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FIRST-IN-HUMAN STUDY DEMONSTRATING PHARMACOLOGICAL ACTIVATION OF HEME OXYGENASE-1 IN HUMANS

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Abstract

Heme-oxygenase 1 (HO-1) degrades heme and protects against oxidative stress, but has not been pharmacologically induced in humans. In this randomized study (10 healthy volunteers), hemin (3 mg/kg. i.v in 25% albumin) increased plasma HO-1 protein concentration by 4–5 fold and HO-1 activity by ~15 fold over baseline at 24 and 48 h (placebo – 56.41 ± 6.31 [baseline], 77.44 ± 10.62 [48h] versus hemin – 71.70 ± 9.20 [baseline], 1192.20 ± 333.30 [48h]) in 4 of 5 subjects compared to albumin ($p \leq 0.03$), thereby overcoming a fundamental challenge to HO-1 research in humans.

Keywords

hemin; heme oxygenase; humans; HO-1

INTRODUCTION

Heme oxygenase (HO) is the rate limiting enzyme in the catabolism of heme, generating equimolar amounts of biliverdin, free iron and carbon monoxide (CO). [1] Of the 2 functional HO isoforms in mammals, HO-2 is a constitutively expressed enzyme with an important role in neuronal function. [1] Normally, expression of HO-1 is low to undetectable except for tissues involved in erythrocyte metabolism (i.e., liver and spleen). However, several substances (e.g., heme, metals, xenobiotics, endocrine factors, and synthetic metalloporphyrins) increase HO-1 expression and activity in many systems, including hemopoietic, hepatic, epithelial, and endothelial cells. When HO-1 is induced, more heme is removed and end products of heme metabolism (i.e., CO, iron and biliverdin) are generated. [1] In addition to inducing inflammation, excess heme causes cytotoxicity by multiple mechanisms (e.g., by perturbing cytoskeletal integrity, impairing cytosolic enzymes, and denaturing DNA). [2] HO-1 is also a

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CONFLICT OF INTEREST/DISCLOSURE

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heat-shock protein. Among the metabolic products of heme, CO exerts vasorelaxant, anti-apoptotic, and anti-inflammatory effects; bile pigments are antioxidant, anti-inflammatory, protect against endothelial damage and reduce atherogenic risk. Together, these data are consistent with the hypothesis that induction of HO-1 is essential for protecting cells from physical, chemical, and biologic stress. HO-1 also modulates cell survival and proliferation. [2–4] Experimental models suggest that induction of HO-1 has beneficial effects in diverse conditions, as detailed elsewhere. [1] For example, induction of HO-1 may prevent atherosclerosis, [5] can mitigate heme-induced renal injury (e.g., due to rhabdomyolysis, cisplatin nephrotoxicity, and nephrotoxic nephritis), [6] decrease myocardial infarct size and incidence of reperfusion arrhythmias after ischemia-reperfusion injury, protect against pancreatic and lung injury in acute pancreatitis, [7] and protect against oxidative stress and endothelial injury in experimental diabetes. [1] Failure to upregulate expression of HO-1 in response to oxidative stress is responsible for the loss of interstitial cells of Cajal and delayed gastric emptying in non-obese diabetic mice. [8] Upregulation of HO-1 can prevent or reverse delayed gastric emptying in this model. [8]

In light of this evidence supporting biological effects of HO-1, the inability to induce HO-1 by pharmacological or genetic approaches in humans poses a fundamental challenge to studies pertaining to HO-1 in humans. [1,9] For some compounds (e.g., aspirin, statins) known to activate HO-1 *in vitro*, it is unknown if drug concentrations required to activate HO-1 can be achieved by pharmacologically relevant doses in humans. Other compounds (e.g., probucol, rapamycin, and heavy metals) have toxic effects limiting their use in humans. [9]

Hemin is the most powerful inducer of HO-1. [10] While intravenous hemin (1–4 mg/kg/day for 3 to 14d) is FDA-approved to treat acute intermittent porphyria, it has also been used to treat thalassemia intermedia, myelodysplastic syndrome, and liver allograft failure in erythropoietic protoporphyria. [11–16] Our hypothesis was that compared to placebo, hemin will increase HO-1 protein expression and activity in humans.

