the cecal tissue and peritoneal fluid

The PGE_2 level in the cecal tissue was determined as previously described (Futaki et al., 1994; Tanaka et al., 2002). Briefly, the cecum was excised on day 7, washed quickly with ice-cold PBS,

weighed and placed in a tube containing 100% methanol plus 0.1M indomethacin. The tissues were homogenized using a Polytron homogenizer, followed by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant of each sample was evaporated under a vacuum, the residue was re-dissolved in the assay buffer, and the PGE_2 concentration was determined using a PGE_2 enzyme immunoassay (EIA) kit (Amersham Biosciences, Piscataway, NJ).

For determination of PGE_2 contents in peritoneal fluid, the peritoneal cavity was washed with 1mL of PBS and the solution collected was directly used in the EIA.

Western blot analyses

To investigate the protein expression levels of COX-1 and COX-2 in the cecum upon adhesion, Western blot analyses were conducted as previously described (Qiu et al., 2003). Band intensity was quantified by densitometry and all protein levels were normalized against those at day 0.

Immunoprecipitation

Immunoprecipitation was conducted as previously described (de Alvaro et al., 2004). Briefly, a part of the cecum (about 100 mg) was homogenized with a Polytron homogenizer and lyzed in 1 ml of lysis buffer (50 mM Hepes, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40,

leupeptin, aprotinin, phenylmethylsulfonyl fluoride, benzamidine sodium vanadate and β-glycerophosphate). The cell extract was normalized for protein content and incubated for 2 hr at 4°C with anti-p38 antibody pre-coupled with Protein G-Sepharose beads. The beads were subsequently washed 3 times with lysis buffer. The protein was released from the beads by boiling the samples for 5 min in sample buffer. The supernatants were run on SDS-PAGE and transferred to nitrocellulose membranes. Western blotting was conducted using an anti-p38 antibody or an antibody specific for the phosphorylated form of p38 and a secondary peroxidase-conjugated anti-rabbit IgG diluted to 1:1000. The Western blots were quantified by densitometry, and phosphorylated p38 MAP kinase levels are presented as relative values normalized against the total p38 MAP kinase levels.

Statistics

All data are expressed as mean ± S.E.M. Adhesion scores were statistically analyzed by a non-parametric Mann-Whitney U-test. Other numeric data were statistically evaluated using an unpaired Student's t-test or by using one-way analysis of variance with Bonferroni's correction (for multiple comparison). A p value less than 0.05 was considered to be statistically significant.

RESULTS

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Time course of peritoneal adhesion formation

Because the extent of peritoneal adhesion largely depends on the experimental conditions, including the severity of the trauma, we first conducted a time course study and assessed the peritoneal adhesion daily. As shown in Fig. 1, slight adhesion was even observed on day 1 in most mice. However, the area of adhesion was localized and the tissues were easy to separate. By day 7, broad intra-cecum adhesion had formed and the tissues were difficult to separate. Adhesion of the cecum to other parts of the gastrointestinal tract, to the liver and to the internal surface of the abdominal wall was frequently observed. The severity of adhesion on day 12 did not differ to that on day 7. In contrast, no adhesion formation occurred in the sham-operated mice at any point during the experiment. Typical examples of the intestine on day 7 are shown in Fig. 1A. As shown in Figs. 1B-D, the three indices used here to evaluate the severity of adhesion showed similar time-course trends.

COX-1 and COX-2 gene and protein expression

COX-1 and COX-2 mRNA expression levels were determined in the intact and traumatized cecum by real-time RT-PCR. In the intact cecum, constitutive expression of COX-1 mRNA was observed, whereas only marginal levels of COX-2 mRNA expression were detected. After mechanical stimulation, expression of COX-2 mRNA increased on day 1 and thereafter, with maximum expression

observed from day 3 to day 7 (Fig. 2A). The expression level of COX-1 mRNA did not change after mechanical stimulation, based on measurements made until day 7.

As shown in Fig. 2B, expression of COX-1 and COX-2 proteins showed a similar trend. COX-2 expression increased in the traumatized cecum, while COX-1 expression did not change, even after mechanical stimulation.

Effects of COX-1 and COX-2 inhibitors on adhesion formation

To examine the respective roles of COX-1 and COX-2 on peritoneal adhesion formation, specific inhibitors for each cyclooxygenase isozyme were administered to mice that had undergone mechanical stimulation. As shown in Figs. 3A and 3B, mofezolac, a selective COX-1 inhibitor, had no effect on adhesion score and adhesion area, even at 30 mg kg/day, the highest dose examined. On the other hand, NS-398 and CAY10404, highly selective COX-2 inhibitors, attenuated adhesion formation in a dose-dependent manner, and led to a significant reduction in adhesion score and adhesion area at doses of more than 3 mg/kg (Figs. 3A and 3B). No side effects, such as ulcer formation in the gastrointestinal tract, were observed in any experimental groups (data not shown). As shown in Figs. 3C and 3D, the PGE_2 levels in cecum tissues and in the peritoneal fluid were significantly increased on day 7 after mechanical stimulation. NS-398 and CAY10404 inhibited PGE_2

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elevation at doses of more than 3 mg/kg, and the PGE_2 concentration returned to its basal level at doses of 30 mg/kg (Figs. 3C and 3D). In contrast, mofezolac inhibited PGE_2 formation only at 30 mg/kg in traumatized mice, and to a much lesser extent than the COX-2 inhibitors.

Effects of an EP receptor agonist and an EP receptor antagonist

To confirm that COX-2 inhibitors attenuated peritoneal adhesion by inhibiting PGE_2 formation in the traumatized cecum, we examined the effects of exogenously administered PGE_2 in mice treated with COX-2 inhibitors. As shown in Fig. 4A, PGE_2 (10^{-7}M solution, i.p.) eliminated the inhibitory effect of NS-398 on peritoneal adhesion, while PGE_2 alone did not induce adhesion in sham-operated mice. Similar results were obtained when the mice were treated with (R)-butaprost (10^{-6} M, i.p.).

As shown in Fig. 4B, AH 6809, an EP receptor antagonist, also attenuated peritoneal adhesion. In contrast, SC-19220, a selective antagonist of PGE_2 at EP1 receptors, had no effect.

Effect of a COX-2 antisense oligonucleotide

To further examine the significance of the role of COX-2 in adhesion formation, an antisense oligonucleotide for COX-2 mRNA was administered to mice that had received mechanical stimulation. As shown in Fig. 5A, expression of COX-2 was largely attenuated in antisense oligonucleotide

(AS)-treated mice, compared with vehicle-treated or control oligonucleotide (CO)-treated mice. Administration of the antisense oligonucleotide significantly ($p < 0.01$ vs. vehicle control) reduced the adhesion score and the adhesion area, whereas a randomized control oligonucleotide had no effect (Figs.5B and 5C).

Peritoneal adhesion in COX-2-deficient mice

To further investigate the significance of the role of COX-2 in peritoneal adhesion in the traumatized cecum, we used mice lacking the COX-2 gene (COX-2 KO mice). As shown in Fig. 6, peritoneal adhesion was significantly attenuated in COX-2 KO mice, based on both the adhesion score and the adhesion area. Exogenous administration of PGE_2 to the peritoneal cavity of these mice produced severity of adhesion at an almost wild-type level, while this procedure resulted in no additional effect in wild-type littermates.

