Ferritin regulates iron levels and, for unknown reasons, serum ferritin concentrations are increased in patients at risk for and with acute lung injury (ALI) and multiple organ failure. Uncomplexed iron could exacerbate the toxicity of the increased oxidative stress that occurs in patients with ALI and multiple organ failure and thereby contribute to disease. In the present investigation, the authors found that serum and lung lavage ferritin concentrations increased in hemorrhaged rats that develop ALI as manifested by increased lung inflammation (increased lung lavage leukocyte counts and lung myeloperoxidase activities) and increased lung leak (increased lung lavage protein concentrations). Treatment with mepacrine, a phospholipase A2 inhibitor, attenuated the increases in serum and lung lavage ferritin concentrations, lung inflammation, and lung leak that occur in rats subjected to hemorrhage. The findings show that serum and lung ferritin levels increase and may play a role in the development of acute lung injury caused by hemorrhage.

Keywords inflammation, iron, neutrophils, oxidants, shock

Acute lung injury (ALI) is a highly fatal respiratory disorder that complicates various predisposing systemic and pulmonary conditions including infection, trauma, pancreatitis, and aspiration [1–4]. Much has been learned about the pathogenesis of ALI over the past 3 decades [4–6], and much remains unknown. Nonetheless, the pathogenesis of ALI appears to involve oxidative stress [7], and this raises the possibility that iron contributes to ALI [8–10]. This speculation is based on the ability of uncomplexed
Iron to enhance the toxicity of oxidative stress by facilitating the formation of the highly toxic hydroxyl radical (·OH) from the relatively less toxic superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$). Iron is usually complexed to a number of proteins, including ferritin, that prevent it from participating in this reaction. Potential protective effects of ferritin have also been suggested. Exposure to heme stimulated a rapid synthesis (1 to 2 hours) of ferritin in cultured endothelium and these ferritin increases exerted a protective effect against oxidative stress [11]. Likewise, adding ferritin decreased lung leak in isolated rat lungs perfused with xanthine oxidase and purine [12]. Additionally, hemorrhage-induced increases in serum ferritin, lung inflammation, and lung leak were attenuated in rats that were fed an iron-deficient diet [13].

For unknown reasons, serum ferritin concentrations are increased in patients with ALI and multiple organ failure (MOF) [14, 15]. Serum ferritin concentrations are also increased in patients at risk for ALI development and, moreover, appear to be increased more in at-risk patients who later develop ALI than in at-risk patient who do not later develop ALI [14, 15]. Additionally, serum ferritin concentrations correlated with the development of MOF [15]. Thus, although ferritin serves as a biomarker of inflammation, the mechanisms responsible for serum ferritin increases and their role in ALI are unclear [16]. It is possible that the increased expression of immunoregulatory and proinflammatory cytokines, including interleukin (IL)-1 and tumor necrosis factor alpha (TNFα), in the lung could lead to the release of ferritin by various cells [17–20]. In addition, oxygen species are generated rapidly during hypoperfusion produced by hemorrhage [21–24], and oxidants from activated neutrophils can damage vascular endothelial cells and release ferritin from tissues, especially gut tissues, which are highly sensitive to hypoperfusion and are rich in ferritin [25]. Although iron bound to ferritin is generally unavailable for participation in the Haber-Weiss reaction, iron may be released from ferritin by superoxide anion (O$_2^-$) and acidosis, which are commonly encountered in ALI and disorders that predispose to ALI [26].

Activation of the arachidonic acid cascade [27–31] is a key feature of inflammatory reactions and part of the process that has been associated with ALI [32–34]. The drug mepacrine, also known as quinacrine, is known to inhibit phospholipase A$_2$ (PLA$_2$) activity and thereby suppress the arachidonic acid cascade [27]. Although mepacrine may exert effects on gene expression [28], activator protein (AP)-1 activation [28, 29], Ca$^{2+}$ flux [30], or NO synthesis [31, 35, 36], the rapid inhibition of PLA$_2$ may comprise its primary effect on inflammation [27–32].

Given the strong association between levels of serum ferritin and inflammation, in conjunction with the rapid lung inflammation induced by hemorrhage, we hypothesized that disruption of the arachidonic acid cascade with
the PLA2 inhibitor mepacrine would suppress hemorrhage-induced lung inflammation and potentially modulate levels of serum ferritin. Thus, in the present study, we evaluated serum and lung ferritin concentrations and lung inflammation and leak in rats subjected to severe hemorrhage. We also examined the effect of treatment with mepacrine on the levels of serum and lung lavage ferritin and the lung inflammation and leak that occur in hemorrhaged rats.

