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EARLY EXPRESSION OF HEME OXYGENASE-1 IN LEUKOCYTES CORRELATES NEGATIVELY WITH OXIDATIVE STRESS AND PREDICTS HEPATIC AND RENAL DYSFUNCTION AT LATE STAGE OF SEPSIS

Hsiao-Ching Jao,* Yi-Tseng Lin,† Li-Yu Tsai,‡ Chao-Chuan Wang,‡ Hong-Wen Liu,§ and Chin Hsu¶

*Departments of *Respiratory Care, †Clinical Biochemistry, ‡Anatomy, and §Rheumatology, Kaohsiung Medical University Chung-Ho Memorial Hospital, Kaohsiung 807, Taiwan; and ¶Department of Physiology, Kaohsiung Medical University, Kaohsiung 807, Taiwan*

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ABSTRACT—Oxidative stress triggered by septic insult may be the major cause of multiple organ dysfunction syndrome (MODS) in intensive unit care patients. The inducible form of heme oxygenase-1 (HO-1) can be induced by cytokines, lipopolysaccharide, and reactive oxygen species during sepsis. These facts raise the question of whether the expression of HO-1 in leukocytes can indicate the level of oxidative stress of multiple organs in sepsis. Clinical peritonitis was simulated in an animal model by cecal ligation and puncture (CLP). The level of oxidative stress was examined by plasma lipid peroxidation (LPO). Liver function was analyzed by plasma aspartate aminotransferase, alanine aminotransferase, total bilirubin, and direct bilirubin. Lung function was evaluated by severity of edema. Renal function was measured by blood urea nitrogen and creatinine. The correlation between early HO-1 induction and LPO level or organ functional indicators of the same rat at late sepsis was analyzed by linear regression. The results showed that the protein content of HO-1 increased at 9 h after CLP, whereas expression of HO-1 mRNA in leukocytes was significantly increased ($P < 0.01$) at 6 h after CLP. Plasma level of LPO and the indices of hepatic, pulmonary, and renal function were significantly increased at 18 h after CLP. Moreover, highly negative correlations were observed between HO-1 mRNA expression at 6 h after CLP and level of LPO or severity of hepatic/renal dysfunction at 18 h after CLP. These results suggest that early HO-1 mRNA expression in leukocytes may represent oxidative stress and may predict the severity of liver and renal dysfunction during sepsis.

KEYWORDS—HO-1, severe inflammation, organ failure

INTRODUCTION

Sepsis, a condition resulting from a harmful or damaging host response to infection, is characterized by decreases in vascular tone and increases in oxidative stress. During the onset of sepsis, the inflammatory system becomes hyperactive, involving cellular and humoral defense mechanisms. In later stages of sepsis, various innate functions are suppressed, especially the functions of neutrophils, leading to a hyporeactive host defense system and immunoparalysis (1). The innate response normally associated with host defense against infection causes cell or tissue damage and leads to multiple organ failure (2). Medical decision-making requires adequate information to select the correct therapeutic option. Therefore, a marker is needed to identify a biological state or to predict the severity of pathologic processes during sepsis. Various markers of inflammation including interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, and IL-8 have been studied. However, these mediators have been found to be insufficient in sensitivity and specificity. The use of these markers can also be time consuming and expensive. Moreover, none of the aforementioned markers can predict functional failure of multiple organs resulting from oxidative stress in polymicrobial sepsis. Recent evidence suggests that an important cellular response to oxidative stress is the expression of heme oxygenase-1 (HO-1). HO-1 degrades heme to generate carbon monoxide (CO; a vasodilatory gas), iron, and the potent antioxidant bilirubin, and confers protection against oxidative tissue injury (3–5). Expression of HO-1 is induced by diverse stimuli such as heme, hyperoxia, hypoxia, heat shock, endotoxin, hydrogen peroxide, cytokines, UV light, heavy metals, and nitric oxide (6), and all of which are produced during sepsis (7). Previous reports indicated that HO-1 mRNA is induced markedly in the buffy coat of the blood at 3 h after lipopolysaccharide (LPS) administration, which coincides with the increased levels of serum IL-6 and suggested that HO-1 may be one of the key markers of multiple organ dysfunction syndrome (MODS) in LPS-induced sepsis (8). In addition, HO-1 production by monocytes may serve as a potent anti-inflammatory agent for controlling excessive cell or tissue injury in the presence of oxidative stress and cytokinemia (9). These facts prompt us to investigate whether HO-1 in leukocytes reflects the level of cellular oxidative stress, which is predictive of organ failure during polymicrobial sepsis.

