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Role of Thromboxane Derived From COX-1 and -2 in Hepatic Microcirculatory Dysfunction During Endotoxemia in Mice

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Although thromboxanes (TXs), whose synthesis is regulated by cyclooxygenase (COX), have been suggested to promote inflammation in the liver, little is known about the role of TXA_2 in leukocyte endothelial interaction during endotoxemia. The present study was conducted to investigate the role of TXA_2 as well as that of COX in lipopolysaccharide (LPS)-induced hepatic microcirculatory dysfunction in male C57Bl/6 mice. We observed during *in vivo* fluorescence microscopic study that LPS caused significant accumulation of leukocytes adhering to the hepatic microvessels and non-perfused sinusoids. Levels of serum alanine transaminase (ALT) and tumor necrosis factor alpha (TNFα) also increased. LPS raised the TXB_2 level in the perfusate from isolated perfused liver. A TXA_2 synthase inhibitor, OKY-046, and a TXA_2 receptor antagonist, S-1452, reduced LPS-induced hepatic microcirculatory dysfunction by inhibiting TNFα production. OKY-046 suppressed the expression of an intercellular adhesion molecule (ICAM)-1 in an LPS-treated liver. In thromboxane prostanoid receptor-knockout mice, hepatic responses to LPS were minimized in comparison with those in their wild-type counterparts. In addition, a selective COX-1 inhibitor, SC-560, a selective COX-2 inhibitor, NS-398, and indomethacin significantly attenuated hepatic responses to LPS including microcirculatory dysfunction and release of ALT and TNFα. The effects of the COX inhibitors on hepatic responses to LPS exhibited results similar to those obtained with TXA_2 synthase inhibitor, and TXA_2 receptor antagonist. In conclusion, these results suggest that TXA_2 is involved in LPS-induced hepatic microcirculatory dysfunction partly through the release of TNFα, and that TXA_2 derived from COX-1 and COX-2 could be responsible for the microcirculatory dysfunction during endotoxemia. (HEPATOLOGY 2004;39:139–150.)

The initial hepatic responses to lipopolysaccharide (LPS) include the activation of the nonparenchymal cells that constitute the hepatic microvascular system. The early events occurring in the hepatic microvasculature, including increases in leukocyte adhesion, reduction of sinusoidal perfusion, and activated Kupffer cells contribute to alterations in liver function caused by endotoxin.[1,2] However, the mechanisms by which LPS induces hepatic microcirculatory dysfunction are not fully understood.

Proinflammatory cytokines released from activated Kupffer cells including tumor necrosis factor alpha (TNFα)[3,4] are involved in the hepatic microvascular inflammatory response to LPS. Inactivation of Kupffer cells with gadolinium chloride ameliorates LPS-induced hepatic microcirculatory dysfunction, and this process was accompanied by a decrease in the plasma TNFα level.[2] The administration of TNFα causes a hepatic microcirculatory derangement similar to that produced by endotoxin.[5] In addition to cytokines, metabolites of arachidonic acid including prostaglandins (PGs) and thromboxanes (TXs) have been suggested to participate in liver injury during endotoxemia.[6] For example, a significant increase in the plasma level of TXB_2 (a stable metabolite of TXA_2) is shown after LPS administration.[7] The TXA_2 receptor antagonist exerts a protective effect on

Abbreviations: LPS, lipopolysaccharide; TNF, tumor necrosis factor; PG, prostaglandin; TX, thromboxane; COX, cyclooxygenase; TP, thromboxane-prostanoid; ALT, alanine aminotransferase; RT-PCR, reverse-transcription polymerase chain reaction; ICAM-1, intercellular adhesion molecule-1; PECAM-1, platelet-endothelial cell adhesion molecule-1; mRNA, messenger RNA.

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liver injury caused by endotoxin.[8] Furthermore, PGs and TXs modulate TNFα synthesis. PGE_2 suppresses TNFα production from Kupffer cells stimulated with endotoxin,[9] while TXA_2 synthase inhibitor suppresses TNFα release from peritoneal macrophages.[7] These results suggest that TXA_2 could augment leukocyte-endothelial interaction during endotoxemia by affecting the production of TNFα.

The synthesis of TXA_2 is regulated by cyclooxygenase (COX), which catalyzes the conversion of arachidonic acid to PGs and TXs. Two isoforms of the enzyme have been identified. The isoform designated COX-1 is constitutively expressed in most tissues, while the isoform designated COX-2 is inducible by a variety of factors, such as cytokines and endotoxin.[10] Indeed, the expression of COX-2 protein was induced in LPS-treated liver from rats.[11] The liver injury caused by LPS sensitized with galactosamine was minimized in COX-2 deficient mice.[12] Inhibition of COX-2 protects the liver[13] against injury from ischemia/reperfusion. These results indicate that COX-2 contributes to liver injury. However, much remains unknown about the involvement of 2 isoforms of COX (COX-1 and COX-2) in hepatic microcirculatory dysfunction during endotoxemia.

The present study was thus conducted to examine the effects of the inhibition of TXA_2 synthase and of the blockade of TXA_2 receptor on the hepatic microvascular response to LPS in mice using *in vivo* microscopic methods. Some of the experiments were performed with thromboxane prostanoid (TP)-receptor knockout mice to elucidate the role of endogenously produced TXA_2 in this response. We also investigated the effects of selective inhibition of COX-1 and COX-2 on LPS-induced hepatic microcirculatory dysfunction.

Materials and Methods

Drugs. Endotoxin (LPS from *Escherichia coli*, serotype 055:B5) was purchased from List Biological Laboratories (Campbell, CA). The selective TXA_2 synthase inhibitor OKY-046 ((E)-3-[p-(1H-imidazolmethyl)-phenyl]-2-propenoic acid) was supplied from Kissei Pharmaceutical (Osaka, Japan). The TXA_2 receptor antagonist S-1452 (14) (calcium (1R, 2S, 3S, 4S)-(5Z)-7(((phenylsulphonyl)-amino)-bicyclo-(2.2.1)-hept-2-yl) hept-5-heptenoate dihydrate) was supplied by Shionogi Pharmaceutical (Osaka, Japan). The selective COX-1 inhibitor SC-560 (15) (5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethyl-pyrazole) was obtained from Searle (St. Louis, MO). The selective COX-2 inhibitor NS-398 (16) (N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide) was obtained from Cayman Chemical (Ann Arbor, MI). The nonselective COX inhibitor indomethacin was purchased from Merck (Rahway, NJ). The NS-398, SC-560, indomethacin, and S-1452 were suspended in 5% gum arabic, while the OKY-046 was dissolved in physiological saline. Anti-mouse lymphocyte function associated antigen (LFA)-1 monoclonal antibody was purchased from Endogen (Woburn, MA).

Animals. Male C57BL/6 mice (6–8 weeks of age), weighing 20–25g, were obtained from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). TP receptor-knockout mice ($TP^{-/-}$, male, 8 weeks of age) were developed by us.[17] A genetic background of TP-receptor knockout mice is similar to that of C57BL/6 mice. They were maintained at a constant humidity (60 $\pm$ 5%) and temperature (25 $\pm$ 1 °C) and were kept continuously on a 12-hour light/dark cycle. All animals were provided food and water *ad libitum*. All procedures on animals were performed in accordance with the guidelines for animal experiments of Kitasato University School of Medicine.