Involvement of p38 kinase in COX-2 induction

We next investigated the molecular signals responsible for up-regulation of COX-2 mRNA in the cecum. When the mice were treated with an inhibitor of p38 MAP kinase, SB202190 (3 and 15 μM), adhesion scores were significantly reduced (54% inhibition at 15 μM) (Fig. 7A), and the adhesion

area was also reduced by treatment with SB202190 (Fig. 7B). PGE_2 elevation in the traumatized cecum was dose-dependently decreased by treatment with SB202190, and COX-2 mRNA expression levels in the cecum were also significantly lower in SB202190-treated mice. The dose-response profiles of the adhesion scores, PGE_2 contents and COX-2 mRNA expression were almost the same.

Activation of p38 MAP kinase in the traumatized cecum was examined by immunoprecipitation with a phosphorylated p38 MAP kinase-selective antibody. As shown in Fig. 8, only a faint level of phosphorylated p38 MAP kinase was detected in the cecum in sham-operated mice (S). In the traumatized cecum, the level of the phosphorylated form of p38 significantly increased on day 1 and thereafter, suggesting activation of p38 MAP kinase in the traumatized cecum.

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DISCUSSION

The formation of abdominal adhesion is a very common phenomenon that represents a major complication in surgery. However, the molecular mechanisms underlying adhesion formation are yet to be fully understood, and for this reason we have investigated the role of COX-2 in peritoneal adhesion in traumatized tissues, since COX-2 is known to be expressed in the inflammatory response to various stimuli, including mechanical stimuli.

The major findings of the present study are as follows: firstly, COX-2 is induced extensively after trauma, while COX-1 is constitutively expressed and its expression level does not change after mechanical stimulation. Secondly, COX-2, but not COX-1, plays a significant role in adhesion formation, because treatment with selective inhibitors for COX-2 and with an antisense oligonucleotide for COX-2 mRNA attenuated adhesion; the COX-1 isoform had only a marginal effect, if any, on adhesion. Thirdly, peritoneal adhesion was much less severe in COX-2 knockout mice, compared with wild-type littermates. Fourthly, PGE_2 is one of the primary COX-2-derived mediators responsible for adhesion formation, and fifthly, the p38 MAP kinase pathway is involved in COX-2 induction in the traumatized cecum. Taken together, these results suggest that a mechanical stimulus can activate the p38 MAP kinase-dependent signaling pathway, resulting in COX-2 induction, which then promotes adhesion formation via increased PGE_2 production.

As shown in Fig. 2, COX-1 is constitutively expressed in the intact cecum and its expression level is not changed after tissue trauma. In spite of the presence of COX-1 in the cecum, mofezolac, a selective COX-1 inhibitor, did not exert any effects on adhesion formation, even at a dose of 30 mg/kg. This dose is thought to be sufficient for COX-1 inhibition in rodents, because mofezolac inhibits COX-1-dependent acute pain in mice, even at 10 mg/kg (Goto et al., 1998; Kusuhara et al., 1998). In a preliminary experiment, we also confirmed that platelet aggregation is inhibited upon administration of mofezolac at 30 mg/kg in mice (85.3 ± 10.8 % inhibition 60 min after drug administration). Therefore, our present results suggest that the contribution of COX-1 to peritoneal adhesion formation is marginal, at most. In contrast, selective inhibition of COX-2 by NS-398 or CAY10404 significantly reduced the severity of peritoneal adhesion, suggesting that COX-2 plays an important role in adhesion formation. We note that COX-independent actions of COX inhibitors have recently been reported by several investigators (Tegeder et al., 2001). However, here we have used three structurally distinct COX inhibitors (Mofezolac, NS-398 and CAY10404) and found that the two COX-2-selective inhibitors attenuated adhesion formation. Importantly, the COX-2 inhibitor-induced reduction in adhesion formation was reversed by simultaneous treatment of the traumatized cecum with exogenous PGE_2 (Fig. 4A). Furthermore, an antisense oligonucleotide for COX-2 mRNA significantly attenuated adhesion (Fig. 5). These results, taken together, strongly suggest a primary

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role of COX-2 in adhesion formation induced by mechanical stimuli.

As shown in Figs. 3C and 3D, mechanical stimuli elevate the PGE_2 contents in the traumatized cecum over those in the intact cecum, and also increase the PGE_2 concentration in the peritoneal fluid. Administration of NS-398 or CAY10404 significantly inhibited PGE_2 elevation, and the PGE_2 concentration was decreased to its basal level at a dose of 30 mg/kg, the highest dose examined. These results suggest that the primary enzyme responsible for elevated PGE_2 production in the traumatized cecum is COX-2. Because the decreased PGE_2 level in the COX-2 inhibitor-treated cecum is clearly correlated with decreased adhesion formation, it is highly plausible that COX-2-derived PGE_2 is involved in the pathogenesis of peritoneal adhesion. This hypothesis is supported by the reversal of the effect of the COX-2 inhibitors by exogenous PGE_2 or a PGE_2 analog, (R)-butaprost (Fig. 4A), and attenuation of adhesion formation by an EP receptor antagonist (AH6809) equipotent for the EP1 and EP2 subtypes (Fig. 4B). Because (R)-butaprost is relatively highly selective for the EP2 receptor subtype, a significant part of the PGE_2 activity may be mediated via EP2 receptor. However, the EP receptor subtypes involved in adhesion formation in the traumatized cecum need to be more closely investigated, using subtype-specific antagonists or mice which are genetically devoid of each subtype.

As shown in Figs. 7 and 8, p38 MAP kinase was activated after mechanical stimulation, and

pharmacological blockade of the p38 MAP kinase-dependent pathway with SB202190 resulted in reduced expression of COX-2 mRNA, with this change showing a strong correlation with attenuation of adhesion formation. These results suggest that p38 MAP kinase is directly involved in mechanical-stimulus-induced adhesion formation, via COX-2 induction. Several previous reports have also shown that p38 MAP kinase can mediate COX-2 expression induced by mechanical stimuli (Dieckgraefe et al., 1997; Pyles et al., 1997; Martineau et al., 2004), and it is possible that the p38 MAP kinase-dependent pathway may be the primary common pathway responsible for mechanical stimulus-induced COX-2 expression in various cells. Involvement of other pathways, such as the NF-□B-dependent pathway, should also be investigated in this respect. In the traumatized cecum in SB202190-treated mice, we observed that phosphorylation of p38 MAP kinase itself was attenuated when compared with that in vehicle-treated mice (data not shown). The reason for the reduced level of phosphorylated p38 is unclear, because SB202190 is not known to inhibit phosphorylation of p38. It is likely that downstream factors (for example, COX-2-derived eicosanoids) are involved in the prolonged activation of p38 through a feedback mechanism.

In conclusion, selective inhibitors of COX-2 and an antisense oligonucleotide for COX-2 mRNA significantly attenuated peritoneal adhesion formation in the mechanically-stimulated cecum, whereas a COX-1 inhibitor had no effect, suggesting that COX-2, but not COX-1, is implicated in adhesion

formation. Up-regulation of COX-2 mRNA expression was observed after mechanical stimulation and this elevated expression was inhibited by treatment with inhibitors of the p38 MAP kinase pathway. Exogenously administered PGE_2 or an EP2-selective PGE_2 analog reversed the inhibitory effect of the COX-2 inhibitors. These results suggest that induction of COX-2 occurs through activation of the p38 MAPK pathway in the mechanically-stimulated cecum, leading to elevation of PGE_2, which then facilitates adhesion formation.

REFERENCES

Celebioglu B, Eslambouli NR, Olcay E, and Atakan S (1999) The effect of tenoxicam on intraperitoneal adhesions and prostaglandin E2 levels in mice. *Anesth Analg* **88**:939-942.

