Ischemia/reperfusion Lung Injury Increases Serum Ferritin and Heme Oxygenase-1 in Rats

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Intestinal ischemia/reperfusion (I/R) is one of common causes of acute lung injury (ALI). Early and accurate diagnosis of patients who are like to develop serious acute respiratory distress syndrome (ARDS) would give a therapeutic advantage. Ferritin and heme oxygenase-1 (HO-1) are increased by oxidative stress and are potential candidates as a predictive biomarker of ARDS. However, the mechanisms responsible for the increases of ferritin and HO-1, and their relationship to ALI, are unclear. In order to elucidate the interactions between ferritin and HO-1, we studied the changes in ferritin and HO-1 levels in serum and bronchoalveolar lavage (BAL) fluid after intestinal I/R injury in rats. Leukocyte number and protein contents in BAL fluid were elevated following I/R, and the increases were attenuated by mepacrine pretreatment. Both serum ferritin and HO-1 concentrations were progressively elevated throughout the 3 h observation period. Mepacrine pretreatment attenuated the increase of serum and BAL fluid ferritin concentrations, but did not suppress the increase of serum HO-1. Moreover, BAL fluid HO-1 levels did not change after I/R or after mepacrine pretreated I/R compared with sham rats. Unlike ferritin, HO-1 levels are not exactly matched with the ALI. Therefore, there might be a different mechanism between the changes of ferritin and HO-1 in intestinal I/R-induced ALI model.

Key Words: Ferritin, Heme oxygenase-1, Ischemia/reperfusion, Acute lung injury, ARDS

INTRODUCTION

Acute lung injury (ALI) and its most severe form, the acute respiratory distress syndrome (ARDS), are frequent complications in critically ill patients and responsible for significant morbidity and mortality (Repine, 1992; Ware and Matthay, 2000). Ischemia/reperfusion (I/R) frequently occurs in human pathological conditions, and it has been considered as a significant cause of ALI and ARDS (Otamiri et al., 1988; Grace, 1994). The pathogenesis of this injury appears to involve the generation of reactive oxygen species (ROS), which can be detected during both ischemia (Minamiya et al., 1998) and reperfusion (Kennedy et al., 1989).

Having been indicated as one of the earliest and most important components of tissue injury after reperfusion of the ischemic organ, ROS and the end-products of lipid peroxidation have been shown to up-regulate a number of cytoprotective genes, including heme oxygenase-1 (HO-1; Choi and Alarm, 1996). It has been suggested that the induction and maintenance of HO-1 expression was a key for protection in an intestinal I/R model (Hassoun et al., 1992; Willis et al., 1996; Attuwaybi et al., 2003). HO-1 induction before injury protects against oxidant-mediated cytotoxicity in

cell culture (Lee et al., 1996) and prolongs survival after lipopolysaccharide (LPS) instillation in rats (Otterbein et al., 1995). HO-1 inhibition also resulted in a loss of endotoxin-induced protection against oxidant injury in vascular smooth muscle cells (Yet et al., 1997).

Heme oxygenase is a heme-cleaving enzyme that opens the porphyrin ring, producing biliverdin, carbon monoxide, and free iron (Tenhunen et al., 1968; Otterbein et al., 2003). HO-mediated release of iron has been shown to influence ferritin expression, such that an increase in HO-1 may lead to increased ferritin expression (Eisenstein et al., 1991; Harrison and Arosio, 1996; Ferris et al., 1999). This sequence of events has been proposed as one of the cytoprotective functions of HO-1 (Vile et al., 1994; Choi and Alarm, 1996).

Previous studies have reported simultaneous induction of ferritin and HO-1 following hyperoxia- and hemoglobin-induced lung injury in rats (Balla et al., 1995; Taylor et al., 1998).

Ferritin plays an important role as a fast-acting endogenous cytoprotectant in cellular antioxidant defense mechanisms by rapidly sequestering free cytosolic iron, which is the crucial catalyst of oxygen-centered radical formation via the Fenton reaction in biological systems (Balla et al., 1992; Cairo et al., 1995; Juckett et al., 1995; Kim et al., 1995). Therefore, the heme degradation products and the metabolic derivatives generated by HO-1 suppress toxic events in cells.

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ABBREVIATIONS: ALI, acute lung injury; ARDS, acute respiratory distress syndrome; HO-1, heme oxygenase-1; BAL, bronchoalveolar lavage.

