Heme oxygenase-1 is induced in peripheral blood mononuclear cells of patients with acute pancreatitis: a potential therapeutic target

Aida Habtezion,2* Raymond Kwan,1,* Alice L. Yang,2 Maureen E. Morgan,2 Ehsaan Akhtar,2 Stephen P. Wanaski,3 Stephen D. Collins,3 Eugene C. Butcher,2 Ahmad Kamal,2,5 and M. Bishr Omary1

1University of Michigan Medical School, Department of Molecular and Integrative Physiology, Ann Arbor, Michigan; 2Stanford University School of Medicine, Department of Medicine, Division of Gastroenterology and Hepatology, Stanford, California; 3NeuroTherapeutics Pharma, Chicago, Illinois; 4Stanford University School of Medicine, Department of Pathology, Stanford, California; and 5Division of Gastroenterology and Hepatology, Department of Medicine, Santa Clara Valley Medical Center, San Jose, California

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ACUTE PANCREATITIS (AP) can follow a severe course that leads to 10–30% mortality in high-risk patients and, in the United States alone, it accounts for over 220,000 hospital admission every year (34). The most common risk factors for developing AP include alcohol and gallstones. Other risk factors include diagnostic and therapeutic instrumentation of the biliary and/or pancreatic ducts such as endoscopic retrograde cholangiopancreatography (ERCP) (34). To date, despite the significant morbidity, mortality, and cost incurred, no effective active treatment aside from supportive therapy (e.g., fluid resuscitation) exists. Thus better understanding of the mechanism(s) of disease and the characterization of potential therapeutic targets are of high priority in the field.

Heme oxygenase-1 (HO-1) is a rate-limiting enzyme that catalyzes heme and is important for the maintenance of iron homeostasis, regulation, and suppression of inflammatory responses (9, 15, 19, 22, 23, 28, 29, 32). Induction of HO-1 expression using hemin or Panhematin (PH), the latter being an approved hemin formulation for the treatment of porphyrias (1, 14), protects mice from pancreatic injury in experimental pancreatitis models (21). Intravenous hemat in has been used clinically to treat patients with acute porphyrias for the past four decades, and PH was the first Food and Drug Administration agency-approved formulation in the United States (27). Other formulations, such as heme arginate, have been used in Europe to treat patients with acute porphyrias and other hematological disorders such as myelodysplastic syndromes (10, 20, 26, 33). More recently, parenteral hemin administration to healthy individuals was shown to induce plasma HO-1 levels (5).

HO-1 induction correlates with the suppression of inflammatory responses (4, 16, 19, 22, 25). The anti-inflammatory effects of HO-1 are believed to be mediated by carbon monoxide and biliverdin, the end products of heme degradation through HO-1-mediated catalysis (22). We previously showed that hemin upregulates HO-1 expression in monocytes and ameliorates pancreatic injury in mouse models of AP (21). Although HO-1 upregulation occurs in the context of experimental pancreatitis, the extent of upregulation is significantly more robust upon administration of hemin (21) or PH (unpublished observations) which is likely contributing to the protective effect. More recently, Chen et al. (6) showed that a carbon monoxide releasing compound protected rats from sodium taurocholate-induced pancreatitis. In addition, induction of HO-1 was shown to improve pancreatic graft survival in a rat model of pancreatic transplantation (4). On the basis of these findings, we reasoned that HO-1 induction in leukocyte populations may play a role in the resolution of human AP and...
investigated the expression of HO-1 over time in patients with AP compared with controls.

As a preclinical first step toward targeting HO-1 as a potential therapeutic intervention in human AP, we investigated the expression and ex vivo inducibility of HO-1 in PMBCs of hospitalized patients with AP compared with hospitalized control (HC) patients who were admitted to the hospital because of etiologies unrelated to pancreatic disease. We also included healthy volunteer controls (VC) as an additional study group. HO-1 levels were assessed in various leukocyte subsets as patients recovered from their AP. In addition, using an experimental model of AP we examined the ex vivo inducibility of mouse splenocyte HO-1 during the course of pancreatitis in response to PH.

