Preconditioning by Low Dose LPS Prevents Subsequent LPS-Induced Severe Liver Injury via Nrf2 Activation in Mice

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ABSTRACT

Background Sepsis is a syndrome triggered by endotoxin lipopolysaccharide (LPS) during bacterial infection. Sepsis sometimes recurs, with the second sepsis giving rise to a different phenotype because of disease modification by the preceding sepsis. Such a protective modification is called a preconditioning (PC) effect. PC is an endogenous protective mechanism by which sublethal damage confers tolerance to a subsequent lethal load. Oxidative stress is one of the important pathogenetic mechanisms that occur in sepsis. The nuclear factor erythroid 2 (NF-E2)-related factor-2 (Nrf2) system is a key regulatory transcription factor that protects organs and cells against oxidative stress and may be associated with the PC effect in repeated sepsis.
Methods The effect of PC induced by low-dose LPS on survival rate and liver injury against subsequent high-dose LPS stimulation was examined using a mouse model of sepsis. In order to understand the detailed mechanism(s) involved in the PC effect within the liver, gene expression array was performed. As a candidate mechanism of PC, the activation of the Nrf2 system was analyzed using Nrf2 reporter mice. Furthermore, the induction of heme oxygenase-1 (HO-1), one of the main targets of Nrf2, in the liver was examined by immunoblotting and immunohistochemistry. The PC effect on liver injury induced by LPS was further examined using Nrf2-deficient mice.
Results PC by LPS (1.7 or 5.0 mg/kg body weight, intraperitoneally) increased the survival rate of mice and decreased liver injury in response to a subsequent injection of a lethal level of LPS (20 mg/kg body weight). DNA array revealed that the gene ontology term "antioxidant activity" as one of the candidate mechanisms of the PC effect by LPS. In Nrf2 reporter mice, PC immediately and intensely enhanced luminescence that indicated Nrf2 activation after subsequent LPS injection. The induction of HO-1 by LPS was also enhanced by preceding PC, and its induction was observed mainly in Kupffer cells of the liver. In Nrf2-deficient mice, the induction of HO-1 in Kupffer cells and the hepatoprotective effect of PC were decreased as compared with wild-type mice.
Conclusion Our results suggest that activation of the Nrf2 system is, at least in part, one of the mechanisms of a PC effect in the mouse liver in the case of repeated LPS stimulation.

Key words heme oxygenase-1; Kupffer cells; lipopolysaccharide; NF-E2-related factor 2; preconditioning

Sepsis is a complex syndrome characterized by an enhanced innate immune response to bacterial infection. Lipopolysaccharide (LPS) endotoxin within the bacterial wall triggers a release of proinflammatory mediators from macrophages and neutrophils through a Toll-like receptor 4 (TLR4) signaling pathway that mediates host damage.[1] Sepsis sometimes recurs where the second sepsis may differ from the first sepsis on the type of organ disorder induced.[2] In this regard, the first sepsis may modify the protective reaction of organs to the pathogenesis of the second sepsis.

Preconditioning (PC) is an endogenous protective mechanism for organs by which a sublethal stress, including ischemia,[3–5] metals,[6] and inflammation,[7, 8] confers tolerance to a subsequent lethal stress. The detailed mechanisms of PC are not yet fully understood, however, experimental and clinical evidence from various sources, especially in the ischemic preconditioning field, have highlighted alterations in gene expression[3–5] and epigenomic modifications[9] as candidate mechanisms of the PC phenomenon.

Oxidative stress contributes, at least in part, to the pathogenesis of LPS-induced sepsis and inflammation.[10] Therefore, reduction of oxidative stress via gene expression of antioxidative molecules may be associated with PC mechanisms. Nuclear factor erythroid 2 (NF-E2)-related factor-2 (Nrf2) is known as a key regulator of an antioxidative response.[11] Nrf2 usually binds to Keap1, an E3 ubiquitin ligase, and is located in the extranuclear space of cells. Under normal conditions, Nrf2 is unstable because it is proteolyzed by an ubiquitin-proteasome system; however, once cells are exposed to oxidative stress, Nrf2 translocates to the nucleus and acts as a transcription factor. Nrf2 binds to the antioxidant response element (ARE) and upregulates ARE-driven genes such as heme oxygenase-1 (HO-1).[11] Indeed, LPS induced Nrf2-related gene expression and Nrf2-deficient mice are vulnerable to LPS-induced sepsis.[12–17]

In the present study, we focused on the role of Nrf2 in PC under recurrent LPS exposure.

MATERIALS AND METHODS
Chemicals and antibodies
LPS from *Salmonella enterica* serotype *enteritidis* was purchased from Sigma (St. Louis, MO). Beetle luciferin for Nrf2 reporter mice was obtained from Promega (Madison, WI). Antibodies against HO-1 and CD163 were obtained from Santa Cruz (Dallas, TX), and anti-β-actin antibody was obtained from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase (HRP)-linked anti-rabbit antibody for western blotting was obtained from GE Healthcare (Buckinghamshire, UK).

