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Original Article

Role of the Transcription Factor BTB and CNC Homology 1 in a Rat Model of Acute Liver Injury Induced by Experimental Endotoxemia

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Hepatic oxidative stress plays an important role in the pathogenesis of several acute liver diseases, and free heme is thought to contribute to endotoxemia-induced acute liver injury. The heme oxygenase 1 (HO-1) gene is upregulated and the δ-aminolevulinate synthase (ALAS1) gene is downregulated in the rat liver following lipopolysaccharide (LPS) treatment. BTB and CNC homology 1 (Bach1) is a heme-responsive transcription factor that normally represses HO-1 expression. In this study, we evaluated the changes in *HO-1*, *ALAS1*, and *Bach1* expression and nuclear Bach1 expression in rat livers following intravenous LPS administration (10 mg/kg body weight). LPS significantly upregulated *HO-1* mRNA and downregulated *ALAS1* mRNA in the rat livers, suggesting that hepatic free heme concentrations are increased after LPS treatment. *Bach1* mRNA was strongly induced after LPS injection. In contrast, nuclear Bach1 was significantly but transiently decreased after LPS treatment. Rats were also administered hemin (50 mg/kg body weight) intravenously to elevate heme concentrations, which decreased nuclear Bach1 levels. Our results suggest that elevated hepatic free heme may be associated with a decline of nuclear Bach1, and induction of *Bach1* mRNA may compensate for the decreased nuclear Bach1 after LPS treatment in the rat liver.

Key words: heme oxygenase-1, BTB and CNC homology 1, heme, lipopolysaccharide, liver injury

Sepsis and septic shock are the most common causes of death in intensive care units worldwide [1] and frequently lead to multiple organ failure. The liver is a major target organ [2], and up to 50% of patients with sepsis experience liver involvement [3]. We previously demonstrated that expression of heme oxygenase-1 (HO-1), the rate-limiting enzyme in heme catabolism, is upregulated, and expression of δ-aminolevulinate synthase (ALAS1), the rate-limiting enzyme in heme biosynthesis, is downregulated in the rat liver following intraperitoneal administration of lipopolysaccharide (LPS) [4]. This suggests that free heme released from hepatic heme proteins enhances oxidative stress and exacerbates hepatic injury in septic liver damage.

The heme-responsive transcription factor BTB and CNC homology 1 (Bach1) represses *HO-1* expression under normal conditions (baseline conditions), but is exported from the nucleus to allow for transcriptional activation of *HO-1* in the presence of excess free heme [5-7]. Reactive oxygen species and free heme, a pro-oxidant, are thought to be involved in the pathogenesis of septic multiple organ failure and LPS-induced tissue injury [8, 9]. However, the mechanisms

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Conflict of Interest Disclosures: No potential conflict of interest relevant to this article was reported.

underlying these effects are not fully understood.

In this study, we evaluated the changes in gene and nuclear protein expression of Bach1 over time after intravenous LPS treatment in association with heme metabolism.

Materials and Methods

Materials. LPS from *Escherichia coli* O127:B8 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Hemin (BioXtra, from pigs) was also purchased from Sigma-Aldrich. Rabbit anti-BACH1 polyclonal antibodies (cat. no. 14018-1-AP) and mouse beta-actin monoclonal antibodies (cat. no. sc-47778) were purchased from Proteintech (Rosemont, IL, USA) and Santa Cruz Biotechnology (Dallas, TX, USA), respectively. Horseradish peroxidase-conjugated goat anti-rabbit IgG (cat. no. sc-2004) and horseradish peroxidase-conjugated goat anti-mouse IgG (cat. no. sc-2005) were purchased from Santa Cruz Biotechnology.

Animal experiments. All animal experiments were approved by the Animal Use and Care Committee of Okayama University Medical School (Okayama, Japan; approval nos. OKU-2014409, OKU-2014528 and OKU-2020546). The care and handling of the animals were carried out in accordance with the National Institutes of Health Guidelines for Animal Research (Guide for the Care and Use of Laboratory Animals).

Male Sprague-Dawley rats, weighing 200-250 g, were purchased from CLEA Japan, Inc. (Tokyo). The rats were housed in a temperature-controlled (25°C) room with a 12-h light/dark cycle and allowed free access to water and chow diet for at least 6-7 days until the start of experiments. All surgical procedures and painful treatments were carried out under anesthesia with ethyl ether.

Experimental protocols and treatments. The rats were randomly divided into the following 4 groups: LPS-treated animals, saline-treated animals, untreated control animals, and hemin-treated animals. The LPS-treated animals were administered LPS (10 mg/kg body weight) intravenously (i.v.) via the tail vein to develop an *in vivo* endotoxemia model. LPS was dissolved in sterile saline to give a final concentration of 10 mg/ml. Saline-treated control animals were administered the same volume of physiological saline (1 ml/kg body weight). In another set of experiments, hemin-treated animals were administered hemin (ferriprotoporphyrin IX chloride; 50 mg/kg body weight, i.v.) to elevate free heme concentrations in liver tissue. Hemin was dissolved in a small volume of 0.1 M NaOH solution and reconstituted in physiological saline. The pH was then adjusted to 7.4 with 0.1 M HCl solution to yield a final concentration of 50 mg/ml.

