Significance and Therapeutic Potential of Prostaglandin E_2 Receptor in Hepatic Ischemia/Reperfusion Injury in Mice

Yukiyasu Kuzumoto,[1] Masayuki Sho,[1] Naoya Ikeda,[1] Kaoru Hamada,[2] Takashi Mizuno,[1] Satoru Akashi,[1] Yoshikazu Tsurui,[1] Hisanori Kashizuka,[1] Takeo Nomi,[1] Atsushi Kubo,[3] Hiromichi Kanehiro,[1] and Yoshiyuki Nakajima[1]

Prostaglandin E_2 (PGE_2) mediates a variety of innate and adaptive immunity through four distinct receptors: EP1-EP4. It has been suggested that each EP plays a unique and pivotal role in various disease conditions. We investigated the pathophysiological role of EP receptors in hepatic ischemia/reperfusion (I/R) injury. In this study, a 70% hepatic ischemic model was used in male C57BL/6 mice. Selective EP agonists were used to clarify the function of each PGE_2 receptor in I/R injury. Although all four receptors were expressed in the naïve liver, EP4 expression was significantly upregulated after hepatic I/R. Although EP1, 2, or 3 agonists did not show any protective effect on liver function, the EP4 agonist significantly inhibited hepatic I/R injury as determined by serological and histological analyses. Furthermore, the EP4 agonist downregulated the local expressions of several proinflammatory cytokines, chemokines, and adhesion molecules in the early phase of reperfusion. In contrast, it augmented the local expression of an anti-inflammatory cytokine, interleukin 10. Additionally, the neutrophil accumulation was also inhibited by EP4 agonist treatment. Finally, to confirm the therapeutic efficacy of the EP4 agonist in hepatic I/R injury, the nonischemic shunt liver was removed after 120 minutes of ischemia, resulting in the death of 86% of control mice within 48 hours. In sharp contrast, 80% of mice treated with the EP4 agonist survived. In conclusion, the PGE_2-EP4 signaling pathway has an inhibitory role in hepatic I/R injury. An EP4 agonist effectively protects against ischemic injury. (HEPATOLOGY 2005;42:608-617.)

Hepatic ischemia/reperfusion (I/R) injury is the main cause of hepatic damage and is an inevitable event after liver resection and transplantation.[1,2] Favorable outcomes of liver transplantation have been increasing the need for hepatic grafts. To correct the imbalance between available organs and the number of patients waiting for grafts, the use of marginal organs which would otherwise be discarded or would be expected to lead to malfunction after transplantation may be required. I/R injury is a critical barrier to the use of these marginal organs and thus needs to be overcome. Therefore, despite recent improvements in liver preservation and surgical techniques, hepatic I/R injury remains an important clinical problem. Previous studies have revealed that the underlying mechanisms of hepatic I/R injury are considerably complex and that multiple factors are involved. The initial phase is associated with the generation of nontoxic oxygen species, the activation of Kupffer cells, and an initial response of neutrophil activation. Activated Kupffer cells release numerous metabolites that cause cellular damage, including superoxide radicals, nitric oxide, eicosanoids, proteases, and proinflammatory cytokines. Cytokines such as tumor necrosis factor α (TNF-α); interleukin (IL)-1β, -6, and -12; and interferon γ (IFN-γ) and chemokines such as macrophage inflammatory protein 2 and KC have been pro-

Abbreviations: PGE_2, prostaglandin E_2; I/R, ischemia/reperfusion; TNF-α, tumor necrosis factor-α; IL, interleukin; IFN-γ, interferon γ; ICAM-1, intracellular adhesion molecule 1; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; PCR, polymerase chain reaction; mRNA, messenger RNA; RT-PCR, reverse-transcriptase polymerase chain reaction; MCP-1, monocyte chemoattractant protein 1; IP-10, interferon-inducible protein 10.

From the [1]Department of Surgery, the [2]Second Department of Internal Medicine, and the [3]Department of Public Health, Nara Medical University, Nara, Japan.

Received December 25, 2004; accepted June 20, 2005.

Supported in part by a research grant from the Uehara Memorial Foundation, Japan (to M.S.) and Grant-in-Aid 15591891 from the Ministry of Education, Science, Sports, and Culture of Japan (to H.K.).

Address reprint requests to: Masayuki Sho, M.D., Ph.D., Department of Surgery, Nara Medical University, 840 Shijo-cho, Kashihara, Nara, 634-8522, Japan. E-mail: m-sho@naramed-u.ac.jp; fax: (81) 744-24-6866.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.20827

Potential conflict of interest: Nothing to report.

posed to play important roles in the process of hepatic I/R injury.[3-8] Furthermore, the increased expression of adhesion molecules (*e.g.*, intracellular adhesion molecule 1 [ICAM-1], vascular cell adhesion molecule 1, E- and P-selectin) on sinusoidal endothelial cells promotes neutrophil infiltration into the liver, contributing to the progression of parenchymal injury.[9-11]

Prostaglandin E_2 (PGE_2) is produced during inflammatory responses and mediates a variety of both innate and adaptive immunity through four receptor subtypes (EP1-4) with distinct and potentially antagonistic signaling cascades.[12,13] Until recently, the precise roles of each receptor were largely unknown. The use of gene-targeted mice and a selective agonist/antagonist responsible for each receptor has gradually revealed that each receptor functions via a distinct signal cascade and plays a unique role in a variety of disease conditions, including rheumatoid arthritis,[14] inflammatory bowel disease,[15,16] and tumor growth–associated angiogenesis.[17] In the liver, endogenous PGE_2 has been suggested to be produced mainly by activated Kupffer cells during hepatic injury.[18,19] Previous studies have demonstrated that both endogenous and exogenous PGE_2 are protective against liver injury caused by I/R as well as the other hepatic disorders.[20-22] This effect may be associated with increased liver perfusion, inhibition of platelet aggregation, and direct cytoprotection by PGE_2.[23] Furthermore, PGE_2 has been suggested to ameliorate liver injury through the regulation of cytokine cascades.[18,22] Although studies have suggested that PGE_2 might play an important role in hepatic injury, the role of each PGE_2 receptor in hepatic injury, including I/R injury, remains unknown. In this study, we investigated the pathophysiological role of PGE_2 receptors and explored the therapeutic efficacy of each highly selective agonist for EP1-EP4 in hepatic I/R injury in mice.

Materials and Methods

Animals. Male C57BL/6 mice (8-12 wk old) were obtained from CLEA JAPAN (Tokyo, Japan). All mice were maintained under specific pathogen-free conditions in the animal facility at Nara Medical University. All experiments were conducted under a protocol approved by our institutional review board.

Model of Hepatic I/R Injury. We used a murine model of 70% partial hepatic ischemia for 90 minutes. Briefly, mice were anesthetized with sodium pentobarbital (60 mg/kg intraperitoneally) and a midline laparotomy was performed. The left lateral and median lobes of the liver were then clamped at its base using an atraumatic clip. After 90 minutes of ischemia, the clip was removed, initiating hepatic reperfusion. Mice were sacrificed at 2 or 6 hours after reperfusion, after which blood and liver samples were collected for analysis. In some mice, to assess animal survival, the nonischemic shunt liver lobes were surgically removed at the end of 120 minutes of ischemia of the left and median liver lobes.

