Hemin Activation Ameliorates HIV-1 Infection via Heme Oxygenase-1 Induction

Krishnakumar Devadas and Subhash Dhawan

J Immunol 2006; 176:4252-4257; doi: 10.4049/jimmunol.176.7.4252
http://www.jimmunol.org/content/176/7/4252

References
This article cites 37 articles, 13 of which you can access for free at:
http://www.jimmunol.org/content/176/7/4252.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Hemin Activation Ameliorates HIV-1 Infection via Heme Oxygenase-1 Induction

Krishnakumar Devadas and Subhash Dhawan

Hemin, a critical component of hemoglobin, is an active ingredient of a biologic therapeutic approved by the Food and Drug Administration for the treatment of acute porphyrias. This report describes a biological function of this molecule in inducing host defense against HIV-1 infection via heme oxygenase-1 (HO-1) induction. Treatment of monocytes with hemin substantially inhibited HIV replication, as evident by nearly undetectable viral RNA and cell-free HIV-1 p24 protein in a dose-dependent manner. Hemin exposure of these cells before infection, at the time of infection, or after infection caused >90% reduction of HIV DNA with substantially low levels of HIV-1 p24 and HIV-associated cytopathic effects. In addition, hemin treatment significantly suppressed infection of both monocytes and T cells inoculated with R5, X4, R5X4 tropic strains, and reverse transcriptase-resistant, azidothymidine-resistant, dDC/ddI-resistant, nivirapine-resistant, and other clinical HIV isolates. Intraperitoneal administration of hemin 4 days after HIV infection reduced viral load in the serum of human PBMC-reconstituted nonobese diabetic SCID mice by >6-fold. Suppression of HIV replication in hemin-activated cells correlated with the induction of HO-1 and was attenuated by tin protoporphyrin IX, an inhibitor of HO-1 activity, suggesting a pivotal role of this endogenous enzyme in the regulation of HIV infection. Hemin-induced HO-1 induction in the CCR-5, CXCR-4, and CD4 coexpressing GHOST(3) cells was consistent with the inhibition of Tat-dependent activation of long terminal repeat promoter leading to reduced GFP expression. These findings suggest an important role of hemin-induced HO-1 activity as a host defense mechanism against HIV-1 infection. The Journal of Immunology, 2006, 176: 4252–4257.

Hemin is the prosthetic moiety for a large number of proteins that play essential roles in the transport of oxygen, mitochondrial functions, and a variety of signal transduction pathways. It up-regulates heme oxygenase-1 (HO-1), an intracellular enzyme that catalyzes the initial and rate-limiting step in the oxidative degradation of heme, and generates biliverdin, free iron (Fe2+), and carbon monoxide (CO) (1). Over the past decades, investigations of the functional role of HO-1 activity have greatly enhanced our understanding of this enzyme as a cellular defense mechanism against stress-related and various other pathogenic conditions (2–5). The beneficial effects of HO-1 have also been reported in a variety of immune responses and inflammation (6). In experimental inflammatory autoimmune disorders such as encephalomyelitis and colitis (7, 8), protective functions of HO-1 have been shown to be associated with the activation of the immune system.

In addition to conditions related to immune and inflammatory responses, the life cycle of HIV-1 is closely associated with the activation state of its host cells. HIV-1 infection is dependent on a variety of host factors for entry, transcription, and viral gene expression and is controlled by cytokines and the host transcriptional machinery (9). Although numerous previous and recently published reports have demonstrated multiple prominent actions of HO-1 induction in various anti-inflammatory processes, its role in the regulation of HIV-1 infection is not known. The present study investigated the role of hemin-induced HO-1 as a putative protective factor against HIV infection. The findings from this study demonstrate that hemin-mediated augmentation of HO-1 activity substantially suppresses HIV replication both in vitro and in vivo with no apparent toxic effects. Since the 1970s, when it was approved by the Food and Drug Administration, hemin has been used to successfully treat a variety of disorders, such as acute porphyrias, control of liver allograft failure due to recurrence of erythropoietic protoporphyria, and thalassemia intermedia (10–12) with minimal side effects. Our results, therefore, may provide new strategies to develop potentially safe therapeutic interventions for the treatment of HIV infection.

Materials and Methods

Reagents

The stock solutions of hemin and HO-1 inhibitor tin protoporphyrin (SnPP IX; Frontier Scientific) were prepared in 0.2 N NaOH, neutralized to pH 7.4 using 1 N HCl, and stored at −70°C until used. Rabbit anti-human HO-1 Ab was purchased from Stressgen. The HIV-1Ba-L strain was purchased from Advanced Biotechnologies. Other clinical virus isolates were obtained through the National Institutes of Health AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Isolation, culture, and infection of cultured monocyte

Human monocytes were isolated from PBMC of donors seronegative for HIV-1 and hepatitis B after leukopheresis and were purified by counter-current centrifugal elutriation (13). Cell suspensions contained >95% monocytes based on cell morphology in Wright-stained cytosmears, granular peroxidase, and nonspecific esterase. The cells were cultured for 5 days in DMEM supplemented with 10% FBS, 20 μg/ml gentamicin, and...
1000 U/ml M-CSF and were infected with HIV-1 \textsubscript{Ba-L} as previously described (14). Cell-free supernatants were assayed for HIV-p24 using a NEN/DuPont ELISA analysis kit (PerkinElmer).