**MATERIALS AND METHODS**

**Animal Procedures**

Male Sprague-Dawley rats weighing 300 to 450 g (Sasco, Omaha, NE) were allowed to acclimate to Denver altitude for at least 7 days before study. Rats were anesthetized by ketamine (80 mg/kg, intraperitoneal [IP]) and xylazine (16 mg/kg, IP). Subsequently, both the left and right femoral arteries were incised and then polyethylene catheters (PE-50, Clay-Adams) filled with heparinized saline (100 U/mL) were inserted into each femoral artery and used as separate ports—one for hemorrhage and the other for measurement of mean arterial blood pressure (MAP). For the latter, the catheter was connected to a pressure transducer (model P23) linked to a polygraph (model 79, Grass Instrument, Quincy, MA). Electronically damped MAP was then continuously recorded throughout the experiment. The rats remained deeply anesthetized for the entire experiment and were euthanized at the conclusion. For some experiments, rats were randomly divided into 2 groups and were then given an IP injection of mepacrine (60 mg/kg), a PLA2 inhibitor, or saline (3 mL/kg). Ten minutes after injection of saline or mepacrine, arterial blood was withdrawn into a syringe at a constant rate of 4 mL/kg/min for 5 minutes (total 20 mL/kg) using a withdrawal pump (Model PHD 2000, Harvard Apparatus). Sham-treated rats underwent the same surgery but without hemorrhage. For measurement of serum ferritin and protein concentrations, 100 μL of arterial blood was collected through the femoral arterial catheter before and 10, 20, 30, 60, 90 and 120 minutes after hemorrhage. Lung lavage was performed by cannulating the trachea and instilling 8.0 mL of cold normal saline with a syringe. The lavage fluid was rinsed in and out three times before collection.

**Lavage Leukocyte Recovery and Assessment**

Approximately 6 mL of lung lavage fluid was recovered from each rat. The fluid was centrifuged at 1000 × g for 10 minutes, then the supernatant was collected and stored at −70°C for additional assays. The cell pellet was then washed with 1.0 mL of distilled water and 1.0 mL of Hanks’ balanced...
salt solution. After the supernatant was discarded, the pellet was resuspended in 0.2 mL of normal saline. Total leukocytes were counted using a hemocytometer. Differentials were performed using Wright-stained preparations.

**Myeloperoxidase Assay**

To assay lung-associated neutrophils, lung tissue was collected and frozen at \(-70\)°C for analysis of myeloperoxidase (MPO) activity [37, 38]. Briefly, lungs were thawed and then homogenized in 4 mL of 20 mM potassium phosphate buffer (pH 7.4), and centrifuged at 30,000 \(\times\) g for 30 minutes at 4°C. The pellet was then resuspended in 4 mL of 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide, sonicated for 90 seconds, and incubated for 2 hours at 60°C. MPO activity was determined using \(\alpha\)-dianisidine as the substrate and \(\text{H}_2\text{O}_2\) to initiate the reaction.

**Protein and Ferritin Assays**

Protein concentrations were measured using a bicinchoninic acid method (Sigma, St. Louis, MO). Ferritin concentrations were quantified with a commercially available Sandwich ELISA kit (RAMCO Laboratories, Houston, TX) following the manufacturer’s instructions.

**Statistical Analysis**

All data are presented as means \(\pm\) SEM. An unpaired \(t\) test was used to compare groups, and a paired \(t\) test was used for paired observations within a group. A \(P\) value of < .05 was considered statistically significant.

**RESULTS**

**Effect of Hemorrhage and Mepacrine Treatment on Hemodynamics**

MAP of saline-treated (control) and mepacrine-treated rats were the same before hemorrhage (Figure 1). Following hemorrhage, the MAP of both control and mepacrine-treated rats decreased rapidly from approximately 100 mm Hg to approximately 35 mm Hg during the initial 5 minutes of hemorrhage. At the conclusion of blood removal, the MAP of both control and mepacrine-treated rats increased progressively and reached approximately 75 mm Hg 2 hours after hemorrhage. These results show that mepacrine did not interfere hemorrhage-induced alteration of the MAP.
Effect of Hemorrhage and Mepacrine on Lung Inflammation and Leak

Two hours after hemorrhage, the lung lavage leukocyte numbers, lung MPO activities, and lung lavage protein concentrations of hemorrhaged rats were all increased compared to values obtained for sham-treated rats (Figure 2). Although the number of recoverable lavage leukocytes increased in hemorrhaged rats, the differential counts of these cells were unchanged before (96% macrophages, 1% neutrophils, and 3% lymphocytes) and after (98% macrophages, 1% neutrophils, and 1% lymphocytes) hemorrhage. Although mepacrine-treated and control rats had the same degree of hypotension induced by hemorrhage (Figure 1), mepacrine-treated rats had decreased lung lavage leukocyte numbers, lung MPO activities, and lung lavage protein concentrations compared to untreated control rats following hemorrhage (Figure 2).