Selective EP Agonists. All selective EP agonists were generous gifts from Ono Pharmaceutical Co. (Osaka, Japan). ONO-DI-004, ONO-AE1-259, ONO-AE-248, and ONO-AE1-329 are highly specific for the EP1, EP2, EP3, and EP4 receptor, respectively. The specificities of the agonists were analyzed by measuring the binding affinity to each EP expressed in CHO cells, as previously reported.[24] The affinity of each selective agonist for its respective EP has been shown to be 100- to 1,000-fold greater than that for the other EP receptors. The structures and properties of each EP agonist have also been previously shown.[25]

Experimental Protocols. Each EP selective agonist of either 30 μg/kg or 100 μg/kg was subcutaneously injected at the indicated time during I/R of the liver. Because of the short half-life of EP agonists, we treated mice with multiple injections during hepatic I/R. Each EP agonist in a total volume of 0.2 mL was injected six times at -2.5, -0.5, and 0 hours before and 1, 3, and 5 hours after the initiation of reperfusion. In some mice, the EP4 agonist was injected three times at 2.5, 0.5, and 0 hours before the initiation of reperfusion. Control mice received normal saline in the same amount.

Analysis of Alanine Aminotransferase, Aspartate Aminotransferase and Lactate Dehydrogenase Activity. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) were used as established markers of hepatocyte injury. At 6 hours after reperfusion following 90 minutes of ischemia, blood samples were obtained via cardiac puncture, immediately centrifuged at 3,000*g* for 10 minutes, and stored at -20°C until analysis. Serum ALT, AST, and LDH activities were measured using a standard clinical automatic analyzer.

Histopathological and Immunohistochemical Analysis. For histological analysis, tissue samples were fixed in 4% formaldehyde/phosphate-buffered saline overnight at 4°C. The samples were dehydrated and embedded in paraffin. Six-micrometer sections were stained with hematoxylin-eosin. For the immunohistochemical analysis of EP4, serial cryostat sections (5 μm) were cut frozen, coated on polylysine-pretreated slides, and air-dried overnight at room temperature. Specimens were fixed in 100% cold acetone for 10 minutes at 4°C and washed for 10 minutes in phosphate-buffered saline. Tissue sections were pretreated for 10 minutes with peroxidase-blocking reagent (DAKO, Carpinteria, CA) to suppress endoge-

nous peroxidase and pseudoperoxidase activity, washed again in phosphate-buffered saline, and then incubated for 1 hour at 37°C in a humid chamber with the anti-EP4 polyclonal antibody (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA). The slides were rinsed twice and then incubated for 60 minutes with goat anti-mouse immunoglobulins conjugated to a peroxidase-labeled polymer (EnVision + TM, Peroxidase, DAKO). After washing, revealing reaction was performed using 3,3′-diaminobenzidine (Liquid DAB$^+$ Substrate-Chromogen System, DAKO) as a chromogen substrate. Slides were then washed again and counterstained with hematoxylin.

Alterations of neutrophil accumulation in the ischemic liver were detected by standard immunohistochemical techniques using the anti-myeloperoxidase polyclonal antibody (NeoMarkers, Fremont, CA) on paraffin sections. The vectastain ABC kit (Vector Laboratories, Burlingame, CA) was used with a 3, 3′-diaminobenzidine substrate kit (Vector Laboratories) according to the manufacturer's instructions for detection. In the immunostained sections, the numbers of neutrophils in the liver were counted in 10 randomly chosen visual fields (magnification ×400) of the sections, and the average of 10 selected microscopic fields was calculated in a blind manner.

Extraction of Total RNAs and Reverse-Transcriptase Polymerase Chain Reaction. Total RNA was isolated from liver samples before and at 2 and 6 hours after I/R injury using ISOGENE (Nippon Gene, Toyama, Japan). RNA (1 μg) was reverse-transcribed using Qiagen Omniscript RT Kit (Qiagen, Hilden, Germany). The RNA was heated for 15 minutes at 65°C and 4°C cooled on ice. One μg of the RNA was diluted in RNase-free water for a volume of 13 μL. Then, 2 μL of 10 × reaction buffer, 1 μL of RNase inhibitor (10 U/μL), 2 μL of a mixture of dNTPs, 1 μL of Oligo-dT primers (10 μmol/L) and 1 μL of Omniscript Reverse Transcriptase (4 U/μL) were added for a total volume of 20 μL. The mixture was incubated for 60 minutes at 37°C. After incubation, the complementary DNA was stored at −80°C before analysis. The complementary DNA solution (1 μL) was amplified in a total volume of 20 μL that contained: 2 μL of 10× PCR buffer, 1 μL of $MgCl_2$ (25 mmol/L) (Toyobo, Osaka, Japan), sense primer (50 μmol/L), antisense primer (50 μmol/L), 0.5 μL of Takara Ex Taq (Takara, Shiga, Japan) and 2 μL of a mixture of dNTPs. The amplification involved an initial step of denaturation at 94°C for 1 minute, followed by cycles consisting of denauration at 94°C for 1 minute, optimal annealing temperature for 1 minute, and chain elongation at 72°C for 1 minute. The sequence of sense and antisense primers and product size for each EP receptor and GAPDH are as follows: EP1, 5′-GCTTAACCTGAGCCTAGCGGA-3′ and 5′-CGCAGTATACAGGCGAAGCAC-3′, 294 bp; EP2, 5′-CTCAACTACGGGGAGTACGTCC-3′ and 5′-AGGAGAATGAGGTGGTCCGTC-3′, 277 bp; EP3, 5′-AGGTGGTGCTTCATCAGCA-3′ and 5′-GCTCAACCGACATCTGATTGA-3′, 324 bp; EP4, 5′-CGTAGTATTGTGCAAGTCGCG-3′ and 5′-CAGATGATGCTGAGACCCGAC-3′, 215 bp; GAPDH, 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′, 452 bp. Amplified products were analyzed via electrophoresis of the PCR product on a 1.5% agarose gel plus ethidium bromide in TBE buffer. Band intensities were measured using NIH Image Analysis Software version 1.61 (National Institutes of Health, Bethesda, MD), and were determined with the use of the ratios to GAPDH. The fold increase in each EP messenger RNA (mRNA) in the ischemic liver was calculated via the comparison of these expressions before I/R injury. Each EP mRNA level before I/R injury was assigned an arbitrary value of 1.

Real-Time Reverse-Transcriptase PCR Analysis to Monitor Hepatic Gene Expression. The mRNA expression of several cytokines (TNF-α, IL-1β, IFN-γ, IL-6, IL-10), chemokines (monocyte chemoattractant protein 1 [MCP-1], macrophage inflammatory proteins 1α and 2, interferon-inducible protein 10 [IP-10], monokine induced by IFN-γ, RANTES, KC, ENA78), and adhesion molecules (ICAM-1, vascular cell adhesion molecule 1, E-selectin) in naïve and ischemic livers were analyzed via quantitative real-time RT-PCR. In brief, amplification and detection were performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) with the following profile: 10 minutes at 95°C, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. All primers and probes were purchased from Applied Biosystems. Each gene expression of cytokines, chemokines, and adhesion molecules was normalized to GAPDH before the fold change was calculated. The fold increase in each gene expression in the ischemic liver was calculated via the comparison of these gene expressions before I/R injury. Each mRNA level before I/R injury was assigned an arbitrary value of 1.