MATERIALS AND METHODS

Patients. A total of 33 patients (18 AP and 15 HC) admitted to either Stanford Hospital, Palo Alto Veteran Affairs Hospital, or Santa Clara Valley Medical Center, and 7 volunteer controls (VC) of similar age and sex were recruited. The VC group consisted of healthy nonhospitalized volunteers. Patients with past history of AP or chronic pancreatitis were excluded. The diagnosis of AP was confirmed by biochemical assessment (serum lipase levels) and in some with past history of AP or chronic pancreatitis were excluded. The diagnoses of the HC group (Table 1). AP patients were admitted with abdominal pain and elevated lipase levels that were at least three times the upper normal limit. In addition, some of the patients had CT evaluation as part of their admission workup with findings consistent with AP. The majority of the pancreatitis cases were thought to be due to gallstones, and, based on the CT Severity Index (Balthazar Score; mean score of 2.4, Table 2) and clinical status, the pancreatitis was categorized as mild. The CT severity index correlates well with the development of local complications and mortality associated with AP (2). Patients with a past history of pancreatic disease (e.g., previous AP or chronic pancreatitis) were excluded. The diagnoses of the HC group were based on edema, inflammation, hemorrhage, and parenchymal necrosis as described previously (21). The animal protocols were approved by the Institutional Animal Care and Use Committee.

Flow cytometry. PMBCs were stained with antibodies to CD19 (B cells), CD3 (T cells), and CD14 and CD11b (monocytes). PMBCs were subsequently fixed and permeabilized by use of Becton Dickinson Cytofix/Cytoperm and stained for intracellular HO-1. Stained samples were analyzed by flow cytometry using a Becton Dickinson LSRII (Supplementary Fig. S1; the online version of this article contains supplemental data). Rainbow Calibration Particles from Spherotech (Lake Forest, IL) were run after each flow cytometry session. HO-1 mean fluorescence intensity (MFI) values for leukocyte subsets were normalized by using Rainbow Calibration Particles to allow for the comparison of data from multiple flow cytometry sessions. Analysis of flow cytometry raw data was performed via Flowjo software (Treestar, Ashland, OR).

Western blotting. PMBCs were lysed in a homogenization buffer (187 mM Tris, 3% SDS, 5 mM EDTA) at a cell density of 1 × 10⁷ cells/50 μl. Protein concentration was determined by the BCA method. Equal amounts of protein were loaded followed by SDS-PAGE then transfer to polyvinylidene difluoride membranes. Western blotting was performed, and membranes were incubated with antibodies to HO-2 (which is constitutively expressed) as a loading control or to HO-1. Equal loading was also confirmed by Coomassie staining of the membranes.

Statistical analysis. Data were analyzed with SAS version 9.1 (Cary, NC). To account for small sample sizes and nonnormal distribution of data, Fisher’s exact test, the Mann-Whitney U-test, and the Wilcoxon’s signed rank test were used for categorical data, continuous data, and paired data, respectively. All P values are two tailed, and a value of 0.05 was considered statistically significant.

RESULTS

Demographics of patients and healthy volunteers. There were no differences in sex, age, or ethnicity between the AP (n = 18) and HC (n = 15) patients and the VC group (n = 7) (Table 1). AP patients were admitted with abdominal pain and elevated lipase levels that were at least three times the upper normal limit. In addition, some of the patients had CT evaluation as part of their admission workup with findings consistent with AP. The majority of the pancreatitis cases were thought to be due to gallstones, and, based on the CT Severity Index (Balthazar Score; mean score of 2.4, Table 2) and clinical status, the pancreatitis was categorized as mild. The CT severity index correlates well with the development of local complications and mortality associated with AP (2). Patients with a past history of pancreatic disease (e.g., previous AP or chronic pancreatitis) were excluded. The diagnoses of the HC group were based on edema, inflammation, hemorrhage, and parenchymal necrosis as described previously (21). The animal protocols were approved by the Institutional Animal Care and Use Committee.