MATERIALS AND METHODS

Animal model of polymicrobial sepsis

The present study was conducted in accordance with National Institutes of Health guidelines for the use of experimental animals. Experiments described in this study were approved by the Animal Committee of Kaohsiung Medical University. Experiments were performed on male Sprague-Dawley rats 300 to 350 g in body

Address reprint requests to Chin Hsu, PhD, Department of Physiology, School of Medicine, Kaohsiung Medical University, No. 100, Shih-Chuan 1st Road, Kaohsiung 807, Taiwan. E-mail: chinhsu@kmu.edu.tw.

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weight. Before the experiment, all animals were fasted overnight with free access to water. Sepsis was induced by cecal ligation and puncture (CLP) according to the method of Wichterman et al. (10) with minor modification. Under halothane anesthesia, a laparotomy was performed, and the cecum was ligated with a 3-0 silk ligature and punctured twice with an 18-gauge needle. The cecum was then returned to the peritoneal cavity, and the abdomen was closed in two layers. Control rats were sham operated (a laparotomy was performed and the cecum was manipulated but was not ligated or punctured). All animals were resuscitated with 4 mL/100 g body weight with normal saline by subcutaneous injection at the completion of surgery and at 9 h postsurgery, individually (11). Animals were fasted but had free access to water after operative procedures. Blood was sampled from the tail vein at various time points after CLP for detection of HO-1 expression and organ function tests. Because the rats die easily if more than two samples are drawn from the same rat, the data associated with HO-1 expression at different time points (i.e., 0, 2, 3, 6, and 9 h after CLP) comes from different rats. The blood sample on the 9-h time point was collected before subcutaneous resuscitation at 9 h after CLP. For evaluation of the correlation of early HO-1 expression and late organ dysfunction, each sample was derived at 6 h after CLP and the same rat was maintained until 18 h after CLP. The blood samples of late stage of sepsis were taken from the survival rats for measuring the indicators of organ dysfunction. The sample of the early stage of sepsis was discarded if the same rat was dead in the late stage of sepsis.

Western blot analysis of HO-1

Leukocytes were isolated from 2 mL of blood sample after adding NH_4Cl lysis buffer and incubating at room temperature for 10 min followed by centrifugation at 400*g* for 10 min. Samples were homogenized in five volumes of cell lysis buffer containing 10 mM/L Tris (pH 7.5), 10 mM/L NaCl, 0.1 mM/L EDTA, 0.5% Triton X-100, 0.02% sodium azide, and 2 mM/L phenylmethylsulfluoride. Homogenates were clarified by centrifugation at 10,000*g*, and total soluble protein concentration was determined, in accordance with the method described by Bradford (12), by using a commercially available dye reagent (protein assay kit II; Bio Rad, Hercules, CA) with bovine serum albumin as a standard. Aliquots of protein (50 μg/lane) from each organ were fractionated by 12% SDS-PAGE. After electrophoresis, the proteins on the gel were transferred to polyvinylidene difluoride (PVDF) membranes (Life Science, Arlington Heights, IL). After transfer and blocking with 5% nonfat dry milk in Tris-buffered saline/Tween (20 mmol/L Tris, pH 7.5, 0.5 mol/L NaCl, and 0.1% Tween 20), the PVDF membrane was incubated with polyclonal rabbit anti-rat HO-1 primary antibodies (dilution 1:500). Unbound primary antibody was removed by washing the membrane with Tris-buffered saline/Tween. A horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Ig) G at 1:5000 dilutions was used as a second antibody. The blot was stripped and reincubated with a monoclonal antibody against β-tubulin (Boehringer Mannheim Biochemica, Manheim, Germany) to confirm that equal sample protein was loaded. The protein was then detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL), exposed to x-ray film (Fuji, Tokyo, Japan), and evaluated using a computerized densitometer scanner (Bio-1DV.97 software; Vilber Lourmat, Marne La Vallee, France) (13).