After treatment, the animals were returned to the cages and allowed free access to food and water. Under light anesthesia with ethyl ether, the animals were sacrificed by bleeding through a catheter in the abdominal aorta at defined time points (0-48 h). Blood was collected for serum isolation to measure serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities, and the livers were excised, immediately frozen in liquid nitrogen, and stored at −80°C until the preparation of RNA and nuclear protein extracts. Liver samples were used for hematoxylin and eosin staining of liver tissue sections and assessment of hepatic gene and protein expression. Hepatic mRNA levels were examined by northern blot analysis. Hepatic protein levels were examined by western blot analysis.

Histological examination. For histological examination, liver tissue was fixed in 10% neutral-buffered formalin, embedded in paraffin, and sectioned at 4-6 μm thickness. After deparaffinization and dehydration, the sections were stained with hematoxylin and eosin for microscopic examination.

Measurement of serum AST and ALT activities. Serum was separated from whole blood by centrifugation at 1,600×*g* for 10 min at 4°C, and serum AST and ALT activities were measured using an automated clinical chemistry analyzer (FUJI DRI-CHEM 7000i; FUJIFILM Corporation, Tokyo).

cDNA probes. The template cDNAs used to generate probes for northern blot analysis included rat pRHO-1 [10], provided by Dr. S. Shibahara; rat pKRA2cA [11] and ALAS1, provided by Dr. M. Yamamoto; and rat Bach1 cDNA, corresponding to base pairs 785-1382, provided by Dr. K. Igarashi (Tohoku University, Sendai, Japan). All cDNA probes used for northern blot analysis were labeled with [α-^{32}P] dCTP (PerkinElmer, Waltham, MA, USA) using High Prime premixed solution (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions.

RNA isolation and northern blot analysis. Total RNA was isolated from rat tissues using TRI-Reagent (Sigma-Aldrich) according to the manufacturer's

instructions. Northern blot analysis was performed as previously described [12]. Briefly, total RNA (20 μg) was separated by electrophoresis on 1.2% (w/v) agarose gels containing 6.5% (v/v) formaldehyde. After blotting on a sheet of Bio-Rad Zeta-Probe GT blotting membrane (Bio-Rad Laboratories, Hercules, CA, USA), the RNA samples were hybridized with the [α-^{32}P] dCTP-labeled cDNA probes, followed by washing under stringent conditions. Each blotted membrane was exposed to a sheet of Fuji Medical X-ray film (FUJIFILM Corporation) with an intensifying screen at −80°C. Target bands and the 18S ribosomal RNA band on autoradiographs were quantified using a ChemiDoc XRS Plus Imaging System and Image Lab Software version 5.0 (Bio-Rad Laboratories). The relative amounts of hybridized radiolabeled cDNAs were normalized to 18S ribosomal RNA levels to correct for differences in gel loading.

Preparation of nuclear protein extracts and western blot analysis. Nuclear protein was isolated from the rat tissues using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Protein concentrations were determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Samples equivalent to 25 μg protein were treated with premixed sample buffer (4× Laemmli sample buffer; Bio-Rad Laboratories) and applied to 12.5% or 7.5% (w/v) polyacrylamide gels (ATTO Corporation, Tokyo). After electrophoretic separation based on the method of Laemmli, proteins were transferred to Amersham Hybond-P polyvinylidene fluoride membranes (GE Healthcare, Little Chalfont, UK). The membranes were then blocked with Block Ace Powder (DS Pharma Biomedical Co., Ltd. Osaka, Japan) at room temperature for 1 h, followed by incubation with rabbit anti-BACH1 polyclonal antibodies diluted at 1 : 1000 with Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) at 4°C overnight. The membranes were then treated with horseradish peroxidase-conjugated goat anti-rabbit IgG diluted at 1 : 20,000 with TBS-T at room temperature for 1 h. Antigen/antibody complexes were incubated with Clarity Western ECL Blotting Substrate (Bio-Rad Laboratories). The obtained chemiluminescent signals were detected and quantified using a ChemiDoc XRS Plus Imaging System and Image Lab Software version 5.0 (Bio-Rad Laboratories). The transfer efficiency and an equal amount of loading per lane were verified by quantifying the chemiluminescent signals of β-actin obtained from the same protein samples after incubating the membranes with mouse beta-actin monoclonal antibodies diluted at 1 : 5000 with TBS-T at 4°C overnight, followed by treatment with horseradish peroxidase-conjugated goat anti-mouse IgG diluted at 1 : 20, 000 with TBS-T at room temperature for 1 h.

Determination of the heme concentration in microsomes. Liver samples were also used for assessment of the heme concentration in the hepatic microsomal fraction. Livers were homogenized in 3 volumes of 1.15% KCl (w/v) and centrifuged at 10,000×*g* for 30 min at 4°C, followed by a 104,000×*g* centrifugation of the supernatant for 60 min at 4°C to obtain the microsomal fraction. The pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 10% glycerol and used for the measurement of the heme concentration by the pyridine hemochromogen method [13, 14], using a spectrophotometer (Ubest-55; JASCO Corporation, Tokyo). The heme concentration was expressed as nanomoles per milligram of protein. Protein concentrations of the hepatic microsomal fraction were determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). The absorbance was measured using a microplate reader (VICTOR Nivo Multimode Plate Reader; PerkinElmer).

Statistical analysis. For statistical evaluation, multiple group comparisons were performed by analysis of variance, followed by Bonferroni correction post-hoc tests. Differences with p values of less than 0.05 were considered statistically significant.

