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Changes of Serum Ferritin in Acute Lung Injury Induced by Intestinal Ischemia/Reperfusion

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Serum ferritin levels are increased in subjects at-risk for or with acute lung injury (ALI), and there are observations to suggest that increases in serum ferritin levels may help predict the development of ALI in at-risk individuals. To deepen our understanding of increases of serum ferritin and their relationship to the development of ALI, we measured serum ferritin levels before and after intestinal ischemia/reperfusion (I/R) injury in rats, and found that serum ferritin levels increased significantly following I/R. Increases in serum and lavage ferritin levels paralleled increases in lung inflammation (lavage leukocyte numbers and tissue myeloperoxidase activities) and lung leak (lavage protein levels). In contrast, pre-treatment of rats with mepacrine (60 mg/kg, i.p.), a phospholipase A_2 inhibitor, attenuated not only I/R-induced serum and lavage ferritin increases, but also the development of ALI. These findings indicate that, besides of human subjects with ALI, serum ferritin levels increase early on also in an animal model of ALI. Therefore, serum and lavage ferritin can be a candidate for early biomarker of ALI.

Key Words: Intestinal ischemia/reperfusion, Acute lung injury, Ferritin, Rat

INTRODUCTION

Since Ashbaugh's early description of the acute respiratory distress syndrome (ARDS) in 1967 (Ashbaugh et al, 1967), morbidity and mortality have remained high for patients with ARDS (Ware & Matthay, 2000). ARDS usually occurs within 24 h of the insult and essentially occurs always within 72 h (Fowler et al, 1983; Hudson et al, 1995; Pepe et al, 1982), therefore, there is little time to enroll patients into studies.

Early and accurate identification of patients who are likely to develop ARDS would give several therapeutic advantages to the patients. Therefore, the development of specific biological markers for predicting ARDS is a desirable goal (Connelly & Repine, 1997). Numerous risk factors for the development of ARDS have been identified, and they are related to both direct and indirect causes of lung injury (Pittet et al, 1997). These markers may improve the prediction of ARDS in high risk clinical conditions and provide new insights into the pathogenesis of ARDS.

The iron storage protein, ferritin, plays a key role in iron metabolism. Its ability to sequester the element gives ferritin the dual functions of iron detoxification and iron reserve. Ferritin normally stores iron, but it may release free iron in the presence of superoxide or acidosis (Rief, 1992). For unknown reason, serum ferritin levels are increased in patients with acute lung injury (ALI) and multiple organ failure (MOF) (Connelly et al, 1997; Sharkey et al, 1999). Serum ferritin levels are also increased in patients at-risk for ALI development and appear to be increased more in at-risk patients who later develop ALI than in at-risk patients who do not develop ALI later. Additionally, serum ferritin levels correlated with the development of MOF (Sharkey et al, 1999), and severe hemorrhage induced ALI increases serum ferritin levels in rats (Park et al, 2003, 2004). However, the mechanisms responsible for the increase of serum ferritin and their relationship to ALI are still unclear. Therefore, the relationship between the pathogenesis of ALI and ferritin is worhty to be elucidated. Ischemia/reperfusion (I/R) occurs frequently in human pathological conditions (Grace, 1994), and intestinal I/R has been considered as a serious cause of ARDS (Otamiri et al, 1988).

In the present study, therefore, we evaluated serum ferritin level as a predictor of ALI in rats which were subjected to intestinal I/R injury. In addition, we examined the effect of pretreatment with mepacrine, a well-known phospholipase A_2 (PLA_2) inhibitor, on the changes of serum ferritin and ALI that occurs in I/R injured rats.

METHODS

Sprague-Dawley male rats, weighing 300~450 g, were used. Rats were anesthetized by ketamine (80 mg/kg, i.p.)