Experimental Protocols for* In Vivo *Microscopic Study. LPS was injected intravenously (0.3 mg/kg in 0.1 ml of physiological saline) into mice through the tail vein. OKY-046 (50 mg/kg in 0.1 ml saline, intravenously), S-1452 (10 mg/kg, per os), SC-560 (10 mg/kg, per os), NS-398 (10 mg/kg, subcutaneous injection), indomethacin (10 mg/kg, per os), and vehicle (10 mg/kg, per os) were administered 30 min before LPS injection. Some animals were treated with 2 mg/kg (intravenously) of the LFA-1 monoclonal antibody simultaneously with LPS injection. The dose regimens of the specific inhibitors, including NS-398 and SC-560, used in the present study were based on their inhibitory effects on activity against COX-1 or COX-2.[15,16]

Preparation for* In Vivo *Microscopy. Four hours after LPS injection, animals anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally) were prepared for *in vivo* fluorescence microscopy according to modifications of methods previously described.[5] After transverse laparotomy, the animals were positioned on their left side and placed on a stage. The left lobe of the liver was pulled gently and covered with a thin cover glass to stabilize its position and limit movement induced by respiration. The hepatic microcirculation was observed at the surface of the liver using a fluorescence microscope (X2-UD, upright type; Nikon, Tokyo) with a 100-W mercury lamp for epi-illumination. The microscopic images were obtained with a long working distance objective lens (M plan 20/0.20 SLWD; Nikon, Tokyo) and a $\times$5 eyepiece lens. Images of the hepatic microcirculation were transmitted through a silicon-intensified target camera (C2400-08; Hamamatsu Photonics; Hamamatsu) to a TV monitor screen (PVM-1444Q; Sony, Tokyo) and

were recorded on videotape with an S-VHS recorder (BR-S600; Victor, Tokyo).

For contrast enhancement of the plasma, FITC-labeled dextran (Sigma, St. Louis, MO) was intravenously administered (4 mg/kg) just before the start of the observation. Leukocytes were labeled with 0.3 μmol/kg of rhodamine-6G (Sigma) at the same time.[18] FITC-dextran and rhodamine-6G were visualized by epi-illumination with filter combination of 420 to 490 nm/ $>$ 520 nm (excitation/emission) and 510 to 560 nm/ $>$ 590 nm, respectively.

Analysis of* In Vivo *Microscopy. To examine the interaction of leukocytes with endothelium, the number of leukocytes adhering was determined off-line during video playback analysis. A leukocyte was defined as adhering to the venular and sinusoidal walls if it remained stationary for more than 20 seconds. With respect to the leukocytes adhering to the venules, 5 to 8 portal or central venules per animal were observed and assessed. The endothelial surface area of each venule was measured from video recordings using an adjustable electric microscaler (Argus-10; Hamamatsu Photonics; Hamamatsu). We determined the adherence of leukocytes in terms of 1) numbers of adhering leukocytes in the sinusoids per microscopic field (X100), and 2) numbers of adhering leukocytes in the portal and central venules per 1000μm^2 of endothelial surface.

The sinusoidal perfusion deficits were evaluated by counting the number of non-perfused sinusoids in the same microscopic field as that in which the number of adhering leukocytes was determined. The percentage of non-perfused sinusoids was calculated as the ratio of the number of non-perfused sinusoids to the total number of all visible sinusoids. The results were expressed as the percentage of non-perfused sinusoids.

Sampling and Assays. In a separate set of experimental animals, blood was collected from the heart. The serum activity of alanine aminotransferase (ALT) was measured by an automated procedure with an analyzer. The plasma concentration of TNFα was measured with an enzyme-linked immunoabsorbent assay kit (Biosource International Inc, Camarillo, CA).

Experimental Procedure for Perfusion of the Isolated Liver. The isolated non-recirculating perfused liver system was prepared according to the method of Suematsu et al.[19] with some minor modification. In brief, mice were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally) and were heparinized (100 units/mouse) to avoid blood coagulation in the liver. After transverse laparotomy, the portal vein was cannulated with a 24-gauge catheter. Immediately after the cannulation, the liver was initially perfused with a sterile, hemoglobin- and albumin-free Krebs-Henseleit bicarbonate buffered solution (pH 7.4, 37 °C) gassed with carbogen (95% O_2, 5% CO_2). After the start of perfusion of the liver, the inferior vena cava was cut, and the liver was removed. The perfusate was pumped through the isolated liver at a constant flow rate (4.0 ml/min) while the portal perfusion pressure was monitored and maintained at a level of 2 to 4 cmH_2O. Before collection of the first sample, the isolated liver was perfused for 20 min to eliminate blood elements and to stabilize it. Samples of the hepatic effluent emerging from the inferior vena cava were collected every 5 min to determine the amount of TXB_2. The levels of TXB_2 were measured with separate enzyme-linked immunoabsorbent assays (Cayman Chemical) as described previously.[20,21]

Experimental Protocols for Perfusion Experiment. Four experimental groups of animal livers were set up to investigate whether the liver is a site of TXA_2 production. In the 1st group, the livers of wild-type mice perfused with buffer solution throughout the experiment served as controls. In the 2nd and 3rd groups, 30 min after the start of perfusion, the administration of LPS (1.25 μg/min for 20 min) was initiated to TP-receptor knockout mice and to their wild-type counterparts and was continued throughout the experimental period. In the 4th group, 30 min before the start of preparation for the perfusion experiment, wild-type mice were treated with OKY-046 (50 mg/kg, intravenously), and OKY-046 (0.05 μg/min for 50 min) was administered simultaneously with the start of the perfusion with buffer. At 30 min after the start of perfusion, LPS (1.25 μg/min for 20 min) was continuously infused until the end of the experimental period.

Reverse-Transcription Polymerase Chain Reaction Analysis. Four hours after LPS injection, approximately 100 mg of the liver tissue was excised and was homogenized in 1 ml of Trizol Reagent (GIBCO BRL, Rockville, MD) with Polytrone (Kinematica GmbH, Luzern, Switzerland). A sample of RNA was extracted from the tissue in accordance with the manufacturer's instructions. Single-stranded complementary DNA was synthesized from 250 ng of total RNA using 0.4 μg of oligo-p(dT)15 primer and 4 units of AMV reverse transcriptase (Roche Diagnostics, Basel, Switzerland). PCR was performed in 10 μl of 20 mM Tris-HCl (pH 8.7) and 0.5 unit of Taq DNA polymerase (Qiagen GmbH, Germany). The oligonucleotide primers used were for reverse-transcription polymerase chain reaction (RT-PCR) analysis as follows: for mouse COX-1, 5′-TTGCACAACACTTCACCCACCAG–3′ (sense), 5′-AAACACCTCCTGGCCCACAGCCAT–3′ (antisense) (276 bp); mouse COX-2, 5′-GGAGAGAAGGAAATGGCTGCA–3′ (sense), 5′-ATCTAGTCTGGAGTGGGAGG–3′ (antisense) (363

bp); intercellular adhesion molecule (ICAM)-1, 5′-CGGGATCCAGGAAAGCCAAGGCCAAA-3′ (sense) and5′-CGGAATTCTTGACTGTCTTAAGTTCC-3′ (antisense) (326 bp); platelet-endothelial cell adhesion molecule (PECAM)-1, 5′-CGGGATCCAGGAAAGCCAAGGCCAAA-3′ (sense) and 5′-CGGAATTCTTGACTGTCTTAAGTTCC-3′ (antisense) (348 bp); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-CCCTTATTGACCTCAACTACATGGT-3′ (sense) and 5′-GAGGGGCCATCCACAGTCTTCTG-3′ (antisense) (470 bp). These PCRs were run for 40 cycles. Cycling conditions were: 94 °C, 30 sec; 54 °C, 45 sec; and 72 °C, 45 sec for COX-1, ICAM-1, PECAM-1 and GAPDH, followed by a final extension for 10 min at 72 °C. For the amplification of COX-2, annealing was performed at 50 °C for 45 sec. Products of PCR were analyzed using 1.8% agarose gel electrophoresis, and the size of products was predicted from the sequences.

