Inhibition of mPGES-1 protects the liver from ischemia/reperfusion injury through prostanoid EP2 receptor

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Objectives: Hepatic ischemia/reperfusion (I/R) injury is a major adverse reaction to liver surgery. This study aims to examine the role of endogenous prostaglandin E₂ (PGE₂) produced by inducible microsomal PGE synthase-1 (mPGES-1), a terminal enzyme of PGE₂ generation, in liver injury after hepatic I/R.

Methods: Male mPGES-1 deficient (mPGES-1⁻/⁻) mice or their wild counterparts (WT) were subjected to 60 minutes of partial hepatic ischemia followed by reperfusion.

Results: During hepatic I/R, the hepatic expression of mPGES-1 was enhanced in WT mice. mPGES-1 was expressed in CD68-positive cells (macrophages) and Gr-1-positive cells (neutrophils). Compared with WT mice, mPGES-1⁻/⁻ mice exhibited attenuated levels of alanine aminotransferase and necrotic area. This was associated with reduced mRNA levels of proinflammatory cytokines. Hepatic neutrophils in mPGES-1⁻/⁻ mice were decreased, with down-regulated expression of neutrophil-attracting chemokines and their receptors. Hepatic I/R enhanced the hepatic expression of PGE receptor subtype, E prostanoid receptor 2 (EP2), in WT mice, when compared with mPGES-1⁻/⁻ mice. Selective EP2 antagonist (PF04418948) reduced liver injury and hepatic neutrophils with down-regulated expression of chemokines and their receptors at 24-hour postreperfusion.

Conclusions: Inhibition of mPGES-1 and EP2 signaling is a potential therapeutic strategy for attenuation of liver injury after hepatic I/R.

Key words: mPGES-1, EP2, neutrophil, ischemia, reperfusion

Introduction

During major liver resection or liver transplantation, the liver inevitably suffers direct mechanical and hypoxic injury with cellular necrosis. Further dysfunction and damage results from excessive activation of a sterile inflammatory response upon restoration of blood flow even in the absence of microbial pathogens. This process of ischemia and reperfusion (I/R) injury significantly contributes to the complications that follow these surgical procedures.

PGE₂, a metabolite of arachidonic acid produced via cyclooxygenase (COX), is an important mediator of pain and inflammation. The final step of PGE₂ generation is catalyzed by specific PGE synthases (PGESs), of which there are at least three isoforms: cytosolic PGES (cPGES), and two types of microsomal PGES, mPGES-1 and mPGES-2. The mPGES-1 type is the dominant source of PGE₂ biosynthesis under basal conditions or during inflammatory states. In hepatic I/R injury, inhibition of COX-2, an inducible isoform of COX, minimizes the injury. By contrast, PGE₂ has been implicated in the prevention of liver injury. The administration of PGE₂ protects rodents against hepatic I/R injury. To date, the implication of PGE₂ in hepatic I/R injury remains controversial. Therefore, the aims of the present study were to examine the role of endogenous PGE₂ in liver injury after hepatic I/R using...
mPGES-1 deficient (mPGES-1−/−) mice, and to identify the mechanisms underlying hepatic I/R injury mediated by PGE2.

Materials and Methods

Animals
Male C57Bl/6 wild-type (WT) mice (8-weeks old) were obtained from Clea Japan (Tokyo). Male mPGES-1 knockout (mPGES-1−/−) mice (8-weeks old) with a C57BL/6 hybrid background were developed previously. All animal experimental procedures were approved by the Animal Experimentation and Ethics Committee of the Kitasato University School of Medicine (2017-079) and were performed in accordance with the guidelines for animal experiments set down by Kitasato University School of Medicine.

Model of hepatic I/R injury
Animals underwent either sham surgery or I/R. Hepatic I/R was induced as described previously. Briefly, the blood supply to the median and left hepatic lobes was occluded for 60 minutes using an atraumatic vascular clamp (Roboz Surgical Instrument Co., Washington, DC, USA), and reperfusion was initiated by removing the clamp. The sham control mice underwent the same protocol without vascular occlusion.

Experimental protocols
Mouse livers were subjected to ischemia for 60 minutes. Blood was drawn and livers were excised at 6 and 24 hours after reperfusion. The serum was used to determine alanine aminotransferase (ALT) activity in a Dri-Chem 4000 Chemistry Analyzer System (Fujifilm, Tokyo). A part of the excised ischemic left lobe of the liver was fixed with periodate-lysine-paraformaldehyde fixative for histological evaluation.

