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Tissue-Specific Gene Expression of Heme Oxygenase-1 (HO-1) and Non-specific δ-Aminolevulinate Synthase (ALAS-N) in a Rat Model of Septic Multiple Organ Dysfunction Syndrome

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ABSTRACT. Reactive oxygen species are thought to be involved in the pathogenesis of septic multiple organ dysfunction syndrome (MODS). It has been reported that heme oxygenase-1 (HO-1) (EC 1.14.99.3) is induced in septic animal models and is thought to confer protection against oxidative tissue injury. In this study, we examined changes in gene expression of HO-1 and non-specific δ-aminolevulinate synthase (ALAS-N) (EC 2.3.1.37), the rate-limiting enzymes in heme catabolism and heme synthesis, respectively, after intraperitoneal administration of bacterial lipopolysaccharide (LPS) to rats. LPS treatment caused the elevation of body temperature, increases in white blood cell counts, and marked elevation of serum interleukin-6 levels associated with liver, lung, and kidney injuries, characteristic of septic MODS. LPS administration significantly induced HO-1 mRNA, protein, and enzyme activity in the liver, lung, and kidney. In contrast, ALAS-N mRNA was decreased rapidly in the liver, followed by an oscillating recovery pattern. Induction of hepatic HO-1 mRNA and rapid suppression of ALAS-N mRNA were likely the result of a rapid increase in hepatic free heme concentration as judged by the increase in heme saturation of tryptophan pyrrolase. In contrast to that in the liver, the ALAS-N mRNA level in the lung and kidney was increased significantly after LPS administration, suggesting a novel mechanism of ALAS-N regulation in these tissues. These findings suggest that HO-1 and ALAS-N mRNA are regulated in a tissue-specific manner in a rat model of septic MODS. BIOCHEM PHARMACOL **60**;2:275–283, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. lipopolysaccharide; heme; heme oxygenase-1; δ-aminolevulinate synthase; sepsis; multiple organ dysfunction syndrome

Gram-negative sepsis remains an important cause of morbidity and mortality in intensive care units despite recent advances in critical care [1]. The arrays of pathophysiologic features that accompany Gram-negative bacterial sepsis appear to be qualitatively similar to those encountered after a LPS§ insult [2, 3]. LPS, a component of the cell wall of Gram-negative bacteria, is responsible for initiating a series of highly complex cascading events leading to damage in multiple organs, including the liver, lung, and kidney [4, 5]. Reactive oxygen species are thought to be involved as important end-effector molecules in LPS-induced tissue injury [6, 7].

HO-1 is the rate-limiting enzyme in heme catabolism [8], and is also known as heat shock protein 32 [9]. HO-1 has also been shown to be an acute phase reactant [10]. HO-1 is induced not only by its substrate, heme [11], but also by oxidative stress [12]. Recently it has been reported that HO-1 is induced in the liver and in the lung after administration of LPS in rodents, and is believed to confer protection against LPS-induced oxidative injury [13–16]. We also have demonstrated that HO-1 induction plays a protective role against heme-mediated oxidative damage in a rat model of ischemic acute renal failure and halothane-induced liver injury, whereas its blockade by tin-mesoporphyrin aggravates tissue injury [17, 18]. In this study, we examined changes in heme metabolism in the kidney as well as in the lung and the liver, in a rat model of LPS-induced MODS. We specifically examined changes in the levels of mRNAs encoding HO-1 and ALAS-N, the rate-limiting enzymes in heme catabolism and heme biosynthesis, respectively [19]. Hepatic and renal dysfunction were assessed by measurements of serum ALT (EC 2.6.1.2)

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§ *Abbreviations:* ALAS-N, non-specific δ-aminolevulinate synthase; ALT, alanine transaminase; HO-1, heme oxygenase-1; IL-6, interleukin-6; IOD, integrated optical density; LPS, lipopolysaccharide; MODS, multiple organ dysfunction syndrome; and WBC, white blood cell.

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activity and creatinine concentration, respectively, and lung injury was evaluated by histological examination. The extent of heme saturation of tryptophan pyrrolase, which reflects hepatic free heme concentration [20], also was examined. We report here that there was a significant HO-1 mRNA induction followed by an increase in HO activity in septic MODS. Hepatic HO-1 mRNA induction and suppression of ALAS-N mRNA expression were preceded by the increase in hepatic free heme concentration, as reflected by the increase in heme saturation of tryptophan pyrrolase. In contrast to the liver, there was a significant increase in the level of ALAS-N mRNA in both the lung and the kidney. These results suggest that HO-1 and ALAS-N mRNAs are regulated in a tissue-specific manner.