Immunohistochemical Studies. Immunohistochemical studies were performed as described elsewhere[22]. The sections from the paraffin-embedded tissues were incubated with rabbit anti-murine COX-1 or COX-2 antiserum (1:200 dilution, Cayman), or with rat anti-murine ICAM-1 antibody (1:100 dilution, 1A29, Biotechnology, Oxford) or rat anti-murine PECAM-1 antibody (1:100 dilution, MEC 13.3, PharMingen) at 4 °C overnight. These immunoreactivities were visualized using avidin-biotin-peroxydase complex (Vectastain ABC Kit, Vector Lab., Burlingame, CA).

Statistical Analysis. All data were expressed as means ± SEM. Multiple comparisons were carried out using 1-way ANOVA followed by Fisher's test. Differences were considered to be significant for P values less than .05.

Results

Figure 1 shows the *in vivo* fluorescence micrographs of hepatic microvasculature 4 hours after LPS injection. Leukocytes were seen adhering to the sinusoids as well as to the central venules (Fig. 1A). The sinusoidal perfusion deficits were also observed (Fig. 1B). In an additional group of experiments, we observed that the mean systemic arterial blood pressure 4 hours after LPS injection (109.3 ± 5.4 mmHg) did not change significantly when compared with that before LPS treatment (107.5 ± 5.6 mmHg) (n = 4).

The administration of LPS caused significant increases in the numbers of leukocytes adhering to the portal venules (8.5-fold) (Fig. 2A), sinusoids (50.2-fold) (Fig. 2B), and central venules (51.0-fold) (Fig. 2C) in comparison with those in saline-treated mice. Pre-treatment with OKY-046 lowered those by 61%, 46%, and 45.0%, respectively. Pre-treatment with S-1452 also suppressed those by 69%, 48%, and 39.0%, respectively. Concomitantly, the percentage of non-perfused sinusoids after LPS injection was increased (7.1-fold) (Fig. 2D). The percentage of non-perfused sinusoids was significantly lowered by OKY-046 (by 61.0%) and S-1452 (by 47.0%), respectively.

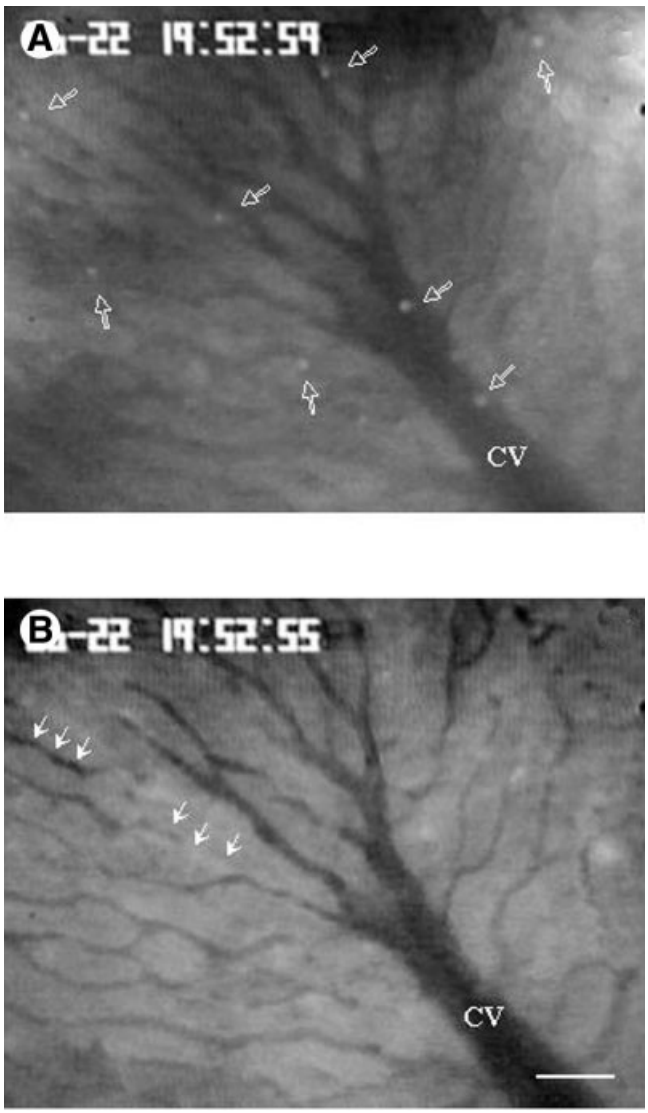


Fig. 1. Representative *in vivo* fluorescence micrographs of the hepatic microcirculation 4 hours after LPS injection. (A) Numerous leukocytes (**open arrows**), which were labeled with rhodamine 6G, adhere to the sinusoids and to the central venules. (B) The sinusoids and hepatic venules were well visualized by the injection of FITC-dextran. The sinusoidal perfusion deficits were detected by direct observation of the microcirculation as evidenced by the cessation of the perfusion (**arrows**) and by the non-visualization of the sinusoids (**arrowheads**). The fluorograph in Fig. 1B shows the same microscopic field as that in Fig. 1A. Bar represents 50 μm.

OKY-046 decreased the levels of plasma ALT activity at 4 hours after and the levels of plasma TNFα at 1 hour after LPS injection by 22% and 31%, respectively.