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are described in Table 3, and only a minority of the patients had “inflammatory” conditions.

AP patients’ PBMCs express higher levels of HO-1 compared with the HC and VC groups. Patients with AP had higher level of circulating monocyte (mean 10,718; range 44 –50,400 arbitrary normalized MFI units) and neutrophil (mean 12,951; range 333–31,200 arbitrary normalized MFI units) HO-1 expression compared with monocytes (mean 2,311; range 1,747–3,281 arbitrary normalized MFI units, \( P = 0.034 \)) and neutrophils (mean 3,085; range 1,616–3,575 arbitrary normalized MFI units, \( P = 0.040 \)) of VCs (Fig. 1, A and B). AP patients also had higher level of neutrophil, T cell (mean 3,716; range 149 –21,990 arbitrary normalized MFI units), and B cell (mean 4,387; range 0–22,440 arbitrary normalized MFI units) HO-1 levels compared with neutrophils (mean 3,634; range 465–18,979 arbitrary normalized MFI units, \( P = 0.004 \)), T cells (mean 514; range 19–2,443 arbitrary normalized MFI units, \( P = 0.002 \)), and B cells (mean 786; range 0–3,680 arbitrary normalized MFI units, \( P = 0.015 \)) from HC patients (Fig. 1, B–D). Although monocyte HO-1 levels were higher in AP (mean 10,718; range 44–50,400 arbitrary normalized MFI units) as opposed to monocytes (mean 3,153; range 465–7,575 arbitrary normalized MFI units, \( P = 0.051 \)) from HC patients, it did not reach statistical significance. VC patients, compared with HCs, had higher T and B cell but not monocyte or neutrophil HO-1 levels (Fig. 1, A–D).

Clinical improvement in AP patients is associated with a significant decrease in monocyte HO-1 levels. PBMCs HO-1 levels were assayed at day 3 following admission to determine whether there were changes in HO-1 expression over time. At day 3, no change in neutrophil, T cell, or B cell HO-1 levels were detected in AP patients or VCs (Fig. 2, B–D). However, there was significant decrease in circulating monocyte HO-1 levels from day 1 to day 3 in AP (mean 6,797; range 754–40,800 arbitrary normalized MFI units, \( P = 0.040 \)) patients as opposed to levels from day 1 to day 3 in VCs (mean 2,745; range 1,495–2,978 arbitrary normalized MFI units, \( P = 0.038 \)) (Fig. 2A). This decrease in HO-1 level was associated with recovery, since all patients with AP recovered by day 3 as determined by improvement of lipase levels and clinical symptoms.