Measurement of HO-1 mRNA expression

Total RNA was extracted from the 2-mL leukocyte sample, which is different from that for HO-1 protein detection, according to a one-step method. Reverse transcription protocols were performed with 5 μg of total RNA in 30 μL (final volume) of reaction buffer. 18S was used as an internal control. Aliquots of the reverse transcription reaction were amplified with the following rat primer sequence of HO-1: forward 5′-AAGGAGGTGCACATCCGTGCA-3′, reverse 5′-ATGTT-GAGCAGGAAGGCGGTC-3′. The PCR products of HO-1 and 18S were 568 and 324 bp, respectively. The kinetic change of HO-1 expression in leukocytes was evaluated at various time points at 0, 2, 3, 6, 9, and 18 h after CLP.

Biochemical measurements of organ functional tests

LPO was examined as severity of oxidative stress. Products of LPO in plasma were estimated by thiobarbituric acid (TBA) method. Malondialdehyde (MDA) is a stable end product of fatty acid peroxidation, which reacts with TBA at acidic conditions to form a complex that has maximum absorbance at 545 nm. Liver function was analyzed by plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, (TBIL), and direct bilirubin (DBIL). AST catalyzes the reversible transamination of L-aspartate and α-ketoglutarate to L-glutamate and oxaloacetate. The oxaloacetate is then reduced to malate in the presence of malate dehydrogenase with the concurrent oxidation of reduced NADH to NAD, which can be detected by the change in the absorbance at 340 nm. ALT catalyzes the reversible transamination of L-alanine and α-ketoglutarate to L-glutamate and pyruvate. The pyruvate is then reduced to lactate in the presence of lactate dehydrogenase with the concurrent oxidation of reduced NADH to NAD, which can be detected by the change in the absorbance at 340 nm. TBIL concentration was measured by an endpoint diazo method. In the reaction, the bilirubin reacts with diazo reagent in the presence of caffeine, benzoate, and acetate as accelerators to form azobilirubin. DBIL concentration was estimated by an endpoint diazo method. In the reaction, direct bilirubin combines with diazo to form azobilirubin. The system monitors the change in absorbance at 520 nm. The change in absorbance is directly proportional to the concentration of total bilirubin in the sample. Renal function was measured by blood urea nitrogen (BUN) and creatinine at 9 and 18 h after CLP. The concentration of BUN was measured by means of an enzymatic conductivity rate method. The reaction converts the nonionic species (urea) to ionic one (ammonium ion and bicarbonate). During the reaction, the timed rate of increase of solution conductivity is directly proportional to the concentration of urea present in the sample. The creatinine concentration was determined by means of the Jaffe rate method. Creatinine from the sample combines with the alkaline picrate solution to produce a red color complex, which was read at 520 nm. All of the AST, ALT, TBIL, DBIL, BUN, and CRE concentrations were determined by the SYNCHRON LX20 System (Beckman Coulter, Fullerton, CA). Ischemic heart injury was detected by plasma troponin T by using Roche Diagnostics Elecsys 2010 to detect the electrochemiluminesence. Pulmonary edema was examined by measuring the lung wet weight-to-dry weight ratio as an index of pulmonary edema and microvascular injury (14). Lung tissue samples were collected and weighed, then dried at 60°C for 72 h. Dry tissue weight was then determined and wet weight-to-dry weight ratio was calculated.

Statistics

The difference of HO-1 expression, plasma level of LPO, and organ functional indicators between sham and septic groups was analyzed using analysis of variance followed by *post hoc* evaluation. Data are presented as mean ± SD. $P < 0.05$ was considered statistically significant. The correlations between early HO-1 mRNA expression and LPO levels or severity of organ dysfunctions at late sepsis were analyzed by linear regression followed by *F* test.

RESULTS

Increase of the protein content of HO-1 in leukocytes 18 h after CLP

To analyze kinetic changes of HO-1 in leukocytes during sepsis, protein content was evaluated at 0, 6, 9, and 18 h after CLP by Western blot analysis with specific antibody for HO-1. As shown in Figure 1, protein content of HO-1 in leukocytes was almost undetectable before CLP operation, slightly elevated at early sepsis, and significantly increased at 18 h after CLP.