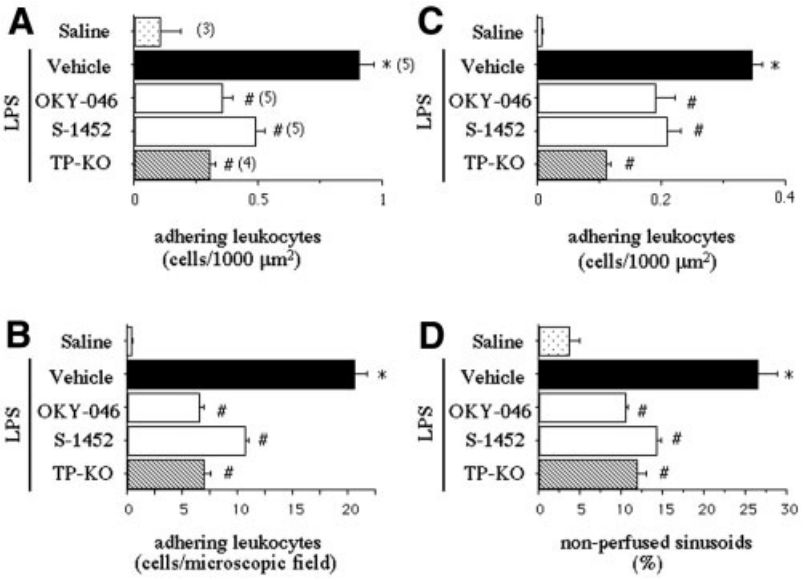


Fig. 2. Effects of LPS on (A) the numbers of leukocytes adhering to the portal venules, (B) the numbers of leukocytes adhering to the sinusoids, (C) the numbers of leukocytes adhering to the central venules, and (D) the percentage of non-perfused sinusoids, all in TP receptor-knockout (KO) mice and in their wild-type counterparts treated with OKY-046 (50 mg/kg, intravenously) and S-1452 (10 mg/kg, p.o.). Numbers in parentheses indicate number of animals. Data are shown as means ± SEM. *$P < .05$ versus Saline-treated mice; #$P < .05$ versus LPS-treated mice.

S-1452 also reduced them by 17% and 40%, respectively (Fig. 3).

To further investigate whether LPS-induced hepatic microcirculatory dysfunction is mediated by endogenously produced TXs, we used TP receptor-knockout mice. As shown in Fig. 2, in wild-type counterparts, LPS caused significant hepatic microcirculatory dysfunction as described above (Figs. 2A–D). In TP receptor-knockout mice, the numbers of leukocytes adhering to the portal venules, sinusoids, and central venules were significantly lower than in wild-type counterparts. Also the percentage of non-perfused sinusoids was lower in TP receptor-knockout mice than in wild-type mice. The levels of ALT and TNFα after LPS administration in TP receptor-knockout mice were decreased by 18% and 28%, respectively (Fig. 3).

Figure 4 illustrates changes in the levels of TXB_2 in the effluent perfusate from isolated perfused liver. The perfusion experiments were performed to determine whether there was a release site of TXA_2 by liver cells. In controls, no significant change in TXB_2 levels appeared (Fig. 4A). Within 15 min of the start of LPS administration, TXB_2 levels were rapidly increased in comparison with the baseline, and then continued to increase (Fig. 4B). Treatment of wild-type mice with OKY-046 completely abolished the increment of TXB_2 in response to LPS (Fig. 4C). In TP receptor-knockout mice, changes in TXB_2 levels after LPS were similar to those in the wild-type counterparts (Fig. 4D). During the perfusion experiment, the perfusion pressure was stable (2~4 cmH_2O) at all time points.

To investigate whether attenuation of hepatic microcirculatory dysfunction by TXA_2 inhibition affected the expression of adhesion molecules, the expression of ICAM-1 and PECAM-1 in the liver was assessed by RT-PCR and by immunohistochemistry (Figs. 5 and 7). LPS resulted in enhanced hepatic expression of messenger RNA (mRNA) of ICAM-1 when compared with that in saline-treated mice (Fig. 5A). OKY-046 partially prevented LPS-induce increase in ICAM-1 mRNA expression. The reduction in ICAM-1 mRNA expression was also seen in TP receptor-knockout mice.

The immunoreactivity with ICAM-1 was demonstrated in the sinusoids of saline-treated mice (Fig. 5B). LPS upregulated ICAM-1 expression in the sinusoids, as well as the hepatic venules (Fig. 5C), and OKY-046 reduced the ICAM-1 immunoreactivity (Fig. 5D).

To examine the significant contribution of ICAM-1 to leukocyte adhesion to the hepatic microvessels, mice were treated with monoclonal antibody against LFA-1, which is a ligand of ICAM-1. As shown in Fig. 6, the anti-LFA-1

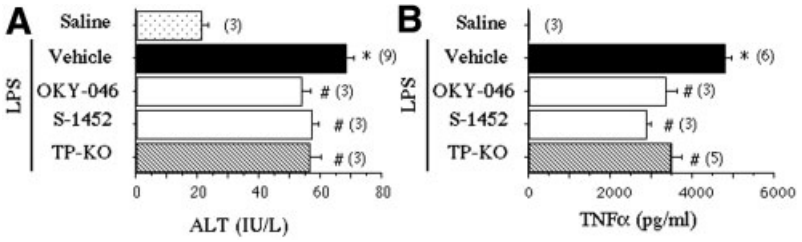


Fig. 3. Effects of LPS on (A) serum ALT activity and (B) the serum concentrations of TNFα in TP receptor-knockout (KO) mice and in their wild-type counterparts treated with OKY-046 (50 mg/kg, intravenously) and S-1452 (10 mg/kg, p.o.). Numbers in parentheses indicate number of animals. Data are shown as means ± SEM. *$P < .05$ versus Saline-treated mice; # $P < .05$ versus LPS-treated mice.

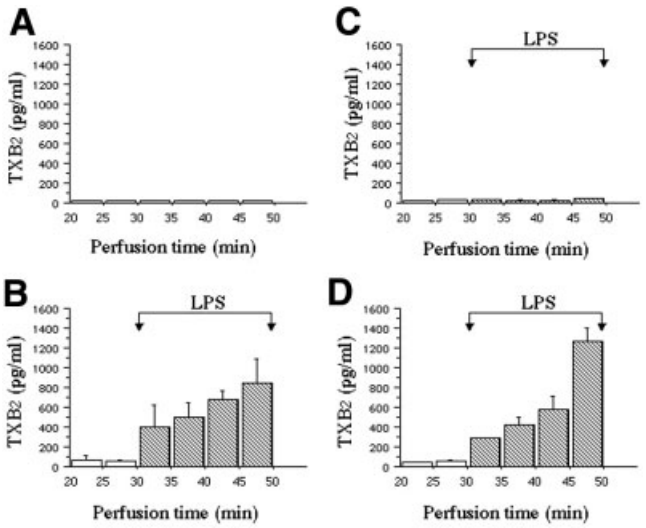


Fig. 4. Changes in the levels of TXB_2 in the effluent perfusate from the isolated perfused livers of wild-type mice (A,B,C) and of TP receptor-knockout mice (D). Livers from LPS-treated wild-type mice (B) and of TP receptor-knockout mice (D). Livers perfused with KH buffer solution alone throughout the experiment served as controls (A). Livers from wild-type mice treated with a combination of OKY-046 (0.05 μg/min for 50 min) and LPS (1.25 μg/min for 20 min) (C). Data are shown as means $\pm$ SEM from 3 animals.

antibody minimized hepatic microcirculatory dysfunction in response to LPS.

LPS resulted in slightly enhanced hepatic expression of mRNA of PECAM-1 when compared with that in saline-treated mice, and OKY-046 slightly reduced the expression of PECAM-1 in response to LPS (Fig. 7A). In results of immunohistochemical study, PECAM-1 was demonstrated in saline-treated mice liver (Fig. 7B). LPS enhanced expression of PECAM-1 weakly in comparison with that of ICAM-1 (Fig. 7C). OKY-046 did not affect PECAM-1 expression induced by LPS (Fig. 7D).