MATERIALS AND METHODS

Animals

Animal experiments were approved by the Animal Care Committee of Okayama University Medical School; care and handling of the animals were in accordance with National Institutes of Health guidelines. Male Sprague–Dawley rats weighing 200–250 g were purchased from Clea Ltd. They were housed in a temperature-controlled (25°) room with alternating 12 hr/12 hr light or dark cycles, and were allowed free access to water and diet prior to experimentation. They were injected with bacterial endotoxin (LPS; *Escherichia coli*, 0127:B8, Difco Laboratories; 10 mg/kg) dissolved in 1 mL of sterile saline to induce sepsis. Control rats received the same volume of sterile saline. Animals were killed by decapitation at each defined time (0–72 hr), under light anesthesia with ethyl ether. Rectal temperature was measured just before killing the animals. Whole blood was collected into a test tube containing EDTA for the measurement of WBC counts, serum ALT activity, creatinine concentration, and IL-6 level, and the livers, lungs, and kidneys were then excised. These organs were frozen immediately in liquid nitrogen and stored at −80° until used for RNA preparation and determination of hepatic free heme concentration. For the determination of HO activity, all organs were perfused *in situ* with physiological saline until the venous effluent became clear, and then removed for the preparation of microsomes.

cRNA Probes

The template cDNAs used were rat pRHO-1 [21] and rat pKRA2cA [19], for rat HO-1 and rat ALAS-N, respectively. All probes used for northern blot analysis were biotin-CTP-labeled antisense riboprobes prepared according to the manufacturer's instructions by using a Nonradioactive RNA Labeling System (Life Technologies, Inc.).

Northern Blot Analysis

Total RNA was isolated from the tissues according to the method of Cathala *et al.* [22]. Twenty micrograms of total RNA was analyzed by northern blot analysis as described previously [23]. After blotting to a sheet of BIODYNE A Nylon membrane (Pall BioSupport Division, Pall Co.), RNA samples were hybridized with biotin-labeled riboprobes, then treated with RNase A (1 μg/mL), followed by washing under stringent conditions. The detection of mRNAs was carried out by using a PHOTOGENE™ Nucleic Acid Detection System Version 2.0 (Life Technologies, Inc.). Chemiluminescent signals were visualized by exposing the membrane to x-ray film. The levels of mRNAs were quantitated by densitometry using a Bioimage Analyzer (Millipore Corp.) as follows. Briefly, after measurement of the IOD of each band, we calculated the ratio of the IOD in hepatic, pulmonary, and renal mRNA at each time point to the IOD in mRNA of the respective tissue at 1 hr for ALAS-N mRNA and 9 hr for HO-1 mRNA after LPS administration at which time hepatic mRNAs revealed maximal expression, respectively. We then multiplied each value by the ratio of the IOD in the pulmonary or kidney mRNA level to the IOD in hepatic mRNA at 1 hr for ALAS-N mRNA and at 9 hr for HO-1 mRNA after LPS administration, respectively. Finally, we converted those ratios to percentages by multiplying by 100.

Determination of Hepatic Free Heme Concentration

Hepatic free heme concentration was estimated as the extent of saturation of tryptophan pyrrolase activity, as described previously [24]. The degree of saturation of the enzyme with heme was expressed as the percentage of the holoenzyme activity (determined without exogenous hemin addition) of the total activity (determined after addition of 1.2 μM hemin).

HO Activity

Tissue was homogenized in 3 vol. of 0.1 M potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose. Microsomal fractions were prepared as described above, and HO activity was measured as described previously [25]. The cytosolic fraction prepared from the livers of adult untreated rats served as a source of biliverdin reductase. HO activity was expressed as picomoles bilirubin formed per 60 min per milligram protein. Microsomal protein concentration was determined by the method of Lowry *et al.* [26].

Measurement of Serum IL-6 Level

Serum IL-6 level was measured by ELISA according to the manufacturer's instructions, using a rat IL-6 ELISA kit (Biosource International, Inc.).