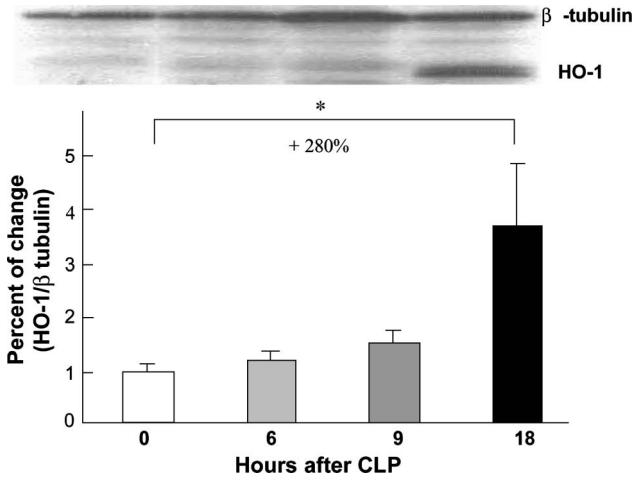

FIG. 1. **Protein content of HO-1 in leukocytes at different time points after CLP.** The blood samples of different time points were derived from different rats, because the rats die easily if more than two blood samples are drawn from the same rat. The molecular weight of HO-1 is 32 kD. β-Tubulin (59 kD) is detected as an internal control. Representative data of Western blot is shown in the top panel. The quantitative result is shown in the bottom panel. Data shown are means ± SD of six samples in each group. $^*P < 0.05$.

Enhancement of HO-1 mRNA expression in leukocytes 6 h after CLP

The expression of mRNA of HO-1 was assumed to occur before the increase of protein content. Therefore, the kinetic alteration was evaluated at 0, 2, 3, 6, 9, and 18 h after CLP. The results shown in Figure 2 indicate that mRNA expression was significantly increased by 230% as early as 6 h after CLP and remained at a high level until 18 h after CLP.

MODS during sepsis

To elucidate organ dysfunction, plasma indicators of liver function (AST, ALT, TBIL, and DBIL), renal function (creatinine and BUN), cardiac injury (troponin T), severity of lung edema, and oxidative stress (LPO) were evaluated at 9 and 18 h after CLP. The results showed that functional indicators of liver dysfunction were significantly ($P < 0.01$) elevated at 18 h after CLP as shown in Figure 3, whereas renal dysfunction occurred as early as 9 h after CLP (Fig. 4, A and B). Cardiac injury and lung edema occurred at 18 h after CLP as shown in Figure 4, C and D. Moreover, the plasma level of LPO was also significantly ($P < 0.01$) increased at 18 h after CLP (Fig. 5).

Correlation between early HO-1 mRNA expression in leukocytes and late organ dysfunction of septic rats

To know the relationship between the early HO-1 mRNA expression in leukocytes and oxidative stress, which leads to tissue damage of the same rat, plasma level of LPO was examined. The correlation between HO-1 mRNA expression and plasma LPO or organ functional index was analyzed by linear regression. As shown in Figure 6, early HO-1 mRNA expression in leukocytes correlated inversely with plasma indicators of liver function at the late stage of sepsis. In addition, a highly and significantly negative correlation was observed between early HO-1 expression in leukocytes and plasma indicators of renal function (Fig. 7, A and B). Although no correlation was observed between HO-1 mRNA expression and cardiac injury or lung edema (Fig. 7, C and D), a high r^2 value was observed between HO-1 mRNA expression and plasma LPO level (Fig. 8).

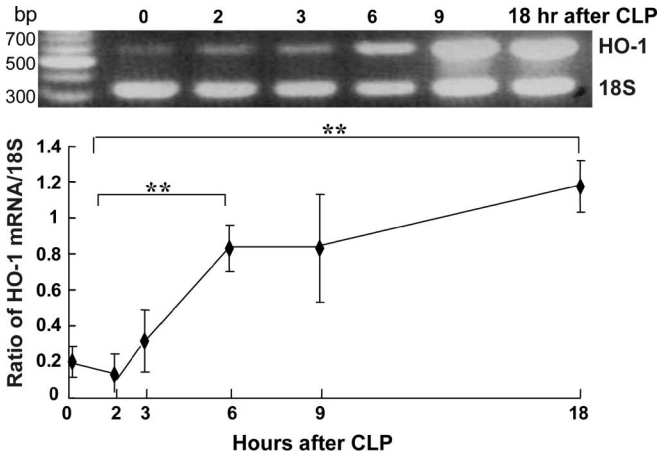

FIG. 2. **Expression of HO-1 mRNA in rat leukocytes at different time points after CLP.** The blood samples of different time points was derived from different rats to avoid death. Expression of HO-1 mRNA is quantified by RT-PCR and normalized by 18S RNA. PCR products of HO-1 and 18S are 568 and 364 bp, respectively. Data shown are mean ± SD of six samples in each group. *$P < 0.05$, **$P < 0.01$.

