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Panhematin provides a therapeutic benefit in experimental pancreatitis

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Abstract

Background and aim—Acute pancreatitis (AP) can result in pancreatic necrosis and inflammation, with subsequent multi-organ failure. AP is associated with increased neutrophil recruitment and rise in pro-inflammatory cytokines such as TNF α . Pretreatment with hemin, results in recruitment of hemoxygenase-1 (HO-1)⁺ macrophages and protects from experimental pancreatitis. It is not clear whether modulation of HO-1 after onset of disease has a protective role. In this study, we tested the utility of Panhematin, a water-soluble hemin formulation, in activating and inducing pancreatic HO-1, and as a therapeutic agent in treating mouse acute pancreatitis.

Methods—We defined the distribution of radiolabeled hemin, then used *in-vivo* HO-1-luciferase bioluminescence imaging and CO₂-release-assay to test Panhematin-induced upregulation of HO-1 transcription and activity, respectively. Using two well-defined AP murine models, we tested the therapeutic benefit of Panhematin, and quantified cytokine release using a luminex assay.

Results—Intravenously-administered Panhematin induces rapid recruitment of HO-1⁺ cells to the pancreas within 2h and *de novo* splenic HO-1 transcription by 12h. Despite high baseline spleen HO-1 activity, the pancreas is particularly responsive to Panhematin-mediated HO-1 induction. Panhematin-treated mice, at various time points after AP induction had significant reduction in mortality, pancreatic injury, together with up-regulation of HO-1 and down-regulation of pro-inflammatory cytokines and CXCL1, a potent neutrophil chemoattractant.

Conclusions—Despite AP-associated mortality and morbidity, no effective treatment other than supportive care exists. We demonstrate that Panhematin leads to: i) rapid induction and activation of pancreatic HO-1 with recruitment of HO-1⁺ cells to the pancreas, ii) amelioration of AP even

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when given late during the course of disease, and iii) a decrease in leukocyte infiltration and pro-inflammatory cytokines including CXCL1. The utility of Panhematin at modest doses as a therapeutic in experimental pancreatitis, coupled with its current use and safety in humans, raises the potential of its applicability to human pancreatitis.

Keywords

Acute pancreatitis; heme-oxygenase 1; CXCL1

INTRODUCTION

Acute pancreatitis (AP) accounts for over 220,000 hospital admission every year in the United States alone, with approximately 20% of these patients suffering a severe disease course and can be associated with significant morbidity and mortality. [1, 2] AP is thought to develop as a result of an injury to the pancreatic acini, leakage and activation of pancreatic enzymes within the tissue, and the subsequent initiation of autodigestion and pancreatic injury. [3–5] The most feared complication of AP and the local inflammatory response are systemic and multiple organ failure, which are associated with high mortality. [2, 6] Despite the burden of disease, aside from supportive management, no effective therapy exists for treating AP. [7] Thus, a search for new treatment modalities for AP remains an important goal in the field.

Hemeoxygenase-1 (HO-1) is a stress inducible enzyme, while its related isoform HO-2 is constitutively expressed. [8] HOs are crucial rate-limiting enzymes that cleave heme into its iron, carbon monoxide (CO), and biliverdin. [9, 10] We previously showed that pre-treatment with hemin, a hemoglobin prosthetic moiety that upregulates HO-1, protects mice from acute experimentally induced-pancreatitis using two independent pancreatic injury models. [11] The protective role of hemin is mediated via HO-1⁺ F4/80⁺ cells that are recruited to the pancreas. [11] Given the utility of hemin as a prophylactic agent for pancreatitis development, we hypothesized that hemin may offer a therapeutic benefit in experimental pancreatitis. Hemin is soluble in alkaline pH, and therefore would have to be given intraperitoneally (i.p.) in mice already experiencing significant peritoneal inflammation. Given the limitation of hemin administration via the i.p. route, the use of Panhematin (PH), a water soluble intravenous (i.v.) formulation of hemin, is a clinically attractive possibility, although protocols for reconstitution of hematin for i.v. administration are available. [12] Notably, PH is an FDA approved drug for the treatment of acute intermittent porphyria. [13, 14]

In the study herein, we show that PH is an effective therapeutic in two experimental models of acute pancreatitis. There is early upregulation of HO-1 and recruitment of HO-1⁺ cells to the pancreas following PH administration. Although hemin administration does not result in its preferential accumulation in the pancreas, there is an increase in HO-1 protein expression and significant upregulation in HO-1 activity in the pancreas after PH therapy. Consistent with the anti-inflammatory effects of PH, there is a decrease in pancreatic and serum TNF α , IL-12p40, and IL-6. Neutrophils and their recruitment to the inflamed pancreas have been shown to play a central role in the pathogenesis of AP. [15] We show here that PH treatment leads to a decrease in pancreatic leukocyte infiltration and CXCL1, a potent neutrophil chemoattractant, thereby providing a potential therapeutic mechanism for PH against pancreatitis.