Charalambous MP, Maihofner C, Bhambra U, Lightfoot T, and Gooderham NJ (2003) Upregulation of cyclooxygenase-2 is accompanied by increased expression of nuclear factor-kappa B and I kappa B kinase-alpha in human colorectal cancer epithelial cells. *Br J Cancer* **88**:1598-1604.

de Alvaro C, Teruel T, Hernandez R, and Lorenzo M (2004) Tumor necrosis factor alpha produces insulin resistance in skeletal muscle by activation of inhibitor kappaB kinase in a p38 MAPK-dependent manner. *J Biol Chem* **279**:17070-17078.

Dieckgraefe BK, Weems DM, Santoro SA, and Alpers DH (1997) ERK and p38 MAP kinase pathways are mediators of intestinal epithelial wound-induced signal transduction. *Biochem Biophys Res Commun* **233**:389-394.

Futaki N, Takahashi S, Yokoyama M, Arai I, Higuchi S, and Otomo S (1994) NS-398, a new anti-inflammatory agent, selectively inhibits prostaglandin G/H synthase/cyclooxygenase (COX-2) activity in vitro. *Prostaglandins* **47**:55-59.

Goto K, Ochi H, Yasunaga Y, Matsuyuki H, Imayoshi T, Kusuhara H, and Okumoto T (1998) Analgesic effect of mofezolac, a non-steroidal anti-inflammatory drug, against phenylquinone-induced

acute pain in mice. *Prostaglandins Other Lipid Mediat* **56**:245-254.

Guan Z, Buckman SY, Miller BW, Springer LD, and Morrison AR (1998) Interleukin-1beta-induced cyclooxygenase-2 expression requires activation of both c-Jun NH2-terminal kinase and p38 MAPK signal pathways in rat renal mesangial cells. *J Biol Chem* **273**:28670-28676.

Guvenal T, Cetin A, Ozdemir H, Yanar O, and Kaya T (2001) Prevention of postoperative adhesion formation in rat uterine horn model by nimesulide: a selective COX-2 inhibitor. *Hum Reprod* **16**:1732-1735.

Habeeb AG, Rao PNP, and Knaus EE (2000) Design and syntheses of diarylisoxazoles: Novel inhibitors of cyclooxygenase-2 (COX-2) with analgesic-antiinflammatory activity. *Drug Develop Res* **51**:273-286.

Jiang Y, Chen C, Li Z, Guo W, Gegner JA, Lin S, and Han J (1996) Characterization of the structure and function of a new mitogen-activated protein kinase (p38β). *J Biol Chem* **271**:17920-17926.

Jiang Y, Gram H, Zhao M, New L, Gu J, Feng L, Di Padova F, Ulevitch RJ, and Han J (1997) Characterization of the structure and function of the fourth member of p38 group mitogen-activated protein kinases, p38δ. *J Biol Chem* **272**:30122-30128.

Kage K, Fujita N, Oh-hara T, Ogata E, Fujita T, and Tsuruo T (1999) Basic fibroblast growth factor induces cyclooxygenase-2 expression in endothelial cells derived from bone. *Biochem Biophys*

Res Commun **254**:259-263.

Kargman S, Charleson S, Cartwright M, Frank J, Riendeau D, Mancini J, Evans J, and O'Neill G (1996) Characterization of Prostaglandin G/H Synthase 1 and 2 in rat, dog, monkey, and human gastrointestinal tracts. *Gastroenterology* **111**:445-454.

Khan I, Al-Awadi FM, Thomas N, Haridas S, and Anim JT (2002) Cyclooxygenase-2 inhibition and experimental colitis: beneficial effects of phosphorothioated antisense oligonucleotide and meloxicam. *Scand J Gastroenterol* **37**:1428-1436.

Kiriyama M, Ushikubi F, Kobayashi T, Hirata M, Sugimoto Y, and Narumiya S (1997) Ligand binding specificities of the eight types and subtypes of the mouse prostanoid receptors expressed in Chinese hamster ovary cells. *Br J Pharmacol* **122**:217-224.

Kumar S, McDonnell PC, Gum RJ, Hand AT, Lee JC, and Young PR (1997) Novel homologues of CSBP/p38 MAP kinase: activation, substrate specificity and sensitivity to inhibition by pyridinyl imidazoles. *Biochem Biophys Res Commun* **235**:533-538.

Kusuhara H, Matsuyuki H, and Okumoto T (1998) Involvement of prostaglandins produced by cyclooxygenase-1 in murine visceronociception induced by phenylquinone. *Prostaglandins Other Lipid Mediat* **55**:43-49.

Langenbach R, Morham SG, Tiano HF, Loftin CD, Ghanayem BI, Chulada PC, Mahler JF, Lee CA,

Goulding EH, Kluckman KD, and et al. (1995) Prostaglandin synthase 1 gene disruption in mice reduces arachidonic acid-induced inflammation and indomethacin-induced gastric ulceration. *Cell* **83**:483-492.

Martineau LC, McVeigh LI, Jasmin BJ, and Kennedy CR (2004) p38 MAP kinase mediates mechanically induced COX-2 and PG EP4 receptor expression in podocytes: implications for the actin cytoskeleton. *Am J Physiol Renal Physiol* **286**:F693-701.

Mazuji MK, Kalambaheti K, and Pawar B (1964) Prevention of Adhesions with Polyvinylpyrrolidone. Preliminary Report. *Arch Surg* **89**:1011-1015.

Muller SA, Treutner KH, Haase G, Kinzel S, Tietze L, and Schumpelick V (2003) Effect of intraperitoneal antiadhesive fluids in a rat peritonitis model. *Arch Surg* **138**:286-290.

Ochi T, and Goto T (2002) Differential effect of FR122047, a selective cyclo-oxygenase-1 inhibitor, in rat chronic models of arthritis. *Br J Pharmacol* **135**:782-788.

Paliege A, Mizel D, Medina C, Pasumarthy A, Huang YG, Bachmann S, Briggs JP, Schnermann JB, and Yang T (2004) Inhibition of nNOS expression in the macula densa by COX-2-derived prostaglandin E(2). *Am J Physiol Renal Physiol* **287**:F152-159.

Pang L, Nie M, Corbett L, and Knox AJ (2003) Cyclooxygenase-2 expression by nonsteroidal anti-inflammatory drugs in human airway smooth muscle cells: role of peroxisome

proliferator-activated receptors. *J Immunol* **170**:1043-1051.

Plummer SM, Holloway KA, Manson MM, Munks RJ, Kaptein A, Farrow S, and Howells L (1999) Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-kappaB activation via the NIK/IKK signalling complex. *Oncogene* **18**:6013-6020.

Pyles JM, March KL, Franklin M, Mehdi K, Wilensky RL, and Adam LP (1997) Activation of MAP kinase in vivo follows balloon overstretch injury of porcine coronary and carotid arteries. *Circ Res* **81**:904-910.

Qiu ZF, Maruyama K, Sunayama K, Kashiwabara H, Shoji T, Nakamura T, Suzuki S, Konno H, and Nakamura S (2003) Piroxicam-induced regression of intestinal adenomatous polyps in APC(delta474) mice. *J Invest Surg* **16**:71-81.

Ridley SH, Dean JL, Sarsfield SJ, Brook M, Clark AR, and Saklatvala J (1998) A p38 MAP kinase inhibitor regulates stability of interleukin-1-induced cyclooxygenase-2 mRNA. *FEBS Lett* **439**:75-80.

Saed GM, Munkarah AR, and Diamond MP (2003) Cyclooxygenase-2 is expressed in human fibroblasts isolated from intraperitoneal adhesions but not from normal peritoneal tissues. *Fertil Steril* **79**:1404-1408.

Scherle PA, Jones EA, Favata MF, Daulerio AJ, Covington MB, Nurnberg SA, Magolda RL, and Trzaskos JM (1998) Inhibition of MAP kinase kinase prevents cytokine and prostaglandin E2 production in lipopolysaccharide-stimulated monocytes. *J Immunol* **161**:5681-5686.