DISCUSSION

Oxidative stress plays an important role in multiple organ failure during sepsis. However, plasma levels of lipid peroxidase oxidation or biochemical indicators of organ dysfunction cannot be detected early enough to prevent organ failure. The present study showed that expression of HO-1 mRNA in leukocytes is significantly elevated as early as 3 h after CLP, suggesting that HO-1 may be an early marker of oxidative stress in polymicrobial sepsis. Moreover, HO-1 expression has a highly negative correlation with liver dysfunction, renal dysfunction, and plasma level of LPO. This suggests that early HO-1 mRNA expression in circulatory blood cells may reflect oxidative stress and predict the severity of hepatic or renal dysfunction during sepsis.

Neutrophils are a major population of leukocytes. Activation of neutrophils, by releasing reactive oxygen species (ROS), cytokines, or chemokines, is one of the major causes of oxidative stress and subsequent liver damage during sepsis (15). Previous studies of HO-1-deficient mice indicated that the high mortality of endotoxemia is related to increased oxidative stress and end-organ dysfunction (16). In addition, hemoglobin-induced protection against endotoxemia in rats is dependent on HO-1 induction (17). The present result revealed a highly negative correlation between HO-1 mRNA expression in leukocytes and serum level of LPO. In addition, a significant negative correlation was observed between early HO-1 mRNA expression in leukocytes and failure of liver or kidney function during sepsis. However, no correlation between HO-1 mRNA expression and heart injury or lung edema was observed. Based on the highly negative correlation between HO-1 mRNA expression in leukocytes and plasma level of LPO, oxidative stress may contribute primarily to liver and renal dysfunction rather than cardiac or lung injury. This negative correlation also suggests that HO-1 mRNA expression may reflect oxidative stress, which leads to tissue damage. In other organs, e.g., the heart, factors other than oxidative stress may contribute to tissue damage during sepsis (18). Further investigation can elucidate the exact role of HO-1 mRNA expression in leukocytes during sepsis.

Inducible HO-1 is an antioxidative stress protein that is mainly induced by ROS, cytokines, and hyperthermia. Among the most critical of cytoprotective mechanisms activated during cellular stress, HO-1 is thought to exert four major protective functions: antioxidation; maintenance of microcirculation; modulation of the cell cycle; and anti-inflammation. The antioxidant function depends on heme degradation, oxygen consumption, biliverdin, and production of ferritin via iron accumulation. The production of carbon monoxide (CO), which has vasodilation and antiplatelet aggregation properties, maintains tissue microcirculation and may be instrumental in antiapoptotic and cell

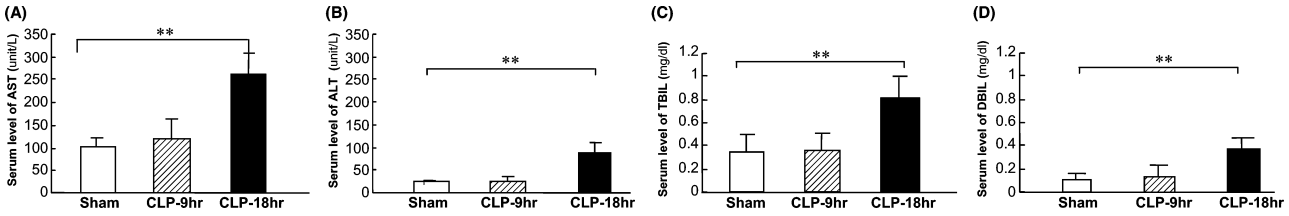

FIG. 3. **Liver functions in rats of sham-operated (Sham), 9 h after CLP (CLP-9h), and 18 h after CLP (CLP-18h).** (A) AST levels; (B) ALT levels; (C) TBIL; (D) DBIL. Data shown are mean ± SD of 10 samples in each group. $^{**}P < 0.01$.

arrest mechanisms. It has also been speculated that CO may mediate cellular cytoprotection via its anti-inflammatory properties. CO inhibited LPS-induced activation of NF-κB has been shown to regulate granulocyte macrophage-colony-stimulating factor transcription by preventing the phosphorylation and degradation of the regulatory subunit IκBα (19). Therefore, heme catabolism and HO-1 overexpression exert profound direct and indirect inhibitory effects on the cascade of host inflammatory responses mediated by neutrophils, macrophages, and lymphocytes (20).