MATERIALS AND METHODS

Mice treatment and induction of experimental pancreatitis

FVB/n were purchased from Taconic. Young female mice (15–19 g) were fasted for 12 h and then fed a choline-deficient diet (CDD; Harlan Teklad) supplemented with 0.5% DL-ethionine (Sigma-Aldrich) or normal chow (control group) for 3 days. In addition, an L-arginine model of pancreatitis was carried out using two intraperitoneal doses of L-arginine (given at time 0 and 1 h) in C56BL/6 mice (Taconic) as described previously.[16] Mice received PH (Ovation Pharmaceuticals) dissolved in sterile water (reported as equivalent to hemin dose, as described on the drug nomogram, per mouse weight) i.v. at various times (Supplementary Table 1). PH was given 24 h following the second dose of L-arginine. Six to eight week-old HO-1-*luc* transgenic mice, whose transgene contains the full-length (15-kb) HO-1 promoter fused to the reporter gene luciferase (*luc*) were used for *in vivo* bioluminescence imaging of HO-1 transcriptional activity. [17] All animal protocols were approved by the Institutional Animal Care and Use Committees.

Western blotting

Mouse pancreata were isolated and frozen immediately in liquid nitrogen. Frozen pancreata were homogenized using a Dounce and 3% SDS/5 mM EDTA/0.187 M Tris-HCl (pH 6.8). Protein concentrations were determined by the Bicinchoninic acid (BCA) Protein Assay. Equal amounts of protein were separated on SDS-PAGE followed by electrophoretic transfer onto Polyvinylidene fluoride (PVDF) membranes. Anti-HO-1 and anti-HO-2 polyclonal antibodies used were purchased from Assay Designs, Inc.

Immunofluorescence and histology

Mouse pancreata were frozen in Tissue-Tek OCT compound and sectioned using a cryostat. Slides were acetone-fixed and incubated with antibodies to HO-1 (Assay Designs) to visualize HO-1⁺ cells. To assess pancreatic injury, mouse pancreata were fixed in 10% neutral buffered formalin (pH 6.8–7.2). Pancreata were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The histologic score and grading were based on edema, inflammation, hemorrhage, and parenchymal necrosis as described previously. [11] To assess leukocyte infiltration, paraffin embedded sections were stained with biotin-labeled anti-mouse CD45 antibody (BD Biosciences) followed by streptavidin-HRP conjugate and DAB chromogen solution (R&D systems).

Biochemical analysis

Blood was collected from mice by cardiac puncture, and serum was isolated from these samples for subsequent lipase and blood urea nitrogen (BUN) level determination using Beckton Dickinson Microtainer Serum Separator Tubes. Pancreatic trypsin activity was determined as described previously. [11].

Bacterial culture

Blood, pancreatic and liver tissues were collected under sterile techniques and submitted for bacterial growth analysis to institutional microbiology lab. Pancreas and liver were first homogenized in sterile saline.

⁵⁵Fe-hemin and tissue homogenization

The indicated amounts of ⁵⁵Fe-hemin (RI consultants) were injected i.v. into mice. Mice were sacrificed at various times, and brain, heart, kidney, pancreas, small intestines, stomach, colon, liver, and spleen were isolated, weighed, and homogenized as described for

immunoblotting. Scintillation counts of homogenized tissue were obtained using a Beckman Coulter.

Tissue sectioning and autoradiography

Whole mice were embedded in Tissue-Tek OCT compound and sectioned using a Leica CM3600 Macrotome. Sections were placed on Kodak Biomax XAR film and exposed for 1 month in order to visualize ⁵⁵Fe-hemin tissue distribution.

HO-1 promoter activity using *in vivo* bioluminescence imaging

HO-1-*luc* transgenic mice with an FVB/n background received 1 dose of PH on day 0. [11, 17] Whole body imaging of *luc* expression (hence HO-1 promoter activity) of the PH- or VE-treated HO-1-*luc* mice were performed at 0, 2, 4, 6, 8, 12, 18, 24 and 30 h post-PH treatment. Luciferin (100 μ L, 30 mg/mL) per 20 g of mouse weight (final 150 mg/kg body weight) was administered i.p. to each mouse 10 min prior to imaging. Mice were then placed in an In Vivo Imaging System (IVIS) and the photons/sec emitted from the tissues were quantified using LivingImage software v3.2 (Caliper Life Sciences, Alameda, CA).