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ABBREVIATIONS: ALI, acute lung injury; ARDS, acute respiratory distress syndrome; I/R, ischemia/reperfusion; MPO, myeloperoxidase.

and xylazine (16 mg/kg, i.p.). Left femoral artery was incised, and then polyethylene catheters (PE-50, Clay-Adams) filled with heparinized saline (100 U/ml) were inserted for blood sampling. The rats remained deeply anesthetized for the entire experiment, and the rats then were euthanized.

Rats were randomly divided into two groups and were given an intraperitoneal injection of PLA_2 inhibitor mepacrine (60 mg/kg, i.p.) or same volume of normal saline as a control. Thirty minutes after injection of saline or mepacrine, acute lung injury was induced by clamping superior mesenteric artery with a bulldog clamp for 30 min, and reperfusion was permitted for 3 hours. Sham-treated rats remained anesthetized after the same surgery without I/R.

For measurement of serum ferritin and protein concentrations, 100 μl of arterial blood was collected through the catheter before ischemia and 0, 30, 60, and 180 min after the reperfusion.

Bronchoalveolar lavage was performed by cannulating the trachea and instilling 8.0 ml of cold normal saline with a syringe. Saline was flushed three times through the lung. Approximately 6 ml of lavage fluid was recovered from each rat. Then, the fluid was centrifuged at 1,000 g for 10 min, and the supernatant was collected and stored at $-70^{\circ}C$ for additional assays. The cellular pellet was resuspended with 1.0 ml of distilled water and 1.0 ml of Hanks'balanced salt solution for a few seconds and centrifuged again. After supernatant was discarded, pellet was suspended in 0.4 ml of normal saline. Total leukocytes were counted with a hemocytometer.

Protein concentrations in serum and lavage fluid were measured using a bicinchoninic acid method (Sigma, St. Louis, MO). The ferritin content in serum and lavage fluid was quantified with commercially available ELISA kit specific for rat ferritin (Panapharm Laboratories, Kumamoto, Japan) by following the instructions.

To confirm pulmonary and intestinal infiltration of leukocytes, lung and intestinal tissues were collected and frozen at $-70^{\circ}C$ for analysis of myeloperoxidase (MPO) activity. MPO activity was measured by the method previously described (Lee et al, 2000). Briefly, the tissues were thawed and then homogenized in 4 ml of potassium phosphate buffer (20 mM, pH 7.4), and the homogenate was centrifuged at 30,000 g for 30 min at $4^{\circ}C$. The pellet was resuspended in 4 ml of 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide, sonicated for 90 s, and incubated for 2 h at $60^{\circ}C$. Then, MPO activity was determined using o-dianisidine as the substrate and H_2O_2 to initiate the reaction.

Statistical analyses: All data are presented as means$\pm$SEM. 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 ischemia/reperfusion induced acute lung injury

Acute lung injury was induced in rats by means of intestinal I/R. The number of leukocyte (Fig. 1), protein content (Fig. 2) in lavage fluid, and lung MPO activity (Fig. 3) were significantly increased by intestinal I/R compared with those of Sham rats. As shown in Fig. 4, mepacrine treatment counteracted the increase of lavage leukocyte numbers, protein contents, and lung MPO activity. Intestinal MPO activity, however, did not show significant change after I/R.

Changes of serum and lung lavage ferritin levels after intestinal ischemia/reperfusion

Figure 5 shows the changes of serum ferritin levels in rats subjected to intestinal I/R. Thirty minutes of ischemia did not change serum ferritin levels. However, the values at 60 minutes after reperfusion showed sharp increases. Serum ferritin concentrations of I/R rats at 180 minutes following reperfusion were significantly higher than other groups. Mepacrine treatment significantly decreased serum ferritin increases in rats subjected to intestinal I/R. In

