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Original Contribution

Pancreatic ascites hemoglobin contributes to the systemic response in acute pancreatitis

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ABSTRACT

Upon hemolysis extracellular hemoglobin causes oxidative stress and cytotoxicity due to its peroxidase activity. Extracellular hemoglobin may release free hemin, which increases vascular permeability, leukocyte recruitment, and adhesion molecule expression. Pancreatitis-associated ascitic fluid is reddish and may contain extracellular hemoglobin. Our aim has been to determine the role of extracellular hemoglobin in the local and systemic inflammatory response during severe acute pancreatitis in rats. To this end we studied taurocholate-induced necrotizing pancreatitis in rats. First, extracellular hemoglobin in ascites and plasma was quantified and the hemolytic action of ascitic fluid was tested. Second, we assessed whether peritoneal lavage prevented the increase in extracellular hemoglobin in plasma during pancreatitis. Third, hemoglobin was purified from rat erythrocytes and administered intraperitoneally to assess the local and systemic effects of ascitic-associated extracellular hemoglobin during acute pancreatitis. Extracellular hemoglobin and hemin levels markedly increased in ascitic fluid and plasma during necrotizing pancreatitis. Peroxidase activity was very high in ascites. The peritoneal lavage abrogated the increase in extracellular hemoglobin in plasma. The administration of extracellular hemoglobin enhanced ascites; dramatically increased abdominal fat necrosis; upregulated tumor necrosis factor-α, interleukin-1β, and interleukin-6 gene expression; and decreased expression of interleukin-10 in abdominal adipose tissue during pancreatitis. Extracellular hemoglobin enhanced the gene expression and protein levels of vascular endothelial growth factor (VEGF) and other hypoxia-inducible factor-related genes in the lung. Extracellular hemoglobin also increased myeloperoxidase activity in the lung. In conclusion, extracellular hemoglobin contributes to the inflammatory response in severe acute pancreatitis through abdominal fat necrosis and inflammation and by increasing VEGF and leukocyte infiltration into the lung.

Under physiological conditions, the concentration of extracellular hemoglobin (Hb) in the circulation is minimal and controlled by specific plasma binding proteins (haptoglobin) and monocyte/macrophage receptors (CD163) [1,2]. However, during pathological conditions associated with hemolysis, such as severe hemolytic anemia, tissue injury, and infection, plasma and cellular binding capacities cannot cope with the increased plasma Hb concentrations and then extracellular Hb becomes a biologically relevant vasoactive and redox-active protein [1,2]. Extracellular Hb causes oxidative stress and eventually cytotoxicity due to its peroxidase activity, which generates free radicals, such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), ferryl heme radical, ferryl protein radical, and reactive derivatives such as lipid peroxides and hypohalous acid [3–5]. Furthermore, large quantities of extracellular Hb present in the circulation often lead to acute renal failure and endothelial dysfunction [1,6].

Extracellular Hb is considered potentially toxic to vascular, myocardial, renal, and central nervous system tissues [7–11]. Infusion of Hb solutions to humans may cause hypertension and acute renal failure [12]. Endothelial cells are highly susceptible to cytotoxicity induced by heme released from Hb [13,14]. Indeed, exposure of endothelial cells to heme triggered cell death mediated by polymorphonuclear leukocytes and other sources of reactive oxygen species (ROS) [13]. Extracellular Hb caused glutathione depletion and necrosis in endothelial cells in the presence of hydrogen peroxide [15].

Abbreviations: EDTA, ethylenediaminetetraacetic acid; eNOS, endothelial nitric oxide synthase; GSH, reduced glutathione; Hb, hemoglobin; HIF, hypoxia-inducible factor; HK-II, hexokinase-2; HO-1, heme oxygenase 1; Hp, haptoglobin; HPAEC, human pulmonary artery endothelial cell; ICAM, intercellular adhesion molecule; IL, interleukin; iNOS, inducible nitric oxide synthase; MDA, malondialdehyde; MALDI-TOF, matrix-assisted laser desorption/ionization–time of flight; MPO, myeloperoxidase; ROS, reactive oxygen species; RT-PCR, real-time polymerase chain reaction; SDS, sodium dodecyl sulfate; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor

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Upon release of Hb into the plasma, it may act as an endogenous damage-associated molecular pattern and it may also release free hemin that has proinflammatory effects. Thus, acute exposure to cell-free Hb in rats increased lung intercellular adhesion molecule (ICAM) protein expression, and chronic exposure to low plasma Hb concentrations induced pulmonary vascular disease via inflammation [16]. *In vitro*, heme induced the expression of ICAM-1, vascular cell adhesion molecule 1, and E-selectin in endothelial cells [17]. *In vivo*, heme induced increased vascular permeability, leukocyte recruitment, and adhesion molecule expression, in particular, enhanced expression of ICAM-1 and P-selectin in mouse liver and pancreas [18]. In leukocytes heme induced, on one hand, tumor necrosis factor-α (TNF-α) secretion by macrophages dependent on TLR4 activation and, on the other hand, oxidative burst and neutrophil recruitment independent of TLR4 [19].