HO activity

Tissues were harvested and prepared as follows: 100 $\pm$ 20 mg of tissue was placed in a 1.5 mL microfuge tube and 9 volumes of phosphate buffer was added, diced, and immediately sonicated at 0°C using an ultrasonic cell disruptor with an ice-cold 3-mm microprobe yielding a concentration of 2 mg fresh tissue weight per 20 μ L of sonicate. Tissue HO activity was determined through measurement of CO as previously described. [18] Briefly, 20 μ L of tissue sonicate was incubated at 37°C for 15 min in CO-free septum-sealed 2-mL vials, containing 20 μ L of methemalbumin (150 μ mol heme/L:15 μ mol BSA/L) and 20 μ L of NADPH (4.5 mmol/L). Blank reaction vials contained 20 μ L of phosphate buffer in place of NADPH. The reaction was stopped by the addition of 5 μ L of 15% sulfosalicylic acid. [18] CO produced and released into the vial headspace was then quantified by gas chromatography (GC). HO activity was calculated as pmol CO generated/h/mg wet weight.

Luminex assay

The Luminex assay was performed as recommended by the manufacturer (Panomics/Affymetrix). Assays were performed in duplicate using the Luminex 200 IS System (Luminex Corporation). Individual cytokines and chemokines were identified and classified by the red laser, and levels were quantified using the green laser. Digital images of the bead array were captured following laser excitation and were processed on a computer workstation.

Standard curves and reports of unknown samples were prepared using BeadView and MiraiBio software.

In situ hybridization

In situ hybridization of pancreatic sections was performed as described. [19] In brief, digoxigenin-labeled sense and anti-sense RNA probes were generated by polymerase chain reaction amplification with the T7 promoter incorporated into the primers. The primers used for CXCL1 were: 5'-ATGATCCCAGCCACCCGCTCGCTT-3' (sense) and 5'-CCGTTACTTGGGGACACCTTTTAGCATC-3' (antisense). *In vitro* transcription was performed with a digoxigenin RNA-labeling kit and T7 polymerase (Roche Diagnostics, Indianapolis, IN). Pancreatic sections from paraffin blocks were used for hybridizing with either sense or anti-sense as described. [19] Sections developed with diaminobenzidine (GenPoint Kit, DAKO) and counterstained in hematoxylin are shown.

Statistical analysis

Student's *t*-test was used to assess statistical significance and a P value of less than 0.05 was considered significant. Values are expressed as mean $\pm$ SEM. Unless indicated, results are from at least 3 independent experiments. Survival curve comparison was based on the Log-rank (Mantel-Cox) test.

RESULTS

Systemic treatment with PH results in early upregulation of HO-1 and recruitment of HO-1⁺ cells to the pancreas

We previously showed that multiple i.p. injections of hemin during a one-week period upregulate HO-1 significantly by days 4 and 5 of starting the injections. [11] Here, we examined whether HO-1 expression can be upregulated with a single i.p. or i.v. dose of PH to ascertain whether PH can be utilized for AP therapy. Interestingly, we found that HO-1 expression was upregulated as early as 2 h following a single-dose administration of PH (Figure 1A). Similarly, immunofluorescence staining revealed increasing number of HO-1⁺ cells at 4 h post-injection, thereby suggesting rapid recruitment of HO-1⁺ cells into the pancreas regardless of the route of PH administration (Figure 1B).

⁵⁵Fe-hemin concentrates preferentially in the liver and spleen

Previous studies using i.v. ⁵⁵Fe-hematin in rhesus monkeys showed a half-life in plasma ranging from 2.2 to 4.8 h. [20] The majority of the injected radioactivity was observed in the liver (48.2%) 21 h after ⁵⁵Fe-hematin injection, but hematin distribution to the pancreas was not examined. [20] Thus, based on the beneficial effects of hemin in preventing pancreatitis and the early upregulation of HO-1 protein expression (Figure 1A) following a single dose of PH, we investigated the distribution of ⁵⁵Fe-hemin in homogenized pancreata and other tissues 4 and 24 h after i.v. injection. [11] Consistent with previous studies, liver, spleen, and colon (in decreasing amounts) had the highest accumulation of radioactivity relative to other tissues at 24 h (Figure 2A). ⁵⁵Fe-hemin accumulation in the pancreas was limited as compared with the three major organs of radiolabeled hemin deposition. Similarly, whole mouse section autoradiography demonstrated high activities in the liver and spleen (Figure 2B).