Stein B, Yang MX, Young DB, Janknecht R, Hunter T, Murray BW, and Barbosa MS (1997) p38-2, a novel mitogen-activated protein kinase with distinct properties. *J Biol Chem* **272**:19509-19517.

Tanaka A, Hase S, Miyazawa T, Ohno R, and Takeuchi K (2002) Role of cyclooxygenase (COX)-1 and COX-2 inhibition in nonsteroidal anti-inflammatory drug-induced intestinal damage in rats: relation to various pathogenic events. *J Pharmacol Exp Ther* **303**:1248-1254.

Tegeder I, Pfeilschifter J, and Geisslinger G (2001) Cyclooxygenase-independent actions of cyclooxygenase inhibitors. *Faseb J* **15**:2057-2072.

Wang XS, Diener K, Manthey CL, Wang S, Rosenzweig B, Bray J, Delaney J, Cole CN, Chan-Hui PY, Mantlo N, Lichenstein HS, Zukowski M, and Yao Z (1997) Molecular cloning and characterization of a novel p38 mitogen-activated protein kinase. *J Biol Chem* **272**:23668-23674.

Wiseman D, Lyachovetsky Y, Keidan I, Trout JR, and Nur I (2004) The effect of tranexamic acid in fibrin sealant on adhesion formation in the rat. *J Biomed Mater Res* **68B**:222-230.

Zhang S, Ren J, Zhang CE, Treskov I, Wang Y, and Muslin AJ (2003) Role of 14-3-3-mediated p38

mitogen-activated protein kinase inhibition in cardiac myocyte survival. *Circ Res* **93**:1026-1028.

FOOTNOTES

Reprint requests

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Figure Legends

Figure 1. Peritoneal adhesion formation in mice. (A) Intestines isolated from mice on day 7 after mechanical trauma. "Intact" intestine, scored as 0, was obtained from sham-operated mice and "moderate" (scored as 2) and "severe" (scored as 4) intestines were obtained from mice that underwent mechanical trauma. (B-D) Time course of peritoneal adhesion. Mechanical trauma in the mouse cecum was achieved by scraping with a swab. Adhesion formation was evaluated 1, 3, 7 and 12 days after the surgical procedure, according to the criteria described in the Materials & Methods. (B) adhesion score, (C) force required for detachment of adhesion, and (D) adhesion area. Mean ± S.E.M., n=10.

Figure 2. COX-1 and COX-2 expression in the traumatized cecum on day 7. Total RNA was extracted from the cecum and the mRNA expression level was examined by semi-quantitative RT-PCR. (A) Time course of expression levels of COX-1 and COX-2 mRNA in the traumatized cecum. Expression levels of COX-1 and COX-2 mRNA were studied by real-time RT-PCR and all data were normalized using mRNA levels on day 0. Open circles, COX-1; closed circles, COX-2. Mean ± S.E.M., n=5. ** $p < 0.01$ versus expression level on day 0. (B) Time course of expression levels of COX-1 and COX-2 proteins in the traumatized cecum. The expression levels of COX-1 and COX-2 were

quantified by Western blot analyses and all data were normalized using the respective protein levels on day 0. Open circles, COX-1; closed circles, COX-2. Mean ± S.E.M., n=5. * $p < 0.05$ and ** $p < 0.01$ versus expression level on day 0.

Figure 3. Effects of cyclooxygenase inhibitors on peritoneal adhesion formation in mice. Mechanical trauma was applied on day 0, and mofezolac (a selective COX-1 inhibitor) or CAY10404 and NS-398 (selective COX-2 inhibitors) was administered orally once daily for 7 days. Adhesion formation (A and B), tissue PGE_2 contents in the cecum (C) and PGE_2 concentration in the peritoneal fluid (D) were evaluated on day 7. Open circles, mofezolac; closed circles, CAY10404; open triangles, NS-398; open squares, vehicle control; closed squares, vehicle-treated sham-operated mice. Mean ± S.E.M., n=7. * $p < 0.05$, ** $p < 0.01$ versus vehicle control.

Figure 4. Effects of an EP receptor agonist and an EP receptor antagonist. (A) PGE_2 (0.5 mL of a 0.1 μM solution) or a PGE_2 analog, (R)-butaprost (0.5 mL of a 1 μM solution), was administered to mice given NS-398 (30 mg/kg/day). Peritoneal adhesion was evaluated on day 7. (B) EP receptor antagonists, AH6809 and SC-19920, were intraperitoneally administered (0.5 mL of a 5 μM solution) once daily for 7 days to mice that had undergone mechanical stimulation. Mean ± S.E.M., n=7. * $p <$

0.05, ** $p < 0.01$ versus vehicle control.

Figure 5. Effect of an antisense oligonucleotide for COX-2. An aqueous solution of the antisense oligonucleotide (AS) or a randomized control oligonucleotide (CO) was administered to mice that had undergone mechanical stimulation, and adhesion was evaluated on day 7. (A) Representative result from Western blot analysis of COX-2. V; vehicle-treated mice, AS; antisense oligonucleotide-treated mice, CO; randomized oligonucleotide-treated mice. (B) adhesion score, and (C) adhesion area. Mean ± S.E.M., n=5. ** $p < 0.01$ versus vehicle control.

Figure 6. Peritoneal adhesion in COX-2 knockout mice. COX-2-deficient mice (COX-2 KO) and wild-type littermates (WT) underwent mechanical stimulation, and PGE_2 (0.5 mL of a 0.1 μM solution) or vehicle was intraperitoneally administered for 7 days. Adhesion was evaluated on day 7. (A) adhesion score, and (B) adhesion area. Mean ± S.E.M., n=5. ** $p < 0.01$ versus vehicle control.

Figure 7. Effects of SB202190. The cecum was traumatized and SB202190, an inhibitor of p38 MAP kinase, was administered intraperitoneally once daily for 7 days. Adhesion score (A) and adhesion area (B) were determined on day 7. (C) PGE_2 contents in the cecum were determined by EIA, as

described in the Materials and Methods. (D) mRNA expression levels of COX-2 in the cecum . The expression levels were quantified by real-time PCR, and are represented as values relative to the values in sham-operated mice. Mean ± S.E.M., n=5. * $p < 0.05$, ** $p < 0.01$ versus vehicle group.

Figure 8. Activation of p38 MAP kinase in the traumatized cecum. The traumatized cecum was excised on days 1, 3 or 7 and the intact cecum was also excised from sham-operated mice on day 7. The extracts were prepared as described in the Materials and Methods. After immunoprecipitation with specific antibodies against p38 MAP kinase, the protein fractions obtained were subjected to SDS-PAGE and Western blotting was performed. (A) Representative results of Western blotting are shown in the inset. Upper; phosphorylated form of p38, lower; total p38. Lane S; intact cecum obtained from sham-operated mice, lanes 2-4; traumatized cecum on day 1, day 3 and day 7, respectively. The graph shows the relative band intensity of the phosphorylated form of p38 normalized against the total p38 band intensity. Open circles represent the intact cecum in sham-operated mice. Mean ± S.E.M., n=7. * $p < 0.05$, ** $p < 0.01$ versus sham-operated mice. (B) Representative results of a Western blot of SB202190-treated and vehicle-treated mice. Lane 1; sham-operated mice, lane 2; SB202190-treated mice, lane 3; vehicle-treated mice.