### Table 3. Diagnosis of HC patients

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Number of Patients</th>
</tr>
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<tbody>
<tr>
<td>Upper gastrointestinal bleed</td>
<td>3</td>
</tr>
<tr>
<td>Congestive heart failure</td>
<td>2</td>
</tr>
<tr>
<td>Cellulitis</td>
<td>2</td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis</td>
<td>2</td>
</tr>
<tr>
<td>Metastatic renal cell carcinoma</td>
<td>1</td>
</tr>
<tr>
<td>Ischial ulcer</td>
<td>1</td>
</tr>
<tr>
<td>Neurosyphilis</td>
<td>1</td>
</tr>
<tr>
<td>Drug overdose</td>
<td>1</td>
</tr>
<tr>
<td>Liver abscess</td>
<td>1</td>
</tr>
<tr>
<td>Cholelithiasis</td>
<td>1</td>
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Fig. 1. Leukocyte subset expression of heme oxygenase-1 (HO-1) using fluorescence-activated cell sorting (FACS) analysis. Peripheral blood mononuclear cells (PBMCs; obtained from patients on the first day of admission) were stained for leukocyte-specific [monocytes (A), neutrophils (B), B cells (C), T cells (D)] surface markers as described in MATERIALS AND METHODS and then stained for intracellular HO-1. The cells were analyzed by FACS and corrected for isotype control antibody and normalized by using Spherotech beads to allow comparison between patients. Statistical analysis was done via the Mann-Whitney U-test. VC, volunteer controls; HC, hospitalized controls; AP, acute pancreatitis; MFI, mean fluorescence intensity.
Clinical presentation and correlation with HO-1 levels. AP patients reported nonspecified and vague abdominal pain ranging in duration between 2 days to 3 mo prior to the day of admission. The patients presented to hospital as their abdominal pain either worsened or had additional associated symptoms such as nausea or vomiting. The only patient we could definitively determine the onset of pancreatitis-related pain with relative accuracy was the patient with post-ERCP pancreatitis. This patient had the highest level of HO-1. Consistent with our cohort of AP patients having mild disease, only one and two of the patients met three and two of admission’s Ranson criteria, respectively. The remaining AP patients met either one or none of Ranson’s criteria. We did not find a correlation between admission Ranson’s criteria and leukocyte HO-1 levels (data not shown).

HO-1 inducibility in response to PH is greater in PBMCs of AP patients compared with HC patients and VC individuals. Hemin treatment in mice induces HO-1 in various tissues such as the liver, spleen, colon, and pancreas (21). HO-1⁺ monocytes are recruited to the pancreas following hemin treatment and confer protection against experimental pancreatitis (21). Thus, as a first step toward considering the targeting of HO-1 as a potential therapeutic intervention in human AP, we investigated whether HO-1 in PBMCs can be induced ex vivo upon treatment with PH. If so, this approach may offer a potential therapy because it suggests that an infusion of PH in this setting would result in further upregulation of HO-1 or, alternatively, that isolation of leukocytes from patients followed by priming with PH to upregulate HO-1 may offer a potential autologous cell-based therapy. PBMCs from AP patients at day 1 readily upregulated HO-1 following 16-, 24-, and 48-h culture with PH (Fig. 3A). Maximal HO-1 upregulation was seen after 24-h culture. The inducibility of HO-1 in response to PH was less during recovery or at day 3 (Fig. 3A). In contrast, minimal HO-1 induction by PH was seen in HC patients or VCs at day 1 following 24-h culture (Fig. 3B). Similarly, analysis by flow cytometry revealed higher HO-1 induction by PH in cultured PBMCs of AP patients as opposed to those of VCs (Fig. 4). This increase in HO-1 expression was highest in monocyte followed by B cell subsets. There was no change in T cell HO-1 levels.
HO-1 induction in leukocytes in response to PH correlates with severity of experimental pancreatitis. To determine whether similar ex vivo induction of human HO-1 by PH (as shown in Figs. 3 and 4) can be seen in an experimental mouse model of pancreatitis, we added PH ex vivo for 24 h to cultured spleen cells isolated from mice with mild, moderate, and severe pancreatitis. To induce experimental pancreatitis, mice were fed a CDE diet. In this model, disease severity increases over time with 100% mortality after 5 day of CDE feeding, and severe pancreatic injury with significant hemorrhage and necrosis is observed after 3 days of CDE feeding (17, 18). We used the CDE model of pancreatitis because it offers an opportunity to study mild to severe disease over time as shown by the progression of pancreatitis based on histological and biochemical assessment (Fig. 5A). Similar to the finding for PBMCs from AP patients at day 1, greater splenocyte PH-mediated HO-1 induction was seen after 60 h (severe disease, lane 9) as opposed to 24 (mild disease, lane 3) or 48 h (moderate disease, lane 6) of CDE feeding (Fig. 5B). In addition, culturing splenocytes from severely ill (lane 8) but not moderately (lane 5) or mildly (lane 2) ill mice with vehicle alone can induce HO-1 (Fig. 5B). Thus splenocyte HO-1 inducibility increases with the severity of experimental pancreatitis. As expected, very little change is observed in HO-2, a heme oxygenase isoenzyme that is constitutively expressed.