PH given 8 and 24 h following CDD initiation, or after L-arginine administration, attenuates pancreatic injury

Pre-treatment with hemin protects against mild to moderate (caerulein model) and severe (CDD model) forms of experimental pancreatitis. [11] In order to evaluate whether PH can treat early or late pancreatitis, we used the CDD-induced pancreatitis model, where mice are fed CDD for 3 days thereby resulting in severe hemorrhagic pancreatitis with peak injury on day 3. Lipase levels in CDD-induced pancreatitis correlate well with pancreatic injury as assessed by histologic scores. [11] We determined the expression of HO-1 and extent of pancreatic injury during CDD feeding over time. After 8 and 24 h of CDD feeding alone, there was no notable HO-1 up-regulation (Figure 3A; lanes 1,2,7,8). A significant increase in serum lipase was observed at 24 h, but not after 8 h (Figure 3B).

We then assessed pancreatic HO-1 expression after 72 h of CDD feeding in mice treated with either vehicle (VE) or PH at 8 or 24 h after initiating the CDD. Notably, a single dose of PH given either at 8 or 24 h following initiation of CDD induced and maintained HO-1 upregulation at 72 h (Figure 3A; lanes 5,6,11,12). This led us to test whether this dosing regimen after feeding with CDD offered a therapeutic benefit. We found that a single dose of PH, equivalent to a hemin dose of 25 μ g/g mouse weight, which previously conferred protection against AP, [11] dramatically suppressed serum lipase elevation (Figure 3C) and

pancreatic injury as determined after scoring of the damage using defined histological criteria (Figure 3D & E) and trypsin activation (Figure 3F) at 72 h. In addition, mice receiving PH at 8 and 24 h had better survival at 72 h (Figure 3G). Importantly, PH was also similarly effective in treating L-arginine induced pancreatitis (Figure 4). Blood, pancreas and liver homogenate cultures did not yield any bacterial growth in either 24 h VE or PH treated mice euthanized at 72 h following initiation of CDD (data not shown).

Late PH treatment during the course of AP requires dose adjustment

Given the therapeutic effects of administering PH 8 and 24 h after CDD feeding, we investigated whether PH can reverse or treat late AP. As expected, there was further rise in serum lipase at 36 and 48 h following CDD initiation (Figure 5A). However, PH at the dose of hemin (equivalent to 25 $\mu\text{g/g}$ weight) given at 36 and 48 h did not protect against AP (Figure 5B). The difference in the protective effect of PH at 36 and 48 h versus 8 and 24 h prompted us to examine whether the dose of PH administered caused potential toxic effects similar to that of high dose hemin, [21] particularly in severely ill mice during late phase pancreatitis. Consistent with this notion, PH treated mice have higher serum blood urea nitrogen (BUN) levels (Supplementary Figure 1). Thus, we examined upregulation of pancreatic HO-1 at lower doses of PH to determine if we could mitigate potential PH toxicity. Dose response studies showed that administration of PH at 6.2 $\mu\text{g/g}$ of mouse weight afforded significant HO-1 overexpression in the pancreas (Figure 6A). Based on this finding and the recommended upper limit dosage in humans [equivalent to 6 mg/kg body weight over 24 h; [14]], we used 6 $\mu\text{g/g}$ weight dosing to test PH effectiveness in late AP. Notably, this lower dose of PH was effective in treating AP when given at 48 h, but not earlier at the 24 h time point (Figure 6B). For comparison, serum lipases from controls (no CDD) and mice fed CDD only for the first 24 or 48 h are shown (Figure 6C).

Effect of PH on HO-1 activity as assessed by *in vivo* bioluminescence imaging and CO release

In order to address the mechanism of PH action, we studied whether there is *de novo* transcription of HO-1 and whether the induction of HO-1 by PH correlates with HO activity by measuring HO-1 promoter activity using HO-1-*luc* transgenic mice (*Luc*-HO-1+/+) and via release of CO from homogenized tissues, respectively. Mice were imaged 0, 2, 4, 6, 8, 12, 18, 24, and 30 h following a single dose of either VE or PH. Significant bioluminescence was observed at 12 h on the left dorsal and lateral areas in mice treated with PH (Figure 7A), likely corresponding to the spleen. Based on this finding, we then treated normal FVB/n mice (similar to those used in induction of AP) with either VE or PH, and assayed HO activity in lung, liver, spleen, and pancreas sonicates after 12 and 24 h. There was significant increase in HO activity in the liver, spleen, and pancreas, but not lung of PH treated mice at both 12 (not shown) and 24 h (Figure 7B). Interestingly, a higher fold increase in HO activity was observed in the pancreas as compared to the spleen following PH treatment (grey bars; Figure 7B).

