Hemin-activated macrophages home to the pancreas and protect from acute pancreatitis via heme oxygenase-1 induction

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Hemin upregulates heme oxygenase-1 (HO-1), a stress-induced enzyme implicated in protection from a variety of injuries while its related isoform HO-2 is constitutively expressed. The role of hemin or HO-1 in the pancreas and their potential modulation of pancreatic injury are unknown. We show that HO-1 is induced in pancreatitis caused by caerulein and more prominently in severe pancreatitis caused by feeding a choline-deficient diet (CDD). Intraperitoneal hemin administration dramatically increases peritoneal and pancreas macrophages that overexpress HO-1 in association with pancreatic induction of the chemoattractants monocyte chemotactic protein-1 and macrophage inflammatory protein-1α but not RANTES or macrophage inflammatory protein-2. Hemin administration before CDD feeding protected 8 of 8 mice from lethality while 7 of 16 controls died. Protection is mediated by HO-1–overexpressing macrophages since hemin-primed macrophages home to the pancreas after transfer to naive mice and protect from CDD-induced pancreatitis. Suppression of hemin-primed peritoneal cell HO-1 using HO-1–specific small interfering RNA prior to cell transfer abolishes protection from CDD-induced pancreatitis. Similarly, hemin pretreatment in caerulein-induced pancreatitis reduces serum amylase and lipase, decreases pancreatic trypsin generation, and protects from lung injury. Therefore, hemin-like compounds or hemin-activated macrophages may offer novel therapeutic approaches for preventing acute pancreatitis and its pulmonary complication via upregulation of HO-1.

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Introduction
Hemin upregulates heme oxygenase-1 (HO-1), a stress-induced enzyme implicated in protection from a variety of injuries while its related isoform HO-2 is constitutively expressed (1–6). HOs play essential roles in oxygen delivery, mitochondrial function, and signal transduction, including hemoglobin, cytochromes, prostaglandin endoperoxide, and nitrous oxide synthases, catalase, and peroxidases. Various formulations of hemin such as hematin and other alternate intravenous formulations (e.g., panhematin) are currently available and have been used in patients since the 1970s with minimal side effects reported to successfully treat acute porphyrias, to control liver allograft failure due to recurrence of erythropoietic protoporphyria, and in patients with thalassemia intermedia (13–16). Hemin is also an established agent for HO-1 induction in several tested cultured cells and in vivo (9, 17–20).

Conversion of trypsinogen to active trypsin within pancreatic acinar cells is an important event in developing acute pancreatitis. Furthermore, it has been well demonstrated that infiltrating neutrophils significantly contribute to this intrapancreatic trypsin activation (21). However, the mechanisms and signals that mediate neutrophil or other inflammatory cell (such as macrophage) recruitment into the pancreas are less well understood. In this report we demonstrate a novel protective role for hemin in experimental mouse pancreatitis and provide a cellular mechanistic basis for such protection. This role is mediated by HO-1 and leads to recruitment of HO-1 expressing macrophages to the pancreas.

Results
Hemin administration protects from injury in experimental acute pancreatitis. We examined HO-1 expression in 2 established mouse pancreatitis models: (a) caerulein hyperstimulation, which causes mild pancreatitis, and (b) feeding with a choline-deficient
diet (CDD), which causes severe pancreatitis (22–24). HO-1 levels increased significantly (particularly in the more severe CDD model) and reversibly in the 2 tested pancreatitis models, while HO-2 levels were minimally altered (Figure 1, A and B). Given the high HO-1 induction we observed in the caerulein and CDD pancreatitis models, we hypothesized that HO-1 induction may be associated with modulation of the extent of pancreatic injury. We tested this hypothesis by first assessing the effect of administering hemin on pancreatic HO-1 levels. Hemin i.p. administration into mice dramatically induced pancreatic HO-1 but not HO-2, though repeat injections blunted HO-1 upregulation (Figure 1C).