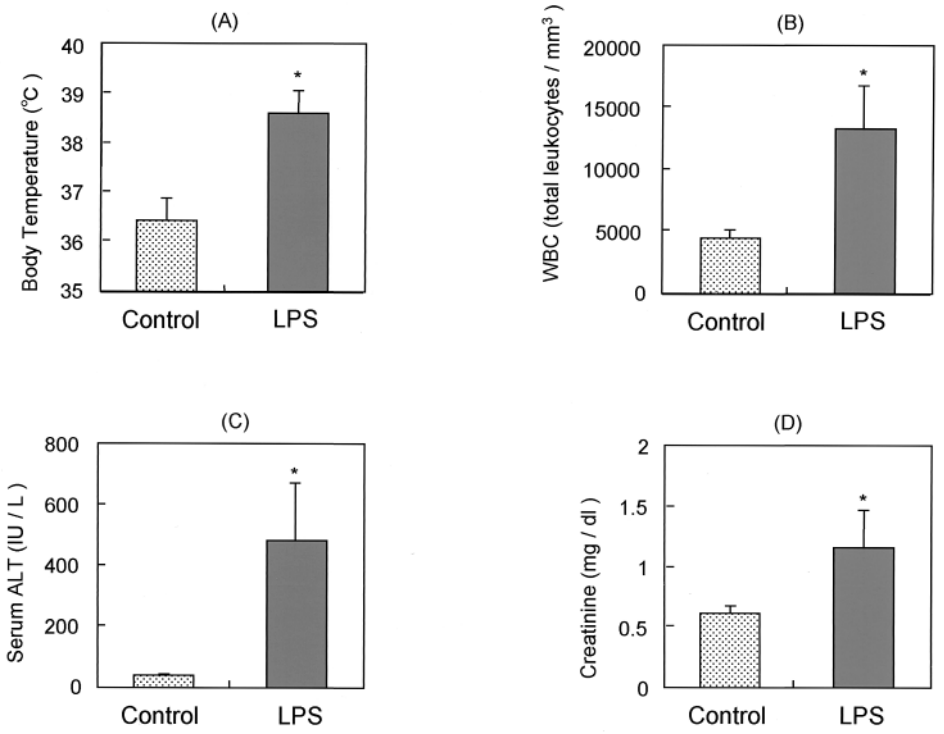

FIG. 1. Effects of LPS administration on body temperature, WBC counts in blood, serum ALT activity, and serum creatinine concentration. Rats were injected intraperitoneally with LPS (10 mg/kg), and were killed 12 hr after the injection. Rectal temperature was measured just prior to killing, and whole blood was collected. WBC counts in the blood, serum ALT activity, and serum creatinine concentration were measured as described in Materials and Methods. Values for body temperature (A), WBC counts (B), serum ALT activity (C), and serum creatinine concentration (D) are shown as means ± SD (N = 6). Key: (*) $P < 0.01$ vs control. Dotted bars, control rats; gray bars, LPS-treated rats.

WBC Counting

The number of WBCs in the blood was counted using a Hematology Analyzer (Beckman Coulter Inc.).

Histological Study

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

Statistical Analysis

Since all the values were distributed normally, statistical evaluation was made by an unpaired Student's *t*-test or ANOVA with Tukey's test using Statview software (Abacus Concepts). Differences were designated as significant when $P < 0.05$.

RESULTS

A Rat Model of Septic MODS

Intraperitoneal injection of LPS resulted in significant increases in body temperature and WBC counts in the blood at 12 hr after the LPS treatment (Fig. 1, A and B). At 3 hr after the LPS treatment, the serum IL-6 level was increased markedly in LPS-treated rats (Fig. 2). Both serum ALT activity and creatinine concentration were increased significantly as compared with the control rats at 12 hr after the LPS treatment (Fig. 1, C and D). Since the lung is the primary target organ of sepsis, we assessed lung injury by

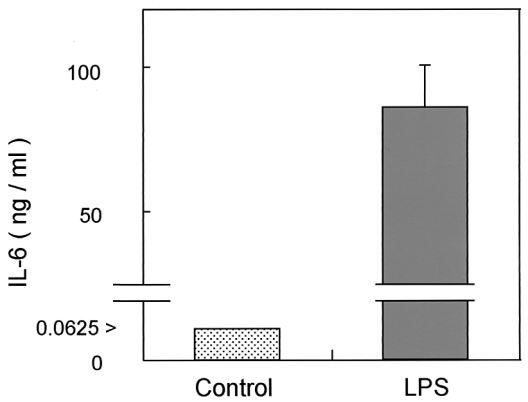

FIG. 2. Effect of LPS administration on serum IL-6 levels in the rat. Rats were injected intraperitoneally with LPS (10 mg/kg), and were killed 3 hr after the injection. Whole blood was collected for determination of serum IL-6 levels as described in Materials and Methods. Serum IL-6 levels in LPS-treated animals are shown as means ± SD (N = 6). Dotted bars, control rats; gray bars, LPS-treated rats.