Animals
C57BL/6 mice ($n = 61$, male) were obtained from CLEA Japan (Tokyo, Japan). Nrf2-deficient mice ($n = 12$, male) and Nrf2 reporter mice (OKD48 mice)[18] ($n = 3$, male) were kindly provided by Dr. Warabi (University of Tsukuba, Tsukuba, Japan) and Dr. Iwawaki (Gunma University, Maebashi, Japan), respectively. The mice were housed under standardized conditions of light (06:00–18:00), temperature (25 °C), and humidity (approximately 50%), and were allowed free access to food and water. The mice used in this study were treated in accordance with the Guidelines for Animal Experimentation of Tottori University (No.14-Y-01). All surgical procedures and collecting blood samples were performed under deep anesthesia with pentobarbital, and in vivo Nrf2 reporter assay was carried out under inhalation anesthesia with isoflurane, and all efforts were made to minimize suffering.

Experimental procedures are shown in Fig. 1. Brief-
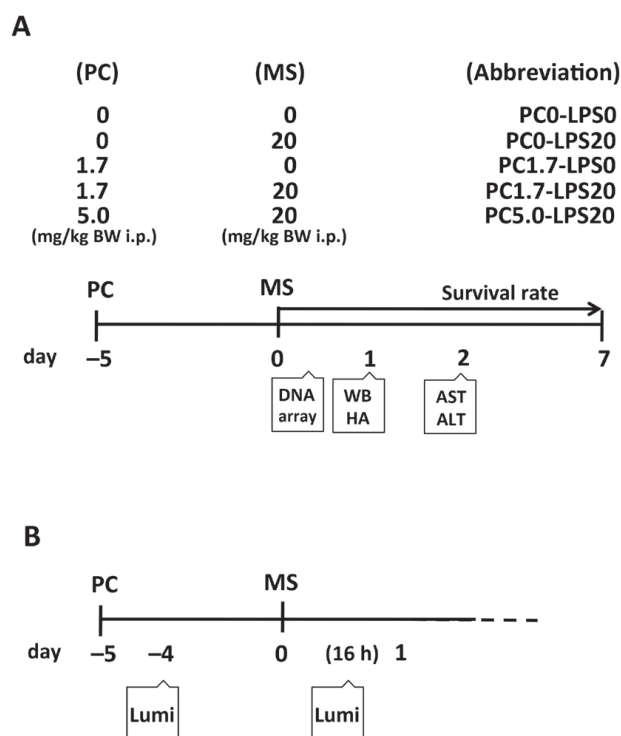
Fig. 1. Experimental protocol performed in the present study. (**A**) PC (0, 1.7, or 5.0 mg/kg BW LPS, i.p.) was carried out at day –5 in C57BL/6 mice. At day 0, a high dose (20 mg/kg BW) of LPS was injected i.p. as a MS. Survival rates were followed for 7 days (days 0–7). Tissue sampling for DNA array was carried out 12 h after MS. Western blotting (WB), histological analyses (HA), and plasma aminotransferase assay (ALT and AST) were carried out at days 1, 1, and 2, respectively. Abbreviations of each condition and how LPS was injected are described in the figure. (**B**) Time course of *in vivo* Nrf2 reporter assay. Luminescence (Lumi) was detected 24 h after PC (on day –4) and 16 h after MS by LPS. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BW, body weight; HA, histological analyses; i.p., intraperitoneal injection; LPS, lipopolysaccharide; Lumi, luminescence; MS, main stimulation; Nrf2, NF-E2-related factor-2; PC, preconditioning; WB, western blotting.

ly, the paired administration of LPS, PC and main stimulation (MS), was carried out. Firstly, 0 (vehicle), 1.7, or 5.0 mg/kg body weight (BW) of LPS dissolved in PBS was administered intraperitoneally (i.p.) in C57BL/6 mice as PC at day –5. Subsequently, after 5 days (day 0), 0 or 20 mg/kg BW of LPS was administered i.p. to each PC group as the MS. Hereafter, these five groups: i) 0 mg/kg BW LPS as PC and 0 mg/kg BW LPS as MS; ii) 0 mg/kg BW LPS as PC and 20 mg/kg BW LPS as MS; iii) 1.7 mg/kg BW LPS as PC and 0 mg/kg BW LPS as MS; iv) 1.7 mg/kg BW LPS as PC and 20 mg/kg BW LPS as MS, and v) 5.0 mg/kg BW LPS as PC and 20 mg/kg BW LPS as MS, were referred to as: i) PC0-LPS0; ii) PC0-LPS20; iii) PC1.7-LPS0; iv) PC1.7-LPS20, and v) PC5.0-LPS20, respectively (Fig. 1A). Af-

ter the time point shown in Fig. 1, DNA array (Fig. 1A), an *in vivo* Nrf2 reporter assay (Fig. 1B), westernblotting (Fig. 1A), histological analyses (Fig. 1A), and aspartate aminotransferase (AST)/alanine aminotransferase (ALT) assays in blood (Fig. 1A) were performed. Survival rates were followed until day 7.