ELISA Quantification of Tissue Cytokines. The protein expression of several cytokines was determined in liver homogenates. Liver homogenates were prepared in 10 volumes of cold lysis buffer containing 1× Laemml sample buffer, 62.5 mmol/L Tris-HCL, 2% sodium lauryl sulfate, and 10% glycerol. After centrifugation at 10,000*g* for 20 minutes at 4°C, aliquots of supernatant were used immediately for measurement. Total protein concentration of each was measured using a BCA protein assay kit (Pierce Chemical Co., Rockford, IL). Hepatic tissue cytokine levels were determined using an ELISA kit from eBioscience (San Diego, CA). Briefly, capture anti-

body was coated onto a 96-well EIA plate overnight, followed by blocking tissue samples that were diluted at 1:3 and then duplicated; the standards provided in the kit were used, followed by one to two serial dilutions. Biotinylated detecting antibodies were added, followed by avidin-HRP. Tetramethylbenzidine was used as the substrate, and the color reactions were stopped by 1 mol/L H_3PO_4. Tissue concentrations were calculated from the standard curve.

Statistical Analysis. The mean and SEM were calculated for all parameters determined in the study. Statistical significance between two groups of parametric data was evaluated using an unpaired Student *t* test. *P* values less than .05 were considered significant. The survival curve as determined with the Kaplan-Meier method was analyzed using a log-rank test.

Results

EP Receptor mRNA Expression During Hepatic Ischemia/Reperfusion Injury. First, we examined the local expression of each EP receptor in the process of hepatic I/R injury via RT-PCR analysis. All four receptors were expressed in both the naïve and ischemic liver (Fig. 1A). Semiquantification of mRNA levels indicated that there were no differences in EP1 and EP2 expression between the naïve and ischemic liver. EP3 mRNA expression was significantly downregulated at 2 or 6 hours after reperfusion in the postischemic liver compared with the naïve liver ($P = .0187$ and .0238, respectively). In sharp contrast, EP4 mRNA was significantly upregulated at 2 hours after reperfusion compared with the naïve liver ($P < .0001$). The EP4 expression levels declined thereafter (Fig. 1B).

EP4 Receptor Protein Expression During Hepatic I/R Injury. In the naïve liver, EP4 protein expression was moderately expressed in hepatocytes. At 2 hours of reperfusion following 90 minutes of ischemia, prominent EP4 expression was observed in the membrane as well as in the cytoplasm of hepatocytes and on sinusoidal cells, mainly in the periportal region (Fig. 2). EP4 expression was relatively low in the perivenous region. In addition, the EP4 expression was absent in bile duct epithelial cells. EP4 expression was declined at 6 hours of reperfusion (data not shown).

Pathophysiological Function of EP Receptor in Hepatic I/R Injury. To explore the function of each PGE_2 receptor in hepatic injury induced by I/R, we used a pharmacological approach using highly specific agonists responsible for each EP. Serum levels of ALT, AST, and LDH were measured after 6 hours of reperfusion following 90 minutes of ischemia. The administration of the

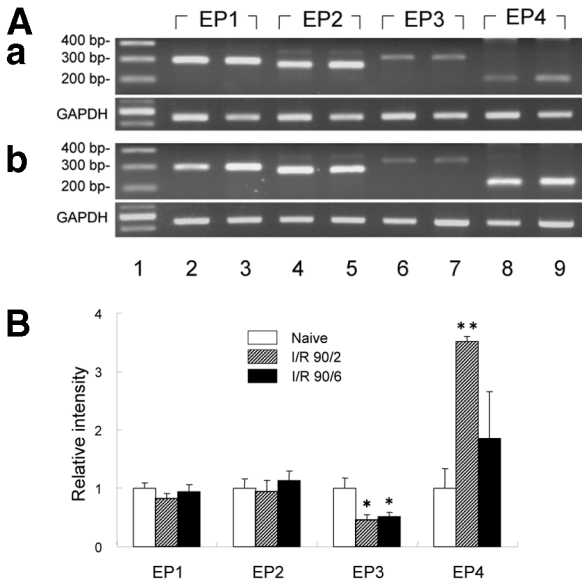

Fig. 1. Each EP mRNA expression in the liver during I/R injury. (A) All PGE_2 receptors were expressed in the naïve liver (panel a) and in the ischemic liver (panel b) at 2 hours after reperfusion following 90 minutes of ischemia. Lane 1, size marker; lanes 2 and 3, EP1; lanes 4 and 5, EP2; lanes 6 and 7, EP3; lanes 8 and 9, EP4. (B) The ratios of EP1, EP2, EP3, and EP4 to GAPDH at 2 and 6 hours after reperfusion following 90 minutes of ischemia were compared with the ratios of the naïve liver. Each EP mRNA level in the naïve liver was assigned an arbitrary value of 1. EP3 receptor expression was significantly downregulated at 2 and 6 hours after reperfusion (*$P = .0187$ and .0238, respectively, vs. naïve control). In contrast, EP4 receptor expression was significantly upregulated at 2 hours after reperfusion (**$P < .0001$ vs. naïve control). Data represent the mean ± SEM of 5 to 7 mice per group. I/R, ischemia/reperfusion.

selective EP1, EP2, and EP3 agonists had no beneficial effect on hepatic injury (Fig. 3). In contrast, the treatment of the selective EP4 agonist significantly reduced all serum levels compared with the control (ALT, 1,977 ± 412 vs. 5,240 ± 1173; AST, 1,556 ± 231 vs. 4,661 ± 1,047; LDH, 8,014 ± 1,634 vs. 17,201 ± 3,707, respectively; EP4 agonist–treated group vs. control group) (Fig. 3). Taken together, the PGE_2-EP4 signaling may be a critical pathway in hepatic I/R injury.

Protective Effect of EP4 Agonist on Liver Function in a Dose-Dependent Manner. We were intrigued with the therapeutic efficacy of the selective EP4 agonist in protecting hepatocytes from I/R injury (Fig. 4A). First, to confirm its protective effect on hepatic I/R injury, we examined a dose–response relationship of the EP4 agonist. Mice received the EP4 agonist three times at 30 μg/kg, six times at 30 μg/kg, or three times at 100 μg/kg. As a result, the serum levels of ALT after 6 hours of reperfusion following 90 minutes of ischemia with each treatment were 3,631 ± 1,166, 1,977 ± 412, and 1,535 ± 292, respectively (Fig. 4B). These data indicate an inhib-