Serum ferritin levels were elevated in an animal model of ALI (Park et al., 2003, 2004; Park and Lee, 2006). Several clinical studies also have reported that serum ferritin level might be a useful predictor of the onset of severe acute respiratory distress syndrome (Connelly et al., 1997; Sharkey et al., 1999; Ji et al., 2007). If the increase of serum ferritin is solely dependent on HO-1 following ALI, HO-1 would be a better predictor for the early diagnosis of ARDS than ferritin. However, met-hemoglobin induces ferritin even after the inhibition of HO-1 activity in rat lung endothelium (Balla et al., 1993). This means there might induction of ferritin independent HO-1. Until now, relatively few studies have addressed the interactions between ferritin and HO-1 and the sequential changes of ferritin and HO-1 in serum and/or bronchoalveolar lavage (BAL) fluid following ALI at the same time (Mumby et al., 2004).

On the other hand, there is a limitation to use serum ferritin level as an indicator of lung injury. In a previous report, no close relationship was seen between serum and BAL ferritin increase in a severe hemorrhage-induced ALI model (Park et al., 2004). Therefore, it is very important to evaluate the reliability of biomarkers and to understand the mechanisms responsible for the changes of HO-1 and ferritin in serum or BAL fluid. In this study, the temporal pattern of the changes of ferritin and HO-1 in serum and BAL fluid following intestinal I/R were examined, while evaluating ferritin and HO-1 levels as a predictor of ARDS in rats subjected to intestinal I/R injury.

METHODS

Experimental protocols were approved by the Ethical Committee of Animal Experiments in Daegu Catholic University, School of Medicine. Male Sprague-Dawley (Hyo Chang Science, Daegu) rats weighing 300~450 g were used. Rats were anesthetized by intraperitoneal injection of mixed ketamine (80 mg/kg) and xylazine (16 mg/kg). The left femoral artery was incised, and a polyethylene catheter (PE-50, Clay-Adams, USA) filled with heparinized saline (100 U/ml) and inserted for blood sampling. The rats remained deeply anesthetized for the entire experiment, booster shots if needed.

Rats were randomly divided into different groups: Mepacrine-treated I/R groups (Mepa + I/R; injection of PLA₂ inhibitor mepacrine, 60 mg/kg i.p.), I/R groups (saline vehicle, i.p.). Thirty minutes after the pretreatment with saline or mepacrine, intestinal ischemia was achieved by clamping the superior mesenteric artery for 30 min using a non-traumatic artery clamp, and recirculation of blood flow was established by releasing the clip. Reperfusion was allowed for 3 hours. Sham-operated rats underwent identical surgical procedures, except that no artery clamp was applied.

For measurement of serum ferritin and HO-1 concentrations, 100 μ l of arterial blood was collected through the catheter just before ischemia and 30, 60, and 180 min after the reperfusion. At the end the experiment, samples of lung and jejunal part of intestinal tissue were excised and frozen at -70°C for various assays.

Bronchoalveolar lavage was performed 3 h after the reperfusion by the method previously described (Park and Park, 2006). Total leukocytes in BAL fluid were counted with a hemocytometer. Protein concentrations in BAL fluid were measured using a bicinchoninic acid method (Sigma,

St. Louis, USA). Ferritin and HO-1 concentrations were quantified with commercially available ELISA kits specific for rat ferritin (Panapharm Laboratories, Japan) and HO-1 (Takara Bio Inc., Japan) following the instructions.

Tissue samples of lung and intestine were homogenized separately in sodium phosphate buffer (100 mM, pH 7.4) and heated at 70°C for 10 min. The homogenates were cooled to 4°C and centrifuged at 12,000 g for 15 min. Subsequently, the supernatants were assayed for tissue ferritin and protein concentrations and the results expressed as ng/mg of tissue protein.

Data were presented in means $\pm$ S.E.M. Unpaired t test was used to compare groups, and paired t test was used for paired observations within a group. A p value of <0.05 was considered statistically significant.

