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Research Paper

Heme attenuates beta-endorphin levels in leukocytes of HIV positive individuals with chronic widespread pain

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ABSTRACT

The prevalence of chronic widespread pain (CWP) in people with HIV is high, yet the underlying mechanisms are elusive. Leukocytes synthesize the endogenous opioid, β-endorphin, within their endoplasmic reticulum (ER). When released into plasma, β-endorphin dampens nociception by binding to opioid receptors on sensory neurons. We hypothesized that the heme-dependent redox signaling induces ER stress, which attenuates leukocyte β-endorphins levels/release, thereby increasing pain sensitivity in people with HIV. Results demonstrated that HIV positive individuals with CWP had increased plasma methemoglobin, erythrocytes membrane oxidation, hemolysis, and low plasma heme scavenging enzyme, hemopexin, compared to people with HIV without CWP and HIV-negative individuals with or without pain. In addition, the leukocytes from people with HIV with CWP had attenuated levels of the heme metabolizing enzyme, heme oxygenase-1, which metabolizes free heme to carbon-monoxide and biliverdin. These individuals also had elevated ER stress, and low β-endorphin in leukocytes. *In vitro,* heme exposure or heme oxygenase-1 deletion, decreased β-endorphins in murine monocytes/macrophages. Treating cells with a carbon-monoxide donor or an ER stress inhibitor, increased β-endorphins. To mimic hemolytic effects in a preclinical model, C57BL/6 mice were injected with phenylhydrazine hydrochloride (PHZ). PHZ increased cell-free heme and ER stress, decreased leukocyte β-endorphin levels and hindpaw mechanical sensitivity thresholds. Treatment of PHZ-injected mice with hemopexin blocked these effects, suggesting that heme-induced ER stress and a subsequent decrease in leukocyte β-endorphin is responsible for hypersensitivity in people with HIV.

1. Background

In the modern treatment era, individuals infected with human immunodeficiency virus-1 (HIV-1) who are diagnosed and treated early can have a near-normal life expectancy. However, chronic widespread pain (CWP) in HIV is associated with a high rate of disability and reduced quality of life [1], with prevalence estimates ranging from 25% to 85% [2–4].

Leukocytes (neutrophils, monocytes/macrophages, and lymphocytes) are a rich source of endogenous opioid peptides [5–13] that inhibit nociceptive transmission by binding to peripheral opioid receptors [14–16]. During inflammation, leukocytes are recruited to the site of damage [17]. Upon stimulation with mediators such as interleukin-1 (IL-1), corticotropin-releasing factor (CRF), or norepinephrine, they release opioid peptides (i.e., β-endorphin which exert an anti-hyperalgesic effect in inflamed tissues [12,18–22], [18,19,21,22]. Blocking the action of endogenous opioids with antibodies or elimination of peripheral immune cells that produce and release them decreases this effect [14,18,22]. Preclinical studies support a role for immune cells in endogenous analgesia. For example, drug-induced immunosuppression in rats increases mechanical and thermal hyperalgesia [23], while and adoptive transfer of allogenic polymorphonuclear leukocytes (PMNs) after innate PMN depletion restores opioid receptor-mediated analgesia during inflammation [24]. Macrophages, in particular,

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generate and release opioid peptides in response to inflammation or injury, with greater opioid content and release by the M2-polarized, pro-resolution phenotype than the pro-inflammatory, M1 phenotype [25]. Promoting the polarization of naive macrophages toward the M2 phenotype [26,27] can attenuate clinical postoperative pain [28] and decrease tactile hypersensitivity in animals [29].

Encapsulated heme plays an essential role in various physiological functions. However, when liberated from red blood cells (RBCs), cell-free heme is deleterious. Cell-free heme is transported from plasma to macrophages in liver by the heme scavenging enzyme, hemopexin, and is then metabolized by the enzyme, heme oxygenase-1, to biliverdin and carbon monoxide. We and others have shown that cell-free heme, a pro-inflammatory molecule, induces endoplasmic reticulum (ER) stress and cellular injury [30–33]. Heme activates toll-like receptor 4 signaling in macrophages [34,35], causing the release of pro-inflammatory, pro-algesic cytokines (e.g., IL-1α, IL-6, TNF-α) [36–38]. In macrophages, heme-induced ER stress promotes apoptosis and polarization to the pro-inflammatory, M1 phenotype [32,39,40]. Clinical and preclinical studies have shown that cell-free heme is correlated with acute painful vaso-occlusive crises in children with sickle cell disease and in transgenic sickle mice [41,42]. Animal studies have demonstrated that heme oxygenase-1, a heme metabolizing protein, ameliorates hyperalgesia due to nerve injury [43] and inflammation [44]. However, whether cell-free heme is directly involved in chronic pain is not known.

The objective of the present study was to examine the role of cell-free heme as a mediator of CWP in HIV, since HIV infection is associated with susceptibility to hemolysis due to secondary sequelae [45–50]. Specifically, we hypothesized that increased cell-free heme impairs peripheral endogenous analgesic mechanisms by increasing ER stress in peripheral immune cells, leading to increased pain. Using a multifaceted approach, the current study provides evidence that elevated cell-free heme and low heme oxygenase-1 contributes to CWP and characterizes mechanisms of this effect.

2. Methodology

Human participants: This study was conducted at the University of Alabama at Birmingham (UAB) and approved by the UAB Institutional Review Board (IRB Protocols 300000860 and 170119003). Participants were categorized as 1) Healthy controls, based on negative HIV status and absence of any pain or chronic disease which may cause hemolysis, 2) HIV-negative with chronic low back pain, 3) HIV-positive individuals without any perceived chronic pain, and 4) HIV-positive individuals with self-report of CWP. HIV-positive and -negative participants were recruited from the UAB Center for AIDS Research Network of Integrated Clinical System (CNICS) site. Pain Patient Reported Outcomes (PROs) were part of CNICS as reported earlier [51]. The pain PROs consisted of a Brief Chronic Pain Questionnaire about the intensity and duration of the pain. Participants with chronic low back pain were recruited via flyers posted at the Pain Treatment Clinic within the UAB Department of Anesthesiology and Perioperative Medicine and the surrounding community. Participants were included if they were active patients at the UAB Pain Treatment Clinic, and reported chronic low back pain that had persisted for at least three consecutive months and was present on at least half the days in the past six months [28]. Participants were only included if they denied any type of low back surgery or significant trauma/accident within the past year. Low back pain was the primary pain complaint reported for all participants with chronic low back pain. Demographic and clinical information was recorded from all participants and blood was drawn. Blood samples were processed, aliquoted, and RBCs and plasma were isolated and stored at −80 °C using Freezer works Sample Inventory Management software (Data works Development, Inc, Mountlake Terrace, WA, USA). No samples underwent freeze-thaw cycles prior to use.

Animals: Adult male C57BL/6 mice (20–25 g) were purchased from Charles River (Wilmington, MA), Heme oxygenase-1 knockout (HO-1$^{-/-}$) mice on a mixed C57BL/6 and FVB background and wildtype (WT) littermates were obtained from Dr. Anupam Agarwal at UAB, details of which have been published earlier [52]. All mice were housed in conventional polycarbonate cages with woodchip bedding under a 12 h: 12 h light/dark cycle with *ad libitum* access to a standard diet and water. Euthanasia protocol based on intraperitoneal injections of ketamine and xylazine was used in the study for mice to minimize pain and distress. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Alabama in Birmingham (Protocol number: 21416).

Chemicals: Hemin (ferric chloride heme; product no. H9039), phenylhydrazine hydrochloride (PHZ) (for induction of hemolytic anemia; product no. 114715), norepinephrine (NE, product no. N5785), biliverdin hydrochloride (product no. 30891), and CORM-A1 (a carbon monoxide donor, product no. SML0315) were obtained from Sigma-Aldrich (St. Louis, MO). Sodium 4-phenylbutyrate (4-PBA; product no. ALX-270-303), a chemical chaperone that reduces ER stress [53], was obtained from Enzo Life Sciences (Farmingdale, NY). Hx (heme scavenger; product no. 16-16-080513) was obtained from Athens Research and Technology (Athens, GA).