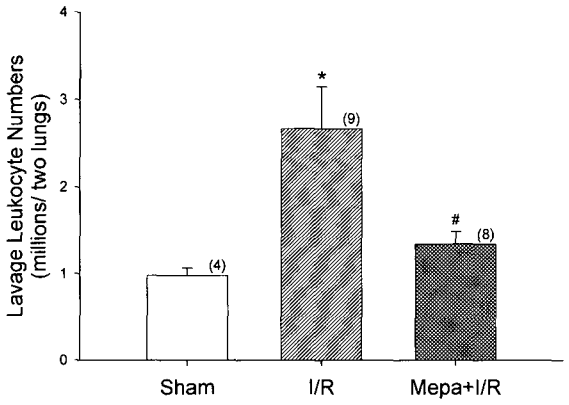

Fig. 1. Intestinal ischemia/reperfusion (I/R) significantly increased the number of leukocyte in bronchoalveolar lavage fluid. Mepacrine pretreatment (Mepa+I/R) effectively prevented the increase. The data shown are means$\pm$S.E.M. for the number of determinations shown in the parenthesis. *p<0.05, compared with Sham; #p<0.05, compared with I/R.

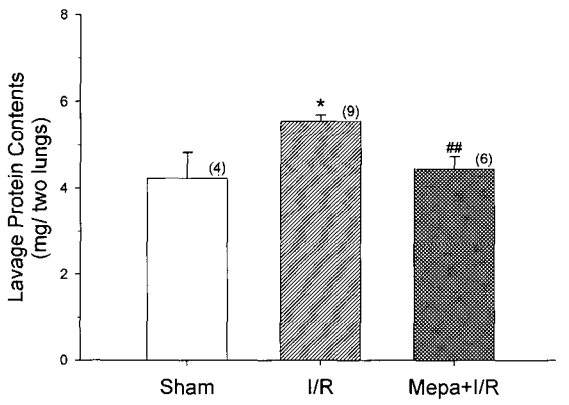

Fig. 2. Rats subjected to intestinal ischemia/reperfusion (I/R) had significantly increased protein contents in bronchoalveolar lavage fluid. This change was significantly decreased by the pretreatment with mepacrine (Mepa+I/R). The data shown are means$\pm$S.E.M. for the number of determinations shown in the parenthesis. *p<0.05, compared with Sham; ##p<0.01, compared with I/R.

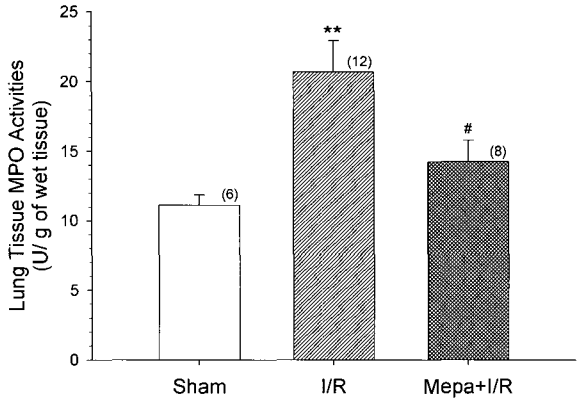

Fig. 3. Intestinal ischemia/reperfusion (I/R) significantly elevated lung tissue myeloperoxidase (MPO) activity. This change was significantly attenuated by the pretreatment of rats with mepacrine (Mepa+I/R). The data shown are means±S.E.M. for the number of determinations shown in the parenthesis. **p<0.01, compared with Sham; #p<0.05, compared with I/R.

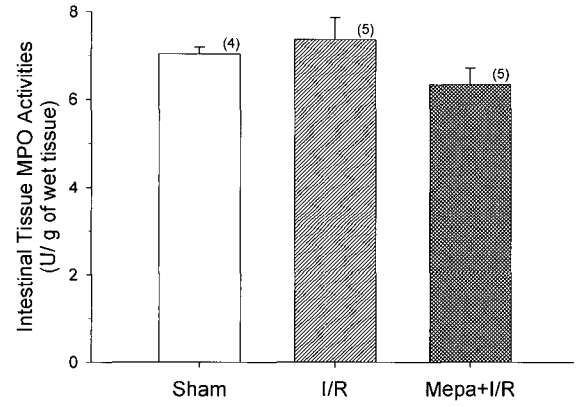

Fig. 4. Intestinal tissue myeloperoxidase (MPO) activity did not change after intestinal ischemia/reperfusion (I/R) and mepacrine pretreated I/R (Mepa+I/R). The data shown are means±S.E.M. for the number of determinations shown in the parenthesis.