RESULTS

Effects of mepacrine on intestinal I/R-induced acute lung injury

ALI was induced following intestinal I/R in rats and effectively attenuated by mepacrine pretreatment (Fig. 1, 2). The number of leukocytes in BAL fluids were significantly increased following intestinal I/R (3.1 ± 0.5 millions/two lungs) compared to the values obtained for sham-treated rats (0.9 ± 0.1 millions/two lungs). Intestinal I/R treated rats also had significantly elevated lung lavage protein contents (5.7 ± 0.1 mg/two lungs) compared to sham rats (4.0 ± 0.4 mg/two lungs). Mepacrine treatment significantly attenuated the increase in leukocyte number and protein content in lung lavage fluids.

Changes of serum ferritin and HO-1 levels after intestinal I/R

The serum ferritin concentrations of all groups were the same before I/R (Fig. 3). From 30 min after I/R, serum ferri-

Fig. 1. Intestinal ischemia/reperfusion (I/R) significantly increased the number of leukocytes in lung lavage fluids. Mepacrine pretreatment (Mepa+I/R) effectively suppressed the increase. The data shown are means $\pm$ S.E.M. for the number of rats shown in the parentheses. * $p < 0.05$, ** $p < 0.01$, compared with Sham; ## $p < 0.01$, compared with I/R.

tin concentration in I/R rats started to significantly increase from the baseline value (from 27 ± 8 to 45 ± 9 ng/ml, $p < 0.01$). After 1 h of reperfusion, serum ferritin levels of I/R rats were significantly higher than the values of mepacrine-treated I/R rats (Mepa+I/R) and sham-treated rats. Serum ferritin levels of mepacrine-treated I/R rats were significantly elevated compared with the levels of sham-treated rats only at 3 h after reperfusion. Serum ferritin concentrations of I/R rats at 180 min following reperfusion

Fig. 2. Rats subjected to intestinal ischemia/reperfusion (I/R) had significantly increased protein contents in lung lavage fluids. This change was significantly attenuated by the pretreatment with mepacrine (Mepa+I/R). The data shown are means $\pm$ S.E.M. for the number of rats shown in the parentheses. ** $p < 0.01$, compared with Sham; ### $p < 0.001$, compared with I/R.

Fig. 3. Rats subjected to ischemia/reperfusion (I/R) had greatly increased serum ferritin concentrations after 60 min following I/R. Serum ferritin concentrations of I/R rats at 180 min of reperfusion were significantly higher than those of other rats. By comparison, mepacrine pretreatment (Mepa+I/R) significantly attenuated the increase following I/R. Serum ferritin concentrations of sham rats did not change through the observation period. Each point represents mean $\pm$ S.E.M. for the number of rats shown in the parentheses. * $p < 0.05$, *** $p < 0.001$, compared with Sham; # $p < 0.05$, ### $p < 0.001$, compared with Mepa+I/R.

(148 ± 15 ng/ml) were significantly higher than the values obtained for Mepa+I/R (65 ± 7 ng/ml, $p < 0.001$) and sham rats (38 ± 8 ng/ml, $p < 0.001$). In contrast, sham rats did not show any significant changes of serum ferritin throughout the experiment.

Fig. 4 shows the changes of serum HO-1 concentrations. Until 30 min of reperfusion, there was no difference in HO-1 levels across groups. However, after 60 min of reperfusion, mepacrine treated I/R rats had increased HO-1 levels compared with I/R and sham-treated rats. At the end of 3 h of reperfusion, I/R treated rats (I/R, 9.8 ± 1.2 ; Mepa+I/R, 12.4 ± 2.3 ng/ml) showed a significant increase of serum HO-1 concentrations compared with sham rats (4.3 ± 0.2 ng/ml). In comparison with the changes of serum ferritin, serum HO-1 levels of mepacrine-treated I/R rats were significantly higher than untreated I/R rats.

Changes of BAL fluid ferritin and HO-1 levels after intestinal I/R

Intestinal I/R significantly increased BAL fluid ferritin levels (Fig. 5). Mepacrine treated rats had a significant decrease in the lavage ferritin concentrations compared to I/R rats. However, HO-1 concentrations in BAL fluid were the same in all groups (Fig. 6).

Ferritin contents of lung and intestinal tissues after intestinal I/R

Ferritin contents of lung and intestinal tissues were elevated following intestinal I/R (Fig. 7, 8). Mepacrine-treated I/R rats showed a tendency to decrease in tissue ferritin contents compared to I/R rats.