In another experiment, mice were treated with either EP2 or EP4 receptor antagonists. The EP2 antagonist (PF04418948) was obtained from Cayman Chemical (Ann Arbor, MI, USA). The EP4 antagonist (ONO-AE3-208) was donated by ONO Pharmaceutical (Osaka). The respective EP antagonists (20 mg/kg body weight, i.p.), dissolved in 40% dimethyl sulfoxide (DMSO) prepared in saline in a total volume of 0.2 ml, were administered at the time of clip removal and every 12 hours following reperfusion. The doses of EP antagonists were selected based on previous results. As a control group, mice were administered with the vehicle DMSO.

Immunofluorescence staining
Sections were then incubated with rabbit anti-mouse mPGES-1 polyclonal antibody (1:50; Cayman Chemical, Ann Arbor, MI, USA), rat anti-mouse Gr-1 IgG2b monoclonal antibody (mAb) (1:100 AbD Serotec, Raleigh, NC, USA), rat anti-mouse CD68 IgG2a mAb (1:100 AbD Serotec, Raleigh), and anti-EP2 rabbit polyclonal antibody (1:50; Cayman Chemical). Images were captured using a fluorescence microscope (Biozero BZ-X710 Series; Keyence, Osaka). After labeling, six low-power optical fields (400× magnification) were randomly selected and the positive cells were counted. At least five animals were analyzed per marker.

Real-time reverse transcript polymerase chain reaction (RT-PCR)
Transcripts encoding COX-2, mPGES-1, tumor necrosis factor-α (TNFα), interleukin 1-β (IL-1β), C-X-C motif

<table>
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<th>Gene</th>
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</tr>
<tr>
<td>EP4</td>
<td>CTGGGACCTTGTGTTGAAGC</td>
<td>ACCCGACAGACCCAGAAG</td>
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mPGES-1 and liver injury
ligand 1 (CXCL1), CXCL2, CXC receptor 1 (CXCR1), CXCR2, EP1,2,3,4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured by real-time reverse transcript polymerase chain reaction (RT-PCR). The primers used for real-time PCR were designed using Primer 3 software (http://primer3.sourceforge.net/) based on data from GenBank; the sequences are listed in Table 1. Data were normalized to the expression level of GAPDH in each sample.

**Statistical analysis**
All results are expressed as the mean ± standard error of the mean (SEM). All statistical analyses were performed using GraphPad Prism version 5.01 (GraphPad Software, La Jolla, CA, USA). Student’s t-test was used for comparisons between the two groups. One-way analysis of variance followed by Bonferroni’s post-hoc test was used for comparisons between multiple groups. A P-value of <0.05 was considered statistically significant.

**Results**

**Hepatic I/R injury in mPGES-1-/- mice**
The mRNA levels of mPGES-1 in livers from WT mice were elevated at 6 and 24 hours postreperfusion as compared sham-controls (Figure 1A). Regarding the up-stream enzyme of PGES, COX-2 in both genotypes, was enhanced, peaking at 6 hours and declining at 24 hours postreperfusion. There were no differences in COX-2 mRNA levels between WT and mPGES-1-/- mice (Figure 1B). The levels of the two other isoforms mPGES-2 and cPGES did not change significantly in either genotype (not shown). Immunofluorescence demonstrated that hepatic I/R caused enhancement of mPGES-1 expression in CD68-positive cells and in Gr-1-positive cells, indicating that mPGES-1 in response to hepatic I/R was derived from macrophages and neutrophils (Figure 1C).

To investigate the functional role of mPGES-1 in hepatic I/R injury, we compared ALT levels in WT and mPGES-1-/- mice (Figure 1D). Both WT and mPGES-1-/- mice experienced maximal injury at 6 hours postreperfusion, then decreased at 24 hours postreperfusion. Compared with mPGES-1-/- mice, WT mice showed aggravation of hepatic I/R injury based on ALT levels (Figure 1D). We also determined the hepatic mRNA levels of proinflammatory mediators. mRNA expressions of TNF-α and IL-1β in WT mice were greater than those in mPGES-1-/- mice (Figure 1E).