Results

Effects of LPS treatment on serum AST and ALT levels. Serum AST and ALT levels were assessed as a measure of LPS-induced hepatic dysfunction. Serum levels of AST and ALT increased sharply immediately after intravenous injection of LPS (10 mg/kg), followed by a more gradual increase, and were significantly elevated by 6 h after LPS treatment compared to the levels in the untreated and vehicle-treated control groups (Fig. 1). The serum AST and ALT levels then remained at high levels until 24 h after LPS treatment (Fig. 1). The marked elevation in these hepatic enzymes indicated the occurrence of LPS-induced hepatic injury.

Effects of LPS treatment on histological findings. We examined histological changes in the hematoxylin

and eosin-stained sections of rat livers to confirm LPS-induced hepatic injury. Sections of livers excised from untreated control and vehicle-treated control rats had a normal histological appearance (Fig. 2A and B), whereas sections excised from LPS-treated rats at 24 h after injection revealed massive and severe hepatic necrosis in the centrilobular zone with hemorrhage (Fig. 2C). These results indicate that i.v. injection of LPS caused endotoxemia-induced liver injury in the rat model.

Effects of LPS treatment on* HO-1 *and* ALAS1 *expression. We confirmed that *HO-1* and *ALAS1* expression was altered after i.v. LPS treatment. *HO-1* mRNA expression was very low in the untreated and vehicle-treated control livers (Fig. 3A and B). However,

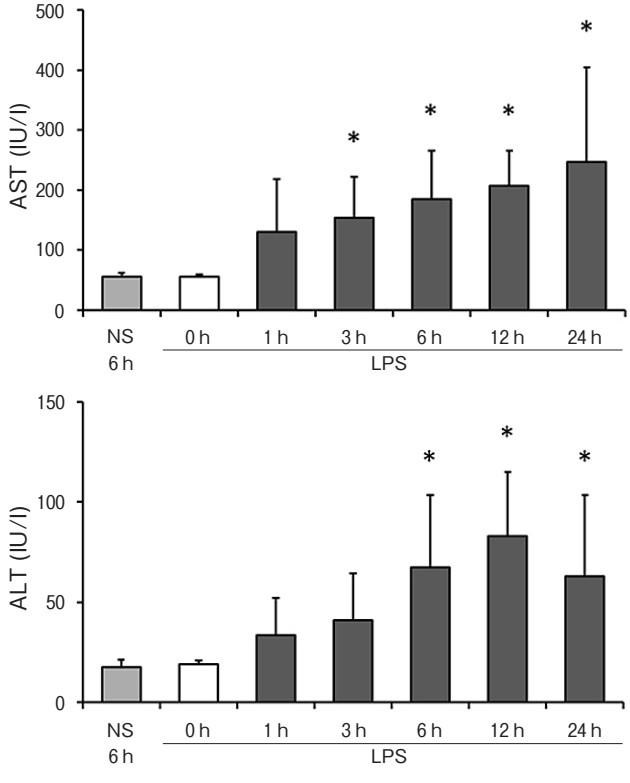

Fig. 1 Effects of LPS treatment on serum AST and ALT activities. The rats were injected i.v. with LPS (10 mg/kg) or saline (1 ml/kg) and were sacrificed at 0, 1, 3, 6, 12, or 24 h after treatment. Whole blood was collected for measurement of serum AST and ALT activities. Data are expressed as the means ± standard deviations (n = 6–10) and were statistically evaluated using ANOVA followed by Bonferroni correction post-hoc tests. $^{*}P < 0.05$ versus the untreated control group (LPS 0 h). NS 6 h, vehicle-treated control group; LPS, lipopolysaccharide; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