More importantly, hemin priming of mice prior to feeding with CDD (Figure 1D) provided complete protection from the severity of pancreatic injury. When tested in 2 independent pancreatitis models, the extent of lung injury after caerulein administration was able to unmask a significant difference in lung MPO activity after caerulein plus saline administration alone had elevated MPO levels (Figure 2C). Notably, hemin priming conferred a dramatic protection from the lung injury that associates with caerulein pancreatitis, as supported by histologic assessment (Figure 2, D–F). Despite baseline elevation of lung MPO activity in hemin plus saline–treated mice, the extent of lung injury after caerulein administration was able to unmask a significant difference in lung MPO activity after caerulein plus hemin administration (Figure 2D). Hence, hemin protected from pancreatitis-associated injury when tested in 2 independent pancreatitis models.

**Peritoneal macrophages home to the pancreas after hemin administration.**

In order to understand the mechanism of protection by hemin, we asked which cells account for the induced HO-1 during pancreatitis. Previous studies showed that hemin induces HO-1 in a variety of epithelial and nonepithelial cell types (9, 17–20). HO-1+ cells that are induced in the pancreas after pancreatic injury are mesenchymal and not epithelial, as determined by HO-1 colocalization with vimentin but not with keratin-8 staining (Supplemental Figure 2, A–F). Interestingly, pancreatic HO-1+ cells (after hemin administration) did not stain with the endothelial marker M7/18 (Supplemental Figure 2, G–I), but most of HO-1 colocalized primarily with F4/80 (>90%) and to a much lesser extent with Gr-1 (Figure 3, A–D) that represent macrophage (F4/80+) and neutrophil (Gr-1+) markers, respectively. Staining of Mac-1 (another macrophage marker) provided similar results to those obtained with F4/80 (not shown). This suggests that HO-1 induction during pancreatic injury occurs preferentially in pancreatic macrophages.

**Figure 1**

Effect of hemin or pancreatic injury on HO-1 induction and inhibition of pancreatic injury by hemin. (A and B) Total tissue homogenates were obtained from pancreata of caerulein-injected (cae-injected) or CDD-fed mice. Two age- and sex-matched mice were used for each time point. Homogenates were tested by blotting, using antibodies to HO-1 or HO-2. (C) Mice (3 mice/condition) were given hemin (H) by i.p. injection (4 times during 1 week or 8 times during 2 weeks). Two control (Co) mice were injected with vehicle alone. Pancreatic homogenates were then obtained and blotted with anti–HO-1 and anti–HO-2. (D–G) Mice were injected with saline (S), hemin, or vehicle (V) (8 mice/group) 3 times (arrows) followed by feeding with CDD for 3 days, then harvesting of the pancreata. HO-1 and HO-2 were analyzed by blotting of pancreatic homogenates (3 mice/group). The number of mice that died in each cohort of 8 mice is shown, and the survival difference was significant ($P < 0.03$) when comparing controls (saline and vehicle) with hemin-injected mice. Representative H&E stainings of pancreata from mice that survived CDD feeding are shown. Scale bar: 50 µm. Note the marked pancreas edema (ED) and necrosis (N) in the saline- (not shown) and vehicle-injected mice as compared with the hemin group.
Given the dramatic effect of HO-1 induction in the pancreas by simple i.p. injection of hemin in the absence of pancreatitis (Figure 1C) and the observed protection from pancreatitis by hemin (Figures 1, E–G, and 2), we quantified the effect of hemin on pancreatic macrophages in the presence or absence of CDD feeding. Comparison between peritoneal cells of hemin- and vehicle-injected mice showed a more than 2-fold increase in total peritoneal cells and a more than 4-fold increase in peritoneal macrophages (Supplemental Table 1). Quantification of the number of pancreatic macrophages showed a significant increase upon hemin administration with or without CDD feeding that paralleled the frequency of hemin injections (Figure 3E). Hemin treatment induced recruitment of HO-1+ macrophages to the pancreas with or without causing pancreatitis (using histological and serologic criteria, not shown), which suggests that hemin is directly or indirectly involved in chemoattraction of macrophages to the pancreas. Earlier studies showed that hemin can induce the chemokine monocyte chemotactic protein-1 (MCP-1) in immortalized rat proximal tubular epithelial cells (18) and can result in leukocyte activation (20). Therefore we tested the hypothesis that hemin upregulates chemokines, particularly those that attract macrophages. Hemin or vehicle were injected i.p. into mice only once followed by harvesting of pancreata from injected or noninjected mice after 24 hours in order to measure the mRNA levels of several chemokines. As shown in Figure 3F, MCP-1 and macrophage inflammatory protein-1α (MIP-1α) but not RANTES or MIP-2 levels increased dramatically after a single i.p. dose of hemin. Hence, the hemin-induced increase in MCP-1/MIP-1α and/or other chemoattractants provides a plausible explanation for the observed increase in pancreas HO-1+ macrophages.