Fig. 4. Rats subjected to ischemia/reperfusion (I/R) had greatly increased serum heme oxygenase-1 (HO-1) concentrations after 3 hours of reperfusion compared to Sham rats. At 180 min following I/R, HO-1 levels of mepacrine-treated rats (Mepa+I/R) was significantly higher than the values of untreated I/R and sham rats. Serum HO-1 concentrations of sham rats did not change over the course of the experiment. Each point represents mean $\pm$ S.E.M. for the number of rats shown in the parentheses. *** $p < 0.001$, compared with Sham; # $p < 0.05$, compared with Mepa+I/R.

Fig. 5. Intestinal ischemia/reperfusion (I/R) significantly increased lung lavage fluid ferritin concentrations. Mepacrine pretreatment (Mepa + I/R) significantly reduced the increase. The data shown are means $\pm$ S.E.M. for the number of determinations shown in the parentheses. *** p < 0.001, compared with Sham; ## p < 0.01, compared with I/R.

Fig. 6. There were no significant differences in lung lavage heme oxygenase-1 (HO-1) concentrations among all groups. Ischemia/reperfusion (I/R) did not change HO-1 levels compared to the values of sham rats. The data shown are means $\pm$ S.E.M. for the number of rats shown in the parentheses.

DISCUSSION

The period of reperfusion after ischemia is thought to be a critical period of oxidant damage in many tissues, including heart, brain, gut, and other organs (Torti and Torti, 2002). Until now, however, there remains limited information about the injurious and protective mechanisms after I/R, and there have been no specific therapies for this disorder. Therefore, if there is a biomarker to predict progressing to ARDS, prophylactic therapy in at risk patients may get better results than administration to those with established disease.

Several previous studies (Park et al., 2003, 2004; Park and Lee, 2006; Park and Park, 2006) reported that mepacrine pretreatment significantly reduced ALI in severe hemorrhage- and intestinal I/R-induced ALI models. In this study, mepacrine pretreatment attenuated I/R-induced

Fig. 7. Intestinal ischemia/reperfusion (I/R) significantly increased lung tissue ferritin content. Mepacrine pretreatment (Mepa + I/R) slightly reduced the increase. The data shown are means $\pm$ S.E.M. for the number of determinations shown in the parentheses. * p < 0.05, compared with Sham.

Fig. 8. Intestinal ischemia/reperfusion (I/R) had tendency to increase intestinal tissue ferritin contents. Mepacrine pretreatment (Mepa + I/R) attenuated the tendency. The data shown are means $\pm$ S.E.M. for the number of rats shown in the parentheses.

ALI. These results were useful in this study to evaluate predictability of biomarkers in three kinds of injury, including normal, I/R, and reduced I/R. In this study, serum and BAL fluid ferritin levels were well matched with the severity of ALI.

The antioxidant properties of ferritin are likely mediated by sequestration and storage of iron, thus preventing ferryl or hydroxyl radical generation (Klausner et al., 1993; Picard et al., 1998; Kakhlon et al., 2001), playing a key role in maintaining iron homeostasis. During postischemic reoxygenation of the rat liver, early ferritin degradation was counteracted by enhanced ferritin transcription. It was suggested that this might act to re-establish ferritin levels and limit reperfusion damage (Tacchini et al., 1997). Indeed, hypotransferrinemic mice, which have high levels of ferritin and lactoferrin, are resistant to hyperoxia-induced lung injury (Yang et al., 1999).

Although serum ferritin levels usually correlate with to-

tal body iron stores, several physiologic conditions such as inflammatory or infectious states (Harrison and Arosio, 1996) can increase serum ferritin as one of the acute-phase proteins (Gabay and Kushner, 1999). Rogers et al. (1990) also reported that the inflammatory induction of ferritin synthesis was different from iron-dependent ferritin gene expression. Serum ferritin levels may increase solely as a consequence of cellular necrosis or damage (Jacobs and Worwood, 1975). This possibility is supported by previous clinical findings of an association between serum ferritin levels and the injury severity score (Connelly et al., 1997; Sharkey et al., 1999; Ji et al., 2007).

In this study, serum and BAL ferritin had increased following I/R, and significantly decreased after mepacrine pretreatment. These results clearly matched with the findings of ALI in BAL fluid (Fig. 1, 2).

In accordance with previous studies, the concomitant increases of ferritin levels and severity of ALI in untreated I/R can suggest a possibility to use serum and/or BAL ferritin levels as a biomarker for predicting ARDS. These findings confirm previous *in vitro* (Balla et al., 1992) and *in vivo* (Park et al., 2003, 2004; Park and Lee, 2006; Park and Park, 2006) studies.