PH suppresses pancreatic pro-inflammatory cytokines and a potent neutrophil chemoattractant

Activated leukocytes during the early phase of pancreatitis release cytokines, which in turn mediate and enhance the inflammatory cascade observed in AP. Particularly, TNF α and IL-1 are among the most prominent and well-studied in AP. [6, 22, 23] We performed Luminex assays to determine if PH treatment affects cytokine levels in the pancreas. Although no difference in IL-1 pancreatic levels was noted between 24 h VE or PH treated mice fed with CDD for 60 h, there was a significant decrease in TNF α in PH treated mice (Figure 8A). Nevertheless, we could detect significant decrease in IL-1 β in serum of PH

treated mice (Supplementary Figure 2). In addition, there were significant decreases in pancreatic IL-12p40 and IL-6 in the PH treated mice.

We previously showed that single dose of hemin given i.p., in non-CDD fed mice, increased pancreatic CCL2 (MCP-1) and CCL3 (MIP-1 α) but not CCL5 (RANTES) mRNA 24 h later. [11] Herein, we did not detect significant differences in pancreatic protein levels of CCL3 or CCL5 after 60 h CDD feeding in mice treated with either VE or PH at 24 h during the feeding (Figure 8B). We selected to harvest the pancreas at 60 h and not 72 h, since all the mice would be alive at 60 h. Based on the fact that CXCL1 is a potent neutrophil attractant and neutrophils play an important role in pancreatic injury during AP, [24] we tested whether PH can modulate pancreatic CXCL1. Consistent with the findings that PH can treat experimental pancreatitis, CXCL1 was significantly decreased in mice treated with PH (Figure 8B).

In order to determine the cellular source for the increased CXCL1, we performed in situ hybridization with CXCL1 sense (Figure 8C, d) and anti-sense (Figure 8C, a–c) probes. Relative to VE-treated mice, pancreata from PH-treated mice had significantly reduced CXCL1 mRNA levels (Figure 8C, b versus c). In VE-treated mice, the CXCL1 mRNA was found primarily proximal to vascular areas (Figure 8, b). Consistent with the reduction in pancreatic CXCL1, pancreata from PH-treated mice had decreased leukocyte infiltration as demonstrated by a decrease in CD45+ cells (Figure 8D; c and d versus a and b).

DISCUSSION

AP remains a challenging clinical problem, particularly in patients with severe disease. Despite a handful of clinical trials with pharmacologic agents, no effective treatment exists for AP. We previously demonstrated that pre-treatment, as a prophylactic, with hemin or hemin-activated cells protects against experimentally-induced pancreatic injury. [11] In the study herein, the beneficial effect of hemin prophylaxis has been extended to therapeutic intervention using the hemin formulation PH in mice undergoing two different models of pancreatitis. Importantly, even at a later time during AP, PH was effective in minimizing the observed pancreatic injury. This finding is likely related to the observation that HO-1 in the pancreas is quickly upregulated and maintained following a single PH dose. Clinically such potency may be critical since patients with AP are a heterogeneous group, presenting to the hospital at different time during the course of the disease.

The limited accumulation of ⁵⁵Fe-hemin in the pancreas suggests that the pancreas is not a major site for hemin uptake. This is consistent with our previous finding that HO-1⁺ F4/80⁺ cells are recruited to the pancreas from elsewhere and that these recruited cells provide the source for pancreatic HO-1 and is responsible for the observed protection. Potentially Panhematin may also affect other organs such as the liver and spleen monocyte/macrophages and suppress systemic inflammatory response. Furthermore, PH not only upregulates HO-1 at the protein level but also functionally as we found significant increases in HO activity in the pancreas. The beneficial effects of HO-1 induction may be mediated via its byproducts, CO and biliverdin/bilirubin, since both have been shown to have anti-inflammatory and anti-oxidant properties. [25–28] In support of our findings, the CO-releasing molecule-2 (CORM-2) demonstrated a protective effect in a rat model of pancreatitis. [29] Potential therapeutic limitations of such compounds lie in their instability and insolubility in aqueous solutions. In addition, our study demonstrated the efficacy of PH against AP at various time points whereas CORM-2 efficacy was demonstrated only at one time point immediately after AP induction. The relative ease of PH administration, its potency in inducing HO-1 expression in the pancreas, and its efficacy using two independent

models of AP at different stages of the disease renders it an attractive potential candidate therapeutic agent for AP.