The increase in HO-1+ peritoneal and pancreatic macrophages in response to hemin raised the hypothesis that hemin administration induces homing of peritoneal macrophages to the pancreas, which in turn are responsible for the observed protection afforded by hemin. We tested this hypothesis by carrying out the transfer experiment outlined in Figure 4A. Whole body in vivo imaging following transfer of Mac-1+ peritoneal cells from hemin-pretreated luciferase-overexpressing mice showed that a significant number of the injected cells remained in the peritoneal cavity of the nonluciferase recipient mice given hemin while recipients given vehicle dissipated (Figure 4, B–D). Importantly, the double-positive luciferase/Mac-1+ macrophages in mice given hemin were specifically localized in the pancreas but not in the liver (Figure 4, B–D) or other abdominal organs (not shown). Presence of luciferase/Mac-1+ cells within the pancreas was confirmed by staining (Figure 4, E–G), indicating that hemin-primed peritoneal macrophages are capable of migrating to the pancreas of hemin-treated but not vehicle-treated recipient mice.

Peritoneal cells protect from CDD-induced pancreatitis via HO-1 induction. Confirmation that peritoneal cells were indeed responsible for imparting the protective effect of CDD-induced pancreatic damage was carried out by transferring peritoneal cells obtained from hemin- or vehicle-treated mice into naive mice, followed by exposure of the recipient mice to CDD (Figure 5A). Significantly lower serum amylase and blood urea nitrogen (BUN) levels (elevated upon dehydration and renal injury) were noted in mice receiving hemin-activated cells (Figure 5B). Half of the mice receiving cells from vehicle-treated animals developed macroscopic hemorrhagic pancreatitis (3 of 6 mice) while none of the mice receiving hemin-activated cells had any macroscopic evidence of hemorrhage.
of the peritoneal cells that are used for cell transfer. siRNA con-  
hemin but rather to hemin-activated macrophages that home to  
then used to pretreat mice (together with hemin on alternate  
ure 6, A and B). Construct 2 and the scrambled construct were  
struct 2 had a dramatic effect in blocking HO-1 induction (Fig-  
blunt the hemin-induced upregulation of HO-1. First, we tested  
2 siRNA constructs for their ability to inhibit HO-1 induction  
chemokine to  
then estimated from pancreata of 3 mice per group as a ratio of the indicated  
and MIP-1α induction. (A–D) Pancreas from CDD-fed mice was triple stained  
Figure 3  
Hemin increases HO-1+ pancreatic macrophages in association with MCP-1 and MIP-1α induction.  
(P < 0.05) (Figure 5C). This was further confirmed by histological  
(P < 0.05) Mice were treated i.p. with hemin or vehicle 1 or 3 times dur-  
unanticipated effect of hemin on leukocyte recruitment  
Discussion  
Acute pancreatitis can be a severely debilitating if not lethal  

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ies will be necessary to determine whether hemin-based therapy can also be protective at specific windows of opportunity after onset of pancreatitis or if the benefit is strictly prophylactic.