Blood plasma and cell concentration measurements: Heme concentration in plasma samples from humans and mice was measured using the QuantiChrom heme assay kit (product no. DIHM-250; BioAssay Systems, Hayward, CA), according to the manufacturer's instructions. Plasma concentration of hemopexin was measured in humans using the human hemopexin ELISA kit (product no. GWB-4B6D1A; GenWay Biotech, Inc. San Diego, CA) and in mice using the mouse hemopexin ELISA kit (product no. GWB-D5D320; GeWay Biotech, Inc). Plasma levels of methemoglobin (product no. LS-F40208; LSBio, Seattle, WA), carboxyhemoglobin (product no. LS-F39492; LSBio), and bilirubin (Bilirubin Assay Kit; Sigma-Aldrich) were analyzed by the human methemoglobin, carboxyhemoglobin, and bilirubin ELISA kits respectively according to the manufacturer's protocol. A panel of 10 cytokines [IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12 (p70), IL-13, and TNF-α] were assessed in plasma from human participants using a V-PLEX Proinflammatory Panel 1 (human) cytokine kit (product no. K15049-1D, Meso Scale Diagnostics, LLC, Rockville, Maryland). Cytokine concentrations were measured using a MESO QuickPlex SQ 120 electrochemiluminescence plate reader. Human plasma and mouse J774A.1 cell concentrations of β-endorphin were measured using QuickDetect beta-Endorphin ELISA kits (human: product no. E4458-100; mouse: product no. E4459-100; BioVision, Milpitas, CA). Human plasma and leukocyte and mouse J774A.1 cell concentrations of the 78-kDa glucose-regulated protein (GRP78) also known as the Binding immunoglobulin protein (BiP) were measured using the GRP78/BiP ELISA kit (product no. ADI-900-214-0001; Enzo Life Sciences). Human lactate dehydrogenase (LDH) activity in plasma was assessed using a LDH cytotoxicity assay kit (product no. 88953, Thermo Scientific, Rockford, IL).

RBC fragility assay: Blood was obtained from participants in the presence of an anticoagulant. Plasma was separated and the RBCs were washed with isotonic solution 3 times to remove traces of plasma. RBCs were then re-suspended in normal saline. The RBC suspensions along with 4x4mm glass beads (Pyrex) in DPBS were then rotated 360° for 2 h at 24 rpm at 37 °C. The RBC suspension was then centrifuged at 13,400g for 4 min to separate the intact or damaged cells from the supernatant containing heme/hemoglobin from the lysed cells during this mechanical stress. Free heme/hemoglobin was transferred into a new tube and the absorbance of the supernatant recorded at 540 nm as described earlier [54]. Subsequently, 100% hemolysis of RBCs was achieved by treating them with 1% Triton x-100 solution. The fractional hemolysis of the sample was then obtained by dividing the optical density of the sample by the optical density of the 100% hemolyzed sample.

Measurement of protein carbonyl adducts: Protein carbonyl adducts in RBC ghosts were measured as previously described [33]. Briefly, RBCs were separated from plasma and hemolyzed with 20 mM hypotonic HEPES buffer. The mixture was centrifuged at 14000×*g* for 20 min

and RBC membrane pellet was dissolved in radio-immuno-precipitation assay (RIPA) buffer (Product number: 89901, Thermo Scientific). Protein was quantified using a BCA kit (product no. 23225, Thermo Scientific, Rockford, IL) and equal amounts of protein (10 μg) were loaded onto each lane of a 4–20% gradient gel. Separated proteins were stained with Amido Black (Sigma-Aldrich, St Louis, MO). The presence of protein carbonyl adducts in RBC ghosts was assessed using an Oxyblot protein oxidation detection kit (product no. S7150; EMD Millipore, Billerica, MA) according to the manufacturer's protocol. The abundance of protein carbonylation was assessed using densitometry and normalization for protein loading by SDS-PAGE gel quantification.

Leukocyte isolation from blood: Blood drawn from human participants or mice was mixed with an equal volume of 3% dextran for 30 min to separate leukocyte-rich plasma. Leukocytes containing supernatant was centrifuged (10 min, 1000 rpm, 4 °C). The supernatant was discarded, and the remaining RBC and leukocytes were collected. The RBCs were lysed by adding hypotonic solution and leukocytes were re-suspended in 1x PBS/glucose [55] and analyzed immediately.

Phenylhydrazine hydrochloride (PHZ) and hemopexin administration: Mice were challenged with an intraperitoneal injection of PHZ (25 or 50 mg/kg BW) or saline (vehicle) on 2 consecutive days. Six hours after the second injection, mice were given a single, intraperitoneal injection of either saline (vehicle) or hemopexin in 1x phosphate buffered saline (PBS) (4 μg/kg final concentration). Hemopexin stocks were prepared daily in sterile PBS with injection volumes of 75 μl.

Cell culture: J774A.1 cells (ATCC), macrophages from adult female BALB/cN mice, were cultured to confluence in DMEM media (product no. 11995–065, Thermo Scientific) and then exposed for 24 h to hemin (25 μM) or vehicle (dimethyl sulfoxide, DMSO) in the presence or absence of 4-PBA (10 μM). Five min before the end of incubation, NE (100 nM) was added to the cells to induce secretion of β-END into the supernatant. In a separate set of experiments, the cellular levels of heme oxygenase-1 were genetically attenuated in J774A.1 cells using mouse siRNA (heme oxygenase siRNA (m); product no. sc-3555; Santa Cruz Biotechnology, Dallas, Tx) for 48 h. Control cells received control siRNA (product no. sc-37007; Santa Cruz Biotechnology). Cellular β-endorphin levels were measured at the end of the experiment.

Western Blot analysis: Mouse liver and human leukocytes were separately homogenized in RIPA buffer containing protease inhibitors. The samples were sonicated for 3×10 s on ice in 1.5 ml Eppendorf tubes using an ultrasonic liquid processor and centrifuged at 14,000 g for 20 min at 4 °C. Protein concentration was measured in cleared supernatants with a BCA assay kit. Equal amounts of protein (25 μg) were loaded in 10% Tris·HCl Criterion precast gels (Product no. 567–1093, Bio-Rad Laboratories, Hercules, CA) and transferred to polyvinylidene difluoride membranes (Product no. 162–0177, Bio-Rad Laboratories) and immuno-stained with an anti-heme oxygenase-1- antibody (1:1000; Product no. ADI-SPA-896-F, Enzo Life Sciences). Bands were detected by the Protein Detector LumiGLO Western blot kit (Product no. 95059–302, Radnor, PA). Protein loading was normalized by re-probing the membranes with an antibody specific to β-actin.

Mechanical sensitivity assay: To examine cutaneous mechanical sensitivity, mice were placed on a mesh floor inside a rectangular plexiglass chamber and allowed to acclimate for 1 h prior to each test session. Then, mechanical allodynia was measured using the simplified up-down method [56] in which calibrated monofilaments (Stoelting, Dale Wood, IL) were applied to the glabrous skin of each hindpaw to determine a paw withdrawal threshold estimate. Filaments numbered 2 through 9 were used and testing always began with filament 5. If no withdrawal was present for a given stimulus, the next highest value monofilament was applied; if a withdrawal occurred, the following stimulus presentation was the next lowest monofilament value. A total of 5 stimulus presentations occurred. Filament number was converted to force and data are expressed in grams.

Statistical analyses: Statistical analysis was performed using GraphPad Prism version 7 for Windows (GraphPad Software, San Diego, CA). Data are presented as mean ± SEM. Statistical significance was determined by unpaired *t*-test for two groups or one-way ANOVA followed by Tukey's post-hoc test for more than two groups. A value of $p < 0.05$ was considered significant.