Previous reports have demonstrated that exhaustive exercise induces expression of iNOS and HO-1 in human leukocytes (21). The rise in the cytoplasmic expression of HO-1 is more pronounced in monocytes and granulocytes than that in lymphocytes after endurance-training exercise, which can produce an inflammatory response such as elevation in the plasma levels of myeloperoxidase, TNF-α, and interleukin 8 (IL-8) (22). Monocytes play key roles in innate and adaptive antigen-specific immunity, and they constitute critical components of the immune responses. Although most of the monocyte-derived cytokines exhibit proinflammatory functions *in vivo*, HO-1 exerts potent anti-inflammatory effects through production of CO and bilirubin. Significantly elevated HO-1 mRNA levels seen in acute inflammatory illnesses suggest that monocyte HO-1 production serve as a potent anti-inflammatory agent for controlling excessive cell or tissue injury in the presence of oxidative stress and cytokinemia. (9). In the present result, the highly negative correlation between early expression of HO-1 in leukocytes and late tissue dysfunction in liver or kidney strongly suggest an anti-inflammatory or antioxidant function of HO-1 in leukocytes. However, the causal relationship and details regarding the molecular mechanism between HO-1 in leukocytes and tissue damages require further investigation.

Previous reports have indicated that the increased mortality rate during endotoxemia in HO-1$-/-$ mice is related to increased oxidative stress indicated by LPO products in liver tissue (MDA+4-HNE) and end-organ (renal and hepatic) damage (16). In sepsis, the liver participates in host defense and tissue repair through hepatic cell cross-talk, which controls most of the coagulation and inflammatory process. The sepsis-induced changes in hepatic function involve cross-talk between Kupffer cells, hepatocytes, and sinusoidal endothelial cells (23). In addition, activated neutrophils, which are recruited in the liver and produce destructive enzymes and oxygen-derived radicals, may further enhance liver injury (24). The HO-carbon monoxide system is a regulator of hepatobiliary function (25), and the liver is the major organ involved in detoxification of heme and biliary excretion of bilirubin (25). Furthermore, HO-1 induction has been shown to inhibit leukocyte adhesion through the action of bilirubin (26). Sepsis also sensitizes hepatic microcirculation to ET-1, and impairment of microcirculatory flow due to ET-1 in sepsis contributes to hepatic injury (27). HO-1-derived CO also appears to be necessary for protecting hepatic microcirculation under conditions of stress (25). Taken together, HO-1 may play an important role in protecting hepatocytes from injury not only by catalyzing heme, a pro-oxidant, or by producing antioxidative bilirubin, but also by releasing CO, a vasodilator for microcirculation.

Peroxidation of lipid in erythrocytes by LPS appears to play a role in inducing septic shock (28). Heme proteins are introduced into tubular epithelial cells after incorporation and degradation of erythrocytes by the renal tubular epithelium, which then leads to a tubulointerstitial injury (29). Induction of