DNA microarray

Total RNAs were prepared from liver tissues using an SV Total RNA isolation kit (Promega). In order to examine the effect of PC with 5.0 mg/kg BW of LPS on the alteration of gene expression, especially in the early stages, RNAs were isolated from liver tissue collected from PC0-LPS20 mice ($n = 1$) and PC5.0-LPS20 mice ($n = 1$) 12 h after MS. We compared gene expression between PC0-LPS20 and PC5.0-LPS20 groups using DNA microarray (GeneChip Mouse Gene 2.0 ST array, Filgen, Nagoya, Japan). Using microarray data, gene ontology (GO) analysis (Filgen) was performed to classify the genes by function.

In vivo Nrf2 reporter assay (OKD48 mice)

In order to clarify when and where Nrf2 was activated in mice after LPS injection, an *in vivo* Nrf2 reporter assay was performed. For this experiment, Nrf2 reporter mice, known as OKD48 mice, were used. OKD48 mice were treated with 0, 1.7, and 5.0 mg/kg BW of LPS (i.p.) as PC, and after 24 h, D-luciferin (0.15 mg/kg BW) dissolved in PBS was administered intraperitoneally. Luminescence was detected using an *in vivo* imaging system IVIS (Xenogen, Alameda, CA). In a similar manner, Nrf2 activation in PC0-LPS20, PC1.7-LPS20, and PC5.0-LPS20 mice groups was examined 16 h after MS.

AST and ALT assays

Blood samples (approximately 200 μL each) from PC0-LPS20 and PC1.7-LPS20 mice were collected from the heart using 25G needles at day 2, and bloods were centrifuged at $1,200 \times g$ to separate plasma and blood cells. Using AST and ALT assay kits (Transaminase CII-test; Wako, Osaka, Japan), plasma AST and ALT levels were measured as an index of liver injury.

Western blotting

Liver tissues for immunoblot analyses were collected from PC0-LPS0, PC1.7-LPS0, PC0-LPS20, and PC1.7-LPS20 groups of mice 24 h after MS. Tissues were lysed in SDS sample buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 2 mM ethylenediaminetetraacetic acid). Protein aliquots (20 μg) were separated on the basis of

molecular size on 12% polyacrylamide gels, transferred onto polyvinylidene difluoride membranes (Hybond-P; GE Healthcare), and hybridized with anti-HO-1 or β-actin antibody in PBS with 0.1% Tween 20 at room temperature for 1 h. The immunoreactive signal was detected using HRP-linked anti-rabbit or mouse IgG and ECL detection reagents (GE Healthcare). The protein content of each sample was measured using a bicinchonic acid (BCA) protein assay system (Thermo Fisher Scientific, Rockford, IL).

Histological studies

Hematoxylin-eosin (HE) staining and immunohistochemistry were performed on paraffin-embedded sections of mouse liver, brain, lung, and kidney 24 after MS. A portion of liver tissue was fixed by perfusion using 4% paraformaldehyde (Wako), and embedded in paraffin. Paraffin sections (4 μm) were deparaffinized by placing slides into three changes of xylene, followed by rehydration in a graded ethanol series. Sections were treated with methanol containing 3% H_2O_2 at room temperature for 30 min to reduce endogenous peroxidase activity. HE staining was performed following a standard protocol.

HO-1 and CD163, as a marker of Kupffer cells, were detected by incubating sections in HO-1 antibody at a 1:1000 dilution or CD163 antibody at a 1:1000 dilution in PBS at 4 °C overnight. The labeled antigens were visualized using a Histofine kit (Nichirei Biosciences, Tokyo, Japan), followed by followed by manufacturer's protocol using HRP/3,3-Diamiobenzidine (DAB) and/or alkaline phosphatase/fuchsin reaction. For double staining for HO-1 and CD163, the signal of HO-1 was visualized by alkaline phosphatase/fuchsin reaction (red) and that of CD163 was visualized by HRP/DAB reaction (brown). Finally, sections were counterstained with hematoxylin and observed by microscopy (BZ-9000; Keyence, Tokyo, Japan).