Because our results indicate that the hepatic microcirculatory dysfunction is mediated by TXA_2, and because TXA_2 is regulated by COX, we detected the expression of mRNA for COXs using RT-PCR. As shown in Fig. 8, in wild-type counterparts, COX-1 mRNA was expressed in both saline- and LPS-treated livers, whereas the expression of COX-2 mRNA was observed in an LPS-treated liver, but not in a saline-treated liver. The same was true for the livers of TP receptor-knockout mice.

The immunoreactivity with COX-1 in the saline-treated liver (Fig. 9A) and LPS-treated liver (Fig. 9B) was

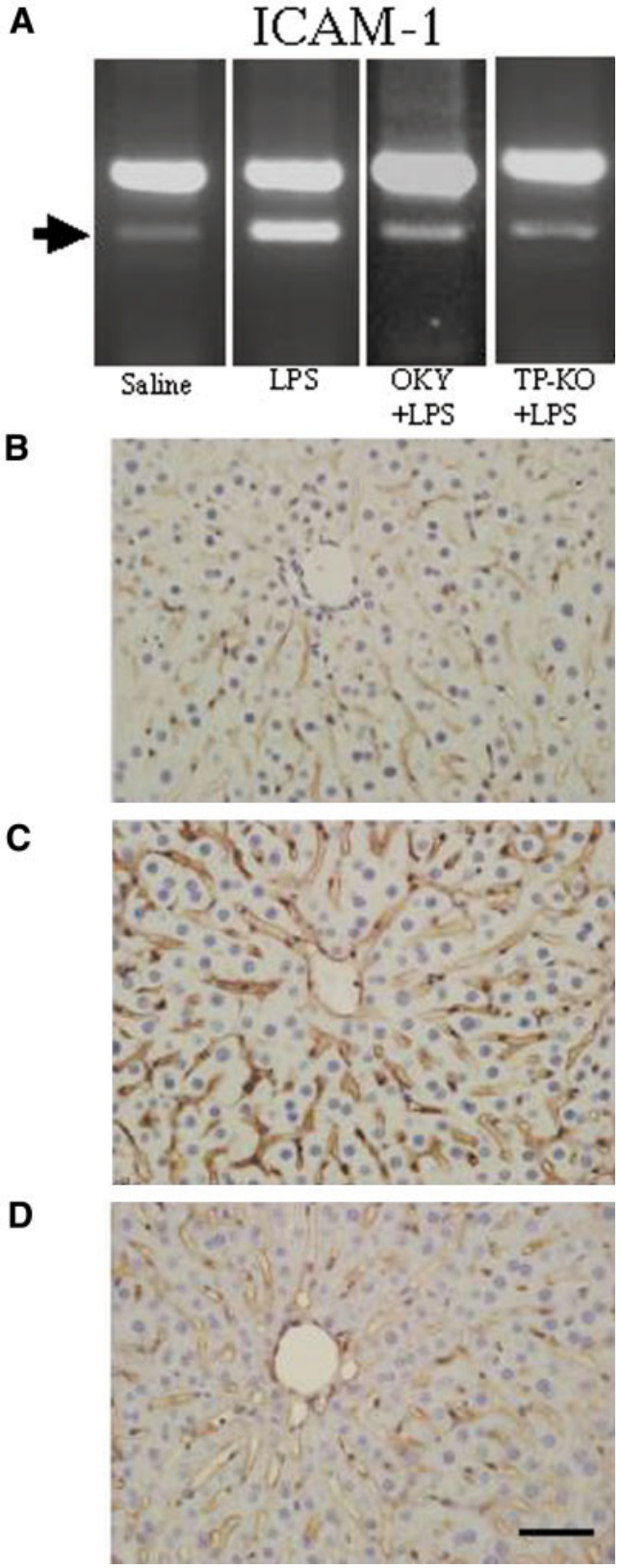


Fig. 5. Hepatic expression of ICAM-1 by RT-PCR analysis (A) and by immunohistochemical staining (B,C,D) 4 hours after LPS administration. LPS enhanced the expression of ICAM-1 mRNA in the liver (A). The hepatic expression of ICAM-1 mRNA after LPS was reduced in mice treated with OKY-046 and in TP- receptor-knockout mice. Immunoreactive ICAM-1 was seen in the sinusoidal lining cells in saline-treated mice (B). LPS enhanced ICAM-1 expression (C), and OKY-046 reduced the expression of ICAM-1 in LPS-treated liver (D). One representative experiment of 2 animals was presented. Bar indicates 50 μm.

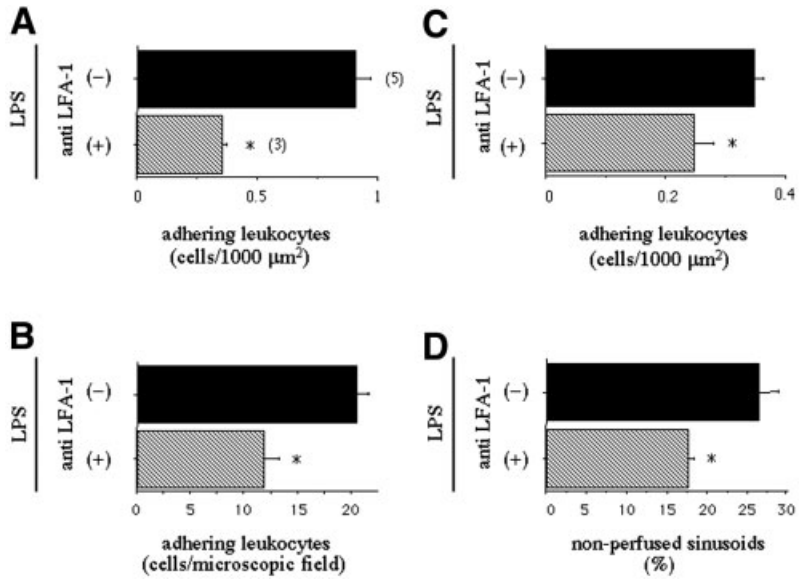


Fig. 6. Effects of an anti-LFA-1 antibody on (A) the numbers of leukocytes adhering to the portal venules, (B) the numbers of leukocytes adhering to the sinusoids, (C) the numbers of leukocytes adhering to the central venules, and (D) the percentage of non-perfused sinusoids at 4 hours after LPS administration. Numbers in parentheses indicate number of animals. Data are shown as means ± SEM. $*P < .05$ versus LPS-treated mice.

detected on the surface of hepatic microvessels. In contrast, the staining of COX-2 was negative in the saline-treated liver (Fig. 9C), however, the immunoreactive COX-2 in the LPS-treated liver was observed on the surface of the sinusoidal lining cells and on the hepatic venules. The most intense staining was shown in the sinusoids (Fig. 9D).

To ascertain which COX isozymes contribute to LPS–induced hepatic microcirculatory dysfunction, mice were pre-treated with SC-560, NS-398, and indomethacin. SC-560, NS-398, and indomethacin significantly lowered the numbers of leukocytes adhering to the portal venules (Fig. 10A), sinusoids (Fig. 10B), and central venules (Fig. 10C), respectively. In sinusoids, indomethacin lowered the numbers of adhered leukocytes more than SC-560 or NS-398 (Fig. 10B). The percentages of non-perfused sinusoids after LPS administration also were lower in mice treated with SC-560, NS-398, or indomethacin than in mice treated with vehicle (Fig. 10D). SC-560, NS-398, and indomethacin themselves did not significantly change leukocyte adhesion and sinusoidal perfusion in saline-treated mice (data not shown).