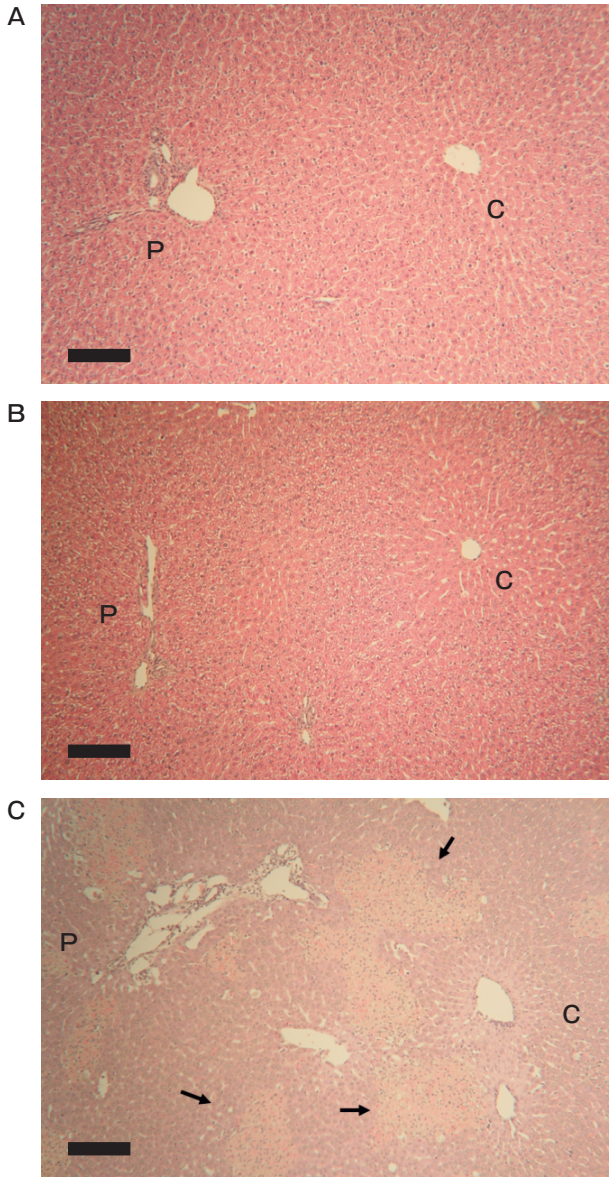

Fig. 2 Histological changes in rat livers after LPS treatment. The rats were injected i.v. with LPS (10 mg/kg) or saline (1 ml/kg) and were sacrificed at 24 h after injection. Livers were removed for histological examination of hematoxylin and eosin-stained liver sections. Representative light microscopic images of livers from at least three independent experiments are shown. **P** and **C** denote the portal tract and central vein, respectively. The bar represents 100 μm. **(A)**, Livers in the untreated control group showed normal liver histology; **(B)**, Livers in the vehicle-treated control group, at 24 h after injection of saline, showed almost normal histology; **(C)**, Livers in the LPS group, at 24 h after injection of LPS, showed massive hepatic necrosis in the centrilobular zone with hemorrhage (arrows).

the *HO-1* mRNA levels began increasing at 1-3 h after LPS injection, reaching a maximum level 7-fold higher than that in untreated control livers at 6 h after treatment prior to a gradual decrease to less than 50% of the maximum by 48 h (Fig. 3A and B). In contrast, *ALAS1* mRNA was highly expressed in the untreated and vehicle-treated control livers (Fig. 3C and D). Following i.v. LPS injection, the levels of hepatic *ALAS1* mRNA were slightly increased just after treatment but decreased to a minimum of 60% of baseline by 3 h, followed by a rapid increase and return to a level above the baseline by 6 h after treatment, which was maintained throughout the observation period (Fig. 3C and D). These changes were consistent with those reported in our previous study [4]. The reciprocal responses of *HO-1* and *ALAS1* suggest an increase in hepatic intracellular free heme following LPS treatment.

Effects of LPS treatment on nuclear Bach1 protein expression. Bach1 is a heme-responsive transcription factor and key regulator of *HO-1* expression. Therefore, we analyzed the expression of Bach1 protein in the nucleus. Nuclear Bach1 protein expression showed a significant but transient decrease and was barely detectable by 1 h after LPS injection, followed by a rapid return to baseline by 3 h (Fig. 4A and B). These changes may reflect Bach1 nuclear export via the direct binding of Bach1 to the increased intracellular heme after LPS treatment.

Effects of LPS treatment on* Bach1 *expression. Similar to hepatic *HO-1* mRNA, *Bach1* mRNA expression was very low in untreated and vehicle-treated control livers (Fig. 5A and B). *Bach1* mRNA then began increasing immediately after LPS injection, reaching a maximum level that was 5-fold higher than the level in