PH treatment resulted in significant reduction in TNF α , IL-6, and IL-12p40 levels in pancreatic tissue. Serum levels of TNF α are increased in patients with AP and mouse models of the disease, and increased levels are correlated with disease severity.[29–31] Blockade of TNF α in experimental mouse models ameliorates AP, indicating the importance of this cytokine in disease pathogenesis.[32] The reduction of TNF α levels in the pancreas may be one of the mechanisms by which PH improves AP. IL-12 in conjunction with IL-18 has been shown to induce necrotizing AP in obese *ob/ob* mice.[33] In humans, IL-12p40 is consistently increased during the course of AP; whereas, IL-12p70 was only increased on day 1, but significantly decreased on the second to fourth day of illness. [34] Our findings are supportive of these results and show that, 60 h post-CDD-feeding, IL-12p40 are much higher as compared to IL-12p70. In addition, PH treatment leads to a decrease in IL-12p40 and an increase in IL-12p70, although the latter was not statistically significant. Increased serum IL-6 levels are also observed in AP patients and in experimental mouse models. [35] Although IL-6 does not appear to play an important role in initiating or mediating the inflammatory cascade in AP, it is considered a useful predictor of disease severity. [35] In support of PH's therapeutic benefit in experimental AP, we find that IL-6 is significantly lower in PH-treated animals as opposed to their VE-treated controls.

IL-1 blockade, via its receptor or via IL-1 converting enzyme (ICE), [36–40] attenuates experimental pancreatitis. PH here did not alter pancreatic IL-1 levels 60 h after the initiation of CDD feeding, suggesting that an IL-1 independent mechanism may account for PH's therapeutic action. However, our results do not rule out a role for IL-1 down-modulation in PH-mediated protection against AP since the effect of PH on IL-1 levels may occur early during the course of pancreatitis. This latter hypothesis is supported by the fact that at 60 h we could detect significant IL-1 β reduction in the serum (Supplementary Figure 2) and IL- β levels in the pancreas are much higher early during CDD feeding (e.g. at 24 h as opposed to 48 h or 60 h, data not shown). For example, CORM-2 suppresses IL-1 β levels 12 h after induction of AP. [29] Similarly and in the same study, levels of the anti-inflammatory cytokine, IL-10, increased 12 h after induction of AP. We did not observe significant changes in pancreatic IL-10 levels 60 h after CDD diet feeding in response to PH treatment. Again, this suggests that PH-mediated protection does not depend on IL-10 solely or, alternatively, that the effect of PH on IL-10 levels occurs at an earlier time point.

In addition to the above-mentioned cytokines, our findings show that the neutrophil chemoattractant, CXCL1, is reduced in PH-treated mice. CXCL1, a potent neutrophil chemoattractant similar to IL-8 in humans, is expressed in fibroblasts, macrophages and endothelial cells particularly in response to inflammatory stimuli. [41, 42] Furthermore, marked reduction in severity of pancreatitis is observed with depletion of neutrophils. [24, 43] The fact that PH suppresses local pancreatic CXCL1 expression supports the notion that PH ameliorates AP by mitigating neutrophil recruitment into the pancreas through reduction in CXCL1 levels. This conclusion is further supported by the reduced leukocyte (CD45+ cells) infiltration that was observed following PH treatment.

One important aspect of our findings is the dosing of PH. For example, higher and lower doses of PH were effective if given earlier and later during the course of AP, respectively. Over time during the CDD feeding, mice become more dehydrated, as shown by the increase in blood urea nitrogen (BUN) levels (Supplementary Figure 1). Treatment with higher doses of PH likely compromises renal function further, since larger doses of PH are known to cause reversible renal failure. [21] In patients with porphyria, PH is given daily for 3 to 14 days based on the resolution of clinical symptoms and appears to be well tolerated.

However, if our findings are translated to patients with AP, care will need to be taken with the dosing. Collectively, our findings show that the use of PH offers a novel potential therapeutic strategy for AP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of non-standard Abbreviations

AP	acute pancreatitis
CDD	choline-deficient diet
HO-1	hemeoxygenase-1
PH	Panhematin
VE	vehicle

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SUMMARY BOX

What is already known about this subject

- Animal studies in acute pancreatitis (AP) show that HO-1 upregulation by hemin, prior to induction of pancreatitis, prevents pancreatic injury.