Figure 1

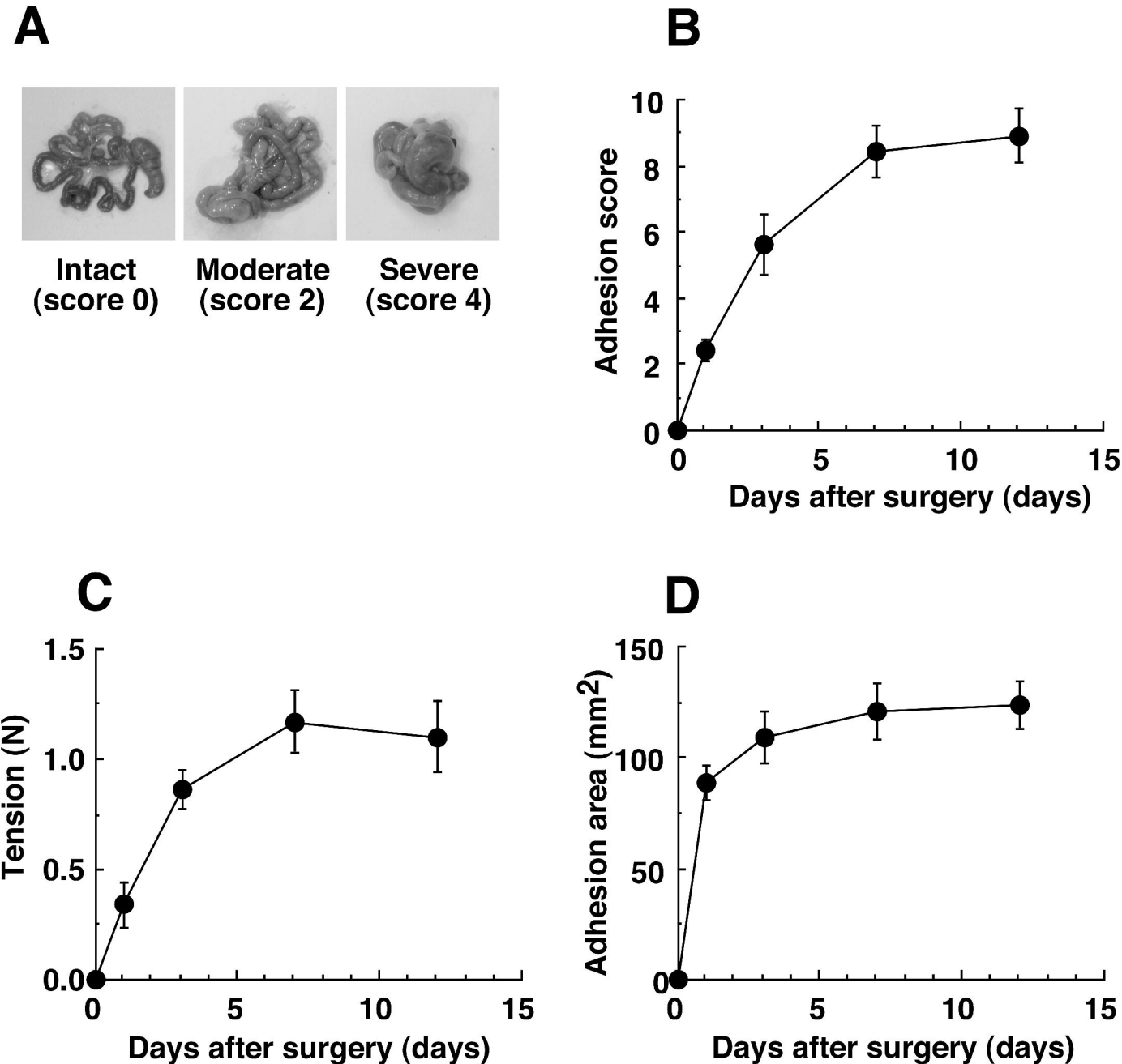
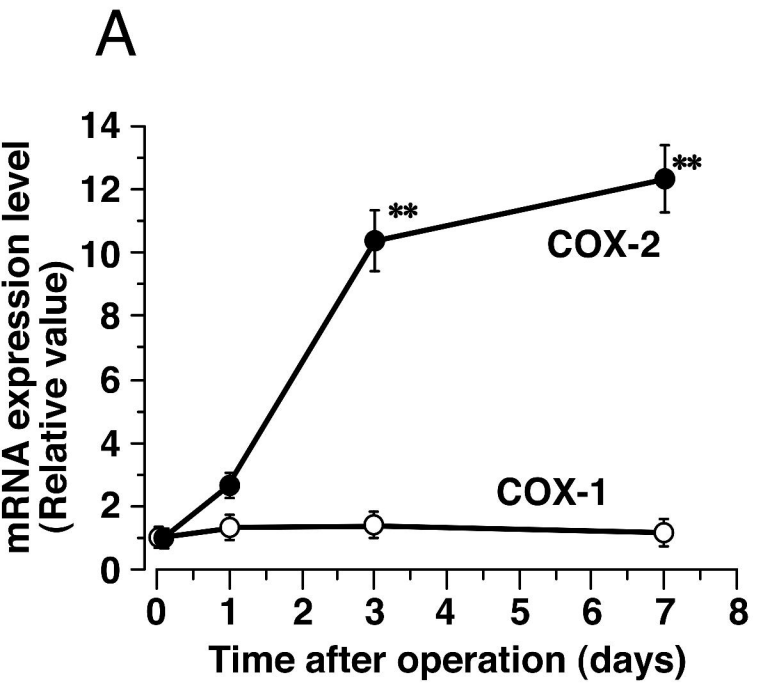

A
Intact
(score 0)
Moderate
(score 2)
Severe
(score 4)
B
Adhesion score
0
2
4
6
8
10
0
5
10
15
Days after surgery (days)
C
Tension (N)
0.0
0.5
1.0
1.5
0
5
10
15
Days after surgery (days)
D
Adhesion area (mm2)
0
50
100
150
0
5
10
15
Days after surgery (days)

Figure 2


A
mRNA expression level
(Relative value)
14
12
10
8
6
4
2
0
0 1 2 3 4 5 6 7 8
Time after operation (days)
**
**
COX-2
COX-1

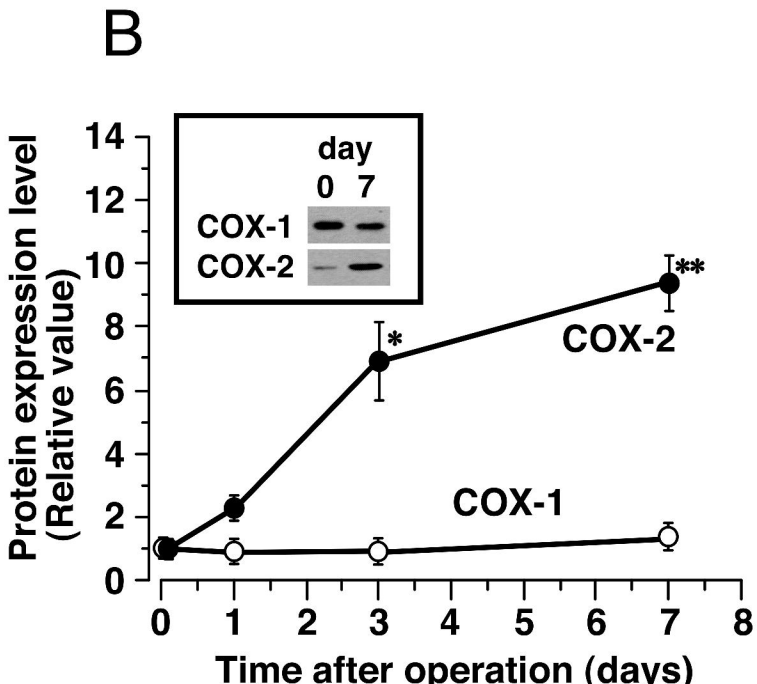

B
Protein expression level
(Relative value)
14
12
10
8
6
4
2
0
0 1 2 3 4 5 6 7 8
Time after operation (days)
day
0 7
COX-1
COX-2
*
**
COX-2
COX-1