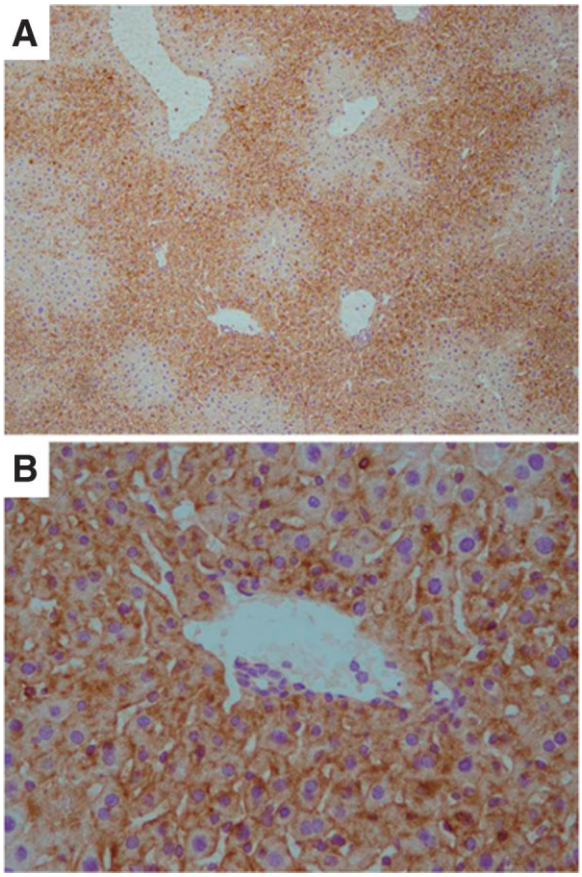

Fig. 2. Immunohistochemistry for EP4 protein expression in the ischemic liver at 2 hours after reperfusion following 90 minutes of ischemia. EP4 was prominently expressed in the membrane as well as in the cytoplasm of hepatocytes and on sinusoidal cells, mainly in the periportal region, whereas the expression was relatively low in the perivenous region. EP4 expression was absent in bile duct epithelial cells. (A) Original magnification: ×100. (B) Original magnification, ×400.

itory effect of the EP4 agonist on hepatic I/R injury in a dose-dependent manner.

Protective Effect of EP4 Agonist in Ischemic Liver Tissue. To further confirm the protective effect of the EP4 agonist on hepatic I/R injury, sections of the liver obtained from the ischemic lobe at 6 hours after reperfusion were evaluated for histopathological analysis. In the control liver, massive cellular infiltration and extensive hepatic cellular necrosis were observed (Fig. 5A-B). In contrast, mild cellular infiltration, little necrosis, and comparatively preserved lobular architecture were seen in the livers treated with the EP4 agonist (Fig. 5C-D).

The EP4 Agonist Suppresses Local Immune Activation. To clarify the underlying mechanisms for the protective effect of the EP4 agonist, we evaluated the local expressions of several cytokines, chemokines, and adhesion molecules in the liver at 2 and 6 hours after reperfusion following 90 minutes of ischemia using quantitative real-time RT-PCR analysis. EP4 agonist treatment significantly downregulated the local expression of several potent proinflammatory cytokines, including TNF-α, IL-1β, and IFN-γ at 2 hours after reperfusion compared with the control ($P = .0032$, .0003, and .0059, respectively) (Fig. 6A). After 6 hours of reperfusion, the expressions of these cytokines in the control liver were declined, and the significant differences between the control and the EP4 agonist–treated group were not sustained. In sharp contrast, IL-10, an anti-inflammatory cytokine, was significantly upregulated at 2 hours after reperfusion ($P = .0365$) (Fig. 6A). Thus, EP4 agonist treatment suppressed some potent proinflammatory cytokines, while it augmented a critical antiinflammatory cytokine at the early phase of reperfusion. Furthermore, we examined the local protein levels of several cytokines in the liver at 2 and 6 hours after reperfusion following 90 minutes of ischemia

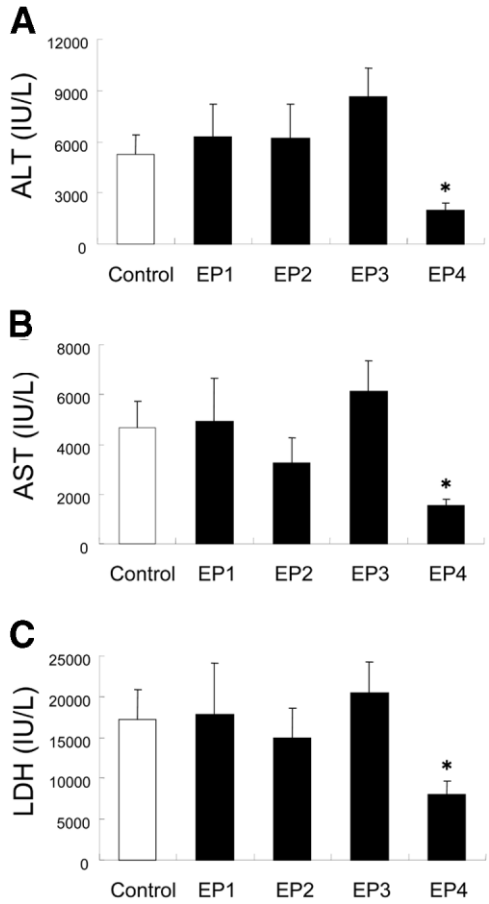

Fig. 3. The serum levels of ALT, AST, and LDH in mice treated with each EP agonist at 6 hours after reperfusion following 90 minutes of ischemia. EP1, EP2, or EP3 agonist treatment (100 μg/kg at 2.5, 0.5, and 0 hours before and 1, 3, and 5 hours after reperfusion) did not reduce any levels. EP4 agonist treatment (30 μg/kg at 2.5, 0.5, and 0 hours before and 1, 3, and 5 hours after reperfusion) significantly decreased all serum levels compared with the control. Data represent the mean ± SEM of 6 to 16 per group. *$P < .05$ vs. control. ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase.

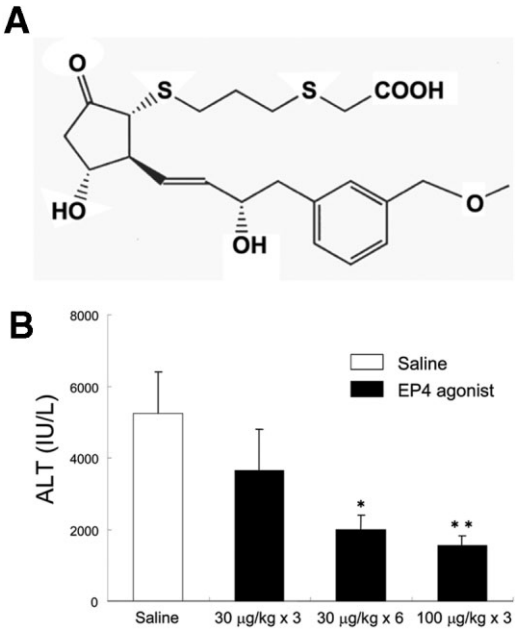

Fig. 4. (A) Molecular structure of ONO-AE1-329, a highly specific agonist for the EP4 receptor. (B) Protective effect of the EP4 agonist on liver function in a dose-dependent manner. The serum levels of ALT at 6 hours after reperfusion of mice that received the EP4 agonist with different doses are shown. The highest dose (100 μg/kg at 2.5, 0.5, and 0 hours before reperfusion) was most effective against hepatic injury. The results showed a dose-dependent decline. Data represent the mean $\pm$ SEM of 11 to 16 mice per group. *$P = .0267$. **$P = .0070$ vs. control. ALT, alanine aminotransferase.

using ELISA analysis. EP4 agonist treatment significantly inhibited the local production of several potent proinflammatory cytokines, including TNF-α and IFN-γ at 6 hours after reperfusion compared with the control ($P = .011$ and .012, respectively) (Fig. 6B). However, the treatment did not significantly augment the local protein expression of IL-10 (data not shown). Next, we also examined the local expressions of several chemokines. In particular, MCP-1 and IP-10 were significantly downregulated by EP4 agonist treatment at 2 hours after reperfusion ($P = .0398$ and .0245, respectively, vs. control) (Fig. 7A). In addition, we also examined the local expressions of some adhesion molecules. Among them, ICAM-1 and E-selectin were significantly downregulated at 2 hours after reperfusion by EP4 agonist treatment ($P = .029$ and .0042, respectively, vs. control) (Fig. 7B). Taken together, the protective effect of EP4 agonist on hepatic I/R injury might be associated with the inhibition of local immune activation.