Table 1
Demographic and clinical data of study participants. The table shows demographic and clinical data describing average age in years, current and nadir $CD4^+$ cell count (cells/mm^3), and the average current and highest viral load (VL). SD: Standard deviation.

Group (Number of participants)	HIV_{neg}, $Pain_{neg}$ (15)	HIV_{neg}, $Pain_{pos}$ (15)	HIV_{pos}, $Pain_{neg}$ (25)	HIV_{pos}, $Pain_{pos}$ (25)
Avg. age (SD)	48.7 (13.3)	45.3 (14.5)	50.1 (8.7)	52.3 (7.5)
Percent Females	66.6	66.6	48	48
Percent Afr. Amer.	66.6	66.6	68	68
Avg. $CD4^+$ (SD)			681.2 (364.3)	785.2 (412.8)
Avg. Nadir $CD4^+$ (SD)			168.4 (249)	221.9 (225.4)
Avg. VL (SD)			34 (63)	42.9 (95.1)
Avg. Highest VL (SD)			180456 (204667)	253200 (885708)

3. Results

People with HIV who self-report CWP have increased plasma levels of cell-free heme and impaired heme scavenging. As a first step in establishing cell-free heme as a contributing factor to HIV-associated chronic pain, plasma concentrations of cell-free heme, plasma methemoglobin, RBC membrane oxidation and fragility, and the heme scavenging protein, hemopexin, were measured in HIV-positive and -negative cohort with or without CWP. The most common sites of pain reported by people with HIV are low back (86%), hands/feet (81%), and knee (66%) [51]; therefore, HIV-negative individuals with chronic low back pain were included as a second control group in addition to people with HIV without chronic pain.

General demographic characteristics of participants were not significantly different among groups (age range 45–52 years; race distribution 66–68% African American), with the exception of sex (M > F) (Table 1). Reflective of the patient population of the clinic from which HIV-positive individuals were recruited, the proportion of men in the HIV-positive group was 52% vs. 33% in the HIV-negative groups. De-identified clinical data showed that the average current and nadir $CD4^+$ cell counts and average current and highest viral load (VL) were not different between HIV-positive individuals with or without CWP.

Whole blood samples were collected from study participants for analysis of plasma levels of total (free and protein-bound) cell-free heme. Data demonstrated that the HIV cohort with CWP had significantly higher (2–3 fold increase) cell-free heme levels than other groups (Fig. 1A). However, hemopexin concentrations were comparatively lower in people with HIV with than without CWP, suggesting that heme scavenging machinery is attenuated in the context of pain (Fig. 1B). *Ex vivo* RBC mechanical fragility was assayed by quantification of heme/hemoglobin (Hb) release in response to mechanical stress using freshly obtained RBCs from participants [54]. Data demonstrated that HIV positive individuals had significantly higher RBC fragility, resulting in hemolysis, compared to HIV-negative individuals (Fig. 1C). Further, hemolysis was also greater in people with HIV infected with CWP compared to individuals without chronic pain. The plasma levels of methemoglobin (a form of oxidized hemoglobin) were also greater in HIV cohort with CWP compared to individuals from other groups (Fig. 1D). In addition, RBC membranes were isolated for measurement of carbonyl (aldehyde and ketone) adducts, a hallmark of protein oxidation. Protein carbonylation in RBCs was significantly higher in people with HIV- with CWP compared to the HIV-negative individuals with low back pain and both pain-free groups (Fig. 1E). Together, these results suggested that people with HIV with CWP were prone to hemolysis and

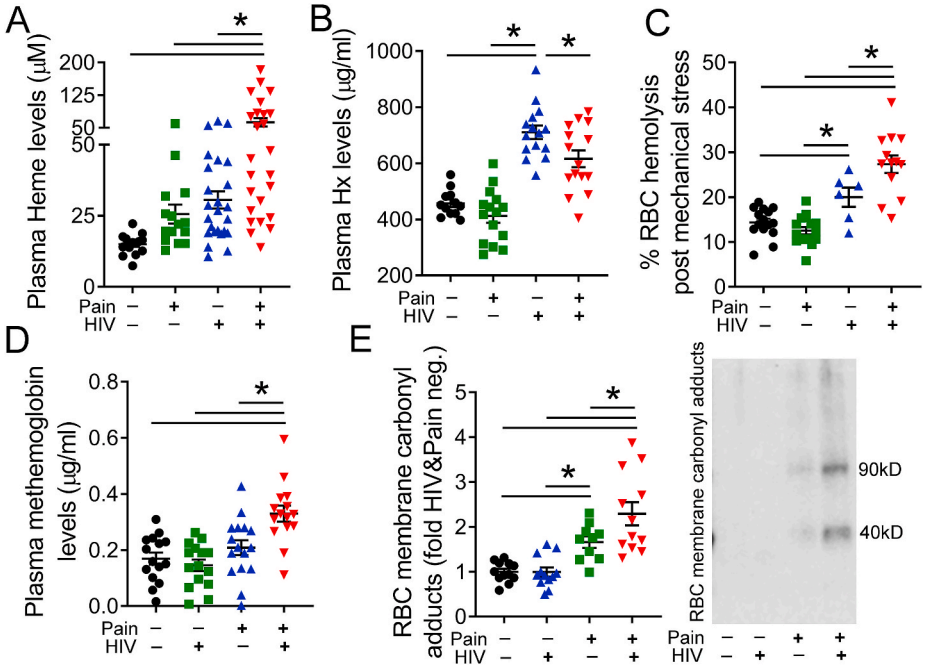

Fig. 1. People with HIV with CWP have increased plasma levels of cell-free heme and impaired heme scavenging. Total (free and protein bound) cell-free heme was measured in plasma. HIV positive individuals with CWP had significantly higher heme than without CWP and HIV-1 negative controls (n = 15–25) (A). Plasma hemopexin (Hx) levels were lower in people with HIV with CWP than without pain (n = 12–15) (B). People with HIV with CWP had increased RBC hemolysis compared to other groups (n = 6–25) (C). HIV positive individuals with CWP had elevated plasma levels of methemoglobin compared to other groups (n = 15) (D). Protein carbonylation in RBC membrane was significantly higher in HIV positive people with CWP compared to the no-pain groups and the HIV-negative group with low back pain (n = 10–12) (E). Individual values and means ± SEM. *$P < 0.05$ vs. groups at the end of individual lines; one-way ANOVA followed by Tukey post-hoc testing.