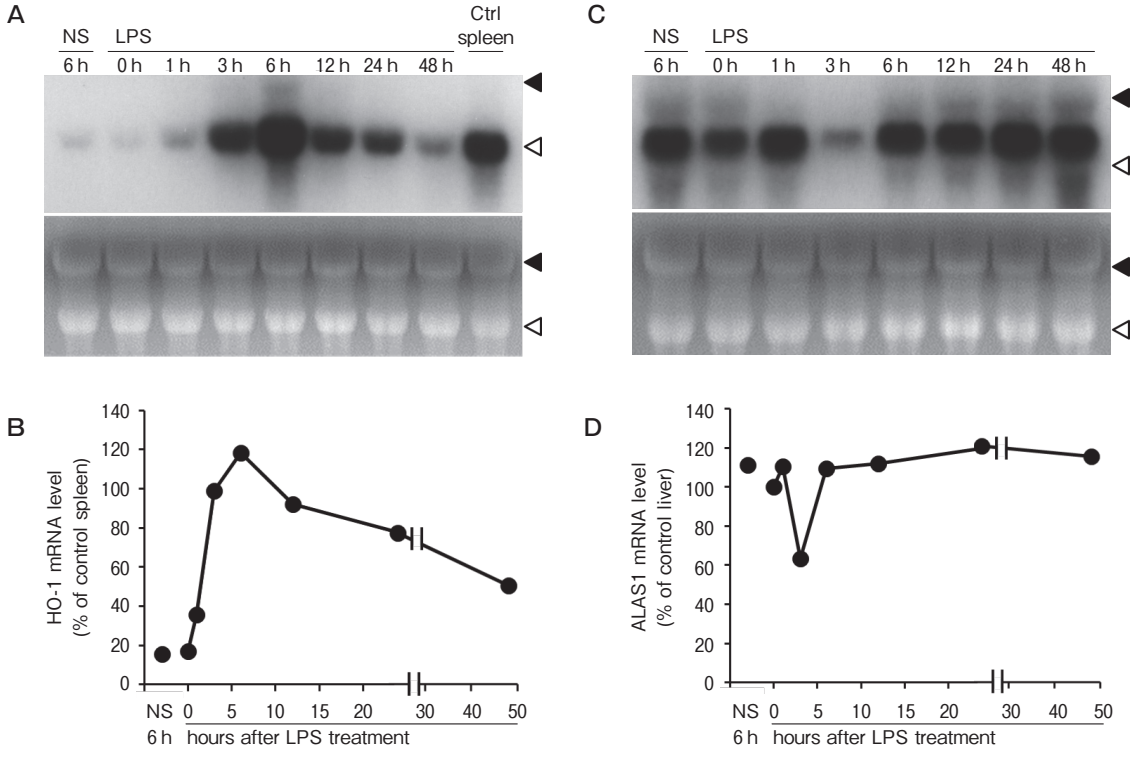

Fig. 3 Effects of LPS treatment on *HO-1* and *ALAS1* gene expression. The rats were sacrificed at 0, 1, 3, 6, 12, 24, or 48 h after i.v. injection of LPS (10 mg/kg), and their livers were excised for RNA isolation. Total RNA (20 μg) was subjected to northern blot analysis. Shown are autoradiographic signals of RNA blots hybridized with [α-^{32}P] dCTP-labeled **(A)** *HO-1* or **(C)** *ALAS1* cDNA probes. Ethidium bromide staining of the same RNA is shown as the loading control. Closed arrowhead, 28S ribosomal RNA; open arrowhead, 18S ribosomal RNA. More than three independent experiments showed similar results, and a typical example is shown; **(B)**, Concentrations of *HO-1* mRNA are expressed as values relative to the concentration of untreated control spleens, in which HO-1 is known to be constitutively expressed; **(D)**, Concentrations of *ALAS1* mRNA are expressed as values relative to the concentration of untreated control livers. HO-1, heme oxygenase-1; ALAS1, δ-aminolevulinate synthase; LPS, lipopolysaccharide; NS 6 h, vehicle-treated control; LPS 0 h, untreated control.

control livers at 3-6 h after LPS injection, shortly after the transient decrease in nuclear Bach1 protein, followed by a rapid decrease to baseline levels by 24 h (Fig. 5A and B). These results suggest that *Bach1* mRNA was highly induced to compensate for the consumption of Bach1 protein, which might be exported from the nucleus by binding to elevated intracellular heme after LPS treatment.

Effects of LPS and hemin treatment on nuclear Bach1 protein expression. Rather than directly detecting intracellular heme in the livers of rats injected with LPS, we developed a rat model of elevated hepatic heme concentrations by i.v. administration of hemin (50 mg/kg). Similar to the observations in rats treated with LPS (Fig. 6A and B), nuclear Bach1 protein decreased 1 h after hemin treatment in the liver (Fig. 6C and D). These results may suggest that Bach1 protein was exported from the nucleus by binding to increased free heme and that Bach1 protein transiently disappeared from the nucleus.

Effects of LPS and hemin treatment on the heme concentration in microsomes. Additionally, we analyzed the heme concentration in LPS-treated and hemin-treated livers. The heme concentration in the hepatic microsomal fraction showed a significant increase 1 h after LPS and hemin treatment compared to the untreated and vehicle-treated control groups (Fig. 7). These results support the idea that the heme concentration in hepatic tissue was elevated after both LPS and hemin treatment.

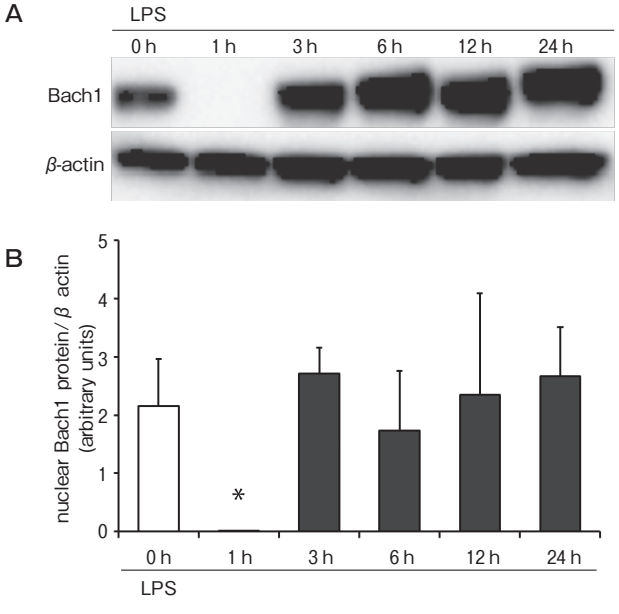

Fig. 4 Effects of LPS treatment on nuclear Bach1 protein levels. The rats were sacrificed at 0, 1, 3, 6, 12, or 24 h after i.v. injection of LPS (10 mg/kg), and their livers were excised for nuclear protein extraction. Nuclear protein (25 μg) was subjected to western blot analysis. **(A)**, Blots of nuclear proteins were incubated with anti-Bach1 or anti-actin antibodies. Blots show one representative example of at least three separate experiments; **(B)**, The intensity of bands of nuclear Bach1 protein was quantified by digital densitometry, normalized to actin, and expressed in arbitrary units. Data are expressed as the means ± standard deviations (n = 3–4) and were statistically evaluated by ANOVA followed by Bonferroni correction post-hoc tests. $^{*}P < 0.05$ versus the untreated control group (LPS 0 h). LPS, lipopolysaccharide; LPS 0 h, untreated control.