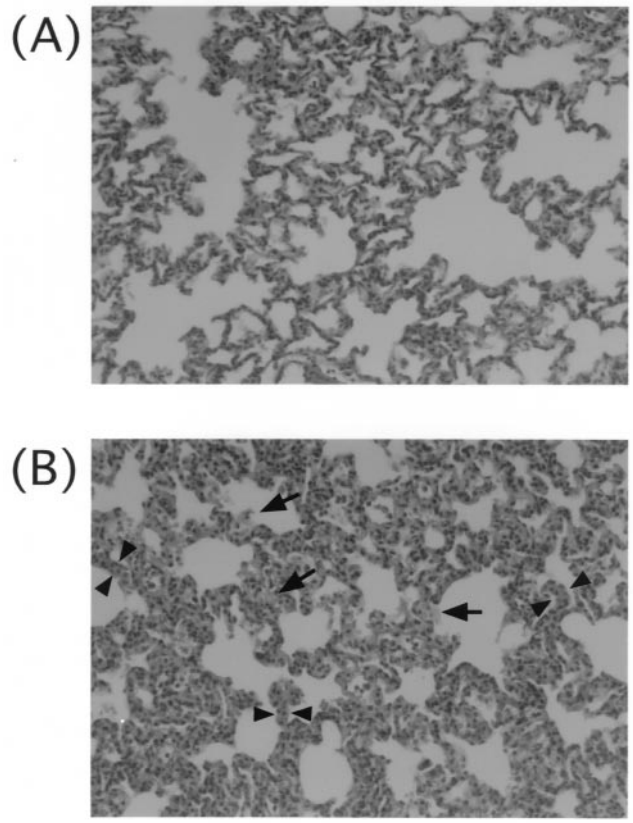

FIG. 3. Histological changes in the lungs after LPS administration. Rats were injected intraperitoneally with LPS (10 mg/kg). Twelve hours after the injection, lungs were removed for histological study as described in Materials and Methods. Three independent experiments showed similar results, and a typical example is shown in the figure. (A) Control rats; (B) LPS-treated rats. Marked congestion and influx of inflammatory cells were observed throughout the field. Edema and alveolar septal thickening typically were observed at the portion indicated by arrows and arrowheads, respectively, although these findings were also observed throughout the field (original magnification x200).

histological examination. Marked lung congestion, edema, alveolar septal thickening, and influx of inflammatory cells were observed at 12 hr after LPS administration (Fig. 3). These results indicate that an intraperitoneal injection of LPS into rats induced a condition compatible with septic MODS as defined by the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference [27].

Time Courses of HO-1 mRNA Expression after LPS Administration

Since MODS was established after LPS treatment, we examined the effect of intraperitoneal injection of LPS into rats on HO-1 mRNA levels in the liver, lung, and kidney. Hepatic HO-1 mRNA started to increase significantly at 6 hr after LPS treatment (70% of maximal level), reached a maximum at 9 hr, then gradually declined to 60% of the maximal level by 24 hr, and finally returned to the control level by 72 hr (Fig. 4, top). In the lung, HO-1 mRNA also significantly increased at 9 hr, reached a maximum at 12 hr, and then declined to the basal level by 48 hr (Fig. 4, middle). In the kidney, HO-1 mRNA started to increase significantly at 6 hr, reached a maximum at 9 hr, and then rapidly declined to 60% of the maximal level by 12 hr, and finally returned to the control level by 48hr (Fig. 4, bottom). Although HO-1 mRNA induction was observed in all organs examined, the maximal induction of renal HO-1 mRNA was relatively small as compared with those in the lung and liver.

Time Courses of ALAS-N mRNA Expression and Changes in Hepatic Heme Saturation after LPS Administration

Since HO-1 is the rate-limiting enzyme in heme catabolism, we also examined the level of another critical enzyme in the heme supply, ALAS-N mRNA, the rate-limiting enzyme in heme biosynthesis [19]. Following administration of LPS, ALAS-N mRNA levels in the liver decreased rapidly, and reached a minimum level (~10% of the level in the control) at 3 hr (Fig. 5, top), followed by a gradual increase to the basal level by 12 hr, and then decreased again by 72 hr, showing an oscillation pattern. Since it is known that HO-1 is induced by heme, whereas ALAS-N is down-regulated by heme in the liver, we determined the hepatic free heme concentration after LPS treatment. Whereas free heme concentration cannot be determined directly for various reasons, such as its low concentration and its extreme dynamic equilibrium, hepatic free heme concentration can be assessed indirectly by the extent of heme saturation of tryptophan pyrrolase activity [20]. Using this assay, we found that there was a significant increase in hepatic free heme concentration within 1 hr, which reached a maximum at 6 hr (Fig. 5, top). The increase returned to the control level by 18 hr. The time course of ALAS-N mRNA up to 12 hr after LPS treatment was a quasi-reverse image of hepatic heme saturation, suggesting that the increased heme concentration down-regulated ALAS-N gene expression [28]. The oscillating pattern of ALAS-N mRNA levels following LPS administration was also consistent with oscillating responses of ALAS activity in the livers of rats treated with intravenous injection of hematin [29].