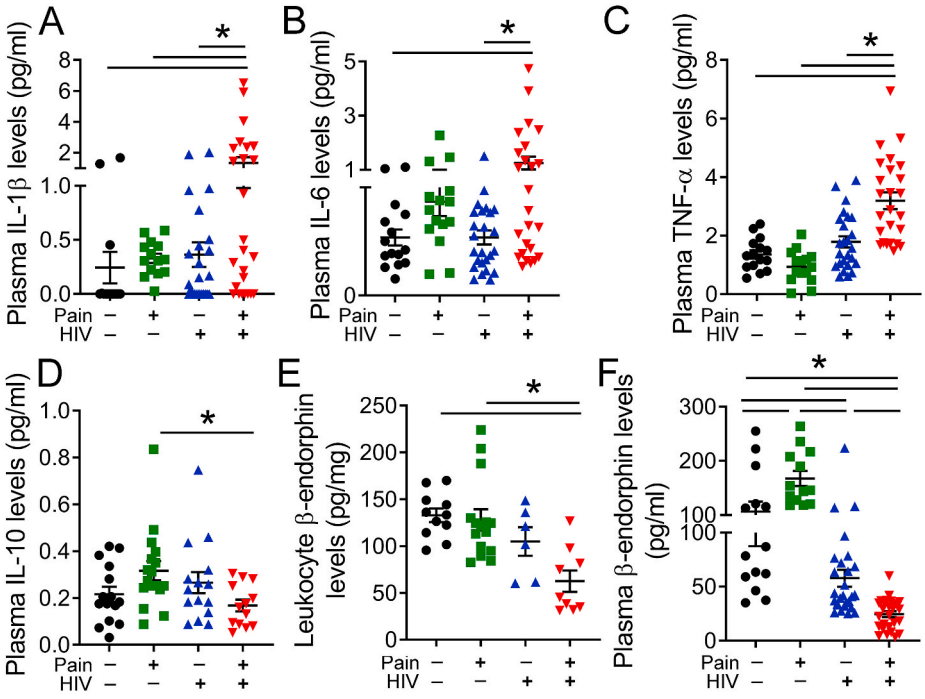

Fig. 2. People with HIV with CWP have increased pro-algetic cytokines and reduced β-endorphins. HIV positive individuals with CWP had significantly higher levels of pro-inflammatory and pro-algetic cytokines in plasma, including IL-1β (n = 15–25) (A), IL-6 (n = 15–25) (B), and TNF-α (n = 15–25) (C). HIV with CWP group also had significantly lower levels of anti-inflammatory cytokine, IL-10 (n = 13–25) than HIV-negative individuals with low back pain (D). Leukocyte levels of β-endorphins were significantly lower in HIV positive individuals with CWP compared to HIV-negative, pain-free controls and HIV positive people without pain (n = 6–15) (E). Circulating β-endorphins in plasma were higher in HIV-negative individuals with low back pain, but lower in HIV-positive group without pain. HIV-positive group with CWP had the lowest concentration of plasma β-endorphins (n = 13–25) (F). Individual values and means ± SEM. *$P < 0.05$ vs. groups at the end of individual lines; one-way ANOVA followed by Tukey post-hoc testing.

thus exhibited elevated plasma levels of cell-free heme. All HIV positive individuals (with or without pain) were on anti-retroviral therapy (ART) and had low VL (mean VL = 34–42 copies/ml of blood) suggesting that RBC hemolysis and elevated cell-free heme are not dependent on VL or ART.

People with HIV with CWP exhibit a pro-algesic immune profile. Immune cells release both pro-inflammatory, algesic, and anti-inflammatory, analgesic mediators, the balance of which contributes to the presence of hyperalgesia or allodynia. We have previously demonstrated that heme increases pro-inflammatory cytokines [33]. In the current study, plasma profile of 10 cytokines was quantified in the participants. Data showed that people with HIV with CWP exhibited significantly higher levels of proalgesic cytokines including IL-1β (Fig. 2A), IL-6 (Fig. 2B), and TNF-α (Fig. 2C), that are produced predominantly by pro-inflammatory, rather than pro-resolution, immune cells [57–59]. In addition, people with HIV with CWP had significantly lower levels of anti-inflammatory, IL-10, compared to HIV negative individuals with chronic low back pain (Fig. 2D). The plasma concentration of other 6 cytokines were below the reading threshold of the measuring kit and are not reported. Since a prolonged or ongoing presence of pro-inflammatory cytokines can reduce endogenous opioid peptides [25], blood leukocytes were also isolated for measurement of β-endorphins. In leukocytes from people with HIV with CWP, significantly lower levels of β-endorphins were detected compared to those

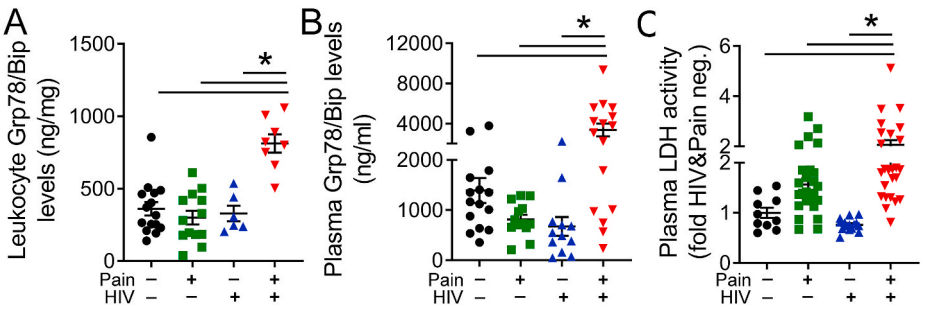

Fig. 3. People with HIV with CWP have increased ER stress. Levels of the ER stress marker, glucose-regulated protein 78 (Grp78/BiP), were significantly higher in leukocytes (n = 6–18) (A) and plasma (n = 12–21) (B) of people with HIV with CWP compared to pain-free HIV-negative individuals and pain-free HIV positive individuals. Lactate dehydrogenase (LDH) activity in the plasma of HIV-positive group with CWP was 2-fold higher than in all other groups (n = 10–25) (C). Individual values and means ± SEM. *$P < 0.05$ vs. groups at the end of individual lines; one-way ANOVA followed by Tukey post-hoc testing.

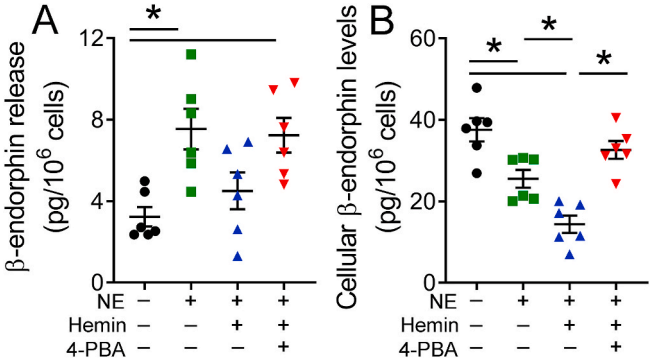

Fig. 4. Heme-induced ER stress attenuates β-endorphin levels/release from immune cells. Cultured J774A.1 cells (murine macrophages) were exposed to (hemin, 25 μM, 24 h) or vehicle (dimethyl sulfoxide, DMSO) in the presence or absence of a chemical chaperone, sodium 4-phenylbutyrate (4-PBA; 10 μM). Norepinephrine (NE; 100 nM) was added 5 min before the end of incubation to stimulate β-endorphin secretion into the supernatant. NE significantly increased β-endorphin secretion in DMSO-treated, but not in hemin-treated, cells (n = 6) (A). 4-PBA restored β-endorphin release in hemin-treated cells. Intracellular β-endorphin levels were depleted in hemin-exposed cells but not in cells treated with hemin + 4-PBA (n = 6) (B). Individual values and means ± SEM. *$P < 0.05$ vs. groups at the end of individual lines; one-way ANOVA followed by Tukey post hoc testing.

isolated from HIV-negative individuals with low back pain and both no-pain groups (Fig. 2E). In addition, the mean circulating β-endorphins concentration in plasma of healthy individuals (100 pg/ml) was twice that of pain-free people with HIV (50 pg/ml) and four-fold higher than that of HIV positive individuals with CWP (25 pg/ml) (Fig. 2F). This was not due to diurnal variation in opioid release [60], since blood was collected between 9am-11am to minimize this effect.

CWP in HIV is associated with increased ER stress. ER stress is one of the adverse effects triggered by the presence of cell-free heme [32]. Given the observed elevation in heme in people with HIV with CWP, the presence of ER stress was examined in plasma and peripheral leukocytes by measurement of glucose-regulated protein 78 (GRP78/BiP), an important regulator of the unfolded protein response (UPR). GRP78/BiP is the only ER stress marker that is secreted from the cell, allowing it to be measured in both cells and in circulation [32]. The results of this experiment demonstrated that HIV positive individuals with CWP had almost 2-fold higher GRP78/BiP in leukocytes (Fig. 3A) and 3-fold higher GRP78/BiP in plasma (Fig. 3B) compared to HIV-negative individuals with pain and both non-pain groups. Since prolonged ER stress causes cell death, plasma activity of lactate dehydrogenase (LDH), a stable enzyme that leaks from cells upon plasma membrane damage [61], was also measured. Consistent with evidence of ER stress, HIV cohort with CWP had significantly elevated plasma LDH activity compared to people in other groups (Fig. 3C).