On the other hand, serum protein levels can be a mirror of the status of systemic inflammation, not the direct product of injured tissues. A previous report indicated that there was no close relationship between serum and BAL ferritin increases in a severe hemorrhage-induced ALI model. In spite of this limitation, serum ferritin concentration was well matched with the severity of lung injury (Park et al., 2004). Therefore, serum as well as BAL ferritin levels might be a useful predictor of the onset of severe ARDS.

In accordance with previous reports, serum HO-1 levels were greatly elevated following intestinal I/R. Unlike serum ferritin, serum HO-1 levels were elevated and higher than the values of untreated I/R rats in spite of reduced ALI caused by mepacrine pretreatment. In addition, the increase of serum HO-1 levels had a slower onset compared with ferritin levels. A potential explanation may be from a supporting report that intratracheal LPS rapidly induces ferritin protein in the lung independently of its mRNA synthesis or HO enzyme activity (Carraway et al., 1998). Therefore, there might be HO-1 independent induction of ferritin or different mechanism to induce these two proteins in an intestinal I/R injury model.

Mumby et al. (2004) reported that HO-1 concentrations were elevated in lung tissue and BAL fluid taken from patients with ARDS compared to controls. In addition, there was a significant correlation between BAL cellular HO-1 and BAL fluid ferritin concentrations in ARDS patients (Mumby et al., 2004). In this study, however, BAL fluid HO-1 levels were same in all groups, whereas serum HO-1 levels were greatly increased in both I/R injury groups. This might be explained by the difference of the severity of injury or measurement. However, there needs be more studies to elucidate the exact regulatory mechanisms between HO-1 and ferritin.

The present study also demonstrates that the elevations of ferritin in injured tissues, including intestines and lung, were parallel with the serum and BAL fluid levels. These results also support the reliability of serum and/or BAL ferritin levels as a predictive biomarker for the onset of severe ARDS in an intestinal I/R-induced ALI model. However, it remains unclear whether this would be constant in other models of ALI or over long-time observations.

It is known that HO-1 induction responds to common causes of oxidative stress to the airways, including hyperoxia, hypoxia, endotoxemia, heavy metal exposure, bleomycin, diesel exhaust particles, and allergen exposure (Lee et al., 2000; Li et al., 2000). Several studies suggest that HO-1, which is induced after ischemia or oxidant stress, may exert protective effects in I/R, oxidant injury, or endotoxic shock (Otterbein et al., 1995, 1997; Lee et al., 1996). In addition, HO-1 may contribute to ischemic preconditioning, a process of acquired cellular protection against I/R injury, as observed in transplanted lungs in guinea pigs (Soncul et al., 1999). Also, HO-1 overexpression provided potent protection against cold I/R injury in a rat model through an anti-apoptotic pathway (Katori et al., 2002).

Although there was some difference in the temporal pattern of inductions of ferritin and HO-1, both had similar cytoprotective functions against oxidative stress. Indeed, HO-1 overexpression by pharmacological or genetic engineering has exerted protective functions in an I/R injury model (Amersi et al., 1999; Kato et al., 2001; Ryter et al., 2006; Scott et al., 2007). In addition, maintenance of low free iron by increased ferritin levels plays an important role in antioxidant cytoprotection. Like HO-1, overexpression of ferritin heavy chain protected rat livers from I/R injury, and prevents hepatocellular damage upon transplantation into syngeneic patients (Berberat et al., 2003). Furthermore, Balla et al. (1992) already reported that ferritin, rather than heme oxygenase, was the ultimate protectant in oxidant mediated endothelial cell injury *in vitro*. These data means ferritin may be a good biological marker as well as a cytoprotectant in I/R injury patients. Therefore, these two stress induced proteins are important for not only as a diagnostic biological marker, but also as part of a new therapeutic regimen for wide inflammatory disorders.

In conclusion, these results suggested that the serum and lung lavage ferritin concentrations were correlated with intestinal I/R-induced lung injury. However, HO-1 levels were not exactly matched with the lung injury in intestinal I/R-induced ALI model. Therefore, there might be a different mechanism in the induction of ferritin and HO-1 following intestinal I/R injury. Further studies will be required to elucidate the precise mechanisms of the changes of these two proteins in the ALI model or other inflammatory diseases.

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