Figure 3

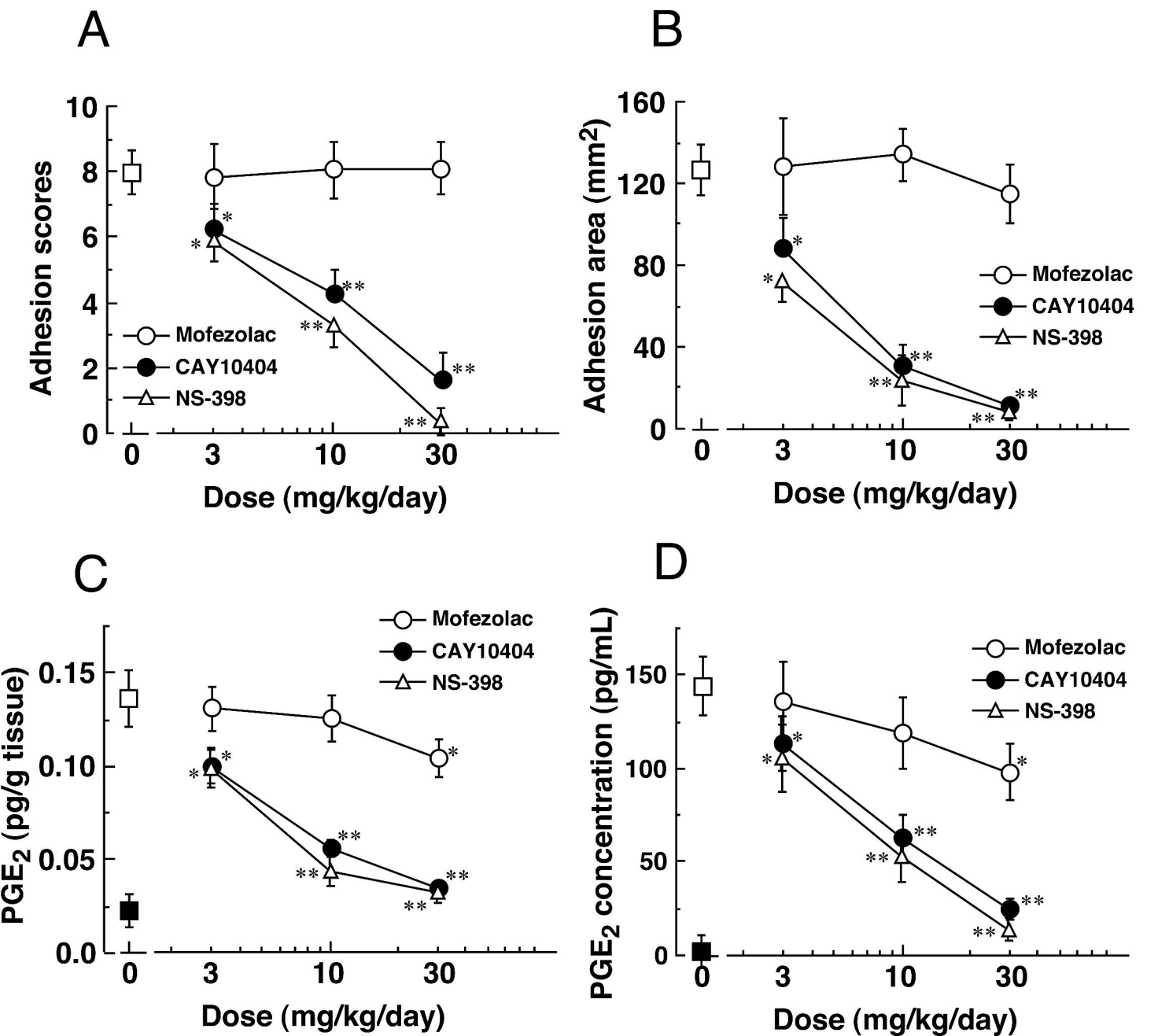

A
Adhesion scores
10
8
6
4
2
0
Mofezolac
CAY10404
NS-398
0
3
10
30
Dose (mg/kg/day)
B
Adhesion area (mm2)
160
120
80
40
0
Mofezolac
CAY10404
NS-398
0
3
10
30
Dose (mg/kg/day)
C
PGE2 (pg/g tissue)
0.15
0.10
0.05
0.0
Mofezolac
CAY10404
NS-398
0
3
10
30
Dose (mg/kg/day)
D
PGE2 concentration (pg/mL)
150
100
50
0
Mofezolac
CAY10404
NS-398
0
3
10
30
Dose (mg/kg/day)

Figure 4

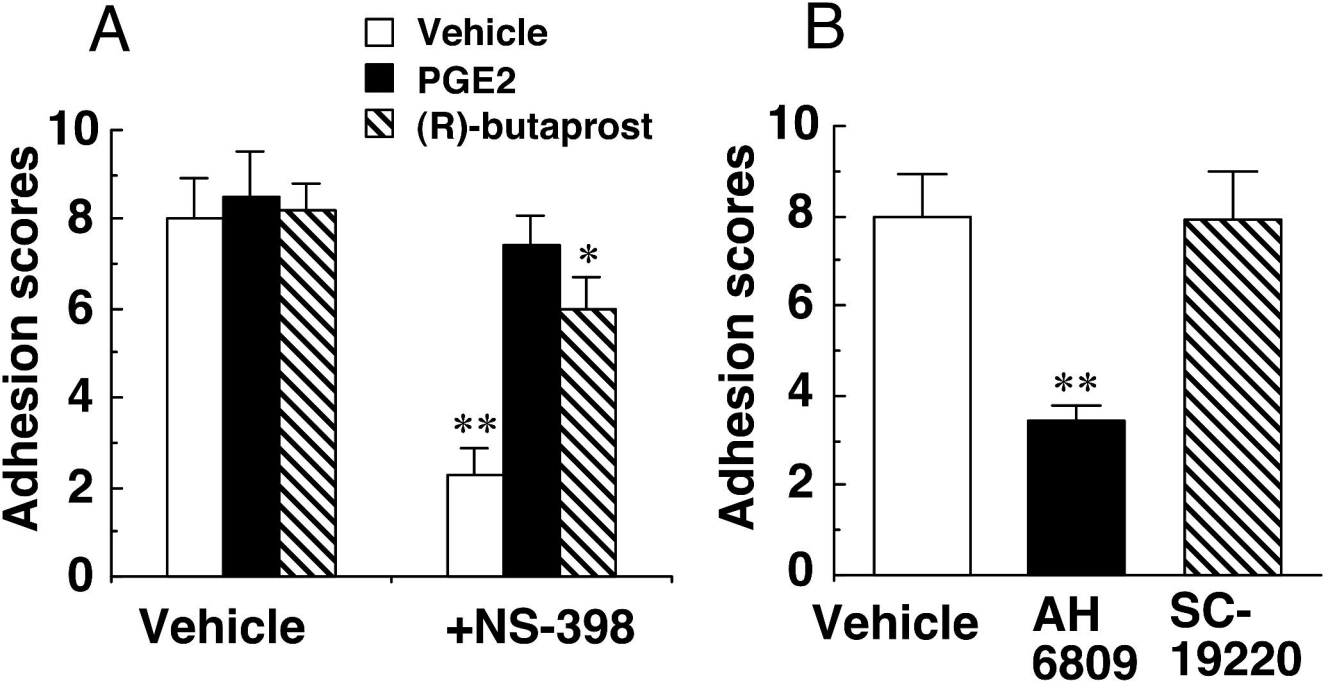
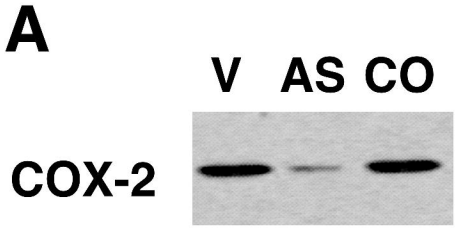
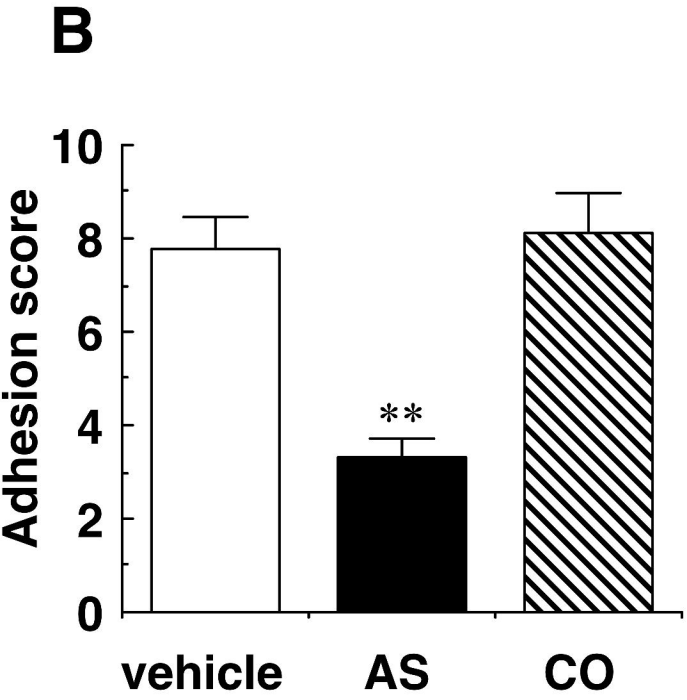