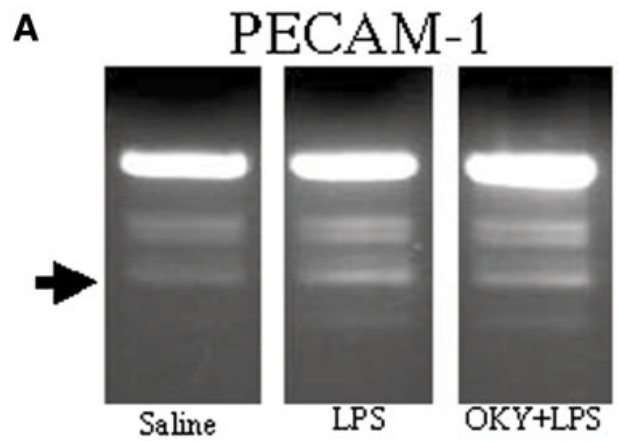
Four hours after LPS injection, the levels of ALT were significantly increased (3.2-fold) in comparison with the saline-treated controls (Fig. 11A). SC-560, NS-398, and indomethacin significantly decreased ALT levels after LPS injection by 35.0%, 42.0%, and 42.0%, respectively. The levels of TNFα 1 hour after LPS injection were significantly increased (Fig. 11B). SC-560, NS-398, and indomethacin significantly decreased TNFα levels, by 49.0%, 30.0%, and 33.0%, respectively.

Discussion

The results of the present study showed that OKY-046, a TXA_2 synthase inhibitor and S-1452, a TXA_2 receptor antagonist attenuated LPS-induced hepatic microcirculatory dysfunction as indicated by an increase of leukocytes adhering to the hepatic microvessels, as well as by impaired sinusoidal perfusion. To rule out the possibility that LPS affects the systemic hemodynamics, we measured the arterial blood pressure. The administration of LPS at a dose of 0.3 mg/kg (intravenously) did not reduce the mean arterial blood pressure. The hepatic microcirculatory dysfunction in response to LPS was accompanied by decreases in the serum levels of ALT and TNFα. These results suggest that TXA_2 enhances hepatic microcirculatory dysfunction during endotoxemia. This possibility was supported by our finding that TP receptor-knockout mice minimized liver injury and hepatic microcirculatory dysfunction in response to LPS by inhibiting TNFα production.

The finding that TXA_2 appears to modulate TNFα production after LPS administration (Fig. 3) is consistent with the findings of others,[7,27,28] that TXA_2 synthase inhibitor and TXA_2 receptor antagonist suppress LPS-induced TNFα release from macrophages[7,27] and from the perfused heart.[28] The inhibitory effects of OKY-046 and S-1452 on TNFα production in response to LPS were partial (30–40% reduction), while OKY-046 completely inhibited TXB_2 release in perfusate from the LPS-treated liver. These results suggest that other factors may be involved in the regulation of TNFα generation.

We observed that OKY-046 prevented the LPS-induced increase in ICAM-1 expression in the intrahepatic



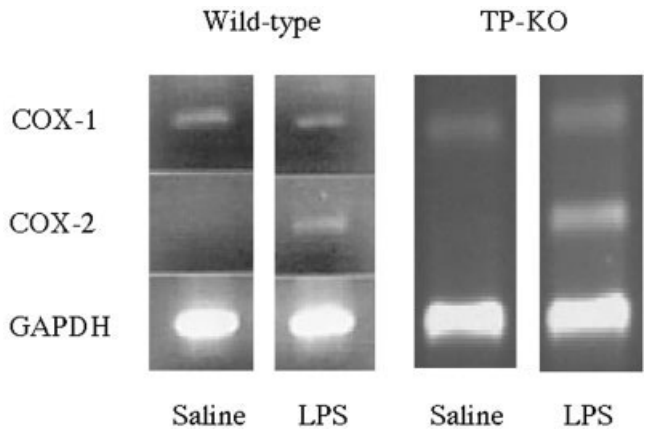
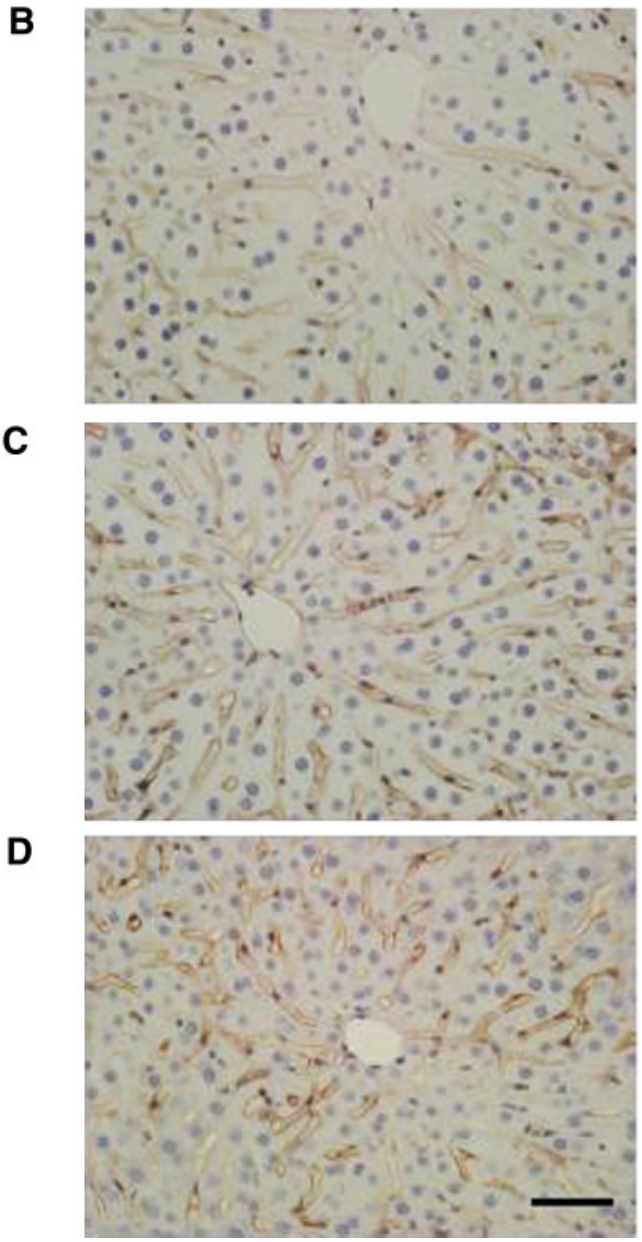


Fig. 8. Expression of COXs in liver tissue by RT-PCR analysis. Liver tissue was excised 4 hours after saline or LPS administration in wild-type and TP receptor-knockout (KO) mice. One representative experiment of 2 animals were presented.