RESULTS

Adverse Effects

Hemin and placebo infusions were well tolerated. Three volunteers (2 placebo and 1 hemin) developed headaches which resolved with acetaminophen; the volunteer randomized to hemin also vomited once. No subjects developed phlebitis. In 2 subjects, the hemin infusion rate declined due to high viscosity. To restore flow, the tubing was transiently disconnected and flushed initially with saline and subsequently with hemin. As a result, these 2 subjects, referred to as “A” and “B”, received 182 of 197mL and 158 of 213mL respectively of the planned infusion volumes.

Effects on HO-1 Protein Concentration and Activity

Compared to placebo, hemin increased HO-1 protein concentration at 24 ($p = 0.008$) and 48 h ($p = 0.008$) but not at 4 or 6 h. (Table 1, Figure 1) Hemin also increased HO-1 activity at 24 ($p = 0.03$) and 48 h ($p = 0.008$) but not venous carboxyhemoglobin concentrations. (Figure 2) In 1 subject (volunteer A), hemin increased HO-1 activity but not protein concentration.. In volunteer B, HO-1 concentrations increased further between 24 and 48h after infusion (baseline 2.1ng/ml, 24h 5.7ng/ml, 48h 7.9ng/ml). HO-1 protein concentrations and activity were correlated at 24 ($r = 0.94$, $p < 0.0001$) and 48 hours ($r = 0.95$, $p < 0.0001$) but not at earlier timepoints.

Effect on Hematological, Biochemical and Coagulation Parameters

Laboratory tests did not reveal any clinically significant changes in hematological or biochemical parameters (data not shown) or coagulation tests (Table 1). Compared to baseline, the only statistically significant changes at 48 hours are as follows: the neutrophil count increased by $706 \pm 200/\text{mm}^3$ (hemin) and decreased by $624 \pm 326/\text{mm}^3$ (placebo) ($p < 0.01$) while creatinine decreased by $0.1 \pm 0.02\text{mg/dL}$ (hemin) and increased by $0.04 \pm 0.02\text{mg/dL}$ (placebo) ($p=0.001$).

DISCUSSION

Although several substances increase expression and/or activation of HO-1 in cell culture systems and other experimental models, this is the first report showing that HO-1 protein concentration and activity can be induced in humans. Since experimental models suggest that increased HO-1 is beneficial in several conditions, these findings are fundamentally important for HO-1 research in humans. [17]

Only hemin prepared in 25% albumin, but not albumin alone, increased plasma HO-1 protein concentrations and activity very substantially in 4 and to a lesser extent in 1 subject, who received less hemin than planned due to delivery problems. Hemin rapidly (i.e., within 3 hours) increases HO-1 protein expression and HO-1 activity. [18–20] Consistent with the effects of heme-albumin on clinical manifestations and porphyrin metabolism in acute porphyria, [16, 21] hemin increased HO-1 protein concentrations and activity by 24 hours after hemin administration. Increased HO-1 protein synthesis and activity were sustained at 48 hours, perhaps partly because hemin is gradually released from albumin, and subsequently bound to hemopexin, a transport protein that has a higher affinity for hemin than albumin and transports hemin to the liver. [22] Hemopexin is saturated and depleted after a single dose of hemin (4 mg/kg). [22] The observed 4–5 fold increase in plasma HO-1 protein concentrations is comparable to the effects of hemin on HO-1 protein concentrations in isolated monocytes and mesenteric arteries. [17,23]

Compared to placebo, hemin did not significantly increase venous carboxyhemoglobin concentrations in this study. This may reflect the lower sensitivity of the assay to identify small changes in CO production.