Heme-induced ER stress reduces β-endorphin in immune cells. To establish a direct link between cell-free heme and ER stress with decreased endogenous opioid production, β-endorphin levels/release in murine immune cells (monocyte/macrophage-like J774A.1 cells) was assessed following exposure to hemin (ferric chloride heme) *in vitro*. Compared to DMSO (vehicle)-treated cells, β-endorphin secretion induced by exposure to norepinephrine (NE) was significantly reduced in cells exposed to hemin (Fig. 4A). This effect was blocked by incubation with sodium 4-phenylbutyrate (4-PBA), a chemical chaperone that reduces ER stress [53]. Further, intracellular β-endorphin content was depleted in hemin-exposed cells, but not in cells treated with hemin+4-PBA (Fig. 4B). Taken together, these results indicate that heme-induced ER stress attenuates β-endorphin levels and release by immune cells.

Heme scavenging ameliorates disrupted opioid homeostasis and mechanical hyperalgesia in a mouse model of hemolysis. To translate our clinical and *in vitro* findings to an *in vivo* model, phenylhydrazine hydrochloride (PHZ) was used to induce hemolysis in adult male C57BL/6 mice and hindpaw mechanical thresholds were assessed over time. Exposure to PHZ for two consecutive days induced a significant reduction in paw withdrawal thresholds compared to baseline, and this effect was blocked by administration of purified human hemopexin 6 h following the second dose of PHZ (Fig. 5A). In the same mice, plasma concentrations of cell-free heme were elevated 24 h following the second dose of PHZ, while the plasma levels of cell-free heme were significantly lower in the mice that received hemopexin (Fig. 5B). Using separate cohorts of mice, we found that PHZ-treated mice had elevated levels of Grp78/BiP (Fig. 5C) and attenuated concentration of β-endorphin in leukocytes (Fig. 5D) and plasma (Fig. 5E) relative to saline-treated mice. However, each of these changes were mitigated in mice treated with hemopexin (Fig. 5C–E). These results directly link elevated heme with reduced β-endorphin levels and mechanical pain threshold.

Mice lacking heme oxygenase-1 exhibit mechanical hyperalgesia and reduced leukocyte β-END. Previous studies have reported that upregulation of heme oxygenase-1, a heme degrading enzyme, mitigates inflammation- and injury-induced hyperalgesia in mice [43,44,62,63]. The underlying mechanisms of this effect are not known. In the present study, we determined whether global knockdown of heme oxygenase-1 in mice was associated with reduced circulating β-endorphins and a predisposition toward increased nociception. To validate this model, heme oxygenase-1 protein was measured in mouse liver homogenates, and complete knockdown in heme oxygenase-1 knockout (HO-$1^{-/-}$) mice was demonstrated (Fig. 6A). The plasma concentration of the heme scavenging protein, hemopexin, was not different in the HO-$1^{-/-}$ mice compared to their wild type (WT) counterparts (Fig. 6B). Next, mechanical sensitivity was assessed in HO-$1^{-/-}$ and WT mice at baseline and two days after administration of PHZ. Because HO-$1^{-/-}$ mice are unable to metabolize heme and thus highly sensitive to hemolysis with very high mortality, half of the PHZ dose given in previous experiments (i.e., Fig. 5) was administered. Interestingly, HO-$1^{-/-}$ mice exhibited significantly lower paw withdrawal thresholds than WT mice at baseline, prior to PHZ treatment (Fig. 6C). Administration of low-dose PHZ did not further increase mechanical hypersensitivity in HO-$1^{-/-}$ mice, and withdrawal threshold in WT mice did not differ from HO-$1^{-/-}$

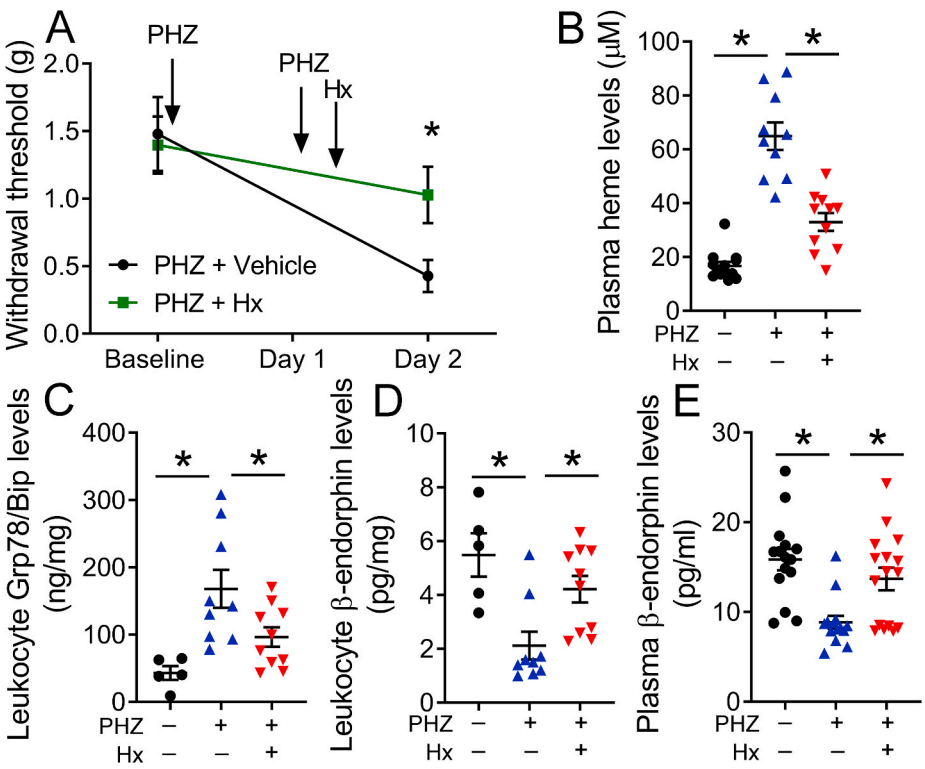

Fig. 5. Heme scavenging increases mechanical pain threshold in a rodent model of hemolysis. Phenylhydrazine hydrochloride (PHZ, 50 mg/kg, IP) or saline was administered to adult male C57BL/6 mice on two consecutive days. Six hours following the second injection, subset of mice were given purified human hemopexin (Hx) (4 mg/kg, IP) or saline. Mechanical hypersensitivity was assessed at baseline and on day two by measuring paw withdrawal thresholds to application of von Frey filaments. PHZ-challenged mice exhibited decreased paw withdrawal thresholds compared to mice that received Hx post-PHZ (n = 9–10) (A). On day two, mice exposed to PHZ had elevated plasma levels of cell-free heme (n = 10–13) (B), higher levels of the ER stress marker, GrpP78/BiP, in peripheral leukocytes (n = 5–10) (C) and lower levels of β-endorphin in both leukocytes (n = 5–10) (D) and plasma (n = 14–16) (E). Hx treatment abrogated the effects of PHZ. Individual values and means ± SEM. *$P < 0.05$ vs. groups at the end of individual lines; unpaired t-tests (A) or one-way ANOVA followed by Tukey post hoc testing (B–E).