**Neutrophil accumulation with hepatic I/R injury**
Inflammatory cells including hepatic neutrophils and macrophages lead to the development of hepatic I/R injury. Therefore, we investigated accumulation of neutrophils and macrophages in the livers after hepatic I/R. Immunofluorescence revealed that Gr-1-positive cells (neutrophils) were substantially accumulated into the WT-livers at 24 hours postreperfusion, and mPGES-1-/- mice exhibited reduced hepatic neutrophils (Figure 2A). There was no significant difference in the number of CD68-positive cells (macrophages) between the two genotypes (Figure 2B). Additionally, the numbers of hepatic neutrophils recruited to the livers were smaller than those of macrophages (Figure 2A, B). We determined the levels of neutrophil-attracting chemokines and their receptors. The hepatic mRNA levels of chemokine CXCL1 and CXCL2 in WT mice were greater than those in mPGES-1-/- mice (Figure 2C). The expressions of CXCR1 and CXCR2 were also up-regulated in WT livers in comparison with mPGES-1-deficient livers (Figure 2D).

**PGE2-EP2 signaling for hepatic I/R injury**
PGE2 acts on target cells through PGE receptor subtypes identified as EP1, EP2, EP3, and EP4.9 To identify the EPs responsible for hepatic I/R injury, we determined the hepatic gene expression levels of each EP (Figure 3A, B, C). mRNA levels of EP2 and EP4 in WT mice were up-regulated at 6 and 24 hours postreperfusion as compared with sham controls. Compared with WT mice, mPGES-1-/- mice exhibited decreases in mRNA levels of EP2 and EP4 at 6 and 24 hours postreperfusion. To further clarify which EP mediates reperfusion injury, WT mice were treated with specific antagonists for EP2 and EP4. ALT levels in mice treated with the antagonists for EP2 (PF04418948), but not for EP4 (ONO-AE3-208) significantly decreased ALT levels (Figure 3D). Double immunofluorescence analysis showed that EP2 was mainly co-localized with expression of Gr-1 (neutrophils) at 24 hours postreperfusion (Figure 3E).

To further explore the role of EP2 in hepatic I/R injury, we determined accumulated Gr-1-positive cells (neutrophils) into livers treated with EP2 antagonist. EP2 antagonist reduced hepatic neutrophils as compared with vehicle (Figure 4A). These were associated with reduced levels of CXCL1 and CXCL2 (Figure 4B) as well as CXCR1 and CXCR2 (Figure 4C).

**Discussion**
The objective of the present study was to examine the
Figure 1. mPGES-1−/− mice show attenuation of hepatic I/R injury.

A, B. Changes in expression of mPGES-1 mRNA and COX-2 in the livers from WT and mPGES-1−/− mice. C. Double immunostaining of livers from WT and mPGES-1−/− mice with antibodies against mPGES-1 (green) and CD68 (red) or Gr-1 (red) at 24 hours postreperfusion. Hepatocyte nuclei are stained with DAPI (blue). White arrows indicate co-localization in double-labeled cells. All images are representative of three independent samples. Scale bars, 50 μm.

D. Changes in ALT after hepatic I/R in WT and mPGES-1−/− mice. E. Hepatic levels of mRNA for TNFα and IL-1β in WT and mPGES-1−/− mice. Data are expressed as the mean ± SEM from 6 mice per group.

*P < 0.05 vs. WT mice, #P < 0.05 vs. Sham-controls.
Figure 2. Decreases in hepatic neutrophils and neutrophil-attracting chemokines and their receptors in mPGES-1−/− mice after hepatic I/R

A, B. (A) Gr-1 and (B) CD68 assessed by immunofluorescence in WT and mPGES-1−/− mice at 24 hours postreperfusion. The numbers of immunoreactive-positive cells were counted. C, D. Changes in mRNA levels of (C) CXCL1/CXCL2 and (D) CXCR1/CXCR2 in livers from WT and mPGES-1−/− mice after hepatic I/R. Data are expressed as the mean ± SEM from 6 mice per group.