In summary, this is the first study to demonstrate that HO-1 can be pharmacologically induced in humans. Further studies, in a larger group of subjects, are necessary to ascertain the duration of effects after a single dose of hemin, the optimal dosage and dose frequency, the effects of long term therapy, and the effects of HO-1 promotor polymorphisms on the response to hemin. [24] While chronic treatment with heme has been shown to lead to the continuous expression of HO-1, lower blood pressure, and modestly decrease cellular heme, [23] a careful appraisal of the benefits and risks of hemin therapy is necessary, because free heme can activate intracellular, vascular, and endothelial leukocyte adhesion molecules leading to leukocyte recruitment. [1]

METHODS

Subjects

Ten healthy volunteers, aged 18–50 years (mean age 31 years; 5 women, BMI $24.4 \pm 0.5 \text{ kg/m}^2$) were recruited by public advertisement and provided written consent to participate. Volunteers were lifetime non smokers and were not taking any medications. All multivitamins were discontinued for 1 week before and for the duration of the study. Subjects were screened by a clinical assessment, laboratory tests, and questionnaires to exclude functional gastrointestinal disorders, anxiety, and depression. [25,26] Laboratory tests included a

complete blood count, serum creatinine, aspartate aminotransferase, alanine aminotransferase, total and direct bilirubin, prothrombin time, and activated partial thromboplastin time. Study participants had negative tests for hepatitis B surface antigen, hepatitis C virus antibody, and HIV 1/2 antibodies. This study was approved by the Mayo Clinic Institutional Review Board.

Study Design

After fasting overnight, participants arrived in the research unit at 7:30 AM on the test day. Subjects were randomized to receive hemin or placebo infusions. Hemin (Panhematin®, 1.25 mL/kg, Ovation Pharmaceuticals, Deerfield, IL) was administered through a large-caliber peripheral vein at a rate of 60 mL/hour. While the product label recommends preparation in sterile water, hemin is very unstable and only 50% of the active ingredient remains after 5 minutes. [22] These degradation products adhere to endothelial cells, platelets, and coagulation factors and have been implicated in causing a transient anticoagulant effect and often phlebitis at the site of infusion. Reconstitution with human albumin enhances stability of lyophilized hemin, significantly reduces the incidence of phlebitis, prevents the anticoagulant effect, and enhances efficacy. [21] Therefore, hemin was diluted in ~ 132 mL of 25% albumin to obtain a hemin concentration of 2.4 mg/mL [21] and administered at a rate of 60 mL/hour. This dose is similar (3–4 mg/kg) to that recommended for treating acute porphyria. [16] The same volume of albumin alone was administered for placebo infusions. All infusions were prepared by standard practice in the Mayo Clinical Research Unit Research Pharmacy using current ASHP class III procedures for sterile preparation. Both albumin 25% and hemin/albumin 25% were filtered using a 5 micron disc filter. The bottle and tubing were shrouded with amber light resistant plastic to assist blinding of the patient and study team. Laboratory tests were repeated at 4, 6, 24, and 48 hours after completing hemin infusions.

Measurement of HO-1 Protein Concentration, HO-1 Activity and Venous Carboxyhemoglobin Concentrations

For measuring plasma HO-1 protein concentrations, blood was collected in EDTA containing tubes and centrifuged at 1000g for 20 min at room temperature (RT). HO-1 concentration was determined using a HO-1 (human) enzyme-linked immunosorbent assay (ELISA) kit (Assay Designs Inc. Ann Arbor, MI). One hundred μ L of plasma was incubated in the wells of the immunoassay plate, which was pre-coated with anti-HO-1, at RT for 30 min. After excess protein was decanted and washed with wash buffer, 100 μ L of anti-human HO-1 was added to each well and the plate was incubated at RT for a further 1 h. After washing, horse radish peroxidase conjugated anti-rabbit IgG secondary antibody (100 μ L) was added and the plate was incubated at RT for 30 min. After washing, the assay was developed by adding of 100 μ L of tetramethylbenzidine substrate. The intensity of the color was measured in a microplate reader at 450nm.

HO-1 activity was assessed as follows. [27] The buffy coat of peripheral blood cells was subject to Ficoll gradient centrifugation (400g, 40min, RT). The cells from the interface layer between the plasma fraction and the Ficoll fraction were collected and washed twice in 1x PBS followed by centrifugation at 100g for 10 min. The cells were then lysed in 50mM Tris/HCl pH 8.0, 150mM NaCl, 1% Nonidet P-40 and 1mM phenylmethylsulfonyl fluoride for 1h on ice. The lysate (3000g, 15min, 4°C) and supernatant (12000g, 20min 4°C) were centrifuged. After ultracentrifugation (105000g, 1h, 4°C), the pellet (representing the microsomal fraction) was resuspended in 100 mM potassium phosphate buffer (pH 7.4) containing 2 mM MgCl₂. In a 100 μ L reaction mixture, 23 μ g of microsome protein was incubated with 50 μ M hemin, 0.5mg human liver cytosol, 0.05U glucose-6-phosphate dehydrogenase, 2mM glucose-6-phosphate and 0.8mM NADPH for 1h at 37°C in the dark. The bilirubin produced was extracted with chloroform, and the absorbance of bilirubin at 464nm was measured against a baseline absorbance at 530nm (extinction coefficient, 40mM⁻¹ cm⁻¹ for bilirubin).