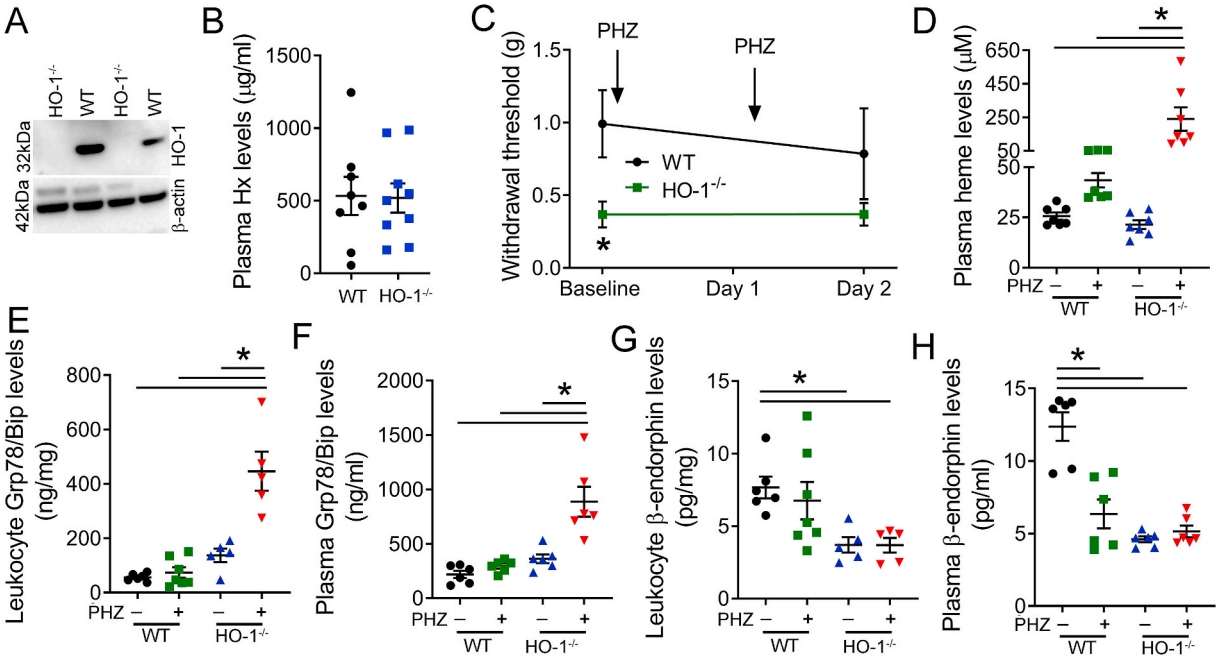

Fig. 6. Heme oxygenase-1 (HO-1) knockout mice have low mechanical pain threshold and β-endorphin levels. Immunoblot from liver homogenate validated that HO-1$^{-/-}$mice (adult male) lack HO-1 expression compared to wild type (WT) mice (n = 4) (A). The plasma levels of the heme scavenging protein, hemopexin, was not different between the WT and HO-1$^{-/-}$ mice (n = 8–9) (B). Mechanical hypersensitivity was assessed at baseline and after administration of phenylhydrazine hydrochloride (PHZ, 25 mg/kg, intraperitoneal) or saline (vehicle) on two consecutive days by measuring paw withdrawal thresholds to application of von Frey filaments. Paw withdrawal thresholds were lower at baseline in the HO-1$^{-/-}$ mice compared to wild type (WT) mice (n = 6) (C). PHZ did not further lower pain threshold in these mice. One day following the 2nd dose of PHZ, HO-1$^{-/-}$ mice had increased plasma levels of cell-free heme (n = 7) (D), higher leukocyte (n = 5–7) (E) and plasma (n = 6) (F) glucose-regulated protein 78 (Grp78/BiP) levels, and lower leukocyte (n = 5–7) (G) and plasma (n = 6) (H) β-endorphin levels. HO-1$^{-/-}$ mice that were not exposed to PHZ also had low β-endorphin levels (G–H). Individual values and means ± SEM. *$P < 0.05$ vs. groups at the end of individual lines; unpaired t-tests (B–C) or one-way ANOVA followed by Tukey post hoc testing (D–H).

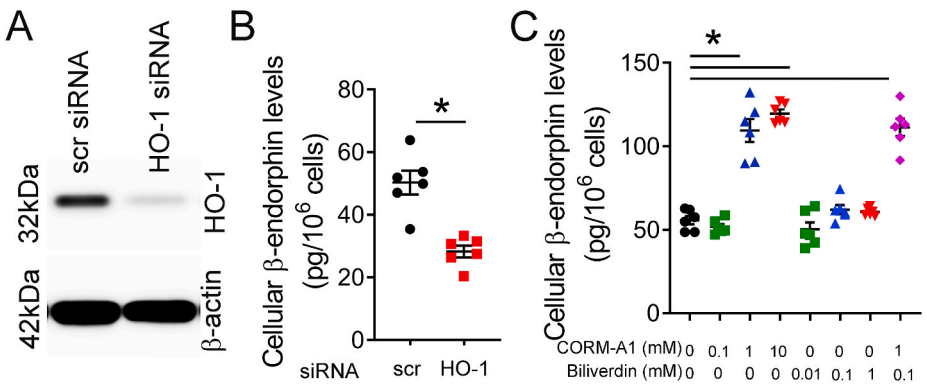

Fig. 7. Carbon monoxide released by heme oxygenase-1 (HO-1) increases β-endorphins in immune cells. J774A.1 cells grown to 50% confluency were treated with either control (scrambled, scr) or HO-1 siRNA *in vitro*. Immunoblot shows attenuation of HO-1 in the cells (n = 4) (A). HO-1 depletion attenuated the cellular levels of β-endorphins (n = 6) (B). The exposure of J774A.1 cells to different doses of carbon monoxide donor, CORM-A1, or biliverdin, demonstrated that at CORM-A1 (1 and 10 mM) increased cellular β-endorphins by more than 2 fold (n = 6) (C). The exposure of cells to a combination of CORM-A1 (1 mM) and biliverdin (100 μM) did not increase β-endorphins more than CORM-A1 alone (C). Individual values and means ± SEM. *$P < 0.05$ vs. groups at the end of individual lines; unpaired t-tests (B) or one-way ANOVA followed by Tukey post hoc testing (C).

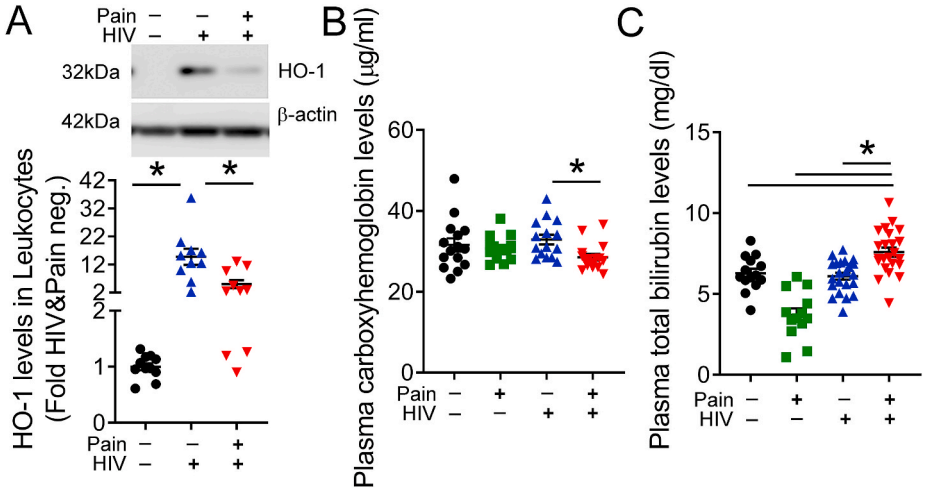

Fig. 8. People with HIV with CWP have impaired heme metabolism. The expression levels of the heme-metabolizing protein, heme oxygenase-1 (HO-1), were measured in the leukocytes of HIV positive and negative individuals. Immunoblot shows that the HO-1 levels were significantly lower in HIV positive individuals with CWP than HIV positive people without pain (n = 10–12) (A). The plasma levels of carboxyhemoglobin were significantly lower in HIV cohort with CWP compared to HIV cohort without pain (n = 15–16) (B). However, the plasma levels of total bilirubin were higher in HIV group with CWP than other groups (n = 15–23) (C). Individual values and means ± SEM. *$P < 0.05$ vs. groups at the end of individual lines; one-way ANOVA followed by Tukey post-hoc testing.

following PHZ (Fig. 6C).