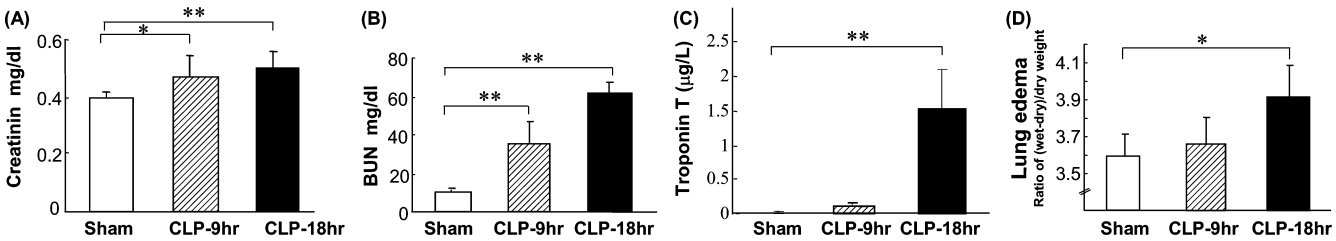

FIG. 4. **Renal functions, serum levels of troponin T, and severity of lung edema in rats of sham-operated (Sham), 9 h after CLP (CLP-9h), and 18 h after CLP (CLP-18h).** (A) Serum levels of creatinine. (B) Levels of BUN. (C) Serum level of troponin T. (D) Severity of lung edema. Severity of lung edema was determined by the ratio of the difference between wet weight and dry weight to dry weight. Data shown are mean ± SD of 10 samples in each group. $^{*}P < 0.05$; $^{**}P < 0.01$.

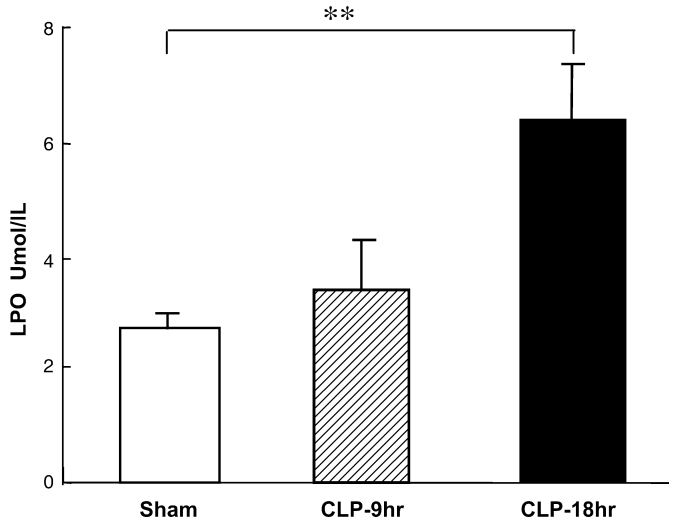

FIG. 5. **Serum levels of LPO in rats of sham-operated (Sham), 9 h after CLP (CLP-9h), and 18 h after CLP (CLP-18h).** Data shown are mean ± SD of six samples in each group. *$P < 0.05$; **$P < 0.01$.

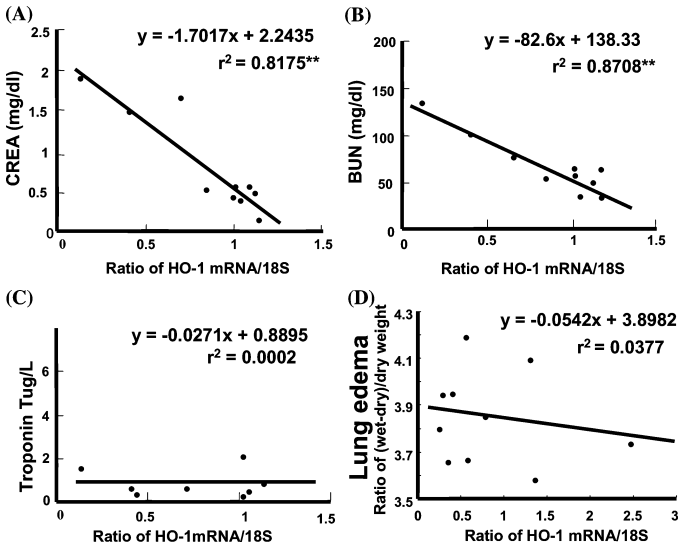

FIG. 7. **Correlation between early HO-1 mRNA expression in leukocytes and renal dysfunction, heart injury, or severity of lung edema at late stage of sepsis.** (A) Creatinine; (B) BUN; (C) troponin T; (D) lung edema. mRNA expression of HO-1 in leukocytes was detected at 6 h after CLP, whereas serum levels of creatinine, BUN, troponin, and ratio of wet/dry weight of lung tissue of the same rat were measured at 18 h after CLP. The correlation between early HO-1 mRNA expression and functional index of the same rat at late sepsis was analyzed by linear regression followed by *F* test. **$P < 0.01$.