In contrast to the liver, ALAS-N mRNA level in the lung maximally increased at 1 hr after LPS administration, rapidly declined by 9 hr, and then gradually returned to the control level by 48 hr (Fig. 5, middle). In the kidney, ALAS-N mRNA levels also increased significantly at 3 hr after LPS treatment and reached a maximum at 6 hr, maintained a high level until 9 hr, and then gradually decreased to the control level by 48 hr (Fig. 5, bottom).

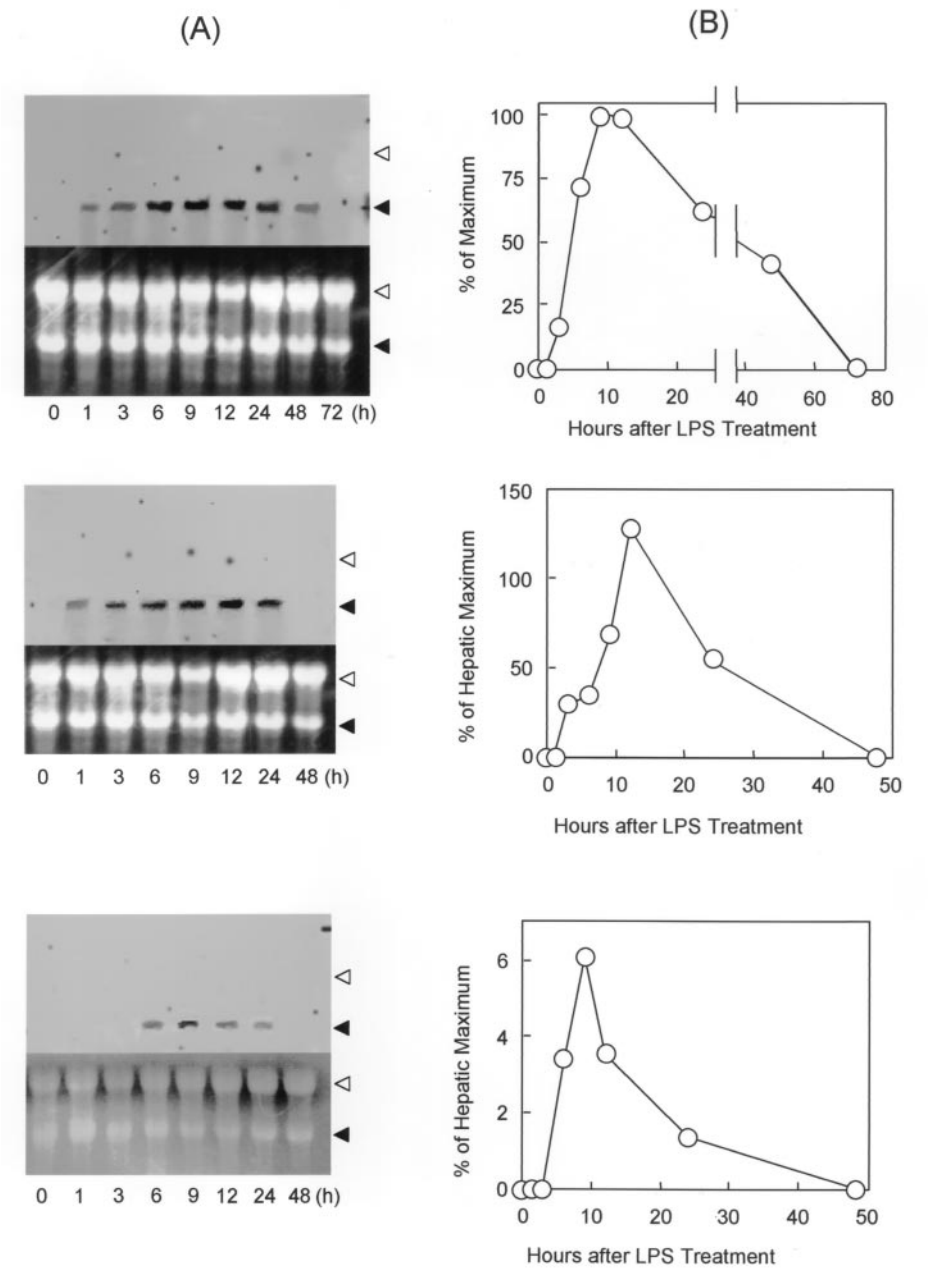

FIG. 4. Time courses of HO-1 mRNA in the rat liver, lung, and kidney following LPS administration. Rats were injected intraperitoneally with LPS (10 mg/kg), and were killed at 0, 1, 3, 6, 9, 12, 24, 48, and 72 hr after the injection. Liver, lung, and kidney were removed for northern blot analysis as described in Materials and Methods. (A) Twenty micrograms of total RNA were subjected to northern blot analysis. Shown are chemiluminescent signals of RNA blots hybridized with biotin-labeled riboprobes of rat HO-1 cRNA in an ethidium bromide-stained gel. Three independent experiments showed similar results, and a typical example is shown in the figure. Closed and open arrowheads indicate 18S and 28S ribosomal RNA, respectively. (B) Levels of HO-1 mRNA (open circles) are expressed as the percentage of the maximal level of hepatic HO-1 mRNA, to permit comparison among the three tissues. Top panel, liver; middle panel, lung; and bottom panel, kidney.

Effect of HO Activity after LPS Administration

To examine whether HO-1 mRNA induction is associated with the increase in HO function, we measured HO activity in the liver, kidney, and lung after LPS administration. Consistent with marked elevation of HO-1 mRNA at 12 hr following LPS administration, HO activity was increased significantly at 12 hr after LPS administration in these organs (Fig. 6).

DISCUSSION

In the present study, we demonstrated an induction of hepatic, lung, and kidney HO-1 mRNA, which was followed by an increase in HO activity in a rat model of septic MODS. Our results were also the first to report the induction of renal HO-1 mRNA after LPS treatment. HO-1 mRNA induction in the liver following LPS treat-