circulation, and that TP-receptor knockout mice exhibited decreased expression of ICAM-1 in the LPS-treated liver (Fig. 5). It has been reported that TXA_2 receptor antagonist suppresses the expression of ICAM-1 on human umbilical vein endothelial cells.[29] These findings suggest that increased ICAM-1 expression mediated by TP-receptor signaling may have facilitated leukocyte adhesion in the liver during endotoxemia. However, it is well known that TNFα released from Kupffer cells in response to LPS induces the transcriptional activation of ICAM-1 expression on the hepatic microvessels independently through TXA_2 signaling,[30] and that TNFα is the central mediator responsible for upregulation of hepatic ICAM-1 expression in response to hepatotoxicants such as carbon tetrachloride[31] and endotoxin.[32]

Enhanced ICAM-1 expression in the liver has been shown to contribute to leukocyte adhesion to the hepatic venules as well as to the sinusoids.[30] Our findings that the anti-LFA-1 antibody attenuated leukocyte adhesion indicates that ICAM-1 is involved in leukocyte adhesion in the endotoxemic liver. These results also suggest that attenuation of hepatic microcirculatory dysfunction is attributable to the reduction of ICAM-1 expression by

Fig. 7. Expression and immunoreactivity of PECAM-1 in liver tissue by RT-PCR analysis (A) and immunohistochemical staining (B,C,D) in liver tissue from mice treated with saline, LPS, and OKY-046/LPS. LPS resulted in slightly enhanced hepatic expression of mRNA of PECAM-1 when compared with that in saline-treated mice, and OKY-046 slightly reduced the expression of PECAM-1 in response to LPS (Fig. 7A). The immunoreactivity with PECAM-1 in saline-treated liver was weak (B), and was slightly enhanced in LPS-treated liver (C). OKY-046 did not change significantly the expression of PECAM-1 in LPS-treated liver (D). Liver tissue was excised 4 hours after LPS administration. One representative experiment of 2 animals was presented. Bar indicates 50 μm.

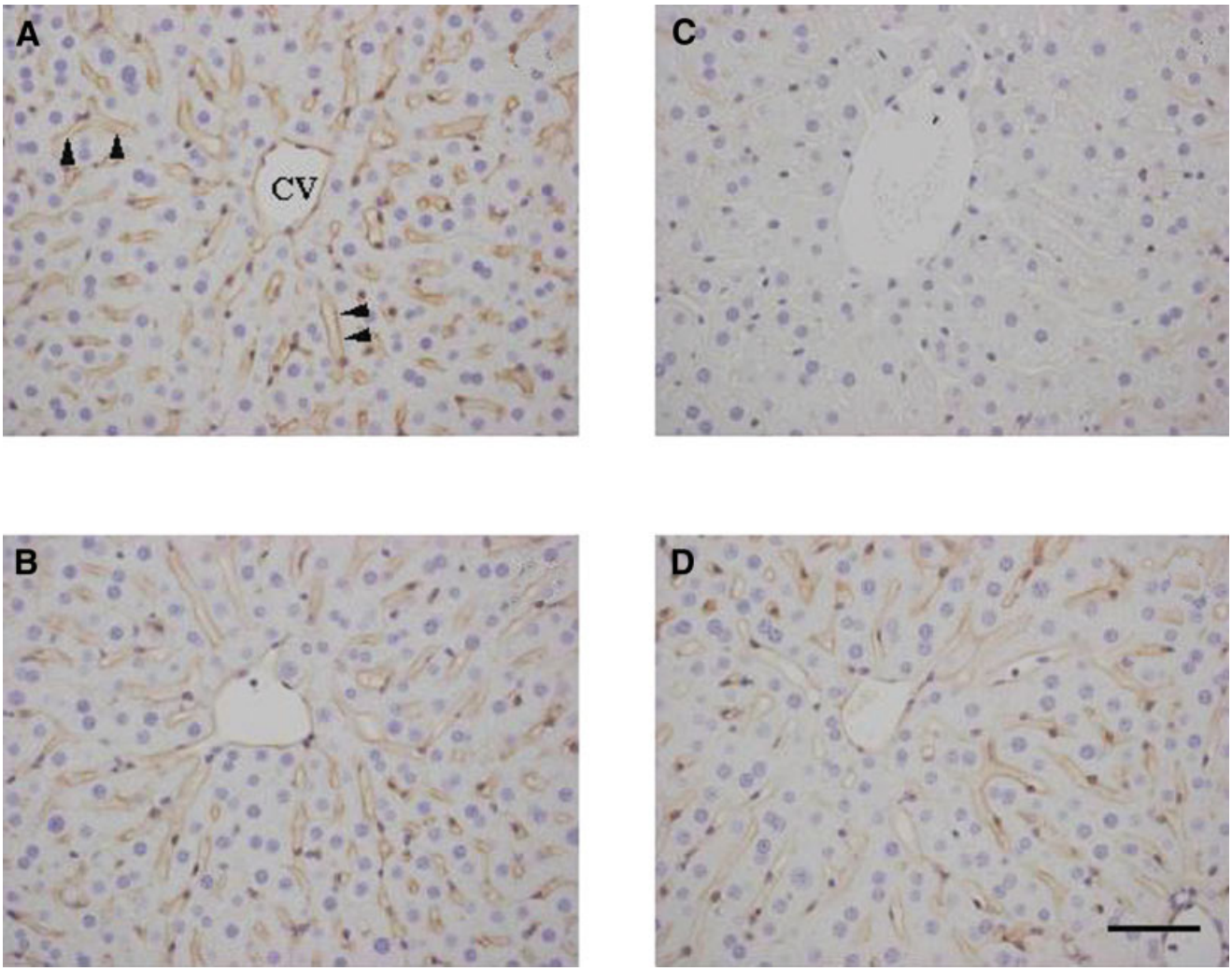


Fig. 9. Immunohistochemical localization of COX-1 (A,B) and COX-2 (C,D) in liver tissue 4 hours after the administration of saline or LPS. Immunoreactive COX-1 was seen in the sinusoidal lining cells in both saline- (A) and LPS-treated mice (B). On the other hand, the immunoreactivity with COX-2 was negative in saline-treated animals (C), whereas it was enhanced in the sinusoids after LPS treatment (D). One representative experiment of 2 animals were presented. Bar indicates 50 μm.

TXA_2 inhibition. However, others[33] have reported that ICAM-1 is involved in accumulation of leukocytes in the hepatic venules, but not in the sinusoids. Furthermore, the present study showed that OKY-046 did not significantly change the LPS-induced increase in the expression of PECAM-1, suggesting that microcirculatory dysfunction improved by TXA_2 inhibition is independent of PECAM-1 expression, and this is consistent with the findings of others.[33]

LPS administration to the isolated perfused liver resulted in a rapid and significant release of TXB_2 into the perfusate, suggesting that the liver is an important source of TXA_2, and that TXA_2 seems to be an inflammatory mediator in the early phase of endotoxemia. We measured the levels of TXB_2 in the effluent perfusate because the levels of TXs and PGs were artificially elevated with ease by mechanical stimulation.[20] In the liver, in response to LPS, TXA_2 is released from nonparenchymal cells (*i.e.*, Kupffer cells[6] and sinusoidal endothelial cells).[34] Of these, Kupffer cells are a major source of TXA_2. However, the possibility that activated platelets are productive sources of TXA_2 *in vivo* cannot be excluded.