Statistics

Quantitative data were assessed by ANOVA using the Stat View software. The criterion for statistical significance was $P < 0.05$ for the AST/ALT assay All values are expressed as means ± S.D. Survival curves were compared by the use of Kaplan–Meiyer analysis and Log–rank test. Values of $P < 0.05$ were considered statistically significant. *P*-value in GO analysis was calculated by two tailed Fisher's extract test using manufacturer's software (Filgen).
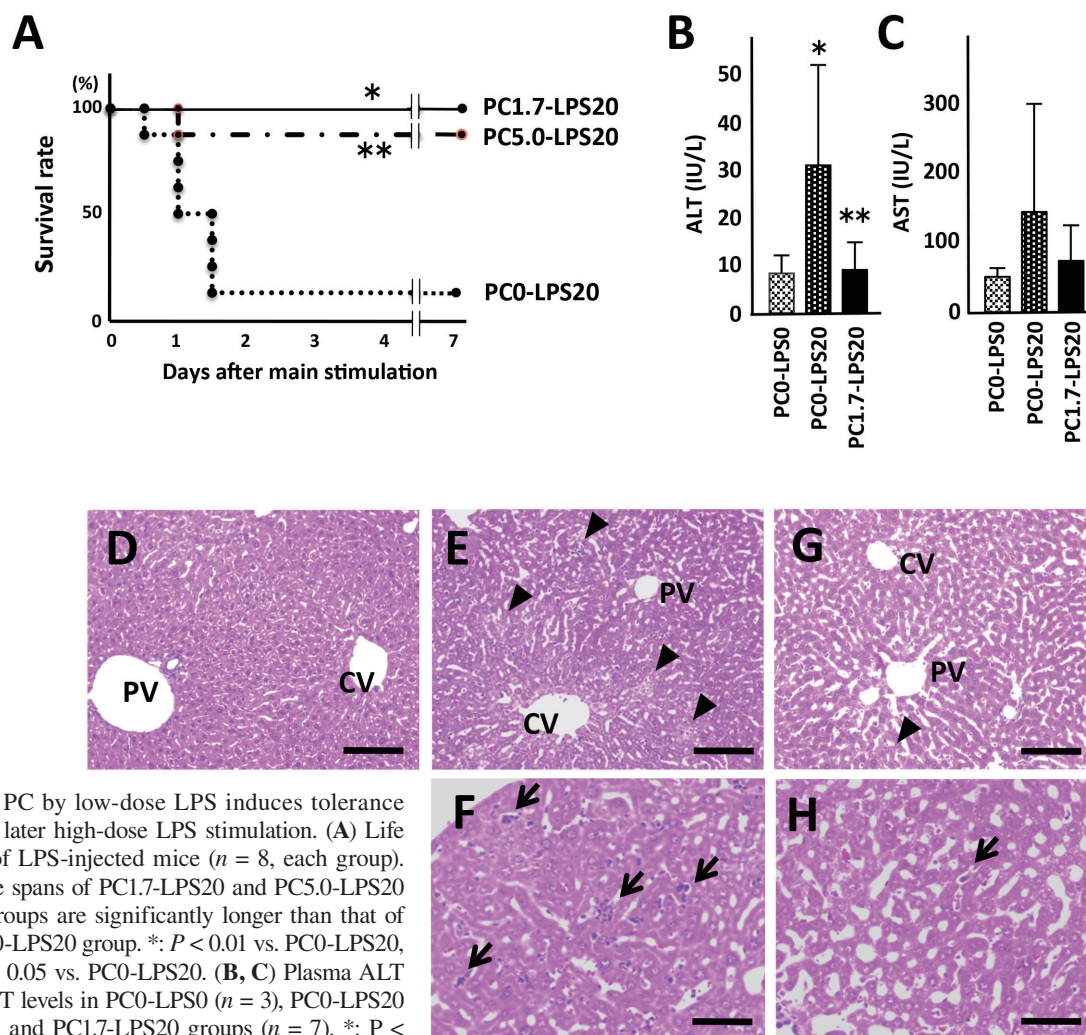
Fig. 2. PC by low-dose LPS induces tolerance against later high-dose LPS stimulation. (**A**) Life spans of LPS-injected mice (*n* = 8, each group). The life spans of PC1.7-LPS20 and PC5.0-LPS20 mice groups are significantly longer than that of the PC0-LPS20 group. *: *P* < 0.01 vs. PC0-LPS20, **: *P* < 0.05 vs. PC0-LPS20. (**B, C**) Plasma ALT and AST levels in PC0-LPS0 (*n* = 3), PC0-LPS20 (*n* = 4), and PC1.7-LPS20 groups (*n* = 7). *: P < 0.05 vs. PC0-LPS0, **: *P* < 0.05 vs. PC0-LPS20. (ANOVA) (**D–H**) HE staining of liver tissues. (**D–H**) HE staining of the liver. (**D**) The liver from a representative PC0-LPS0 mouse. (**E**) The liver from a representative PC0-LPS20 mouse. Sites of focal necrosis are scattered within the liver (arrowheads). (**F**) The liver from a representative PC0-LPS20 mouse at higher magnification. Neutrophil infiltration is observed in the sinusoids (arrows). (**G**) The liver from a representative PC1.7-LPS20 mouse. A small area of focal necrosis (arrowhead) is observed in the liver. (**H**) The liver from a representative PC1.7-LPS20 mouse at higher magnification. A small number of infiltrating neutrophils is observed in a sinusoid (arrow). Scale bars (**D**)(**E**)(**G**) represent 200 μm and (**F**)(**H**) 100 μm. ALT, alanine aminotransferase; AST, aspartate aminotransferase; CV, central vein; HE, hematoxylin-eosin; LPS, lipopolysaccharide; PC, preconditioning; PV, portal vein.

RESULTS

PC by low-dose LPS gives the tolerance against subsequent stimulation by high-dose LPS

We examined the effect of PC by LPS on survival rate after the subsequent administration of a high- dose (20 mg/kg BW) of LPS. Although the administration of high-dose LPS (20 mg/kg BW) decreased the survival rate of mice (to approximately 12.5% after 1.5 days), PC using medium or low doses of LPS (5.0 or 1.7 mg/kg BW, respectively) improved the survival rate to 87.5 % and 100%, respectively (Fig. 2A).