The observed effects of hemin are related to HO-1 activation, which can unveil several potential protective mechanisms consequent to 1 or more of the HO-1 downstream byproducts carbon monoxide, biliverdin, or ferritin (4–6). For example, HO-1–drived carbon monoxide has antiinflammatory effects mediated by IL-10 signaling and p38 mitogen-activated protein kinase activity (3, 28). The effects of the 3 major HO-1 by-products on pancreatitis and its progression remain to be investigated. Our findings suggest a model whereby hemin upregulates several pancreatic chemokines (e.g., MCP-1 and MIP-1α, with the relevant in vivo chemokines remaining to be defined) and causes a significant increase in HO-1+ peritoneal macrophages and other cells (Figure 7). Chemokine induction, in turn, triggers the recruitment of HO-1+ peritoneal macrophages to the pancreas. Our data also demonstrate that the peritoneal activated cells and not a direct hemin effect are responsible for imparting the observed protection from pancreatitis-related injury.
Methods

Hemin, vehicle, and antibodies. Hemin (Sigma-Aldrich) was dissolved in 10% ammonium hydroxide in 0.15 M NaCl to prepare a stock solution of 100 mg/ml, then further diluted 1:40 with sterile 0.15 M NaCl and injected into mice (10 µl/g). Vehicle-injected mice received an identical NH₄OH-containing solution lacking hemin. The antibodies used were directed to the following: HO-1 and HO-2 (Stressgen Biotechnologies); vimentin, smooth muscle actin, and luciferase (NeoMarkers); allophycocyanin-conjugated Gr-1 and Mac-1 (BD Biosciences — Pharmingen); and FITC-conjugated F4/80 (Serotec). Panhematin was kindly provided by Stephen Collins (Ovation Pharmaceuticals, Inc., Deerfield, Illinois, USA).

Mice and pancreatitis models. All animal protocols were approved by the Institutional Animal Care and Use Committees of Stanford University and the VA Palo Alto Health Care System. BALB/c mice were housed under pathogen-free conditions and utilized in 2 established models of pancreatitis (22–24, 29, 30). For caerulein-induced pancreatitis, age- and sex-matched mice were fasted for 12–16 hours but allowed water ad libitum. Mice then received 7 hourly i.p. injections of saline (control group) or 50 µg/kg caerulein (Research Plus) in saline and were followed up to 12 hours. For the pancreatitis induced by feeding a choline-deficient diet (CDD), young female mice (15–19 g) were fasted, then fed CDD (Harlan Teklad) supplemented with 0.5% DL-ethionine (Sigma-Aldrich) or normal chow (control group) for 3 days, then switched to a normal diet for 1, 2, or 5 days.

Animal procedures. Mice were euthanized by CO₂ inhalation, then pancreata and lungs were rapidly removed, divided into 3–4 pieces, and blood was collected by intracardiac puncture. Individual lung and pancreas fragments were immediately fixed in 10% formalin, embedded in Optimum Cutting Temperature medium (Sakura Finetek Inc.) or snap-frozen in liquid N₂ for subsequent protein, enzyme, and RNA analysis (29, 30). Fixed tissues were sectioned then stained using H&E (performed by Histo-Tec Laboratory). Immunofluorescence staining was performed as described (29), and images were analyzed using confocal microscopy. Total tissue lysates were prepared by homogenization in Laemmli sample buffer and analyzed by SDS-PAGE followed by transfer to membranes for blotting and protein visualization by enhanced chemiluminescence. Trypsin activity was determined using a fluorimetric assay and the substrate Boc-Gln-Ala-Arg-MCA (PepTides International) as well as a comparison to a standard curve generated with purified trypsin as described (31). For MPO activity, lung and pancreas tissues were processed as described (32) followed by the use of an