A
Vehicle
PGE2
(R)-butaprost
Adhesion scores
10
8
6
4
2
0
**
*
Vehicle
+NS-398
B
Adhesion scores
10
8
6
4
2
0
**
Vehicle
AH 6809
SC-19220

Figure 5


A
V AS CO
COX-2


B
Adhesion score
10
8
6
4
2
0
**
vehicle
AS
CO

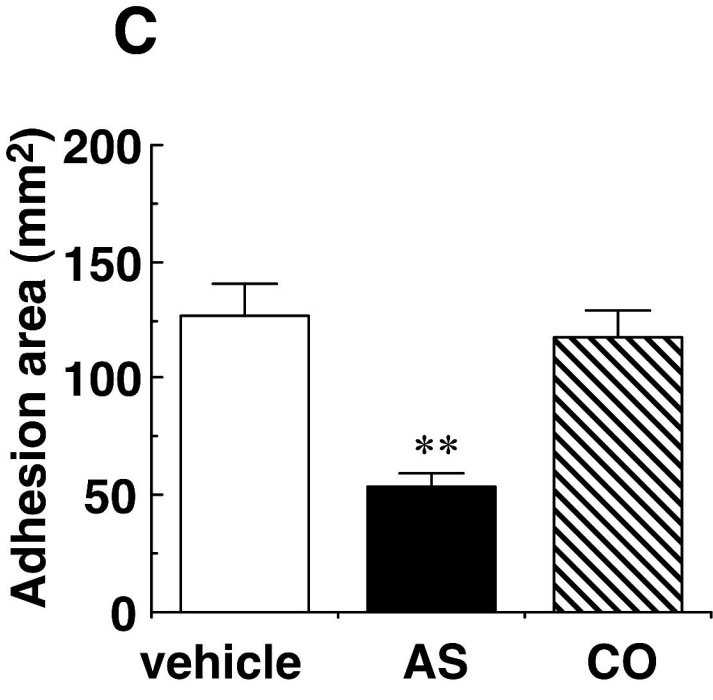
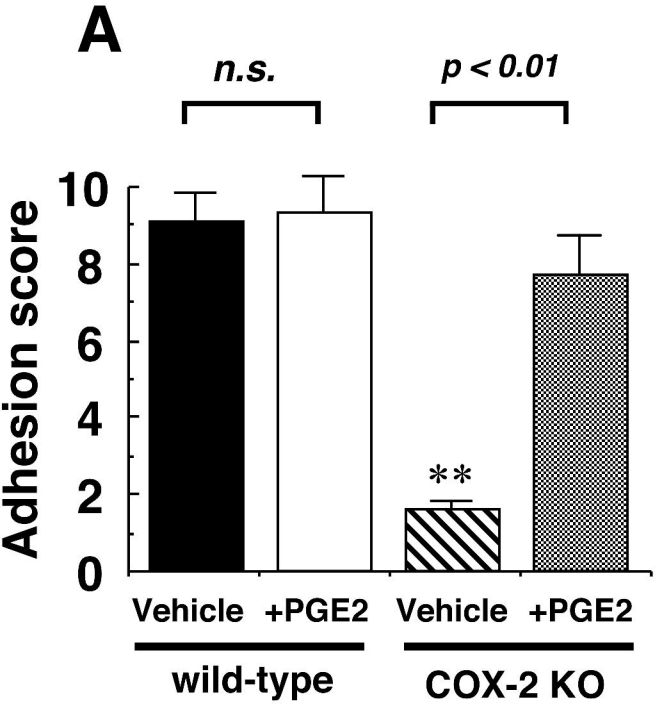

C
Adhesion area (mm2)
200
150
100
50
0
**
vehicle
AS
CO

Figure 6


A
n.s.
p < 0.01
Adhesion score
10
8
6
4
2
0
**
Vehicle
+PGE2
Vehicle
+PGE2
wild-type
COX-2 KO

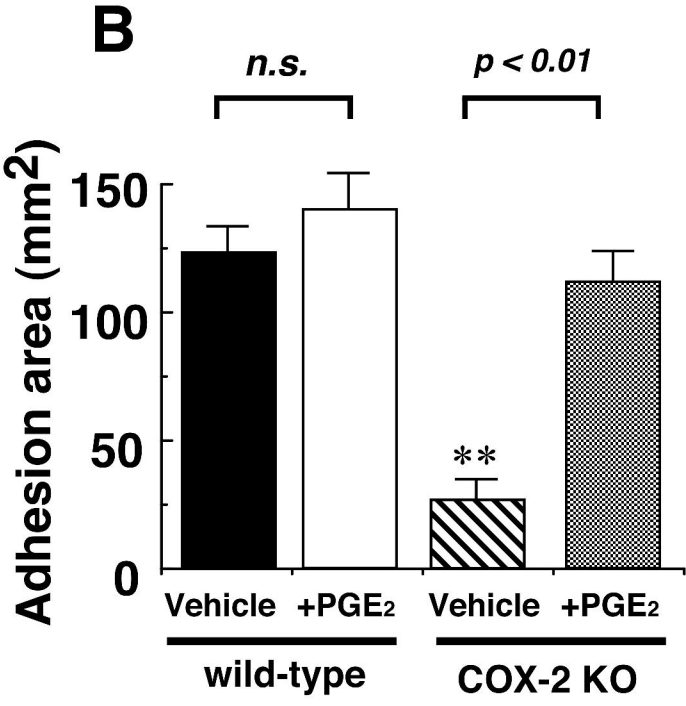

B
n.s.
p < 0.01
Adhesion area (mm2)
150
100
50
0
**
Vehicle
+PGE2
Vehicle
+PGE2
wild-type
COX-2 KO