In order to clarify the effect of PC by low-dose LPS on liver injury in response to a subsequent high dose of

LPS, we measured plasma ALT and AST levels in mice 2 days after MS. Although the plasma ALT level was significantly elevated in the PC0-LPS20 mice group, the ALT level in the PC1.7-LPS20 mice group was significantly decreased compared with that of the PC0-LPS20 group (Fig. 2B). The plasma AST level showed a similar pattern to the ALT level, however the difference between the PC0-LPS20 and PC1.7-LPS20 group was not statistically significant (Fig. 2C).

We performed histological analyses of liver tissues 24 after MS (Figs. 2D–H). In livers from the PC0-LPS20 mice group, small areas of focal necrosis (Fig. 2E) and infiltrating neutrophils were observed in the

sinusoids of the liver (Fig. 2F). On the other hand, livers from the PC1.7-LPS20 mice group showed milder focal necrosis and cellular infiltration compared with the PC0-LPS20 group (Figs. 2G and H). We also investigated the effect of PC on its protective role against sepsis in the brain, lungs, and kidneys. Microhemorrhages in the cerebral cortex 7 days after MS were reduced in brains from PC1.7-LPS20 mice compared with those in PC0-LPS20 mice (data not shown). In lungs from PC1.7-LPS20 mice, the number of inflammatory cells 24 h after MS was reduced compared with that for PC0-LPS20 mice (data not shown). In kidneys from PC1.7-LPS20 mice, kidney tubular necrosis 7 days after MS was milder than that in PC0-LPS20 mice (data not shown).

PC by low-dose LPS enhances Nrf2 activation during later stimulation by high-dose LPS

In order to identify the mechanism(s) which contributed to a PC effect, we performed gene expression array and compared gene expression patterns between PC0-LPS20 and PC5.0-LPS20 groups. GO analysis using microarray data highlighted several classified genes that were activated by PC. We chose 5 molecular functions in GO terms by cutting off the line that met both a Z-score > 0 and a P value < 0.05: antioxidant activity, binding activity, catalytic activity, electron carrier activity, and transporter activity (Table 1). We then focused on "antioxidant activity" in GO terms, and investigated Nrf2 as a key regulator of antioxidant molecules.

Using Nrf2-reporter mice (OKD48 mice), we analyzed Nrf2 activation on the point of time and spatial elements. PC by intraperitoneal injection of 1.7 or 5.0 mgk/g BW LPS mildly activated Nrf2 in the abdominal cavity; luminescence was detected 24 h after PC (Fig. 3A; upper panel). An intraperitoneal administration of 20 mg/kg BW LPS as a MS enhanced the activation of Nrf2 not only in the abdominal cavity but also in the lungs 16 h after MS (Fig. 3A; lower panel, right). Furthermore, activation of Nrf2 in PC1.7-LPS20 and PC5.0-LPS20 mice was more intense and more immediate than that in the case of a single LPS injection (shown in the upper panel; Fig. 3A, middle and left).

We next examined the induction of HO-1 protein in the liver by western blotting since this enzyme is one of the major targets of Nrf2 24 h after MS. The expression of HO-1 in liver from a single LPS injection of PC1.7-LPS0 or PC0-LPS20 mice was minimal (Fig. 3B). In contrast, the induction of HO-1 was more intense in liver from PC1.7-LPS20 mice, than that from other groups, 24 h after MS (Fig. 3B).

In order to confirm what type of cells express HO-1 in the liver, we performed immunohistochemi-

cal analyses at the same time of western blotting. The inducible expression of HO-1 was observed mainly in the CD163-positive Kupffer cells but not in hepatocytes in liver from PC1.7-LPS20 mice (Fig 3D). In liver from PC0-LPS20, the expression of HO-1 in the CD163 positive cells was faint (Fig. 3C).

Table 1 . GO terms that are upregulated in PC5.0-LPS20 compared with PC0-LPS20

Molecular function (GO term)	Z-score	P-value
Antioxidant activity	2.458	0.0395
Binding	2.547	0.0003
Catalytic activity	6.788	< 0.0001
Electron carrier activity	2.668	0.0196
Transporter activity	3.084	0.0036

GO, gene ontology; LPS, lipopolysaccharide; PC, preconditioning.

Nrf2 deficiency cancels the protective effect of PC on liver disturbances

We further analyzed the PC effect on liver injury using Nrf2-deficient mice. In these mice, the induction of HO-1 in livers from PC1.7-LPS20 group mice was abolished 24 h after MS as compared with the PC1.7-LPS20 wild-type mice (Fig. 4A). Immunohistochemistry also showed a reduced expression of HO-1 in Kupffer cells (Figs. 4B and C). In Nrf2-deficient mice with PC0-LPS20 stimulation, liver tissue was severely damaged with microhemorrhage, and numerous neutrophils infiltrated the liver, 24 h after MS (Fig. 4D). In contrast, the PC1.7-LPS20 group showed a mild improvement in histological changes in the liver in terms of focal necrosis, microhemorrhage, and neutrophil infiltration (Figs. 4E and F), however, the protective effect of PC in Nrf2-deficient mice (Figs. 4E and F) was weaker than what was seen in wild-type mice (Figs. 2G and H).