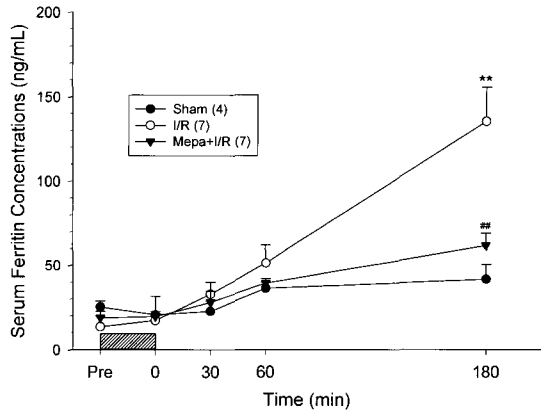

Fig. 5. Serum ferritin concentrations of Sham rats did not significantly change throughout the experiment. Rats subjected to intestinal ischemia/reperfusion (I/R) had greatly increased serum ferritin concentrations after 60 minutes of reperfusion. Serum ferritin concentrations of I/R rats at 180 minutes following reperfusion were significantly higher than those of other rats. By comparison, mepacrine pretreatment (Mepa+I/R) significantly attenuated the increase following I/R. Each point represents mean±S.E.M. for the number of determinations shown in parenthesis. **p<0.01, compared with Sham; ##p<0.01, compared with I/R.

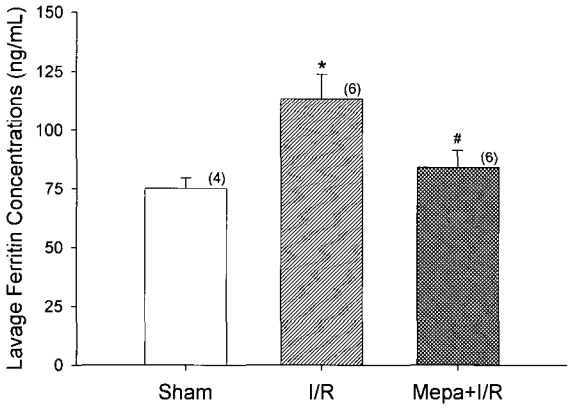

Fig. 6. Intestinal ischemia/reperfusion (I/R) significantly increased bronchoalveolar lavage fluid ferritin concentrations. Mepacrine pretreatment (Mepa+I/R) significantly counteracted the increase. The data shown are means±S.E.M. for the number of determinations shown in the parenthesis. *p<0.05, compared with Sham; #p<0.05, compared with I/R.

contrast, sham rats did not show any significant changes of serum ferritin throughout the experiment. Rats subjected to intestinal I/R had significantly increased lavage ferritin concentrations which were effectively decreased when rats were pretreated with mepacrine (Fig. 6).

DISCUSSION

Oxidative stress, defined as an imbalance caused by overproduction of free radicals or significant diminution in antioxidant defenses, is characterized by excessive levels of reactive oxygen species (ROS) that can cause cell injury and death in several organs (Halliwell, 1987). The reperfusion after severe but non-necrogenic ischemia generates ROS (Kirschner & Fantini, 1994). Intracellular free iron can catalyze the conversion of superoxide anion and hydrogen peroxide, produced by xanthine oxidase during reoxygenation, into more potent oxidants, such as hydroxyl radicals (McCord, 1998), with deleterious effects on postischemic reperfused tissues. Indeed, several therapeutic strategies using iron chelation have proven to be useful to counteract the harmful effects of iron as a potentiator of I/R injury (Arora & Gores, 1996; Hybertson et al, 2002). Although the importance of iron in exacerbating the I/R damage is well recognized, the source of this catalytically active iron is not known. Ferritin, as an iron storage protein, can either represent a potentially harmful iron donor or an iron detoxicant (Harrison & Arosio, 1996).