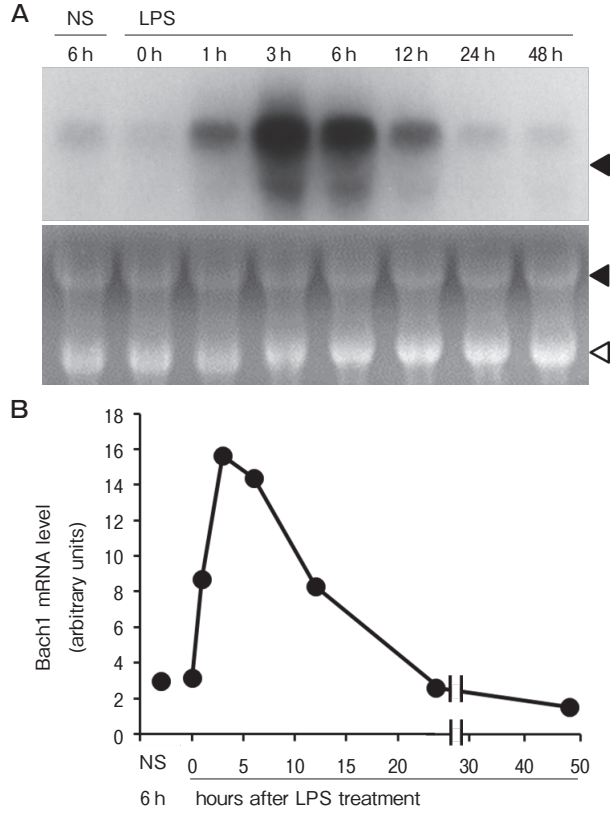

Fig. 5 Effects of LPS treatment on *Bach1* gene expression. The rats were sacrificed at 0, 1, 3, 6, 12, 24, or 48 h after i.v. injection of LPS (10 mg/kg), and their livers were excised for RNA isolation. Total RNA (20 μg) was subjected to northern blot analysis. **(A)**, Representative autoradiographic signals of RNA blots hybridized with the [α-^{32}P] dCTP-labeled *Bach1* cDNA probe are shown. Ethidium bromide staining of the same RNA is shown as the loading control. Closed arrowhead, 28S ribosomal RNA; open arrowhead, 18S ribosomal RNA; **(B)**, *Bach1* expression levels are given in densitometric arbitrary units. More than three independent experiments showed similar results, and a typical example is shown. LPS, lipopolysaccharide; NS 6 h, vehicle-treated control; LPS 0 h, untreated control.

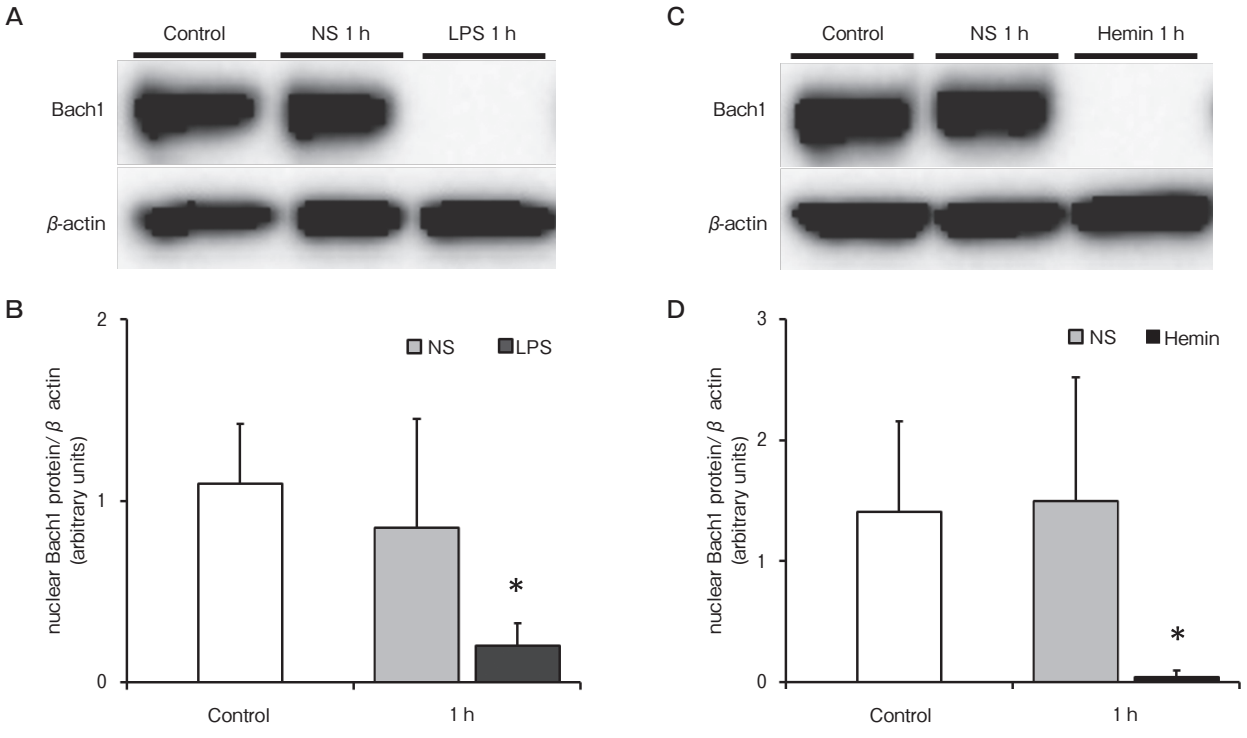

Fig. 6 Effects of LPS and hemin treatment on nuclear Bach1 protein expression. The rats were sacrificed at 1 h after i.v. injection of LPS (10 mg/kg) or hemin (50 mg/kg), and their livers were excised for nuclear protein extraction. Nuclear protein (25 μg) was subjected to western blot analysis to quantify nuclear Bach1 protein levels. Blots were incubated with anti-Bach1 or anti-actin antibodies. (**A** and **C**) Blots show one representative example of three independent experiments. (**B** and **D**) The intensity of bands of nuclear Bach1 protein was quantified by digital densitometry, normalized to actin, and expressed in arbitrary units. Data are expressed as the means ± standard deviations (n = 3) and were statistically evaluated by ANOVA followed by Bonferroni correction post-hoc tests. $^{*}P < 0.05$ versus the untreated control group. Control, untreated control; NS 1 h, vehicle-treated control.