DISCUSSION

Recently, increased attention has been given to the protective effect of PC against adverse effects such as sublethal ischemia, exposure to mild toxin, and inflammation.[3–8] Numerous reports suggest that PC prevents severe organ damage induced by later, more deleterious events.[3–8]

Sepsis is a syndrome characterized by an enhanced innate immune response to bacterial infection. LPS usually triggers a release of proinflammatory mediators, such as interleukins and nitric oxide, from macrophages and neutrophils through a TLR4/NF-ϰB signaling pathway that mediates host damage.[1] Sepsis often occurs
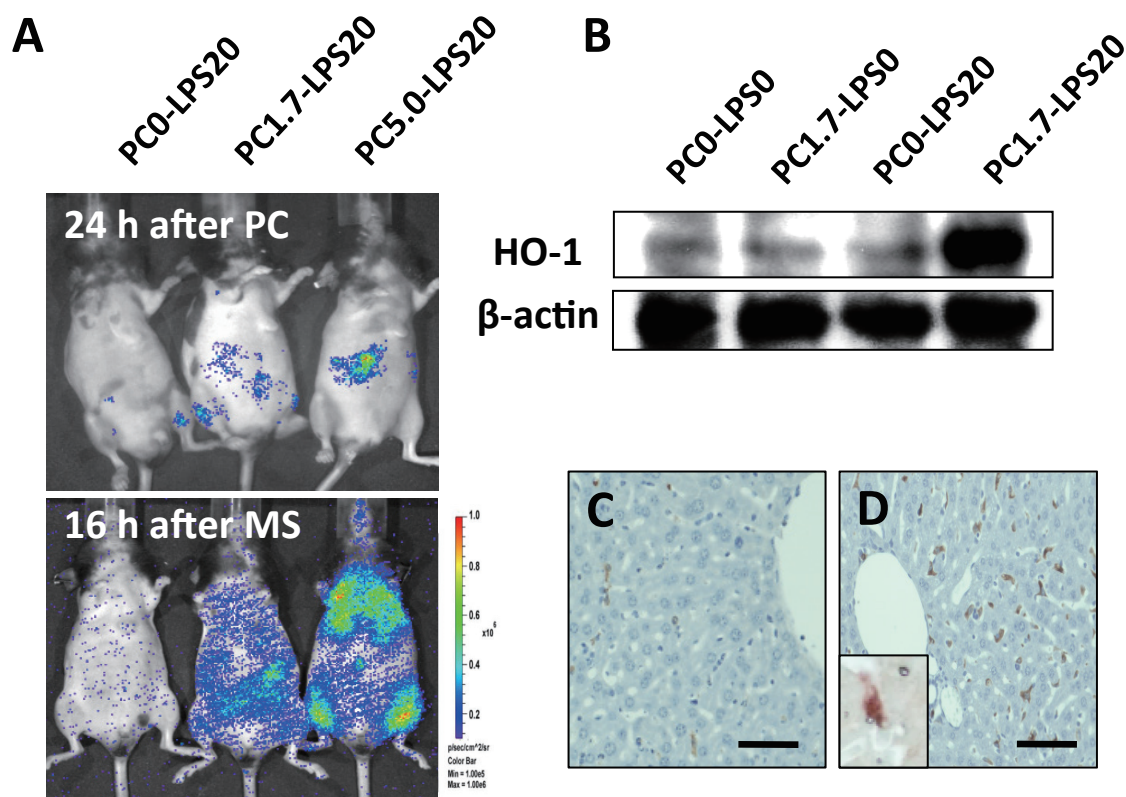
Fig. 3. PC by low-dose LPS enhances Nrf2 activation during subsequent stimulation by high-dose LPS. (**A**) An i*n vivo* Nrf2 reporter assay using OKD48 mice. The upper photograph shows luminescence 24 h after PC (day −4). The lower photograph shows luminescence 16 h after MS with LPS (20 mg/kg BW). An immediate and intense luminescence is observed in PC1.7-LPS20 and PC5.0-LPS20 mice. (**B**) The induction of HO-1 protein is shown. Immunoblots are representative of three independent experiments. HO-1 protein bands in liver samples from PC0-LPS0, PC1.7-LPS0, and PC0-LPS20 mice appear faint. The induced expression of HO-1 protein in liver from PC1.7-LPS20 mice is intense. (**C, D**) Immunohistochemistry using anti-HO-1 and anti-CD163 antibodies in the liver from PC0-LPS20 (**C**) and PC1.7-LPS20 mice (**D**). The expression of HO-1 (brown) is observed mainly in CD163-positive Kupffer cells. Small window in (**D**) represents a double staining for HO-1 (red) and CD163 (brown). Scale bars represent 100 μm. BW, body weight; HO-1, heme oxygenase-1; LPS, lipopolysaccharide; MS, main stimulation; Nrf2, NF-E2-related factor-2; PC, preconditioning.

repeatedly, with a second sepsis that may differ from the first sepsis in terms of organ disturbance.[2] For example, organs may sometimes gain a tolerance against sepsis when exposed to an initial, LPS-induced sepsis, which prevents severe organ disorders on the occurrence of a second sepsis.[2, 7, 8] Such a phenomenon, whereby a tolerance is gained by a preceding mild load, has been termed PC. Several PC phenomena have been reported in various organs and animal species.[3–8] Ischemia and ischemia–reperfusion are the most investigated PC events to confer tolerance against later, more severe ischemia.[3–5] Research on a PC effect by ischemia or ischemia–reperfusion may be useful for understanding underlying mechanisms in cases of organ transplantation in various clinical stages. In a similar manner, research on a PC effect by low-dose LPS may also be useful for understanding how to prevent severe organ damage in repeated sepsis.