Figure 7

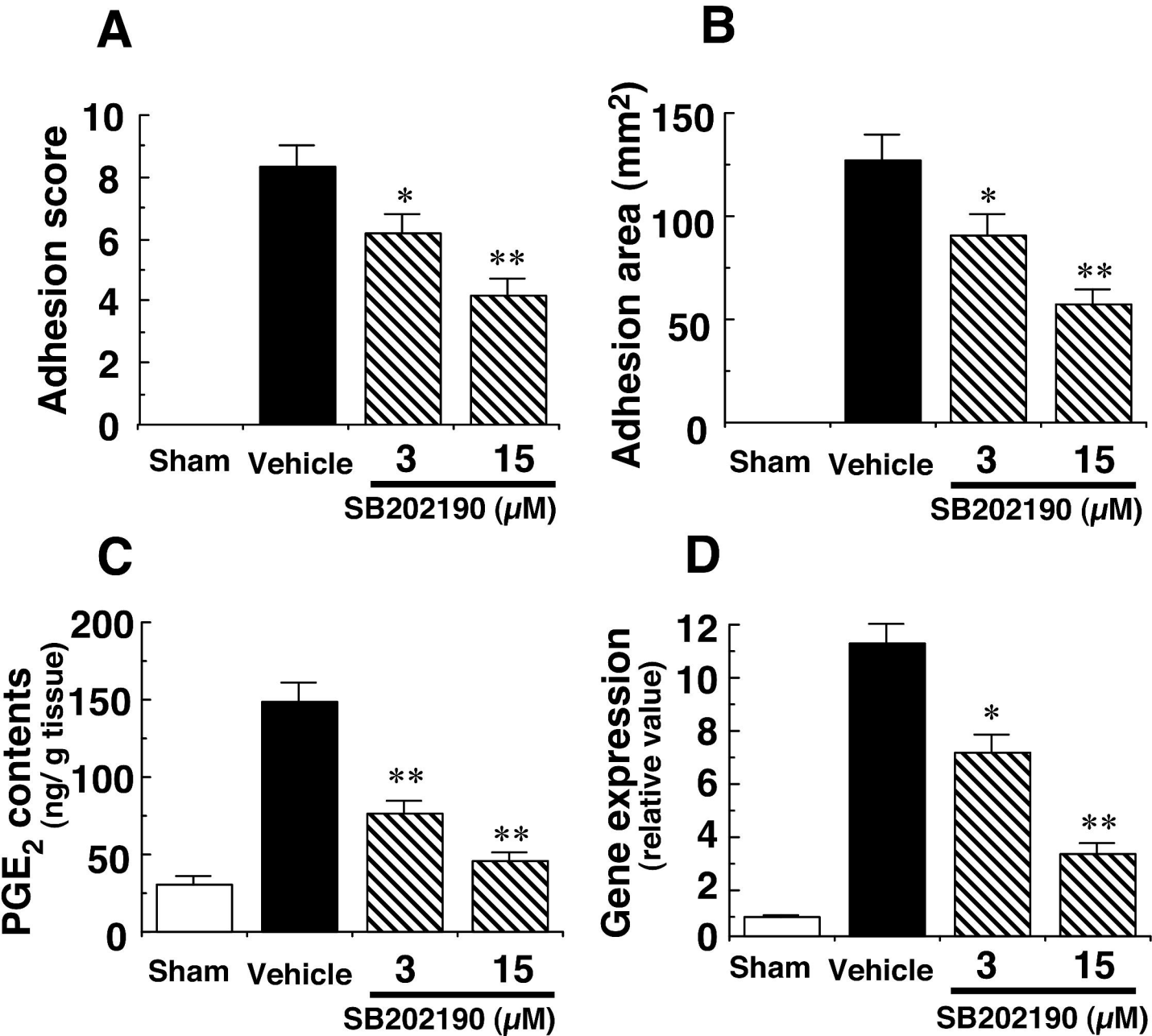
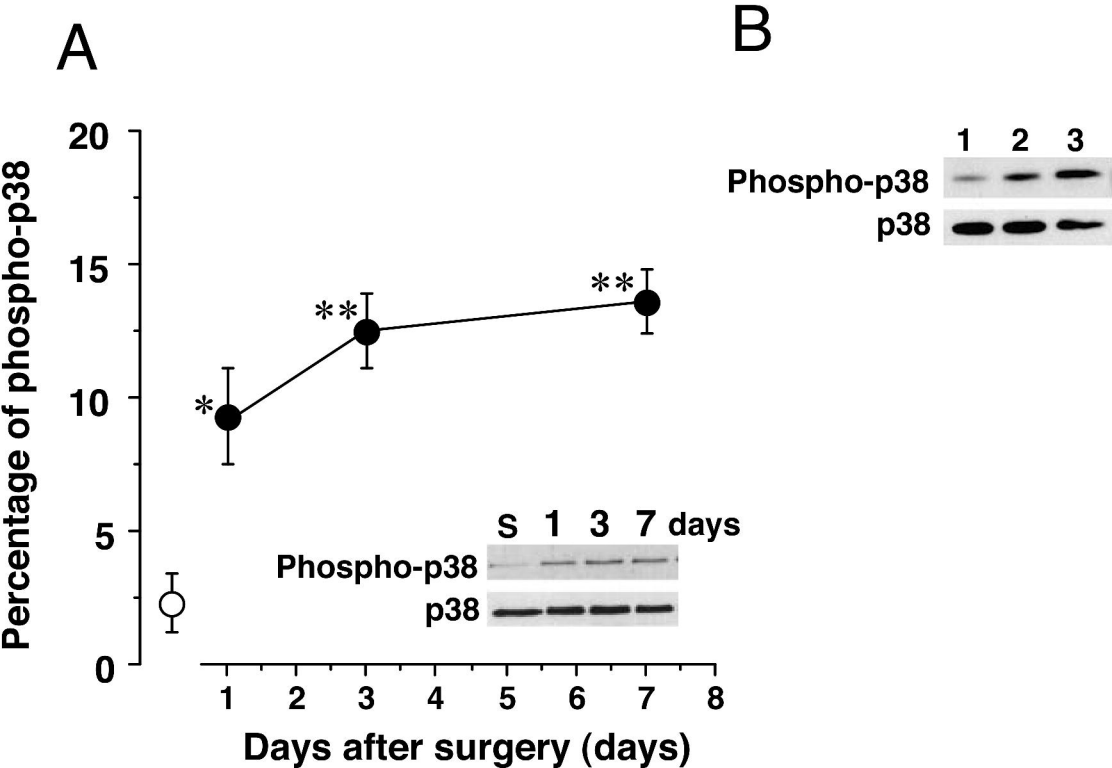

A
Adhesion score
10
8
6
4
2
0
*
**
Sham
Vehicle
3
15
SB202190 (μM)
B
Adhesion area (mm2)
150
100
50
0
*
**
Sham
Vehicle
3
15
SB202190 (μM)
C
PGE2 contents
(ng/ g tissue)
200
150
100
50
0
**
**
Sham
Vehicle
3
15
SB202190 (μM)
D
Gene expression
(relative value)
12
10
8
6
4
2
0
*
**
Sham
Vehicle
3
15
SB202190 (μM)

Figure 8


A
B
20
15
10
5
0
Percentage of phospho-p38
*
**
**
S 1 3 7 days
Phospho-p38
p38
1 2 3 4 5 6 7 8
Days after surgery (days)
1 2 3
Phospho-p38
p38