EP4 Agonist Inhibits Neutrophil Accumulation in the Liver. Because it is widely recognized that neutrophils play a central role in hepatic I/R injury, we directly examined the neutrophil accumulation in the ischemic liver using myeloperoxidase staining analysis. In control livers at 6 hours after reperfusion following 90 minutes of ischemia, a considerable number of neutrophils were identified (Fig. 8A). In contrast, comparatively few neutrophils were identified in livers treated with EP4 agonist (100 μg/kg at 2.5, 0.5, and 0 hours before reperfusion) (Fig. 8B). By counting the stained cells, we found a significant reduction of accumulated neutrophils in the EP4 agonist–treated livers compared with control livers (6.01 ± 0.795 vs. 20.2 ± 6.025; $P = .0282$) (Fig. 8C).

The EP4 Agonist Improves Survival in Lethally Injured Mice. Finally, to confirm the therapeutic efficacy of EP4 agonist on hepatic I/R injury, we used a lethal model. In this model, after 2-hour ischemia of 70% of the liver, the nonischemic right lobe shunt was surgically resected at the initiation of reperfusion. In this model, 86% of mice in the control group died within 48 hours after reperfusion. In sharp contrast, EP4 agonist treatment significantly improved the survival rate, and 80% of treated mice survived ($P = .016$) (Fig. 9). These data clearly demonstrate that the selective EP4 agonist had a therapeutic efficacy and dramatically improved survival after severe hepatic I/R injury.

Discussion

It has been previously shown that endogenous PGE_2 released mainly from activated Kupffer cells via the upregulation of COX-2 expression protects hepatic I/R injury.[18,19] In addition, exogenous PGE_2 has been reported

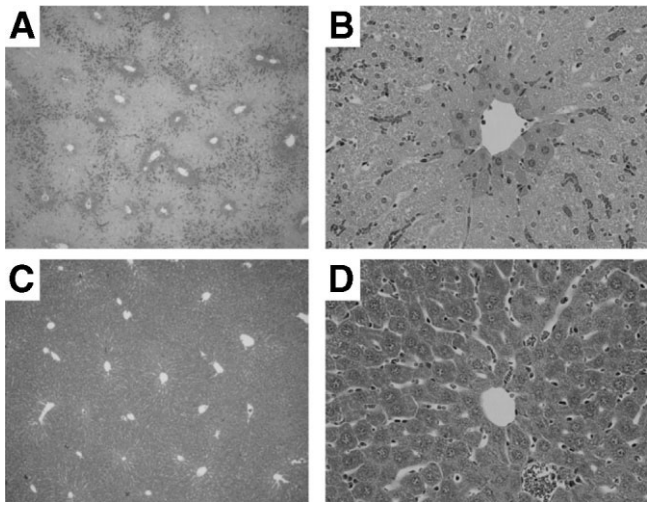

Fig. 5. Representative histological appearances of the liver from (A-B) untreated and (C-D) EP4 agonist–treated mice (100 μg/kg at 2.5, 0.5, and 0 hours before reperfusion) at 6 hours after reperfusion following 90 minutes of ischemia (hematoxylin-eosin stain) (original magnification $\times$40 [A,C] and $\times$400 [B,D]). (A-B) Massive cellular infiltration and extensive hepatic cellular necrosis were observed in control livers. (C-D) In contrast, mild cellular infiltration, few necrosis as well as comparatively preserved lobular architecture were seen in EP4 agonist–treated livers.

to have a beneficial effect on hepatic injury in a variety of experimental models, including liver failure induced by endotoxin and certain drugs.[20-22] Although these studies suggest that PGE_2 plays an important role in hepatic injury, the underlying mechanisms for the protective effect of PGE_2 signal transduction in the liver have not been fully elucidated. In the present study, we investigated the roles and functions of PGE_2 receptors in regulating hepatic injury induced by I/R. PGE_2 acts through four distinct receptors encoded by different genes known as *EPs* (EP1-EP4). EP receptors are G protein–coupled heptahelical proteins. Each receptor subtype has a distinct pharmacological property based on its pharmacophore and PGE_2-evoked signal transduction.[12,13] The EP1 receptor is coupled to intracellular calcium and mediates the elevation of free Ca^{2+} concentration, while the EP2 and EP4 receptors are coupled to G_s and mediate increases in cyclic adenosine monophosphate concentration. Signal transduction by the EP3 receptor is more complex, because multiple EP3 isoforms generated by alternative splicing couple to different signaling pathways.

To clarify the role of EP in hepatic injury, we first examined the local expression of EP in the liver. We detected mRNAs for all four subtypes in the naïve liver and found no differences in EP1 and EP2 expression in the process of I/R injury. On the other hand, EP3 receptor expression was downregulated after reperfusion. Although we cannot presently elucidate the function of the EP3 receptor, it may have some role in hepatic injury. In sharp contrast, the EP4 receptor was significantly upregulated at 2 hours after reperfusion, suggesting that the PGE_2-EP4 signaling pathway may play some role during hepatic I/R injury. To investigate the pathophysiological function of each EP, we used a pharma-