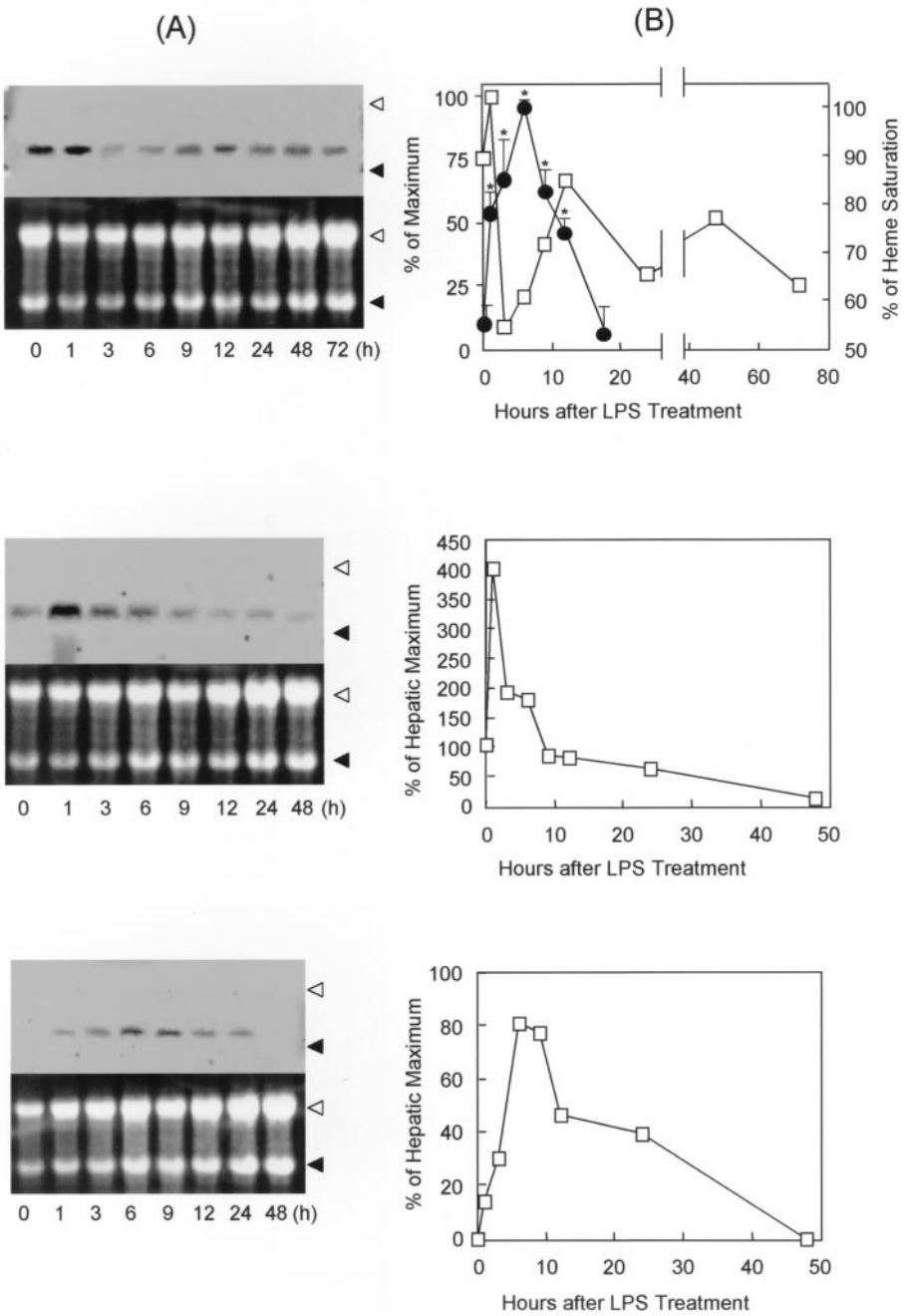

FIG. 5. Time courses of ALAS-N mRNA in the rat liver, lung, and kidney, and hepatic heme saturation following LPS administration. Rats were injected intraperitoneally with LPS (10 mg/kg), and were killed at 0, 1, 3, 6, 9, 12, 24, 48, and 72 hr after the injection. Livers were removed for northern blot analysis and measurement of hepatic heme saturation, and lungs and kidneys were removed for northern blot analysis as described in Materials and Methods. (A) Twenty micrograms of total RNA was subjected to northern blot analysis. Shown are chemiluminescent signals of RNA blots hybridized with biotin-labeled riboprobes of rat ALAS-N cRNA with an ethidium bromide-stained gel. Three independent experiments showed similar results, and a typical example is shown in the figure. Closed and open arrowheads indicate 18S and 28S ribosomal RNA, respectively. (B) Levels of ALAS-N mRNA (open squares) are expressed as the percentage of the maximal level of hepatic ALAS-N mRNA, to permit the comparison among the three tissues. The levels of hepatic heme saturation (top panel; closed circles) are shown as the means ± SD (N = 6). Key: (*) $P < 0.05$ vs control. Top panel, liver; middle panel, lung; and bottom panel, kidney.