**DISCUSSION**

We show here that HO-1 levels are significantly lower in circulating monocytes and neutrophils of VCs and neutrophils, B cells, and T cells of HC patients compared with AP patients at the time of admission. These findings are consistent with the fact that HO-1, also known as heat shock protein 32, is induced during oxidative stress (3). Although our AP population was comprised of mildly ill patients, significant upregulation of HO-1 was detected, which is consistent with the findings of Takaki and coworkers (30) of increased MFI monocyte HO-1 levels in patients with septic shock compared with healthy volunteers and less ill patients. However, unlike the Takaki et al. study, we used HO-1 MFI normalized to Spherobeads so we can more accurately compare different patient samples as well as differences over time (8). The findings by Takaki and coworkers as well as others using various models of sepsis support the conclusion that HO-1 upregulation is associated with less severe damage and a better outcome (12, 30, 31).

![Fig. 3. Panhematin (PH)-mediated HO-1 induction in AP patients, HC patients, and VC PBMCs. A: PBMCs from AP patient blood samples obtained on hospital admission day 1 and day 3 were isolated by density centrifugation. PBMCs were cultured for 16, 24, or 48 h with PH or vehicle (VE) alone. B: PBMCs were isolated from VC healthy individuals and HC patients and cultured for 24 h as in A. For both A and B, cultured PBMCs were lysed in a homogenization buffer as described in MATERIALS AND METHODS, and 10 μg total protein from each sample was separated by SDS-PAGE for Western blotting to compare HO-1 levels. Duplicate gels were also stained with Coomassie blue to confirm equal protein loading. Bar graphs show HO-1 density quantification normalized to protein loaded. *P < 0.05 compared with VE.](http://ajpgi.physiology.org/doi/10.220.33.3)
Monocyte HO-1 levels were highest at the onset of pancreatitis (defined by hospital admission) and decreased significantly with clinical improvement, since all of the AP patients were recovering by day 3. Although only one of the patients had ERCP-induced pancreatitis, the observation that this patient had the highest level of HO-1 (and not lipase), and an acute presentation supports the hypothesis that HO-1 levels are likely to be highest early on during the disease course. We did not assess HC patient HO-1 levels at the later date. A limitation of our study is that the HC patient population comprised a heterogeneous group with different nonpancreatic disease diagnoses and different degrees of disease severity. However, in contrast to AP patient monocytes, VC monocyte HO-1 levels remained constant and AP patient neutrophil, T and B cell HO-1 levels also did not change. We are not able to discern the exact time of pancreatitis onset prior to the time of admission to the hospital since the majority of our patients had gallstones with nonspecific pain from a few days to several weeks in duration prior to admission. However, our results suggest that early HO-1 upregulation in leukocytes and particularly in monocytes may help limit or dampen the inflammatory response associated with pancreatic injury. Given the animal data showing that HO-1<sup>+</sup> monocytes are rapidly recruited to the pancreas and confer protection against experimental pancreatitis (21), high-HO-1-expressing monocytes may be able to access the inflamed pancreas and could contribute to resolution of disease and patient recovery. However, this latter hypothesis and whether there is a cause and an effect between HO-1 upregulation and recovery from AP remain to be proven in humans. Presently, we do not know whether monocyte HO-1 levels remain the same, increase, or decrease in patients who fail to improve since all our AP patients recovered. However, rodent studies show that splenocyte HO-1 levels in response to exogenous PH continue to increase as the severity of pancreatitis increases (e.g., Fig. 5B).