Venous carboxyhemoglobin concentrations were analyzed by a blood gas analyzer (Radiometer ABL 735, Copenhagen, Denmark). Arterio-venous carboxyhemoglobin gradients are comparable in health and conditions associated with increased CO production (e.g., ICU patients), indicating that arterial and venous measurements are equally accurate for detecting increased CO production. [28]

Statistical Analysis

Because there were no previous reports of the primary responses, the sample size was based on practical considerations. All subjects were included in this intent to treat analysis.

Non-parametric alternative (Wilcoxon's rank sum test) was used to compare the effects [i.e., the difference (after-before) treatment] of hemin and placebo on HO-1 concentrations, venous carboxyhemoglobin concentrations, and safety parameters (i.e., complete blood count, serum biochemistry, and coagulation tests). Because there were comparisons at 4 time points (4, 6, 24, and 48 h), the level of significance was set at $\alpha = 0.0125$. Data are reported as Mean $\pm$ SEM and with unadjusted (for multiple comparisons) p-values.

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Figure 1.

Effect of hemin and placebo on venous plasma HO-1 protein concentrations plotted for each participant. Hemin increased HO-1 protein concentrations in 4 of 5 volunteers. However, placebo (albumin) did not increase HO-1 in any subjects.

Figure 2.

Effect of hemin and placebo on venous plasma HO-1 activity plotted for each participant. Only, hemin and not placebo substantially increased HO-1 activity, i.e., in the same 4 volunteers in whom hemin increased HO-1 protein. The exception was subject A (see text), in whom venous plasma HO-1 protein concentration increased from 1.8 to 3.0 ng/mL while activity increased from 43 to 129 pmol bilirubin/mg/h.

Table 1

Comparison of Hemin and Placebo Infusions on Heme-Oxygenase-1 and Coagulation Parameters.

	Placebo	Hemin
Venous plasma HO-1 protein concentrations (ng/mL)		
Baseline	2.38 ± 0.26	1.95 ± 0.98
4h	2.43 ± 0.25	1.88 ± 0.07
6h	2.18 ± 0.18	1.88 ± 0.07
24h	2.20 ± 0.15	9.90 ± 3.00*
48h	2.31 ± 0.26	11.16 ± 2.82*
Venous leukocyte HO-1 activity (pmol bilirubin/mg/h)		
Baseline	56.41 ± 6.31	71.70 ± 9.20
4h	83.17 ± 17.60	69.79 ± 8.50
6h	64.05 ± 7.34	80.31 ± 17.33
24h	69.79 ± 13.00	1126.20 ± 293.30 [†]
48h	77.44 ± 10.62	1192.20 ± 333.30*
Venous carboxyhemoglobin concentration (%)		
Baseline	0.66 ± 0.09	0.52 ± 0.10
4h	0.94 ± 0.10	0.96 ± 0.07
6h	0.88 ± 0.10	0.86 ± 0.07
24h	0.74 ± 0.07	0.80 ± 0.15
48h	0.74 ± 0.12	0.60 ± 0.13
Coagulation parameters		
Prothrombin time (s)		
Baseline	9.8 ± 0.1	9.9 ± 0.4
48 hours	10.0 ± 0.1	10.0 ± 0.4
Activated partial thromboplastin time (s)		
Baseline	27.0 ± 1.5	28.6 ± 1.6
48 hours	28.0 ± 1.7	29.6 ± 1.6

Data shown as mean ± SEM

*
p = 0.008[†]
0.03 vs placebo for corresponding differences from baseline, unadjusted for multiple time points compared.