Effect of Mepacrine Treatment on Serum Ferritin Concentrations in Hemorrhaged Rats

Before hemorrhage, control and mepacrine-treated rats had the same serum ferritin concentrations (Figure 3). Following hemorrhage, the serum ferritin concentrations of hemorrhaged rats increased progressively from...
their baseline levels of approximately 50 ng/mL to posthemorrhage concentrations of approximately 180 ng/mL 2 hours later. By comparison, the serum ferritin concentrations of sham-treated rats remained at their...
baseline levels throughout the 2-hour observation period. The serum ferritin concentrations of rats that had been treated with mepacrine and then subjected to hemorrhage were decreased at 1, 1.5, and 2 hours after hemorrhage compared to the responses of untreated control hemorrhaged rats (Figure 3). Notably, although serum ferritin concentrations increased in rats subjected to hemorrhage (Figure 3), the total serum protein concentrations of both untreated and mepacrine-treated hemorrhaged rats decreased compared to their initial baseline values. The values for both untreated and mepacrine-treated hemorrhaged rats were similarly decreased compared to the values for sham-treated rats, which did not change during the 2-hour period of observation (Figure 4).

**Effect of Mepacrine Treatment on Lung Lavage Ferritin Concentrations in Hemorrhaged Rats**

Lung lavage ferritin concentrations were increased in hemorrhaged rats compared to sham-treated rats (Figure 5). By comparison, lung lavage ferritin concentrations were not increased in mepacrine-treated rats subjected to hemorrhage (Figure 5). The values for mepacrine-treated rats subjected to
hemorrhage were decreased compared to hemorrhaged control rats (Figure 5). In hemorrhaged rats, lung lavage ferritin concentrations correlated with lung lavage protein concentrations (Figure 6a) and lung lavage leukocyte counts (Figure 7), but not with serum ferritin concentrations (Figure 6b).

FIGURE 4 Before hemorrhage, serum protein concentrations of all rats were the same ($P > .05$). The serum protein concentrations of sham-treated rats (○) did not change throughout the 2-hour period of observation. Following hemorrhage, the serum protein concentrations of hemorrhaged rats (●) and of mepacrine-treated rats subjected to hemorrhage (▲) were both decreased compared to sham-treated rats (○). Each point is the mean ± SEM for the number of determinations shown in the parentheses. *$P < .05$ compared to sham-treated rats.

FIGURE 5 Rats subjected to hemorrhage had increased lung lavage ferritin concentrations compared to sham-treated rats and compared to mepacrine-treated rats subjected to hemorrhage. *$P < .05$ compared to control sham-treated rats, #$P < .05$ compared to control rats subjected to hemorrhage.
DISCUSSION

We found that serum ferritin concentrations increased rapidly in rats subjected to hemorrhage [13]. Serum ferritin concentrations increased approximately 4-fold, from about 50 ng/mL to about 200 ng/mL in the initial 2 hours following hemorrhage. By comparison, in one study [14], patients with established acute respiratory distress syndrome (ARDS) had serum ferritin concentrations that were about 10-fold higher than values for control subjects. In that study, the median values were 293 ng/mL for

**FIGURE 6** At 2 hours following hemorrhage, lung lavage ferritin concentrations correlated \( r = .72, n = 30, P < .01 \) with lung lavage protein concentrations, but not with serum ferritin concentrations.
ARDS females and 1450 ng/mL for ARDS males compared to 31 ng/mL for control females and 108 ng/mL for control males. Moreover, serum ferritin concentrations of women (420 ng/mL) and men (925 ng/mL) who were at risk (for less than 6 hours) and would later develop ARDS were also elevated compared to control values. In a second study, at-risk subjects who would later develop ARDS had 3-fold increases in serum ferritin concentrations, averaging approximately 638 ng/mL compared to 185 ng/mL in at-risk patients who did not later develop ARDS [15]. Thus, serum ferritin concentrations are elevated in both rats and humans with ALI. Serum ferritin levels increased while serum total protein concentrations decreased in both untreated and mepacrine-treated rats subjected to hemorrhage. Serum protein concentration decreases most likely occurred as a result of hemodilution caused by the release of fluid into blood vessels following hemorrhage [32].