*P < 0.05 vs. WT mice.
Figure 3. Hepatic expression of EPs and effect of EP2 antagonist on hepatic I/R injury

A-C. Changes in hepatic expression of mRNA encoding EP1, EP2, EP3, and EP4 from WT and mPGES-1−/− mice subjected to (A) sham or (B) reperfusion for 6 hours and (C) 24 hours. D. Effect of EP2 and EP4 antagonists on ALT levels at 24 hours postreperfusion. The antagonists EP2 (PF04418948) and EP4 (ONO-AE3-208) were administered i.p. As a control group, mice were administered with vehicle (DMSO). E. Double immunostaining of livers from WT mice and mPGES-1−/− mice with antibodies against EP2 (green) and CD68 (red) or Gr-1 (red) at 24 hours postreperfusion. Hepatocyte nuclei are stained with DAPI (blue). White arrows indicate co-localization in double-labeled cells. All images are representative of three independent samples. Scale bars, 50 μm. Data are expressed as the mean ± SEM from 6 mice per group.

*P < 0.05 vs. WT mice.
Figure 4. Effects of EP2 antagonist on neutrophils recruitment and neutrophil-attracting chemokines and their receptors after hepatic I/R

A. Immunofluorescent pictures of Gr-1 in livers treated with vehicle (upper panel) and EP2 antagonist (PF04418948) (lower panel) Bar indicates 50 μm. Effect of EP2 antagonist (PF04418948) on the numbers of hepatic neutrophils at 24 hours postreperfusion. B, C. Treatment with EP2 antagonist (PF04418948) down-regulated the expression of chemokines (B) (CXCL1 and CXCL2) and their receptors (C) (CXCR1 and CXCR2) at 24 hours postreperfusion. Data are expressed as the mean ± SEM from 6 mice per group.

*P < 0.05 vs. Vehicle.
role of mPGES-1 in hepatic I/R injury and to elucidate the mechanisms by which endogenous PGE2 aggravates the injury. Here, we demonstrated that mPGES-1-deficient mice exhibited attenuation of hepatic I/R injury associated with reduction in proinflammatory cytokines and neutrophils infiltration through inactivation of EP2 signaling. These results suggest that inhibition of mPGES-1 attenuated hepatic I/R injury with neutrophil accumulation via prostanoid EP2 receptor.

During hepatic I/R, neutrophils are extensively recruited into the injured livers, and hepatic neutrophils mediate hepatic I/R injury. 8 The accumulated hepatic neutrophils produce proinflammatory cytokines, which in turn amplify hepatocellular damage. The present study demonstrated that increases in hepatic neutrophils and proinflammatory cytokines were correlated with hepatic I/R injury. In addition, we have reported that activated neutrophils can cause cell damage through generation of reactive oxygen species (ROS). 9 Therefore, it is likely that hepatic neutrophils mediate hepatic I/R injury through production of inflammatory mediators. Inhibition of mPGES-1 attenuated reperfusion injury associated with reduction of neutrophils and cytokines, indicating a crucial role of mPGES-1 in the liver injury. This result is consistent with recent studies showing that PGE2 synthetized by mPGES-1 enhances the inflammatory response. 10

The present study revealed that mPGES-1 is also expressed in resident macrophages, Kupffer cells (KCs), suggesting that mPGES-1 in KCs contributes to hepatic I/R injury. In particular, KCs are involved in early phase of hepatic I/R injury. 8 Although we did not demonstrate the conditions of KCs at 6-hour postreperfusion, it is suggested that reduced liver injury and proinflammatory cytokines in mPGES-1-/- mice at 6-hour postreperfusion would be caused by inactivation of KCs.

The function of mPGES-1-derived PGE2 is mediated by four G protein-coupled PGE2 receptor subtypes, EP1-EP4. 9 Out of the four EP receptors, hepatic EP2 and EP4 expressions were found to be up-regulated during hepatic I/R. However, the findings that inhibition of EP2 but not EP4 minimized the injury indicate that hepatic I/R injury is dependent on EP2 signaling. In addition, EP2 is upregulated in neutrophils, and EP2 antagonist reduces hepatic neutrophil accumulation, which is associated with attenuation of neutrophil-attracting chemokines and their receptors. Consistent with our results, PGE2/EP2 signaling pathway in peritoneal neutrophils contributes to abdominal inflammation. 12 EP2-mediated proinflammatory responses also have been demonstrated in models of Parkinson's disease, 13 endometriosis, 14 and asthma. 15

In conclusion, these findings suggest that PGE2 derived from inducible mPGES-1 exerts proinflammatory action in hepatic I/R injury through EP2 signaling. Inhibition of mPGES-1 or EP2 provides potential for the attenuation of inflammatory processes and may be a good target for the treatment of acute liver injury elicited by hepatic I/R.

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