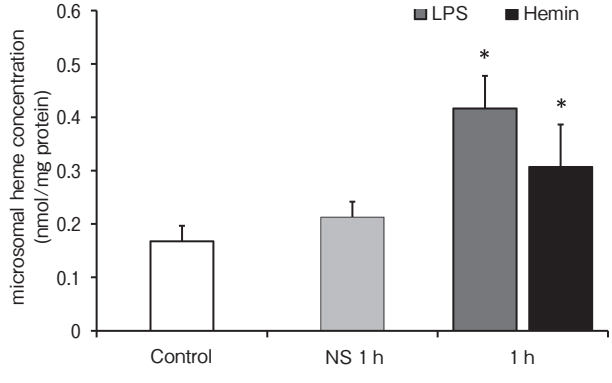

Fig. 7 Effects of LPS and hemin treatment on the heme concentration in microsomes. The rats were sacrificed at 1 h after i. v. injection of LPS (10 mg/kg) or hemin (50 mg/kg), and their livers were excised for microsomal protein extraction. Microsomal protein was used for the measurement of the heme concentration by the pyridine hemochromogen method. The heme concentration was expressed as nanomoles per milligram of hepatic microsomal protein. Data are expressed as the means ± standard deviations (n = 3–6) and were statistically evaluated by ANOVA followed by Bonferroni correction post-hoc tests. $^{*}P < 0.05$ versus the untreated control group. Control, untreated control; NS 1 h, vehicle-treated control.

Discussion

In the present study, we demonstrated that hepatic *HO-1* mRNA expression was upregulated, and hepatic *ALAS-1* mRNA expression was downregulated in the liver in a rat model of experimental endotoxemia following i.v. LPS injection. We also demonstrated that nuclear Bach1 protein was transiently and significantly decreased after i.v. LPS administration. This is the first study showing a decline in nuclear Bach1 protein in a septic animal model. The decline in Bach1 protein was followed by an immediate increase in hepatic *Bach1* mRNA expression. Because *HO-1* and *ALAS-1* are heme regulatory genes and Bach1 is a heme-responsive transcription factor of *HO-1* expression, these results suggest that increased intracellular free heme concentrations in the liver are involved in the pathogenesis of acute liver injury in LPS-treated animals.

We first evaluated changes in heme regulatory genes in the livers of LPS-treated rats. Following i.v. LPS treatment, hepatic *HO-1* mRNA expression was significantly upregulated, whereas hepatic *ALAS-1* mRNA expression was transiently downregulated. These results are consistent with those in our previous reports on septic liver injury after intraperitoneal administration of LPS (10 mg/kg) [4]. Notably, HO-1 is upregulated and ALAS-1 is downregulated in the presence of excess heme, a potent pro-oxidant [15]. Thus, the reciprocal responses of *HO-1* and *ALAS1* in this study suggest an increase in hepatic intracellular free heme concentrations following LPS treatment.

Bach1 is a heme-responsive transcription factor that normally represses the expression of *HO-1* mRNA [5-7]. In this study, we demonstrated that Bach1 protein transiently disappeared from the nucleus of rat liver cells immediately after i.v. LPS injection. This result indicates that Bach1 protein might be exported from the nucleus, likely by directly binding to increased intracellular free heme released from disrupted hepatic heme proteins, such as cytochrome P450. In several *in vitro* studies, the transcription factor Bach1 has been shown to be released from the promoter region of *HO-1* and exported from the nucleus by binding to heme in the presence of excess free heme and oxidative stress; in turn, *HO-1* transcription is activated by nuclear factor (erythroid-derived 2)-like 2 (Nrf2) [16-18] (Fig. 8). Raval *et al.* reported nuclear export of Bach1 protein by heme-mediated oxidative stress evoked by ultraviolet A irradiation in their cell culture study [16]. Although this translocation has been demonstrated in cell culture studies, few reports have described these findings in animal models [19].