The pathophysiological relevance of extracellular Hb *in vivo* is not well understood [1], particularly in inflammatory disorders. Indeed, panhematin, a water-soluble hemin formulation, ameliorated experimental acute pancreatitis through upregulation of protective heme oxygenase 1 [20]. Severe acute pancreatitis is associated with a reddish ascitic fluid, and this clinical observation together with the finding of hemorrhagic ascites in experimental necrotic pancreatitis [21] prompted us to initiate the present study. The aim of this work has been to determine the role of extracellular hemoglobin present in ascitic fluid on the local and systemic inflammatory response during severe acute pancreatitis in rats as an experimental model of systemic inflammatory response.

Materials and methods

Animals

Young male Wistar rats (250 ± 25 g) were used in the experiments. They were fed a standard laboratory diet and tap water *ad libitum* and were subjected to a 12-h light–dark cycle. Rats received humane care according to the guidelines for animal experimentation of the European Union (NEAUE 1999) and also to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the U.S. National Academy of Sciences and published by the National Institutes of Health (NIH Publication 86-23, revised 1985). The Ethics Committee of the University of Valencia (Spain) approved the study protocol.

Study design

A first series of experiments was designed to identify and quantify the hypothesized presence of extracellular hemoglobin in the ascitic fluid and plasma during acute pancreatitis. The experimental groups used in this series of experiments are shown in Table 1.

In a second series of experiments the hemolytic capacity of ascitic fluid was tested to determine whether the presence of extracellular hemoglobin in ascites caused an increase in extracellular hemoglobin in plasma during acute pancreatitis. The experimental groups used in this series of experiments are shown in Table 1.

In a third series of experiments, we assessed the local and systemic effects of extracellular hemoglobin present in ascitic fluid during acute pancreatitis. The experimental groups used in this series of experiments are shown in Table 1.

Rats were anesthetized before sacrifice. Blood was extracted and plasma was obtained after centrifugation at 1800 rpm for 20 min. Plasma samples were stored at −80 °C until analysis. Immediately after sacrifice, abdominal fat, pancreas, liver, and lungs were removed and specimens were frozen by freeze-clamp in liquid nitrogen and stored at −80 °C until analysis.

Experimental model of acute pancreatitis

Taurocholate-induced acute necrotizing pancreatitis was used as an experimental model in rats. Induction and maintenance of anesthesia were performed with isoflurane (Isoflo) inhaled at 3%. The biliopancreatic duct was cannulated through the duodenum and the hepatic duct was closed by a small bulldog clamp. Acute necrotizing pancreatitis was induced by retrograde injection into the biliopancreatic duct of sodium taurocholate (3.5%; Sigma) in a volume of 0.1 ml/100 g of 0.9% NaCl using an infusion pump (Harvard Instruments) [22]. Rats were sacrificed at 0, 0.5, 1, 3, 6, and 24 h after the infusion of taurocholate and were anesthetized as previously mentioned before sacrifice. Plasma lipase activity was measured and histological studies were performed to confirm the appropriate induction of necrotizing pancreatitis.

Peritoneal lavage

Drainage of ascites from rats with pancreatitis was performed using an input cannula of 1.3 mm external diameter placed on the right side of the abdomen and an output cannula of 3 mm external diameter taken out through the upper limit of the laparotomy used for induction of acute pancreatitis, once sutured. Sterile saline serum was infused at 37 °C through the input cannula at a flux of 1.4 ml/min. Peritoneal lavage was maintained for 6 h after pancreatitis induction until animal sacrifice. Induction of anesthesia was performed with isoflurane (Isoflo) inhaled at 2% and anesthesia was maintained continuously during the experiment.

Purification of rat hemoglobin

Ninety milliliters of blood was obtained from 12 control Wistar rats. Hemoglobin was purified from lysed erythrocytes by anionic interchange chromatography as previously described [23], using polysaccharide Q-Sepharose XL (GE Healthcare, Little Chalfont, UK) packed in XK 26/40 columns (GE Healthcare). The eluate was lyophilized and stored at −80 °C.

Hemolytic capacity of ascitic fluid

To test the hemolytic capacity of ascitic fluid, blood extracted from rats with pancreatitis was used to obtain erythrocytes after centrifugation at 1800 rpm for 15 min. Erythrocytes were incubated with ascitic fluid of the same rat in a 1:1 proportion at 37 °C for 45 min. Samples were then centrifuged and the absorbance of supernatants was measured at 540 nm. The same aliquots of blood incubated with saline solution and sonicated were used as controls for 100% hemolysis.

Assays

Reduced glutathione (GSH)

GSH levels were determined spectrophotometrically at 340 nm using glutathione *S*-transferase and 1-chloro-2,4-dinitrobenzene as in [24].

Pancreatic lipase activity

Pancreatic lipase activity was determined in plasma using the Lipase-LQ kit (Spinreact, Girona, Spain).