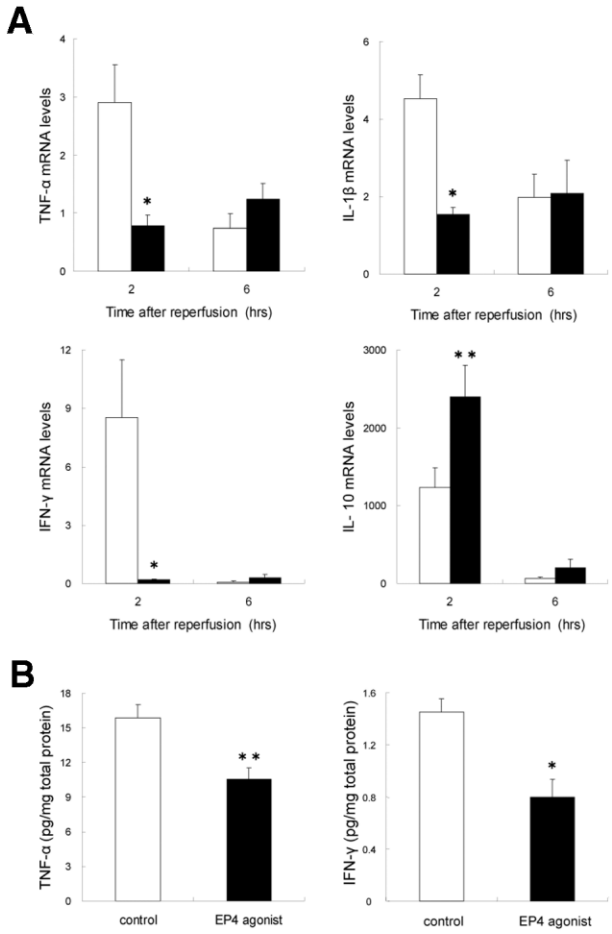

Fig. 6. Effect of the EP4 agonist on cytokine expression in ischemic livers at 2 and 6 hours after reperfusion following 90 minutes of ischemia. (A) EP4 agonist treatment (100 μg/kg at 2.5, 0.5, and 0 hours before reperfusion) significantly inhibited the mRNA expression of several proinflammatory cytokines such as TNF-α, IL-1β, and IFN-γ at 2 hours after reperfusion. In contrast, the treatment significantly augmented local IL-10 mRNA expression. (B) EP4 agonist treatment significantly inhibited the protein production of several proinflammatory cytokines, including TNF-α and IFN-γ, at 6 hours after reperfusion. Data represent the mean $\pm$ SEM of 5 to 7 mice per group. $^*P < .01$, $^{**}P < .05$ vs. control. White bars represent untreated control livers; black bars represent EP4 agonist–treated livers. TNF-α, tumor necrosis factor-α; mRNA, messenger RNA; IL, interleukin; IFN-γ, interferon γ.

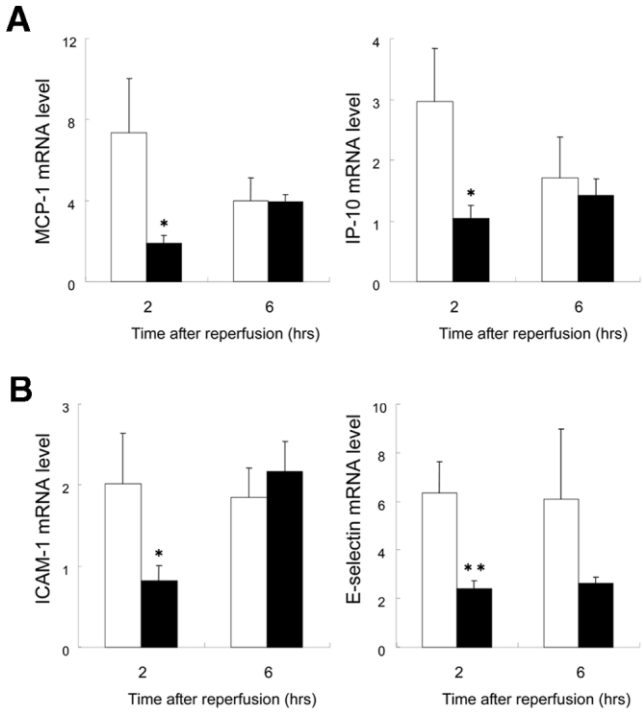

Fig. 7. Effect of the EP4 agonist on expression of (A) chemokines and (B) adhesion molecules in the liver at 2 and 6 hours after reperfusion following 90 minutes of ischemia. EP4 agonist treatment (100 μg/kg at 2.5, 0.5, and 0 hours before reperfusion) inhibited MCP-1 and IP-10 expression at 2 hours after reperfusion; it also inhibited ICAM-1 and E-selectin expression. Data represent the mean $\pm$ SEM of 5 to 7 mice per group. $^*P < .05$. $^{**}P < .01$ vs. control. White bars represent untreated control livers; black bars represent EP4 agonist–treated livers. MCP-1, monocyte chemoattractant protein 1; mRNA, messenger RNA; IP-10, interferon-inducible protein 10; ICAM-1, intracellular adhesion molecule 1.

cological approach using highly specific agonists for the EPs. In this model, only the EP4 agonist significantly attenuated hepatic I/R injury in a dose-dependent manner. Taken together, our data suggest that PGE_2 may exert its protective function in hepatic injury through the EP4 receptor. Although a recent report has also demonstrated that endogenous PGE_2 is protective against cardiac I/R injury through the EP4 receptor,[26] several other studies have indicated a cardioprotective effect of EP3 agonists against I/R injury.[27,28] On the other hand, McCullough et al. have recently reported the neuroprotective function of PGE_2 through the EP2 receptor in cerebral ischemia in a cyclic adenosine monophosphate–dependent manner.[29] Thus, the role of each EP for PGE_2 function may be injured organ–dependent or cell type–dependent.

Next, we were intrigued by the therapeutic efficacy of the EP4 agonist and tried to reveal the underlying mechanisms for its beneficial effect on hepatic I/R injury. Although previous studies have suggested that the diverse function of PGE_2 might be responsible for its protective effect, the regulation of cytokine cascades may be a key mechanism.[18] PGE_2 induces the downregulation of several proinflammatory cytokines and also the upregulation of anti-inflammatory cytokines, including IL-10.[22,30] Moreover, several *in vitro* studies have shown that PGE_2 regulates the production and release of proinflammatory cytokines by macrophages or neutrophils via the EP2 or EP4 receptors.[31-33] To clarify the *in vivo* dynamics of cytokines induced by EP4 agonist treatment in this study, we analyzed the local expression of cytokines in the process of hepatic injury. As a result, we found a significant reduction of several proinflammatory cytokines. In contrast, the EP4 agonist induced significant upregulation of mRNA, but not of IL-10 protein levels. Taken together, the protective effect of the EP4 agonist on the ischemic liver may be mainly dependent on the inhibition of proinflammatory cytokines. These findings are consistent with previous data showing a protective function of PGE_2. Therefore, such cytokine dynamics may further support our interpretation that PGE_2 exerts its beneficial effect through the EP4 receptor in hepatic I/R injury. These cytokine milieu induced by the EP4 agonist in the early phase of I/R injury may be crucial for protecting the ischemic liver from reperfusion injury.

Several studies demonstrated the importance of chemokines in hepatic I/R injury. Because of their potent chemotactic activity for neutrophils, it is generally assumed that CXC chemokines recruit neutrophils into the postischemic liver. However, the significance and contribution of CXC chemokines to I/R injury are somewhat controversial. In this study, one of the CXC chemokines, IP-10, was downregulated by EP4 agonist treatment. In addition, our *in vivo* data indicated that both MCP-1 and IP-10 were inhibited by treatment in the early phase of I/R injury. Both clinical[34,35] and experimental[36] studies have indicated that hepatic levels of MCP-1 and IP-10 are markedly enhanced during various types of liver injury. At present, the role of MCP-1 and IP-10 in the liver has been examined only in terms of its ability to promote and maintain the leukocyte infiltration during liver disease.[37] However, MCP-1 and IP-10 were also reported to have immunomodulatory and anti-inflammatory proper-

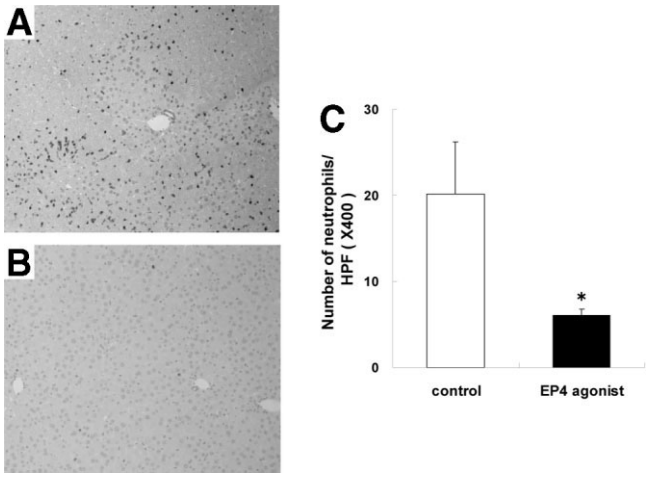

Fig. 8. Inhibitory effect of the EP4 agonist on the accumulation of neutrophils in the liver at 6 hours after reperfusion following 90 minutes of ischemia. Paraffin-embedded sections were stained with a monoclonal antibody against myeloperoxidase (original magnification ×200). (A) A considerable number of neutrophils were observed in the control livers. (B) Relatively fewer neutrophils were seen in livers treated with the EP4 agonist (100 μg/kg at 2.5, 0.5, and 0 hours before reperfusion). (C) Quantitative analysis revealed that EP4 agonist treatment significantly inhibited neutrophil accumulation. Data represent the mean ± SEM of 7 mice per group. *$P = .0282$ vs. control. HPF, high power field.