Aside from the beneficial effects of upregulating HO-1 and/or carbon monoxide in animal models, intensive-care-admitted septic survivors are found to have higher arterial blood carbon monoxide levels as opposed to septic nonsurvivors (30). Thus, one postulate is that a relatively higher degree of HO-1 inducibility is associated with resolution of AP. As such, we tested whether PBMCs could respond to exogenous PH, an approved drug that induces HO-1, and whether differences in induction exist between the three cohorts we studied. Findings herein showed that PBMCs from AP patients respond more readily to PH-mediated HO-1 upregulation as opposed to
HC and VC PBMCs, thereby suggesting that leukocytes from AP patients are primed for HO-1 induction and may respond readily to treatment with agents such as PH.

Analysis of leukocyte subsets in cultured PBMCs showed that monocytes followed by B cells had higher HO-1 induction in response to exogenous PH. Unlike the role of HO-1 in monocytes, HO-1's role in B cells is not well studied, and the significance of HO-1 induction in B cells here is not clear. We did not observe significant HO-1 induction in T cells, which suggests that T cells are less likely to play a role. Alternatively, a subset of T cells such as HO-1+ regulatory T cells (Tregs), which have been shown to suppress airway inflammation (35), may have been missed in our study given their low number and the use of the pan-T cell marker, CD3. However, a more recent study showed that HO-1 is neither required for mouse Treg development or function (37). Our results are also consistent with a previous study that showed significant HO-1 induction in cultured monocytes but not lymphocytes in acutely ill children compared with healthy controls (36).

Overexpression of HO-1 by macrophages (by adenovirus-mediated transfer) leads to increased macrophage-derived IL-10 and attenuated lung inflammation (11) whereas macrophages from HO-1-deficient mice were shown to release increased amounts of proinflammatory cytokines upon stimulation (13). Thus, AP patients are likely to have therapeutic benefit from PH-mediated monocyte induction of HO-1.

The studies herein were performed in patients with mild self-limited disease to show proof of principle that HO-1 is upregulated in relatively mild, acute, self-limited pancreatitis, resolves with recovery, and could be induced further by PH in monocytes from these patients. This adds support to the potential of testing PH therapy in patients with more severe AP, which might be performed in future clinical studies.

Although the AP group we studied had mild and self-limited disease that may not require nor benefit from hemin therapy, an important finding herein is the differential responsiveness to PH depending on the stage of illness, since PBMCs from acutely ill patients (day 1) had more enhanced HO-1 induction...
than PBMCs from recovering patients (day 3). Additional studies in more severe AP patients will be required, but the findings in experimental pancreatitis covering the range of mild to severe involvement are supportive since, splenocytes from mice experiencing severe AP had higher responsiveness to PH-mediated HO-1 induction compared with those with less severe disease. These findings suggest that leukocytes from acute or severely ill AP patients have the capacity to respond to pharmacological induction of HO-1, which may render cell-based therapy a viable option. In support of this, we previously showed that transfer of in vivo hemin-primed cells ameliorates the disease in mice with pancreatitis (21). Our study does not explain why monocytes are differentially affected. However, the animal data (21) indicates that monocytes during the early phase of disease are the primary cells that migrate to the pancreas and upregulate their HO-1 levels either before or upon migration.

In conclusion, our data suggest that differential HO-1 upregulation in leukocyte subpopulations is observed in patients admitted with AP. Further upregulation of HO-1 could be induced by ex vivo treatment with PH. As patients recovered from AP, HO-1 expression in monocytes decreased as well as PBMC responsiveness to exogenous PH. PBMCs from acutely ill AP patients, as well as splenocytes from mice with severe pancreatitis, had enhanced PH-mediated HO-1 induction compared with the corresponding cells from recovering patients and mice with less severe pancreatitis, respectively, indicating that PH may offer a therapeutic benefit in patients with AP. The potential effectiveness of PH, as shown in mouse models of pancreatitis (21), awaits testing in clinical trials.

GRANTS
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DISCLOSURES
The supplier of Panhematin (Ovation Pharmaceuticals) provided support for this study and has a pending patent application for the possible use of Panhematin in human pancreatitis.

REFERENCES