Biochemical analysis of plasma and leukocytes from WT and HO-$1^{-/-}$ mice was performed 24 h following two consecutive daily treatments with PHZ. We observed a significant increase in the plasma concentrations of cell-free heme (40 μM vs. 250 μM) (Fig. 6D), and in leukocyte (Fig. 6E) and plasma (Fig. 6F) concentrations of Grp78/BiP in HO-$1^{-/-}$ mice, relative to WT mice. Further, the mean plasma concentration of cell-free heme in HO-$1^{-/-}$ mice following 25 mg/kg of PHZ was higher than that of C57BL/6 mice given 50 mg/kg of PHZ (Fig. 5), likely due to increased sensitivity to hemolysis in the context of heme oxygenase-1 knockdown. The concentrations of β-endorphin in leukocytes (Fig. 6G) and plasma (Fig. 6H) were lower in the HO-$1^{-/-}$ mice irrespective of PHZ treatment, suggesting that heme oxygenase-1 may regulate β-endorphins independently of heme-induced ER stress.

Therefore, to determine the mechanism responsible for low β-endorphins in HO-$1^{-/-}$ mice under basal conditions, we treated J774A.1 cells with the heme oxygenase-1 siRNA *in vitro,* to deplete the expression of the protein (Fig. 7A). Cells lacking the heme oxygenase-1 enzyme had lower levels of β-endorphins than the cells treated with the control (scrambled) siRNA (Fig. 7B) suggesting that the reduction in heme oxygenase-1 attenuates β-endorphin. Further, the treatment of J774A.1 cells with different concentrations of carbon monoxide donor, CORM-A1, or biliverdin, which are end products of heme metabolism by heme oxygenase-1, demonstrated that CORM-A1 (1 and 10 mM) increased cellular β-endorphins by more than 2 fold (Fig. 7C). Biliverdin did not alter the levels of β-endorphins in the cells suggesting that heme oxygenase-1 mediated analgesia may be mediated by carbon monoxide dependent increase in β-endorphins.

In parallel experiments, we demonstrated that the expression of leukocyte heme oxygenase-1 was significantly lower in HIV positive cohort with CWP than HIV positive cohort without pain (Fig. 8A), even though these individuals had significantly higher levels of cell-free heme (Fig. 1A). Further, HIV positive individuals with CWP had lower carboxyhemoglobin levels than HIV positive people without pain (Fig. 8B), potentially suggesting lower levels of heme-oxygenase-1 derived carbon monoxide. However, these individuals had significantly elevated plasma levels of total bilirubin (Fig. 8C), which is formed from biliverdin in liver, suggesting that these individuals may also have concurrent hepatic dysfunction. (Please note that the bilirubin levels measured in the experiment were higher in all the groups (normal are less than 1.2 mg/dl), potentially due to the commercially available kit.

4. Discussion

This study demonstrates a relationship between self-reported CWP in people with HIV, with elevated cell-free heme and ER stress markers in plasma, and increased ER stress and diminished levels of β-endorphins in leukocytes. Furthermore, it demonstrates that *in vitro* exposure to heme attenuates β-endorphin content/release in murine monocyte/macrophage-like J774A.1 cells. This effect was reversed by the inhibition of ER stress. Similarly, the induction of hemolysis in C57BL/6 mice increased ER stress and reduced β-endorphin levels in leukocytes, and was accompanied by a decrease in mechanical sensitivity thresholds. The data also demonstrated that cells and animals, which lacked heme

oxygenase-1, had lower β-endorphins, while cells treated with carbon monoxide donor had significantly higher β-endorphins. This novel mechanism of β-endorphin regulation by cell-free heme-induced ER stress and heme oxygenase-1 in peripheral immune cells may contribute to hypersensitivity in a subset of HIV positive individuals.

Plasma levels of cell-free heme are elevated in several hemolytic and chronic inflammatory diseases, such as sickle cell disease [35,41,42], lupus arthritis [64], malaria [65], and following treatment with widely used platinum-based anticancer drugs [66]. However, the role of heme in HIV associated co-morbidities has not been yet elucidated. There have been few case reports suggesting that hemolysis, heme scavenging, and heme metabolizing mechanisms are impaired in a subset of HIV-positive individuals. For instance, in a recent case report, hemolysis was the main presentation of acute HIV infection in a 22-year-old patient with G6PD-deficiency [67], although the presence or absence of pain in this case was not reported. It is to be noted that the prevalence of G6PD deficiency in HIV-positive individuals is estimated to be 6.8%–13% [68,69]. HIV also confers a 15–40 fold higher risk of acquired thrombotic microangiopathy [45–47], which is an important cause of hemolysis. Further, HIV positive individuals are at risk of hemolysis secondary to the use of ART [48] or other drugs for HIV-associated infections such as the use of amphotericin B and co-trimoxazole for cryptococcal meningitis [49] and use of interferon and ribavirin for hepatitis C [50].

People with HIV have high reactive species production in peripheral immune cells [70,71] and elevated lipid peroxidation products in plasma [72]. We and others have previously shown that RBCs are especially susceptible to oxidative damage by lipid peroxidation [32,33,73], resulting in release of hemoglobin (Hb), metHb, free heme, and free iron [74]. In this study we also found that HIV positive individuals who self-report CWP had elevated levels of methemoglobin in plasma and carbonyl adducts in RBC membranes. Elevated levels of methemoglobin in the plasma indicate that these individuals have elevated oxidative stress within the RBCs and the oxygen carrying ferrous ion (Fe^{2+}) in the hemoglobin is oxidized to the ferric state (Fe^{3+}), which can predispose the RBCs to oxidative damage [75]. This could be due to either deficiency of methemoglobin reductase enzyme or exposure to chemicals or drugs such as nitrobenzene, nitrites, dapsone, chloroquine etc. Oxidative stress-induced carbonylation of proteins creates neoantigens against which autoantibodies may develop, as earlier reported in diseases such as COPD [76]. Therefore, it is possible that people with HIV with CWP have these autoantibodies that contribute to hemolysis. These individuals also had increased inflammatory cytokine profiles, suggesting that persistent inflammation may be driving hemolysis. In this regard, environmental and social factors or HIV-associated inflammatory diseases may be responsible for higher oxidative stress seen in HIV-positive individuals with CWP. Moreover, cell-free heme released post-hemolysis is highly inflammatory and may further cause hemolysis, leading to a feed-forward loop of hemolysis-heme-hemolysis [77].

Physiologically, heme concentrations in the blood are maintained at low levels [78] by the high binding affinity of serum heme scavenging proteins, hemopexin and haptoglobin [79–82]. Cell-free hemoglobin binds with circulating haptoglobin, resulting in a heterodimeric complex that is internalized by the transmembrane CD163 receptor on monocytes/macrophages [83]. Similarly, cell-free heme binds with hemopexin and the complex is internalized by the CD91 receptor [84]. Once inside the cell, heme and hemoglobin are degraded by heme oxygenase-1 [85]. Immune cells from heme oxygenase-1-deficient mice are more sensitive to heme-induced oxidative stress [86]. A recent case report described impaired CD163-mediated heme scavenging with non-detectable hemopexin in an HIV-positive individual with severe inflammation [87]. In our study, we found that hemopexin levels in plasma and heme oxygenase-1 levels in leukocytes were diminished in HIV positive individuals with CWP compared to the people without pain. These results suggested that the increased hemolysis and diminished heme metabolism are responsible for elevated plasma cell-free heme in HIV cohort with CWP.