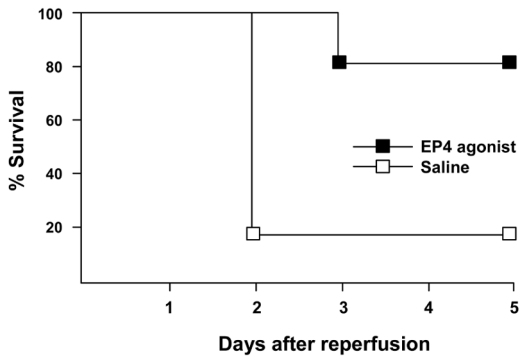

Fig. 9. The therapeutic efficacy of the EP4 agonist in mouse survival after severe hepatic I/R injury. The nonischemic shunt lobe was surgically removed at the time of reperfusion following 120 minutes of ischemia. In the control group, 86% of the mice died within 48 hours after reperfusion (n = 7). By sharp contrast, EP4 agonist treatment (100 μg/kg at 2.5, 0.5, and 0 hours before reperfusion) significantly improved survival: 80% of treated mice survived (n = 5; $P = .016$ vs. control).

ties.[36,38] Therefore, to clarify the responsibility of these chemokines for I/R injury, further investigation will be required. Furthermore, we confirmed that certain adhesion molecules, including ICAM-1 and E-selectin, were also downregulated by the EP4 agonist. The interactions between the expressions of adhesion molecules and PGE_2/EP4 have not been reported so far. Taken together, PGE_2-EP4 inhibitory signaling and the effect of the specific EP4 agonist may depend on the restraints of local immune activation in the liver. Such downregulation of local immune activation can lead to less infiltration and accumulation of neutrophils, resulting in the significant protection from hepatic injury.

Highly specific EP4 agonists have been demonstrated to regulate several inflammatory responses and to have therapeutic potential in various disease conditions.[15,32,39,40] As shown in our mouse survival data, this study may further support the promise of its clinical application into hepatic surgery and transplantation. In fact, PGE_1, which is one of prostaglandins and has properties similar to PGE_2, is known to have some hepato-protective function. However, randomized clinical studies failed to demonstrate a beneficial effect of PGE_1 analog treatment on the incidence of primary liver failure after transplantation, although some clinical benefits were observed.[41,42] This may be related to diverse functions of prostaglandins *in vivo*. Therefore, the treatment targeting downstream molecules of prostaglandins may exert a more specific therapeutic effect while avoiding unfavorable effects. However, before the clinical application of the selective EP4 agonist, further studies may be required to evaluate its therapeutic efficacy in other models, including cold ischemia and fatty liver.

In conclusion, we have demonstrated an inhibitory role of PGE_2-EP4 signaling pathway in the process of hepatic I/R injury. Furthermore, the selective EP4 agonist had a therapeutic efficacy for the protection of the liver from ischemic injury. Therefore, EP4 may be a potent target for protecting hepatic injury, and the clinical application of the selective EP4 agonist can be considered in major liver surgery as well as transplantation.

Acknowledgment: The authors thank Noriko Fujiwara and Shinichiro Orita for their kind support and assistance.

References

1. Huguet C, Gavelli A, Bona S. Hepatic resection with ischemia of the liver exceeding one hour. J Am Coll Surg 1994;178:454-458.
2. Lemasters JJ, Thurman RG. Reperfusion injury after liver preservation for transplantation. Annu Rev Pharmacol Toxicol 1997;37:327-338.
3. Colletti LM, Remick DG, Burtch GD, Kunkel SL, Strieter RM, Campbell DA Jr. Role of tumor necrosis factor-alpha in the pathophysiologic alterations after hepatic ischemia/reperfusion injury in the rat. J Clin Invest 1990;85:1936-1943.
4. Suzuki S, Toledo-Pereyra LH. Interleukin 1 and tumor necrosis factor production as the initial stimulants of liver ischemia and reperfusion injury. J Surg Res 1994;57:253-258.
5. Langdale LA, Wilson L, Jurkovich GJ, Liggitt HD. Effects of immunomodulation with interferon-gamma on hepatic ischemia-reperfusion injury. Shock 1999;11:356-361.
6. Lentsch AB, Yoshidome H, Cheadle WG, Miller FN, Edwards MJ. Chemokine involvement in hepatic ischemia/reperfusion injury in mice: roles for macrophage inflammatory protein-2 and KC. HEPATOLOGY 1998;27:1172-1177.
7. Lentsch AB, Kato A, Yoshidome H, McMasters KM, Edwards MJ. Inflammatory mechanisms and therapeutic strategies for warm hepatic ischemia/reperfusion injury. HEPATOLOGY 2000;32:169-173.
8. Jaeschke H. Molecular mechanisms of hepatic ischemia-reperfusion injury and preconditioning. Am J Physiol Gastrointest Liver Physiol 2003;284:G15-G26.
9. Farhood A, McGuire GM, Manning AM, Miyasaka M, Smith CW, Jaeschke H. Intercellular adhesion molecule 1 (ICAM-1) expression and its role in neutrophil-induced ischemia-reperfusion injury in rat liver. J Leukoc Biol 1995;57:368-374.
10. Yadav SS, Howell DN, Steeber DA, Harland RC, Tedder TF, Clavien PA. P-Selectin mediates reperfusion injury through neutrophil and platelet sequestration in the warm ischemic mouse liver. HEPATOLOGY 1999;29:1494-1502.
11. Martinez-Mier G, Toledo-Pereyra LH, Ward PA. Adhesion molecules in liver ischemia and reperfusion. J Surg Res 2000;94:185-194.
12. Breyer RM, Bagdassarian CK, Myers SA, Breyer MD. Prostanoid receptors: subtypes and signaling. Annu Rev Pharmacol Toxicol 2001;41:661-690.
13. Narumiya S, Sugimoto Y, Ushikubi F. Prostanoid receptors: structures, properties, and functions. Physiol Rev 1999;79:1193-1226.
14. McCoy JM, Wicks JR, Audoly LP. The role of prostaglandin E2 receptors in the pathogenesis of rheumatoid arthritis. J Clin Invest 2002;110:651-658.
15. Kabashima K, Saji T, Murata T, Nagamachi M, Matsuoka T, Segi E, et al. The prostaglandin receptor EP4 suppresses colitis, mucosal damage and CD4 cell activation in the gut. J Clin Invest 2002;109:883-893.
16. Kabashima K, Sakata D, Nagamachi M, Miyachi Y, Inaba K, Narumiya S. Prostaglandin E2-EP4 signaling initiates skin immune responses by promoting migration and maturation of Langerhans cells. Nat Med 2003;9:744-749.
17. Amano H, Hayashi I, Endo H, Kitasato H, Yamashina S, Maruyama T, et al. Host prostaglandin E(2)-EP3 signaling regulates tumor-associated angiogenesis and tumor growth. J Exp Med 2003;197:221-232.
18. Wanner GA, Muller P, Ertel W, Busch CJ, Menger MD, Messmer K. Differential effect of cyclooxygenase metabolites on proinflammatory cytokine release by Kupffer cells after liver ischemia and reperfusion. Am J Surg 1998;175:146-151.
19. Arai M, Peng XX, Currin RT, Thurman RG, Lemasters JJ. Protection of sinusoidal endothelial cells against storage/reperfusion injury by prostaglandin E2 derived from Kupffer cells. Transplantation 1999;68:440-445.
20. Stachura J, Tarnawski A, Ivey KJ, Mach T, Bogdal J, Szczudrawa J, et al. Prostaglandin protection of carbon tetrachloride-induced liver cell necrosis in the rat. Gastroenterology 1981;81:211-217.
21. Miyazaki M, Makowka L, Falk RE, Falk JA, McDonell M, Venturi D. Protection of thermochemotherapeutic-induced lethal acute hepatic necrosis in the rat by 16,16-dimethyl prostaglandin E2. J Surg Res 1983;34:415-426.
22. Takano M, Nishimura H, Kimura Y, Washizu J, Mokuno Y, Nimura Y, et al. Prostaglandin E2 protects against liver injury after Escherichia coli infection but hampers the resolution of the infection in mice. J Immunol 1998;161:3019-3025.
23. Masaki N, Ohta Y, Shirataki H, Ogata I, Hayashi S, Yamada S, et al. Hepatocyte membrane stabilization by prostaglandins E1 and E2: favorable effects on rat liver injury. Gastroenterology 1992;102:572-576.