In this study, mepacrine treatment was found to significantly reduce ALI in rats subjected to intestinal I/R and attenuate the increase of serum ferritin concentration. Park

et al. (2003, 2004) also reported recently that, in severe hemorrhage induced ALI, lung injury and increase of serum ferritin were attenuated in mepacrine-treated rats. These results confirm earlier study on the effects of mepacrine on intestinal I/R, intratracheal IL-1, and severe hemorrhage-induced lung injury (Lee et al, 1997, 1999; Park et al, 2004).

Serum ferritin started to increase at 60 min and reached about 10 times of basal values at 3 h after reperfusion. Mepacrine treatment significantly attenuated the increase of serum ferritin. Balla et al. (1992) also reported that the 1~2 h time lag was followed by rapid synthesis of ferritin in cultured endothelium which was exposed to heme.

Although serum ferritin levels correlate usually with total body iron stores, several physiological conditions such as inflammatory or infectious states (Harrison & Arosio, 1996) can increase it as one of the acute-phase proteins (Gabay & Kushner, 1999). Rogers et al. (1990) also reported that inflammatory induction of ferritin synthesis was different from iron dependent ferritin gene expression.

In general, hypoxia increases intracellular generation of ROS, and oxidative stress has been shown to mobilize iron from intracellular ferritin, thus increasing cell content of unbound iron and thereby promoting additional ferritin synthesis (Cairo et al, 1995; Chandel et al, 2000). Ferritin synthesis has been shown to increase cell resistance to oxidative stress by sequestering intracellular catalytic iron (Epsztejn et al, 1999; Garner et al, 1998), although various causes can elevate ferritin.

Serum ferritin levels may increase solely as a consequence of cellular necrosis or damage (Jacobs & Worwood, 1975). This possibility is supported by earlier clinical finding of an association between serum ferritin levels and injury severity score (Connelly et al, 1997; Sharkey et al, 1999). Furthermore, it is well established that proinflammatory systemic mediators, including TNF-α and IL-1, are elevated in intestinal I/R. Therefore, it is suggested that these inflammatory cytokines can increase the synthesis of ferritin (Rogers et al, 1990; Kwak et al, 1995) as one of the host defense mechanisms.

The importance of iron availability in amplifying I/R injury is well established. Elevated serum ferritin levels may also reflect an increase of ferritin synthesis as a protective response to increased oxidative stress, thereby increasing the ability to sequester iron and increasing antioxidant activity (Bolann & Ulvik, 1990; Balla et al, 1992, 1995; Vile & Tyrrell, 1993). Several studies also showed the protective effect of the iron chelator desferrioxamine (Arora & Gores, 1996; Hybertson et al, 2002). Although the origin of iron during in vivo postischemic reperfusion has not yet been identified, ferritin has been considered as a likely candidate source. Iron bound to ferritin is generally unavailable for the Harber-Weiss reaction, however, proteolysis, superoxide anion and acidosis, which are commonly encountered in disorders at risk for ARDS, may release iron from ferritin (Reif, 1992). Consequently, ferritin-derived iron may worsen oxidative damage in critically ill patients, contributing to the pathologic derangements seen in ARDS. Therefore, as described above, the exact role of increased serum ferritin is not clear at present, and additional studies may be required to explain these questions.

In conclusion, we found that serum ferritin levels were correlated with lung injury. Therefore, serum ferritin concentration in conjunction with other markers could be of use in identifying individual patients at high risk of progressing to ARDS.

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