ment appears to be mediated by an increase in hepatic free heme concentration, since there was an increase in heme saturation of tryptophan pyrrolase, and also a reduction in ALAS-N mRNA expression. This study also showed for the first time that, in contrast to what is seen in the liver, the expression of ALAS-N mRNA is up-regulated in the lung and kidney after LPS treatment. Our findings thus suggested that HO-1 and ALAS-N mRNAs are regulated in a

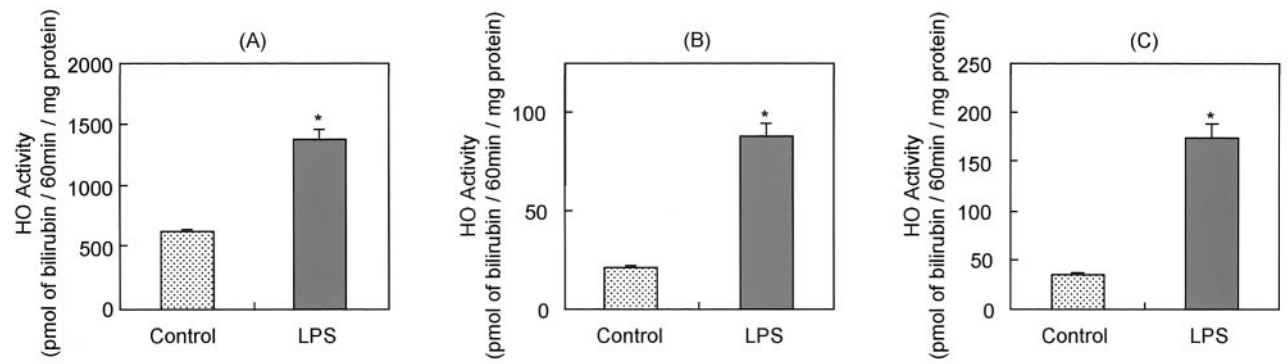

FIG. 6. Effects of LPS administration on HO activity in the rat liver (A), lung (B), and kidney (C). Rats were injected intraperitoneally with LPS (10 mg/kg) dissolved in 1 mL of sterile saline. Control rats received the same volume of sterile saline. These animals were killed at 12 hr after the injection, and livers, lungs, and kidneys were removed for measurement of HO activity as described in Materials and Methods. Data are expressed as the means ± SD (N = 6). Key: (*) $P < 0.01$ vs control. Dotted bars, control rats; gray bars, LPS-treated rats.

highly tissue-specific manner in LPS-induced oxidative tissue injury.