In addition to the serum ferritin changes, we also found that ferritin concentrations increased in the lung lavages of rats subjected to hemorrhage. The mechanism responsible for this finding is unknown. It is possible that lung lavage ferritin increases may reflect leak of ferritin from the blood into the lung. This possibility was suggested by the correlation of lung lavage ferritin and protein concentrations, which also increased in hemorrhaged rats. On the other hand, lavage ferritin levels did not correlate with serum ferritin levels, indicating that some of the lavage ferritin may have originated in the lungs, not simply from leaking in with plasma proteins. Lung lavage ferritin increases might reflect increased release of ferritin from leukocytes, which increase in the lung following hemorrhage. The correlation between

**FIGURE 7** At 2 hours following hemorrhage, lung lavage ferritin concentrations correlated \( r = 0.67, \ n = 21, \ P < .001 \) with lung lavage leukocyte numbers.
lung lavage ferritin concentrations and leukocyte numbers supports this possibility. More leukocytes were recovered from the lung lavages of hemorrhaged rats compared to control rats. In both cases, the recovered cells were predominantly macrophages with similar intracellular ferritin concentrations. The increase in lung MPO activity following hemorrhage likely represents increased numbers of neutrophils adherent within the lung vasculature during the first 2 hours after hemorrhage [37].

Mepacrine treatment attenuated serum and lung lavage ferritin concentration increases, lung lavage leukocyte numbers, lung tissue MPO levels, and lung leak in rats subjected to hemorrhage. The greater inhibition by mepacrine of hemorrhage-induced lung lavage leukocyte counts than hemorrhage-induced increases in lung tissue MPO levels is consistent with the possibility that mepacrine treatment partially inhibits adhesion and retention of circulating leukocytes in the lung, and then also inhibits their migration into the airways. Mepacrine inhibits PLA2, and PLA2 levels are increased in the lungs of ARDS patients [39]. The mechanism by which mepacrine inhibits leukocyte accumulation or serum and lavage ferritin increases in the hemorrhaged lung is unknown, and these may be dependent or independent processes. Activation of ferritin gene expression by leukotriene stimulated AP-1 [40] would be expected to be sensitive to mepacrine inhibition [28, 29], and this may occur during hemorrhage in the present model. Concrete evidence now links leukotriene-induced adhesion molecule expression to both neutrophil [41–44] and monocytes/macrophage recruitment [45, 46], and adhesion molecule expression and activity are sensitive to inhibition of leukotriene expression [41, 44, 47]. Thus, the most tenable speculation is that mepacrine inhibition of hemorrhage-induced PLA2 resulted in down-regulation of leukotriene expression, which in turn modulated leukocyte accumulation through an effect on adhesion molecule expression. Mepacrine treatment was previously shown to decrease N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP)-induced neutrophil superoxide production, adherence, and lysozyme release in vitro [27]. In addition, mepacrine treatment decreased lung neutrophil influx and lung leak in rabbits following intravenous injection of phorbol myristate acetate [48] and in rats following instillation of IL-1 intratracheally [49]. Mepacrine treatment also decreased alveolar type II cell abnormalities and peroxide formation in lungs of rats given IL-1 intratracheally [50]. Our current results support this previous work and indicate that mepacrine treatment is also protective against the development of ALI in a model that uses a different predisposing insult—hemorrhage.

The ability of mepacrine to inhibit both ferritin increases and ALI in these hemorrhaged rats effectively links these 2 phenomena. Although it is not clear how these 2 events are connected, one possibility is that ferritin increases reflect a release of ferritin from injured tissues [51]. Mepacrine
treatment may be decreasing hemorrhage-induced tissue injury, which accounts for both ALI and serum ferritin increases. This possibility parallels a previous finding of an association between serum ferritin concentrations and the injury severity score in human subjects [14, 15]. A second possibility is that the ferritin increases are part of an acute-phase response that is inhibitable by mepacrine and that mepacrine decreases lung injury by blunting this response [52, 53], possibly through effects on alveolar macrophage ferritin secretion [54]. Alternatively, mepacrine treatment could decrease lung injury directly and then, as a result of decreased lung injury, decrease the inflammatory response.

Based on the present and prior observations [13, 14], serum ferritin should be considered a candidate biomarker and possible mediator of ALI. Ferritin increased rapidly in situations that led to ALI and did not increase when ALI was attenuated with mepacrine pretreatment (present work) or feeding an iron-deficient diet [13]. Our data support a role for mepacrine sensitive PLA2 in the inflammation and ferritin release stimulated by severe hemorrhage. However, additional studies are required to determine whether and by what mechanism ferritin participates in the development of ALI.

REFERENCES