It is well known that Nrf2 and antioxidant molecules are upregulated by single LPS stimulation, however, it has not been clarified whether Nrf2 and antioxidant molecules are also associated with PC effects by low dose LPS.[12–17] In order to clarify which molecular mechanism(s) was associated with such a PC effect, we performed DNA microarray and compared gene expression between PC0-LPS20 and PC5.0-LPS20 treated mice. Since the microarray highlighted the induction of genes associated with antioxidant activity as a point of difference between the two mice groups examined, we hypothesized that increased antioxidant activity was one of the candidate mechanisms whereby PC induced a protective effect in the liver. Oxidative stress is one of the most important factors in the pathogenesis of sepsis.[10] Therefore, we focused on Nrf2, a key transcription factor for the induction of endogenous antioxidant molecules under oxidative stress,[11] as one of the candidate targets for PC mechanisms. DNA array revealed an induction of HO-1 in PC5.0-LPS20 mice (more than two
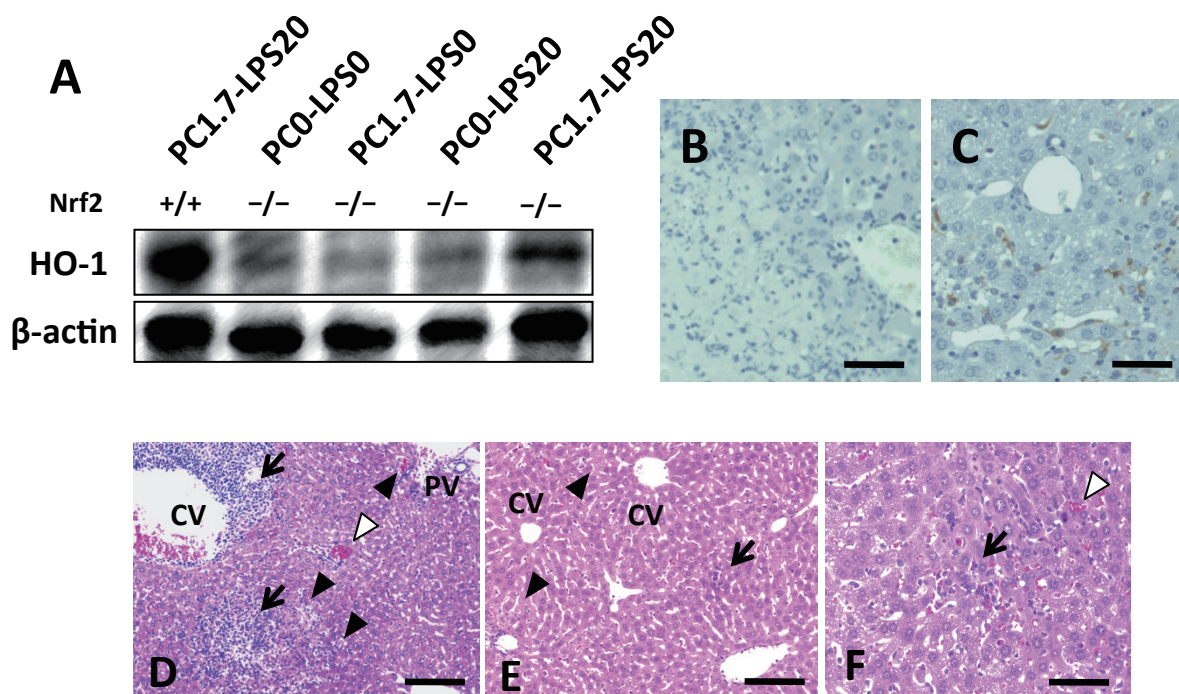
Fig. 4. Nrf2 deficiency abolished the protective effect of PC against liver damage by high-dose LPS in mice. (**A**) Immunoblot using anti-HO-1 antibody. The immunoblot shown is representative of three independent experiments. In Nrf2-deficient mice, the expression of HO-1 protein bands in liver samples from PC0-LPS0, PC1.7-LPS0, and PC0-LPS20 mice are faint, unlike the case for wild-type mice (far left lane). Liver samples from PC1.7-LPS20 treated Nrf2-deficient mice show a lower induction of HO-1 protein (far right lane) than PC1.7-LPS20 treated wild-type mice (far left lane). (**B, C**) Immunohistochemistry using anti-HO-1 antibody at the same time of western blotting. (**B**) The liver from the Nrf2-deficient mice with PC0-LPS20, (**C**) The liver from the Nrf2-deficient mice with PC1.7-LPS20. In Nrf2-deficient mice, the induction of HO-1 in Kupffer cells is reduced even if samples are from PC1.7-LPS20 treatment group. (**D–F**) HE staining of the liver from Nrf2-deficient mice. (**D**) The liver from Nrf2-deficient mice with PC0-LPS20 treatment shows severe necrosis in a large area (arrowheads), severe neutrophil infiltration (arrows), and microhemorrhage (white arrowhead). (**E, F**) In a liver from an Nrf2-deficient mouse after PC1.7-LPS20 treatment, the protective effect of PC is abolished, and focal necrosis (arrowheads), neurotrophil infiltration in sinusoids (arrows), and microhemorrhage (white arrowhead) are observed. Scale bars (**D**)(**E**) represent 200 µm and (**B, C, F**) 100 µm. CV, central vein; HE, hematoxylin-eosin; LPS, lipopolysaccharide; MS, main stimulation; Nrf2, NF-E2-related factor-2; PC, preconditioning.