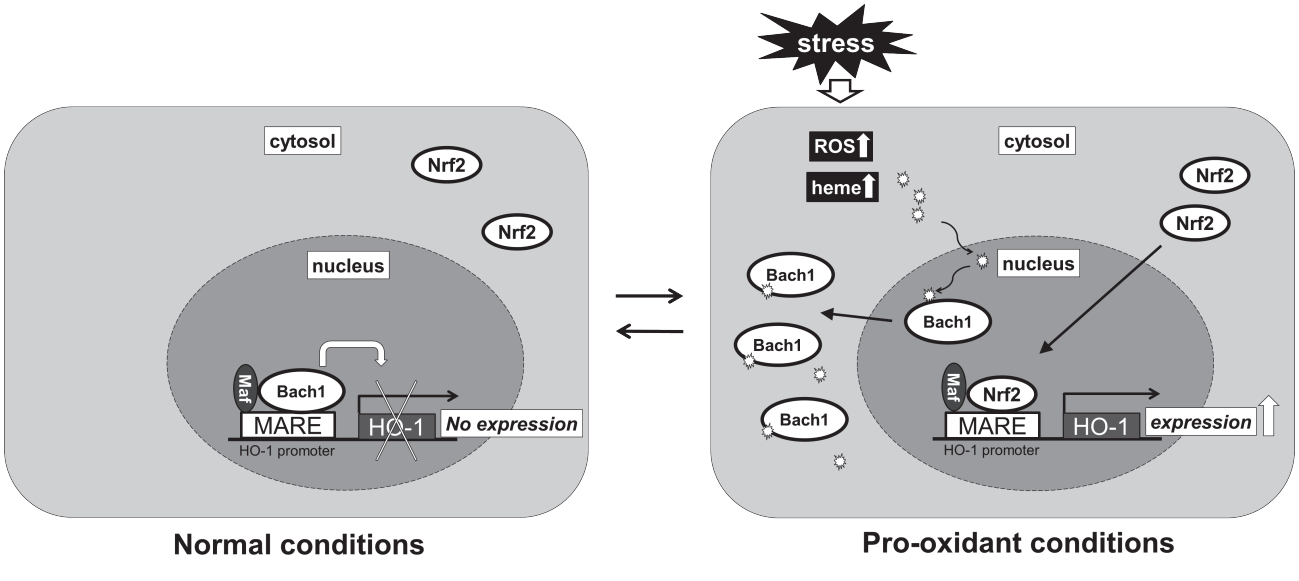

Fig. 8 *HO-1* gene regulation by transcription factors Bach1 and Nrf2. Diagrammatic explanation of *HO-1* gene regulation under the normal and pro-oxidant conditions. The heme-responsive transcription factor Bach1 normally represses expression of the *HO-1* gene. However, in the presence of excess free heme and oxidative stress, several *in vitro* studies report that Bach1 protein is exported from the nucleus by binding with free heme, after which Nrf2 protein is imported into the nucleus and the *HO-1* gene expression is induced.

Following the transient decline in nuclear Bach1 protein, *Bach1* mRNA was strongly induced in hepatic tissue after LPS treatment. Additionally, nuclear Bach1 protein quickly returned to baseline levels. Raval *et al.* also reported that ultraviolet A irradiation of cultured human skin fibroblasts enhanced *Bach1* mRNA expression to control HO-1 overexpression [16]. They speculated that it would be crucial to restore intracellular homeostasis because prolonged HO-1 overexpression is likely detrimental once excess free heme is catabolized and heme levels return to normal. In this study, *Bach1* was upregulated, presumably to compensate for the consumption of nuclear Bach1 protein, which had been exported from the nucleus by binding to elevated intracellular heme. Based on previously reported *in vitro* findings, these results suggest that induction of *Bach1* is important for preventing prolonged HO-1 overexpression to maintain intracellular homeostasis.

We predicted that the presence of free heme released from disrupted hepatic heme proteins can lead to nuclear export of Bach1 protein. Although measurement of the concentration of free heme is crucial for determining the involvement of free heme, free heme is thought to be structurally unstable and technically difficult to measure accurately [20, 21]. The concentration of free heme in living cells is considered to be very low, making it difficult to determine by existing quantitative methods. Rather than quantifying free heme in hepatic tissue, we administered hemin i.v. to mimic the conditions of elevated heme concentrations in hepatic tissue. In the presence of artificially elevated heme concentrations, nuclear Bach1 protein also declined immediately after hemin treatment. Similar declines in nuclear Bach1 protein after both LPS and hemin treatment suggested that free heme released from hepatic heme proteins binds to Bach1 protein for export from the nucleus.

Our study had several limitations. First, we only detected a decline of nuclear Bach1 protein and we did not detect significant changes in cytosolic Bach1 protein or a significant increase in nuclear Nrf2 protein. Nuclear export of Bach1 and nuclear import of Nrf2 are thought to be essential for activating *HO-1* transcription [16, 17]. However, these transcription factors are reported to be rapidly degraded by the ubiquitin proteasome pathway in the cytoplasm [22-24]; therefore, assessing changes in these proteins may be difficult. Regarding Bach1 protein, we did not determine its subcellular localization by immunostaining under either normal or septic conditions. In addition, we did not directly measure the concentrations of free heme in hepatic tissues. As described above, detection of free heme at low concentrations in biological samples is quite difficult [20, 21], and we could not find a suitable method. Although we determined that the heme concentration in the hepatic microsomal fraction was elevated in LPS-treated and hemin-treated rat livers by the pyridine hemochromogen method, it is not clear whether these responses directly reflected the increase of free heme concentration in hepatic tissue. In this study, we predicted that the mechanism of endotoxemia-induced liver injury involves the release of free heme from disrupted hepatic heme protein; however, we only evaluated changes in heme regulatory genes and heme-responsive transcription factor in LPS-treated and hemin-treated animals. There are several reports demonstrating that free heme plays an important role in the pathogenesis of severe sepsis, and that hemopexin, a plasma protein that binds heme with extraordinary affinity, suppresses the cytotoxic effects of free heme, suggesting that targeting free heme may be useful for the treatment of severe sepsis [25].

In conclusion, we demonstrated that the transcription factor Bach1 disappeared from the nucleus of liver cells in an experimental endotoxemia rat model. We also directly demonstrated that nuclear Bach1 protein declined in a similar manner in the presence of elevated heme concentrations.

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