24. Suzawa T, Miyaura C, Inada M, Maruyama T, Sugimoto Y, Ushikubi F, et al. The role of prostaglandin E receptor subtypes (EP1, EP2, EP3, and EP4) in bone resorption: an analysis using specific agonists for the respective EPs. Endocrinology 2000;141:1554-1559.
25. Inoue H, Takamori M, Shimoyama Y, Ishibashi H, Yamamoto S, Koshihara Y. Regulation by PGE2 of the production of interleukin-6, macrophage colony stimulating factor, and vascular endothelial growth factor in human synovial fibroblasts. Br J Pharmacol 2002;136:287-295.
26. Xiao CY, Yuhki K, Hara A, Fujino T, Kuriyama S, Yamada T, et al. Prostaglandin E2 protects the heart from ischemia-reperfusion injury via its receptor subtype EP4. Circulation 2004;109:2462-2468.
27. Zacharowski K, Olbrich A, Thiemermann C. Reduction of myocardial injury by the EP3 receptor agonist TEI-3356. Role of protein kinase C and of K(ATP)-channels. Eur J Pharmacol 1999;367:33-39.
28. Hohlfeld T, Meyer-Kirchrath J, Vogel YC, Schror K. Reduction of infarct size by selective stimulation of prostaglandin EP(3)receptors in the reperfused ischemic pig heart. J Mol Cell Cardiol 2000;32:285-296.
29. McCullough L, Wu L, Haughey N, Liang X, Hand T, Wang Q, et al. Neuroprotective function of the PGE2 EP2 receptor in cerebral ischemia. J Neurosci 2004;24:257-268.
30. Strassmann G, Patil-Koota V, Finkelman F, Fong M, Kambayashi T. Evidence for the involvement of interleukin 10 in the differential deactivation of murine peritoneal macrophages by prostaglandin E2. J Exp Med 1994;180:2365-2370.
31. Yamane H, Sugimoto Y, Tanaka S, Ichikawa A. Prostaglandin E(2) receptors, EP2 and EP4, differentially modulate TNF-alpha and IL-6 production induced by lipopolysaccharide in mouse peritoneal neutrophils. Biochem Biophys Res Commun 2000;278:224-228.
32. Shinomiya S, Naraba H, Ueno A, Utsunomiya I, Maruyama T, Ohuchida S, et al. Regulation of TNFalpha and interleukin-10 production by prostaglandins I(2) and E(2): studies with prostaglandin receptor-deficient mice and prostaglandin E-receptor subtype-selective synthetic agonists. Biochem Pharmacol 2001;61:1153-1160.
33. Kuroda E, Yamashita U. Mechanisms of enhanced macrophage-mediated prostaglandin E2 production and its suppressive role in Th1 activation in Th2-dominant BALB/c mice. J Immunol 2003;170:757-764.
34. Marra F, DeFranco R, Grappone C, Milani S, Pastacaldi S, Pinzani M, et al. Increased expression of monocyte chemotactic protein-1 during active hepatic fibrogenesis: correlation with monocyte infiltration. Am J Pathol 1998;152:423-430.
35. Afford SC, Fisher NC, Neil DA, Fear J, Brun P, Hubscher SG, et al. Distinct patterns of chemokine expression are associated with leukocyte recruitment in alcoholic hepatitis and alcoholic cirrhosis. J Pathol 1998;186:82-89.
36. Bone-Larson CL, Hogaboam CM, Evanhoff H, Strieter RM, Kunkel SL. IFN-gamma-inducible protein-10 (CXCL10) is hepatoprotective during acute liver injury through the induction of CXCR2 on hepatocytes. J Immunol 2001;167:7077-7083.
37. Marra F. Chemokines in liver inflammation and fibrosis. Front Biosci 2002;7:d1899-d1914.
38. Ajuebor MN, Hogaboam CM, Le T, Swain MG. C-C chemokine ligand 2/monocyte chemoattractant protein-1 directly inhibits NKT cell IL-4 production and is hepatoprotective in T cell-mediated hepatitis in the mouse. J Immunol 2003;170:5252-5259.
39. Nitta M, Hirata I, Toshina K, Murano M, Maemura K, Hamamoto N, et al. Expression of the EP4 prostaglandin E2 receptor subtype with rat dextran sodium sulphate colitis: colitis suppression by a selective agonist, ONO-AE1–329. Scand J Immunol 2002;56:66-75.
40. Kasai K, Sato S, Suzuki K. A novel prostaglandin E receptor subtype agonist, 0N0–4819, attenuates acute experimental liver injury in rats. Hepatol Res 2001;21:252-260.
41. Henley KS, Lucey MR, Normolle DP, Merion RM, McLaren ID, Crider BA, et al. A double-blind, randomized, placebo-controlled trial of prostaglandin E1 in liver transplantation. HEPATOLOGY 1995;21:366-372.
42. Klein AS, Cofer JB, Pruett TL, Thuluvath PJ, McGory R, Uber L, et al. Prostaglandin E1 administration following orthotopic liver transplantation: a randomized prospective multicenter trial. Gastroenterology 1996; 111:710-715.