We have previously reported that cell-free heme induced cellular toxicity is mediated by ER stress in both animals and humans after bromine gas exposure [32,33]. Accumulation of misfolded proteins in the ER leads to ER stress and activation of the unfolded protein response (UPR) which serves to reduce translation of proteins in order to prevent further accumulation of mutant proteins [88,89]. Recent studies have showed significantly higher levels of misfolded proteins and UPR markers such as Grp78/BiP in people infected with HIV-1 [90–93]. We found that the levels of the Grp78/BiP, a master regulator of UPR [94], were higher in HIV-positive individuals with CWP, which corresponded with high levels of cell-free heme in this subset of individuals. However, heme may not be the only inducer of ER stress in people with HIV. For instance, the HIV-1 transactivator of transcription (Tat) and glycoprotein (gp) 120 proteins have been shown to induce ER stress and cytotoxicity contributing to HIV-associated neuropathogenesis [95,96]. Whether, these HIV proteins increase ER stress independent of cell-free heme or they cause hemolysis and thereby induce heme-dependent ER stress need further investigation.

ER stress has been implicated in inflammatory pain [97] and diabetic peripheral neuropathic pain [98]. However, the mechanism by which ER stress modulates pain sensation is unknown. Our *in vitro* analysis showed that heme-induced ER stress attenuated the levels and release of β-endorphins from immune cells. The data also showed that people with HIV who self-report CWP with high ER stress have 4-fold lower plasma levels of β-endorphins compared to the healthy controls and 2-fold lower β-endorphins than HIV cohort without CWP. We also found that the leukocytes, which are an important source of opioids, have diminished capacity to produce β-endorphins in HIV-positive individuals with CWP. An earlier study compared plasma and brain β-endorphins in 48 HIV-positive and 19 healthy subjects and found that HIV-positive individuals had significantly low levels of β-endorphins compared to the controls and that the decrease was not correlated to the $CD4^+$T lymphocytes number [99]. Similarly, β-endorphins in our study did not correlate with $CD4^+$ cells as all HIV-positive individuals had low viral load and high $CD4^+$ cells. However, the low levels of β-endorphins were inversely proportional to cell-free heme and ER stress.

To determine, whether enhancing heme scavenging by hemopexin or heme metabolism by heme oxygenase-1 would increase β-endorphins and attenuate hypersensitivity, we generated an animal model of PHZ-induced hemolysis. Adult, male C57BL/6 mice challenged with the hemolytic agent, PHZ (50 mg/kg body weight), over two consecutive days had a significant increase in plasma concentration of cell-free heme. A similar dosing regimen of PHZ has been shown previously to induce hemolysis and not increase mortality in mice [100]. We found that the increase in hemolysis and ER stress correlated with a subsequent decrease in β-endorphins and hindpaw withdrawal threshold in mice. The treatment of the PHZ challenged mice with hemopexin prevented the decline in β-endorphins and the induction of hyperalgesia.

In our heme oxygenase-1 KO ($HO\text{-}1^{-/-}$) mice, the plasma and leukocyte levels of β-endorphins were significantly lower at baseline compared to the corresponding WT mice which correlated with low paw withdrawal threshold in these mice. At baseline, cell-free heme and ER stress was not elevated in $HO\text{-}1^{-/-}$ mice. However, β-endorphins were still lower suggesting that heme oxygenase-1 may directly influence endogenous opioid levels in cells independent of heme. The induction of heme oxygenase-1 enzyme has been historically associated with reduction in inflammatory pain [44,101,102]. Heme oxygenase-1 has been shown to potentiate the analgesic effects of morphine [62], probably by increasing the expression of the μ-opioid receptors [103]. However, the exact mechanism by which heme oxygenase-1 reduces pain has not been elucidated till now. In our *in vitro* study, we found that attenuating heme oxygenase-1 by siRNA, reduced β-endorphins in immune cells. However, treating the cells with the carbon monoxide donor, CORM-A1, increased the levels of β-endorphins by more than 2 fold. It is to be noted that a minimum concentration of 1 mM of CORM-A1 was required to increase β-endorphins. Together, these results indicate that heme oxygenase-1

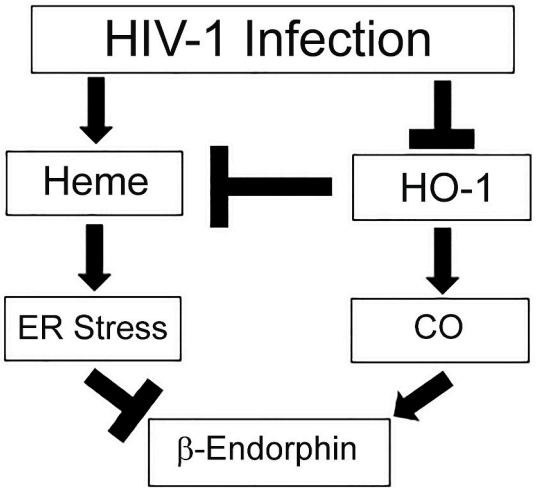

Fig. 9. Schema showing β-endorphin regulation in HIV. In HIV-1 infection, increased hemolysis and low heme oxygenase-1 (HO-1) increases plasma levels of cell-free heme. The combined effect of heme-induced ER stress and diminished production of carbon monoxide by low HO-1 levels, attenuates β-endorphins in immune cells.

dependent carbon monoxide may reduce pain by upregulating β-endorphins and this could be related to the anti-inflammatory properties of carbon monoxide. Although, CORMs have earlier been reported to reduce pain in rats [104,105], this is the first study to report that they increase endogenous opioids.

Interestingly in our HIV cohorts, people with CWP had much lower levels of heme oxygenase-1 in leukocytes compared to people without pain. These individuals also had a small but significant reduction in plasma carboxyhemoglobin levels compared to their HIV positive counterparts without pain. The physiological relevance of this reduction in carboxyhemoglobin may not be significant but taken together with low heme oxygenase-1 levels, it may be postulated that this cohort has diminished endogenous carbon monoxide production, which may influence the inflammatory status of these individuals and also reduce β-endorphins.

Our data also demonstrated that people with HIV with CWP had elevated plasma levels of total bilirubin. Although, it may be impossible to make a clear association, the data suggests that these individuals may have some degree of concurrent liver abnormalities like viral or drug-induced hepatitis, which is quite common in HIV and is not clinically evident in most cases. This may also explain, why this cohort had low levels of hemopexin (produced by liver), compared to the HIV positive group without pain, despite having higher levels of cell-free heme. In conclusion, this study has identified that HIV positive people with CWP may have attenuated levels of β-endorphins secondary to heme-induced ER stress and also due to the dysregulation of heme oxygenase-1 levels/activity (Fig. 9). Strategies aimed at reducing cell-free heme burden and increasing endogenous opioids may mitigate CWP in HIV. One such strategy was used by Pannell et al. [25], where transfer of macrophages containing higher levels of endogenous opioid to mice ameliorated mechanical hypersensitivity, which was reversed by the opioid receptor antagonist, naloxone methiodide [25]. Similarly, peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists, which increase endogenous opioids have been shown to elevate nociceptive threshold in inflammatory pain models [28,106].

Authors contributions

SA: Study design, data acquisition, analysis, interpretation, writing, and quality control; **JD**: Study design, data acquisition, analysis, and interpretation; **IA**: Data acquisition and analysis; **PL, CD, & SM:** Data acquisition; **JSM & BRG**: Study design, recruiting of human participants and participant sample acquisition; **SLH**: Study design, recruiting and acquisition of patient samples, interpretation of the data, and quality control; **SM**: Study design and data interpretation and quality control.

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Declaration of competing interest

The authors have declared that no conflict of interest exists.

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