To explore whether COX-1 and COX-2 activities are involved in hepatic microcirculatory dysfunction during endotoxemia, mice were treated with the selective COX-1 inhibitor SC-560 and the selective COX-2 inhibitor NS-398. Both SC-560 and NS-398 significantly reduced liver injury and hepatic microcirculatory dysfunction in response to LPS. These results suggest that COX-1 and COX-2 are involved in liver injury. In fact, the levels of ALT activity after LPS combination with galactosamine were significantly decreased in COX-2-deficient mice.[12] A selective COX-2 inhibitor, FK3311, limited liver injury in dogs caused by ischemia/reperfusion and restored he-

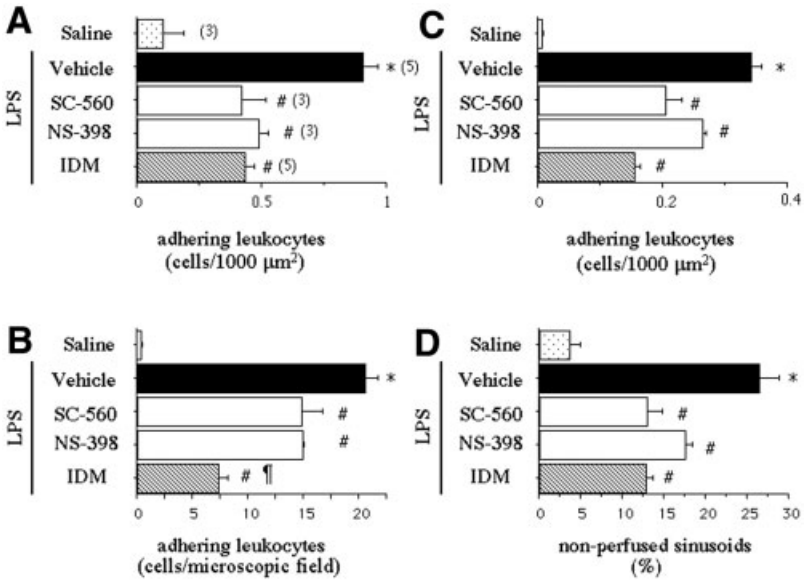


Fig. 10. Effects of SC-560 (10 mg/kg, p.o.), NS-398 (10 mg/kg, s.c.), and indomethacin (IDM) on (A) the numbers of leukocytes adhering to the portal venules, (B) the numbers of leukocytes adhering to the sinusoids, (C) the numbers of leukocytes adhering to the central venules, and (D) the percentage of non-perfused sinusoids after LPS administration. The numbers of adhering leukocytes and of sinusoids were determined 4 hours after LPS injection. Numbers in parentheses indicate number of animals. Data are shown as means ± SEM. $*P < .05$ versus Saline-treated mice; $\#P < .05$ versus LPS-treated mice; $\P P < .05$ versus NS-398-treated mice and SC-560-treated mice.

patic tissue blood flow.[13] Inhibition of COX-2 with NS-398 improved survival from sepsis induced by cecal ligation and puncture in mice.[35] In contrast, Leach et al.[36] reported that inhibition of COX-2 with NS-398 failed to attenuate liver injury elicited by LPS in rats. The reasons for these discrepancies remain unclear, though they may be caused by differences in the animals and the doses of drugs used.

The contribution of PGs generated through the action of COX-1 to hepatic microcirculatory dysfunction and leukocyte adhesion can be estimated from the reduction in the leukocyte adhesion variables after the administration of a COX-1 selective inhibitor, SC-560. This agent used in the present experimental conditions exhibits good selectivity to COX-1. The same was also true in the case of COX-2 inhibition using the COX-2 inhibitor NS-398. In the portal venules, the adhesion of leukocytes were reduced equally by SC-560 and NS-398. This indicates that the PGs generated through COX-1 and COX-2 enhanced the adhesion equally. But, indomethacin did not inhibit the adhesion more intensely than SC-560 and NS-398 in portal venules in the present experiment, although this inhibitor blocks both COX-1 and COX-2. We supposed that there might be a threshold concentration of TXA_2 generated in the microvasculature to induce leukocyte adhesion. Without treatment with COX inhibitors, the amount of TXA_2 generated may be more than the threshold concentration of TXA_2 when either COX-1 or COX-2 is inhibited. If PGs are generated through the action of COX-1 and COX-2, and if no alternative pathway for PG generation is present, the inhibition of either COX-1 or COX-2, or both, certainly reduces the PG levels, which may be lower than the threshold concentration. If the inhibition of either COX-1 or COX-2 is enough to lower the TXA_2 levels below the threshold concentration, the effects of indomethacin are not different from those observed under the inhibition of COX-1 or COX-2. Judging from the results of the perfusion experiment, sites of TXA_2 generation were certainly present in the liver. So, the concentration of TXA_2 may be higher in the sinusoids or in the central venules than in the portal venules. TXA_2 at a concentration over the threshold concentration may induce adhesion in a concentration-dependent manner. In fact, we can observe the effects of indomethacin to be the sum of the inhibition of COX-1 and that of COX-2 in the sinusoids or central venules, where the TXA_2 concentration may be higher than that in portal venules.

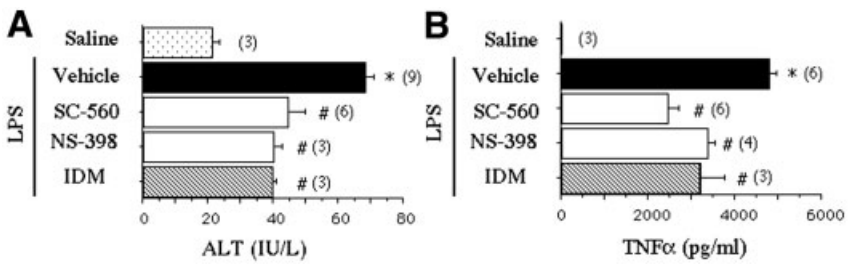


Fig. 11. Effects of SC-560 (10 mg/kg, p.o.), NS-398 (10 mg/kg, s.c.), and indomethacin (IDM 10 mg/kg, p.o.) on (A) serum ALT activity and (B) the serum concentrations of TNFα after LPS administration. Numbers in parentheses indicate number of animals. Data are shown as means ± SEM. $*P < .05$ versus Saline-treated mice; $\#P < .05$ versus LPS-treated mice.

In conclusion, our present study clarifies the important role played by TXA_2 in hepatic microcirculatory dysfunction elicited by LPS administration, although factors other than TXA_2 were reported previously.[37–42] LPS-induced hepatic microcirculatory dysfunction was associated with TXA_2 generation in the liver. The inhibitory effects of TXA_2 synthase inhibitor and TXA_2 receptor antagonist on the hepatic response to LPS were similar to the effects of selective COX-1 and COX-2 inhibitors, suggesting that endogenous TXA_2 derived from both COX-1 and COX-2 participate in LPS-induced liver injury by enhancement of TNFα production. TP receptor signaling may be related to the upregulation of expression of an adhesion molecule, ICAM-1.

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