What are the new findings

- Panhematin, a water-soluble formulation of hemin that is used to treat porphyrias, induces rapid upregulation of pancreatic HO-1 within 2 h of dosing.
- Panhematin offers therapeutic benefit not only when given early during AP course but also when given late during the course of experimental pancreatitis.
- The therapeutic benefit of Panhematin is associated with reduction in pro-inflammatory cytokines and in a potent neutrophil chemoattractant (CXCL1). Neutrophils play a central role in disease pathogenesis and depletion of neutrophils is already known to reduce AP in experimental models.

How might it impact on clinical practice in the foreseeable future?

Despite significant morbidity and mortality associated with AP, no effective treatments exist aside from supportive care such as fluid resuscitation. The therapeutic benefit of Panhematin in experimental pancreatitis raises the hypothesis that pharmacologic administration of Panhematin may ameliorate disease severity in patients with AP.

Figure 1.

Induction of HO-1 by Panhematin (PH). (A) Western blots were performed on pancreatic lysates from mice at time 0, 2 or 4 hours (h), after receiving intraperitoneal (IP) or intravenous (IV) PH. Note, PH upregulates HO-1 (upper band, arrow) in the pancreas as early as 2 h whereas HO-2 remains unchanged. (B) Frozen pancreas sections at 2 h and 4 h following treatment with PH were stained anti-HO-1 (red) antibody. Note, PH causes influx of HO-1⁺ cells into the pancreas.

A.

B.

Figure 2.

⁵⁵Fe-hemin tissue distribution. (A) FVB/n mice were intravenously injected with 2 microcuries of ⁵⁵Fe-labeled hemin. The hemin dosage was adjusted to 6 μ g/g with cold PH. After 4 and 24 h, mice were euthanized, and the specified organs were isolated. A portion of each organ was homogenized and subjected to scintillation counting. The data is presented as cpm/mg of wet weight tissue. Each value represents an average of three mice. Note that ⁵⁵Fe-hemin does not concentrate in the pancreas. (B) Similarly mice that received 2 microcuries of ⁵⁵Fe-labeled hemin as in (A) were euthanized after 24 h and quickly frozen and embedded in Tissue-Tek OCT compound. Whole body sections (50 μ m) were placed on a piece of tape and developed on Kodak Biomax XAR film. Two individual mouse images (a & c) are shown with their corresponding autoradiograph (b & d, respectively). Liver and spleen (arrows) show high activity.

Figure 3.

Effect of Panhematin (PH) in experimental pancreatitis. (A) Female mice on a FVB/n background were fed a choline deficient diet (CDD) to induce acute pancreatitis. Some mice were fed CDD but did not receive PH or vehicle (VE) and were euthanized at 8 and 24 h respectively. Other mice were fed CDD for 72 h and treated with single i.v. dose of PH or VE at 8 and 24 h respectively following the initiation of CDD. Total pancreas lysates were analyzed by SDS-PAGE and Western blotting for HO-1 and HO-2 proteins. (B) Mice were fed either normal diet (white bar) or CDD for 8 (grey bar) and 24 h (black bar) and then euthanized. Serum was collected for lipase determination. (C) Some mice received either VE (white bar) or PH (black bar) treatment at 8 h following initiation of CDD and other group of mice received either VE (white bar) or PH (black bar) treatment at 24 h following initiation of CDD and were then euthanized at 72 h. Serum was used for determination of lipase levels at 72 h. (D) Mice were fed CDD for 72 h and euthanized. The mice were treated with either vehicle (VE) control (a) or PH (b) at 24 h following the initiation of CDD. Pancreata were isolated and fixed in 10% formalin, and sections were stained with

hematoxylin & eosin. Black and green arrows indicate areas of hemorrhage and necrosis respectively. (E) Histologic scores of mice treated with VE or PH at either 8 h or 24 h after the initiation of CDD are shown. Mice were euthanized at 72 h and pancreas processed as in D and scored. (F) Pancreatic tissues from VE or PH treated mice at 24 h after the initiation of CDD and euthanized at 72 h were used to determine trypsin (pmol/mg protein) enzyme activity. $*p < 0.05$. Results shown are from 4 independent experiments with a total of 6–8 mice per group. (G) Survival curve from 8 and 24 h VE or PH treated mice following the initiation of CDD is shown. Results shown are from 4 independent experiments with total of 22 mice per group. $*p < 0.05$ using Log-rank (Mantel-Cox) test.