HO-1 is an essential response for protecting against acute heme protein toxicity *in vivo* (30). Zurovsky and Haber reported (31) that decline in renal function was markedly slower in rats given antioxidants. Therefore, the antioxidant effect of HO-1 may contribute to diminishment of renal dysfunction. In addition, microcirculatory failure is one of the ultimate causes of septic shock. In this context, it is prudent to re-emphasize the roles played by heme protein-related tubular obstruction and renal vasoconstriction. To some extent, protection afforded by HO-1 may depend simply on greater systemic clearance of heme and removal of accumulating heme from tubular lumina by catabolism. On the other hand, generation of CO from oxidation of heme by HO-1 promotes vasodilation. Therefore, HO-1 expression in leukocytes may improve renal function by decreasing LPO and heme overloading as well as vasodilation.

In summary, our results suggest that early mRNA expression of HO-1 may diminish the level of oxidative stress and play a beneficial role for hepatic and renal functions during sepsis. However, identification of the biological significance and the exact mechanisms responsible for HO-1 induction in leukocytes during sepsis require further elucidation.

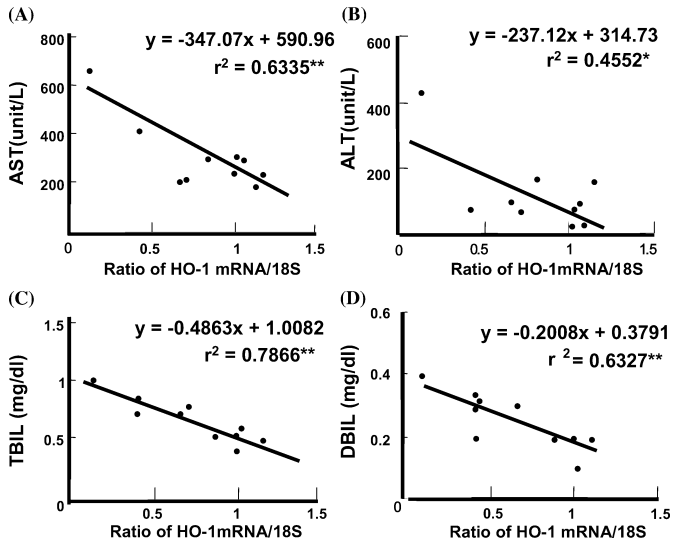

FIG. 6. **Correlation between early HO-1 mRNA expression in leukocytes and hepatic dysfunction at late stage of sepsis. (A) AST levels; (B) ALT levels; (C) TBIL; (D) DBIL**. The expression of HO-1 mRNA in leukocytes was detected at 6 h after CLP, whereas liver functional markers of the same rat were measured at 18 h after CLP. The correlation between early HO-1 expression and liver functional index of the same rat at late sepsis was analyzed by linear regression followed by *F* test. *$P < 0.05$; **$P < 0.01$.

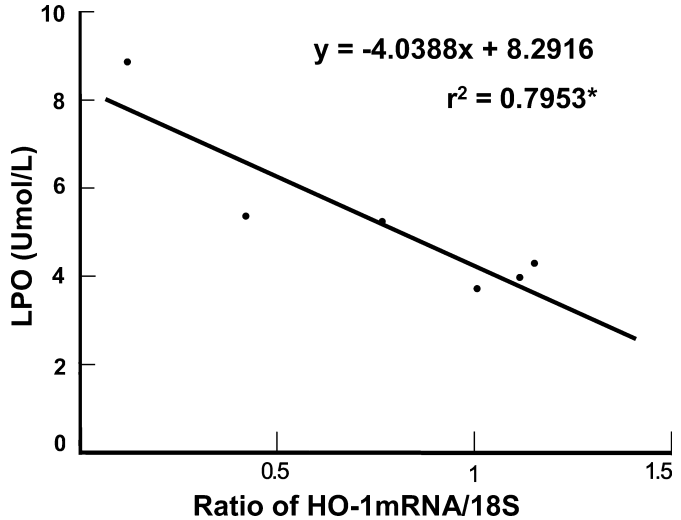

FIG. 8. **Correlation between early HO-1 mRNA expression in leukocytes and serum levels of LPO at late stage of sepsis.** The expression of HO-1 mRNA in leukocytes was detected at 6 h after CLP, whereas serum level of LPO in the same rat was measured at 18 h after CLP. The correlation between early HO-1 expression and plasma level of LPO of the same rat at late sepsis was analyzed by linear regression followed by *F* test. *$P < 0.05$; **$P < 0.01$.

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