times vs. PC0-LPS20). Therefore, we used the induction of HO-1 as an index of the activation of Nrf2.

In the present study, we demonstrated the enhanced activation of Nrf2 by PC with low-dose LPS administration *in vivo* using Nrf2 reporter mice (Fig. 3A). An *in vivo* Nrf2 reporter assay using OKD48 mice showed that an intense and immediate activation of Nrf2 was observed throughout the whole body, including in the lungs and liver, in PC1.7-LPS20 or PC5.0-LPS20 mice. This suggests that PC by low-dose LPS induced the enhanced activation of Nrf2 when mice were exposed to severe LPS-induced toxicity after 5 days. Indeed, HO-1, one of the major targets of Nrf2, was dramatically induced in the PC1.7-LPS20 mouse liver. Furthermore, we also demonstrated that an enhanced induction of HO-1 was observed, predominantly in Kupffer cells, but not in hepatocytes or neutrophils 24 h after MS by LPS. This suggests that Kupffer cells may play an important role in the PC effect via an Nrf2 system.

Kupffer cells are the resident macrophages of the liver. These cells are located in sinusoidal spaces and are in contact with portal blood draining the intestinal tract, during which they are exposed to bacteria and bacterial products such as LPS.[19] Kupffer cells play an important role in clearing LPS.[20] Moreover, the activation and induction of Nrf2/HO-1 in Kupffer cells and hepatocytes is important for hepatoprotection against LPS-induced toxicity.[21] Previous reports and our present studies suggest that PC by low-dose LPS enhances Nrf2 activation and prevents severe damage of the liver.

The Nrf2/Keap1 system is the primary sensor in the cellular response to oxidative stress. The cytoplasmic protein and ubiquitin E3 ligase, Keap1, interacts with Nrf2 and continuously leads it to an ubiquitin–proteasome-dependent proteolysis.[11] However, once cells are exposed to oxidative stress, signals associated with this process disrupt binding between Nrf2 and Keap1, resulting in the transcription of Nrf2 in the nucleus. Within

the nucleus, Nrf2 forms a heterodimer with a small-Maf protein and activates the transcription of oxidative stress-related genes via an ARE.[11] Recently, PC by mild ischemia in the brain was found to confer tolerance against later severe ischemic events via epigenomic modification on the promotor area of the *Nrf2* gene.[9] Moreover, evidence suggests that Nrf2 and its related systems are controlled by epigenomic modifications following several conditions.[22, 23] These facts suggest that an epigenomic change of the *Nrf2* gene may also be included in an LPS-induced PC phenomenon. Intense and immediate upregulation of Nrf2 by PC may bring the resistance against oxidative stress. Furthermore, it has been reported recently that Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription.[24] These facts suggest that PC effect against LPS-induced injury in our experiments may include not only antioxidant activity but also suppression of cytokine transcription.

In order to confirm the role of Nrf2 on the protective effect of PC, we examined this using Nrf2-deficient mice. A severe vulnerability against a preceding stimulation by LPS was noted in Nrf2-deficient mice, with more than a half of the mice dying after a single stimulation for PC (data not shown). Therefore, we could not compare survival rates of PC0-LPS20, PC1.7-LPS20, and PC5.0-LPS20 groups after MS. Since we used tissue samples from the limited numbers of mice that survived during our experimental procedures, we should consider that other mechanisms for tolerance may have been enhanced in the surviving Nrf2-deficient mice.

The decreased induction of HO-1 (Fig. 4A), and increased focal necrosis (Fig. 4E) and neutrophil infiltration (Fig. 4F) in the liver were observed in surviving Nrf2-deficient mice, even in the PC group as cpmpared with wild-type mice. These results strongly suggest that Nrf2 plays an important role in hepatoprotection during PC and subsequent LPS stimulation. Even in Nrf2-deficient mice, a mild induction of HO-1 was still observed in Kupffer cells (Fig. 4C). Since the expression of HO-1 is also controlled by other transcription factors such as AP-1 in addition to Nrf2,[25] this may explain why HO-1 continued to be induced in the liver by LPS in Nrf2-deficient mice.

Our results suggest that Nrf2 plays key role in the hepatoprotective effect induced by PC with low-dose LPS, however, the detailed mechanism of how Nrf2 is enhanced by PC remains unknown. Therefore, further studies, such as on the epigenomic modifications of the *Nrf2* gene, are required in future.

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