Figure 7
Schematic of the effect of hemin on mouse HO-1 induction and homing of peritoneal macrophages to the pancreas. Hemin induced the expression of several pancreas chemokines and increased peritoneal macrophages and their HO-1 expression. Peritoneal macrophages that overexpress HO-1 homed to the pancreas and were able to provide significant protection from pancreatic injury.
was injected in the intervening days (an additional 3 injections). After 7 days, peritoneal cells were harvested and analyzed by immunoblotting using antibodies to HO-1/2. Since significant inhibition of HO-1 occurred with construct 2, construct 2 was used for subsequent transfer of peritoneal cells (pretreated with heme and scrambled siRNA or with heme and HO-1 siRNA) to naive recipient mice that were then fed CDD for 2.5 days followed by isolation of the pancreata. The siRNAs were synthesized by Dharmacon and reported previously (37). The sense and antisense strand sequences of the 2 HO-1 siRNA and nonspecific siRNA scrambled duplex were as follows: 1′, 5′-UUGCUUCUUGA-UAAUAdTdT-3′ (sense) and 5′-AUAGUGAAGGAAAGCCAdTdT-3′ (antisense); 2′, 5′-GCCACACAC-
CACAUAGUAAdTdT-3′ (sense) and 5′-UUACAUUGUGUGUGGCGCdTdT-
3′ (antisense); and scrambled, 5′-GCCGCUUUUGAAGAUUCGdCdTdT-
3′ (sense) and 5′-CAGAUCCUAACAGCGCgCdTdT-3′ (antisense).

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research article

MPO kit according to manufacturer guidelines (Calbiochem). Lung injury after caerulein administration was assessed as previously described based on neutrophilic infiltration, pulmonary edema, alveolar distension, and collapse (32). P values were calculated for histology, enzyme, and serum test comparisons using the paired 1-tailed Student’s t test and for survival comparisons using the χ2 method.

Luciferase transgenic mice and imaging. The luciferase-overexpressing transgenic mice (in an FVB/n background) and their nontransgenic counterparts (35) received 3 doses of heme on days −5, −3, and −1. On day 0, peritoneal cells were harvested, and the Mac-1+ cells were selected using Mac-1−coated (anti-CD11b) microbeads (Miltenyi Biotec). Luciferase/Mac-1 double-positive cells (2 × 10⁶) were transferred i.p. to wild-type FVB/n mice pretreated a day earlier with 1 dose of heme or vehicle in order to prime cellular homing. Bioluminescent in vivo images of the recipient mice were obtained 5 minutes and 24 hours after cell transfer as described (35). Mice that were imaged 24 hours after transfer were then killed followed by isolation of several organs for luciferase imaging and immunofluorescence staining.

Hemin-pretreated peritoneal cell transfer into CDD-fed mice. Peritoneal cells were harvested from hemo- or vehicle-pretreated (3 doses given every other day) young female BALB/c mice (15–19 g). Isolated cells were washed with PBS, and equivalent cells were transferred into naive BALB/c littermates i.p. followed by initiation of CDD feeding. Mice were sacrificed after 2.5 days of CDD feeding, followed by isolation of the pancreata (for histological and fluorescence staining) and blood. Serum was isolated from clotted blood followed by measurement of amylase and BUN using standard methods. The severity of pancreatitis was scored using established histological criteria that assigned individual numerical scores (1, mild; 2, moderate; and 3, severe) to edema, inflammation, and hemorrhage and alternate scores for parenchymal necrosis (3, focal; 5, sublobular; and 7, lobular) (36).

HO-1 siRNA and hemin-pretreated cell transfer. Mice were injected i.p. every other day (3 total injections) with either saline or with 2 HO-1 siRNA constructs (2 mg/kg body weight) or with a scrambled siRNA sequence. Hemin