Following LPS administration, hepatic HO-1 mRNA levels started to increase after 6 hr, reaching a maximum at 9 hr, and then returned to the control level by 72 hr (Fig. 4, top). Prior to induction of HO-1, there was a rapid and significant increase in hepatic heme saturation of tryptophan pyrrolase (Fig. 5, top), suggesting that intracellular free heme concentration may elicit HO-1 mRNA induction [30]. Consistent with an increase in free heme concentration, ALAS-N mRNA levels in the liver were also decreased rapidly after LPS administration (Fig. 5, top). This finding is consistent with the well known heme-mediated repression of ALAS-N gene expression in liver [28]. It has also been reported that intraperitoneal injection of LPS stimulates HO activity and blocks induction of ALAS in the liver, which is thought to be due to redistribution of heme from cytochrome P450 to a regulatory heme pool [31, 32]. It should be also noted that ALAS-N mRNA levels showed an oscillating response up to 72 hr following LPS administration. This pattern of response is essentially similar to that reported for ALAS activity in the livers of rats after infusion of hematin, reflecting an oscillating free heme concentration in the liver [29]. Our results are entirely consistent with these findings, and confirm the previous observations at the transcriptional level.

As shown in Fig. 4, HO-1 mRNA levels in the lung and the kidney increased in a linear fashion, reaching a maximum at 12 and 9 hr, respectively, and then returned to the control level by 48 hr. In contrast to those in the liver, ALAS-N mRNA levels in the lung abruptly increased, reaching a maximum at 1 hr after LPS treatment, and then gradually declined to the control level by 48 hr (Fig. 5, middle). ALAS-N mRNA levels in the kidney also increased significantly at 3 hr after LPS treatment, reaching a maximum at 6 hr, and then declined to the control level by 48 hr (Fig. 5, bottom). It has been reported that IL-6 up-regulates ALAS [33] and HO-1 [10] in hepatocytes in culture. In contrast, our study in rat MODS showed increases in ALAS-N mRNA in the lung and the kidney, but not in the liver. These findings thus suggest that free heme-mediated suppression of ALAS-N expression in the liver may have overwhelmed the IL-6-mediated up-regulation of ALAS-N that may occur in these tissues.

LPS administration to rats has been reported to increase both HO and ALAS activity and to decrease microsomal heme content and cytochrome P450 levels in the lung [34, 35]. In the rat kidney, gene expression of cytochrome P450 isozymes has been reported to increase after LPS administration [36]. Taken together, our findings suggest that heme metabolism in LPS-induced sepsis is regulated in a tissue-specific manner. We have reported recently that heme-mediated oxidative injury induces HO-1 and suppresses ALAS-N gene expression in the kidney after ischemia/reperfusion [17]. However, in our septic MODS model, not only HO-1 but also ALAS-N mRNA were induced in the kidney (Fig. 4, bottom and Fig. 5, bottom). Although the precise mechanism for induction of both HO-1 and ALAS-N mRNAs is yet unknown, the pattern of changes in renal heme metabolism in LPS-induced renal injury is distinct from those observed in ischemia/reperfusion injury.

Recent observations suggested that HO-1 may provide cellular protection against oxidative injury caused by LPS administration. Otterbein *et al.* [16] have reported protective effects of HO-1 induction in a rat model of endotoxin shock. Poss and Tonegawa [14] have observed the increased susceptibility of HO-1-null knockout mice to LPS-induced oxidative stress. HO-1 oxidatively cleaves heme to produce carbon monoxide, iron, and biliverdin IXα, which is then reduced to bilirubin IXα by biliverdin reductase [37]. In this manner, HO-1 induced in the liver would reduce the concentration of free heme, a potent prooxidant [38, 39], and may confer major protection against oxidative injury. In addition, bilirubin IXα functions as an endogenous antioxidant [40], which may contribute further to the cellular defense system against oxidative stress [41].

In conclusion, significant induction of HO-1 mRNA, associated with elevation of HO activity, were demonstrated in the rat kidney, as well as in the lung and the liver following intraperitoneal administration of LPS, which caused septic MODS. In this model, a rapid increase of hepatic free heme concentration, as judged by the increase in hepatic heme saturation of tryptophan pyrrolase, accompanied suppression of ALAS-N gene expression and induction of hepatic HO-1 mRNA expression. While ALAS-N mRNA was down-regulated in the liver after LPS treatment, both pulmonary and renal ALAS-N mRNA expression were up-regulated following LPS administration. These results also indicate that HO-1 and ALAS-N mRNA are regulated in a tissue-specific manner.

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