Figure 4.

Effect of Panhematin (PH) in L-arginine model of pancreatitis. (A) Mice were either treated with VE or PH at 24 h following last L-arginine dose (time 1 h) and euthanized 72 h after the first dose of L-arginine (time 0) and serum was collected for lipase determination. (B) Bar graph denotes pancreatic histologic scores from the same mice in (A). (C) Representative pancreata isolated from the same mice in (A), fixed in 10% formalin and hematoxylin & eosin stained sections are shown. * $p < 0.05$.

Figure 5.

Late PH treatment during AP. (A) Mice were fed either normal diet (white bar) or CDD for 36 (grey bar) and 48 h (black bar) and then euthanized. Serum was collected for lipase determination. (B) Mice were fed CDD for 72 h and then euthanized. Groups of mice received either VE or PH treatment at 36 h or 48 h following initiation of CDD. Serum was used for determination of lipase levels. * $p < 0.05$. Results shown are from 4 independent experiments with a total of 6–8 mice per group.

Figure 6.

Effect of incremental and lower dose of Panhematin (PH) on pancreas HO-1 induction and treatment of AP respectively. (A) Western blots were performed on pancreatic lysates from mice at 24 h after receiving either no or 1.6, 3.2, 6.2 and 25.0 $\mu\text{g/g}$ intravenous PH. Note, significant HO-1 induction with 6.2 $\mu\text{g/g}$ PH, which is comparable to the upper end of the dose used in humans. (B) Mice were fed CDD for 72 h and received either VE or 6 $\mu\text{g/g}$ PH treatment at 24 h following initiation of CDD. 48 h mice received either VE or 6 $\mu\text{g/g}$ PH treatment at 48 h following initiation of CDD. Serum was used for determination of lipase levels. (C) Mice were fed either normal diet (white bar) or CDD for 24 (grey bar) and 48 h (black bar) and then euthanized. Serum was collected for lipase determination. The levels of serum lipase in these studies were much higher as compared to previous studies due to the use of a different assay. * $p < 0.05$. Results shown are from 4 independent experiments with a total of 6–8 mice per group.

Figure 7.

Effect of Panhematin (PH) on *in vivo* HO-1 tissue distribution. (A) A single dose of either PH or VE was injected i.v. into HO-1 *luc* transgenic (HO-1-Luc) mice. Whole body images of anesthetized mice in dorsal (upper panels) and lateral (lower panels) positions were taken at various time points (shown t = 0, 6, 12, and 24 hours). The signal intensity scale bars are shown on the right. (B) A single dose of PH or VE was injected i.v. into female FVB/n mice. Mice were sacrificed at 24 h, and liver, lung, spleen, and pancreata were sonicated in PBS. Tissue sonicates were incubated at 37°C for 15 min with methemalbumin plus NADPH. CO produced was quantitated by gas chromatography (GC). HO activity was calculated as pmol CO generated/h/mg wet weight. Percent control indicates the percent difference between tissues from VE versus PH treated mice. Groups of 3 mice were used to obtain mean values. *p < 0.05.

B.

Figure 8.

PH suppresses pro-inflammatory cytokines and neutrophil chemoattractant, CXCL1. Pancreata were isolated from FVB/n mice that were fed CDD for 60 h and treated with VE (white bars) or PH (black bars) at the 24 h time point. Pancreatic homogenates were used for determining cytokine levels using preconfigured kits, standards, and Luminex reader for quantitative analysis. (B) Similarly, chemokine levels were determined as in (A). * $p < 0.05$. (C) Pancreata were isolated from FVB/n mice that were (a) untreated or (b–d) fed CDD for 72 h and treated with (b, d) VE or (c) PH at the 24 h time point and embedded in paraffin. Pancreatic sections from paraffin blocks were used for in situ hybridization with digoxigenin-labeled (d) sense or (a–c) anti-sense RNA probes as described in Material and Methods. Sections were developed with diaminobenzidine (GenPoint kit, DAKO) and

counterstained in hematoxylin. Note the perivascular CXCL1 staining in VE treated (b) sections and the reduction in staining in PH treated (c) sections. (D) Pancreata were isolated from FVB/n mice that were fed CDD for 72 h and treated with (a, b) VE or (c, d) PH at the 24 h time point and embedded in paraffin. Pancreatic sections from paraffin blocks were stained with CD45 antibody as described in Material and Methods. Scale bar 50 μ m for (a) and (b), magnified views are shown in (b) and (d). A representative of 3 groups of mice is shown.