**Isolation and infection of PBL**

PBMC, isolated from whole blood by Ficolldiatrizoate density gradient centrifugation, were cultured in RPMI 1640 (BioSource) with 10 μg/ml PHA (Sigma-Aldrich), 10% purified human IL-2 (Advanced Biotechnologies), and 15% heat-inactivated FBS (Invitrogen Life Technologies). Cells cultured for 3 days were exposed to T cell tropic viral strains at a multiplicity of infection (MOI) of 0.01 infectious virus/target cell. Culture supernatants were examined for p24 by ELISA (PerkinElmer). All culture reagents were tested using the Limulus lysate assay (Associates of Cape Cod) for endotoxin contamination, and the levels were <0.06 endotoxin U/ml.

**Culture of GHOST(3) cells**

GHOST(3) cells (15–17) were cultured in DMEM supplemented with 10% FBS, 500 μg/ml gentamicin, 100 μg/ml hygromycin, 1 μg/ml puromycin, and 100 U/ml penicillin/streptomycin at 37°C in 5% CO\textsubscript{2}.

**Electroporation of cells**

Cells were electroporated as previously described (18). GHOST(3) cells were cultured at a density of 0.5–0.8 × 10\textsuperscript{6} cells/ml in complete medium in the presence or absence of 100 mM hemin for 24 h. Typically 5 × 10\textsuperscript{6} cells were electroporated with 5 μg of either HIV-Tat or heat-inactivated HIV-Tat (Δ Tat) protein at 960 μF and 240 V in RPMI 1640 medium without serum. After electroporation, cells were plated in six-well plates in 4 ml of complete medium, incubated at 37°C for 48 h, and examined for GFP expression by fluorescence microscopy.

**Detection of HO-1 expression by Western blot analysis**

Monocytes and GHOST(3) cells were cultured for 24 h in complete medium. Cells were washed once with PBS (BioSource) and incubated with hemin in DMEM supplemented with gentamicin. After treatment, cells were collected and washed three times with PBS (pH 7.4). After centrifugation, cell lysis was performed at 4°C in RIPA buffer (50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 1% Nonidet P-40, 1% SDS, 0.5% sodium deoxycholate, 20 mM EGTA, and a protease inhibitor mixture (Complete tablets, mini, EDTA-free; Roche). After centrifugation at 15,000 × g for 30 min, the supernatant was separated and stored at −70°C until used. The protein concentration was determined using the BCA protein assay kit (Pierce). After addition of loading buffer, equal protein amounts (30 μg) of each lysate preparation were subjected to electrophoresis using 10–20% polyacrylamide gradient gel (Invitrogen Life Technologies). Proteins were transferred to nitrocellulose by electroblotting, and nonspecific sites were blocked with 5% nonfat milk in PBS containing 0.1% Tween 20 (pH 7.4;PBS containing 0.1% Tween 20 (PBS-T)) for 18 h at 4°C. After washing three times with PBST, the blots were incubated for 1 h at room temperature with rabbit anti-human HO-1 antiserum (1/2000) and washed three times with PBST, and transferred proteins were incubated with the ECL Western blotting detection system (Amersham Biosciences) for 1 min according to the manufacturer’s instructions, followed by visualization with x-ray film.

**DNA and RT-PCR**

Cellular RNA and DNA were extracted using a DNA isolation kit (Qia-gen). First-strand cDNA synthesis was performed with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies) using 1–2 μg of total cell RNA. Total cellular DNA and cDNA samples were analyzed by PCR amplification (19) using the AccuPrime Taq DNA Polymerase System (Invitrogen Life Technologies) and HIV-1 \textit{env}-specific PCR primers (5'-TCTTAAGGACGACGCAAGCAGCATATTGG-3' (forward) and 5'-AAGCAGAAGTGTATCCTGCTAA-3' (reverse)). The PCR products were separated on a 2% agarose gel using Trisborate buffer and were visualized by ethidium bromide staining.

**Reconstitution of human NOD-SCID (hu-NOD-SCID) mice with human PBMC and infection with HIV-1**

Hu-NOD-SCID mice at 6–8 wk of age (The Jackson Laboratory) were reconstituted with human PBMCs and infected with HIV-1 as previously described (20) with slight modifications. Briefly, human PBMCs were isolated from a healthy HIV-1-seronegative donor and injected i.p. into 20 NOD-SCID mice (40 million cells/mouse). After 2 wk, all 20 mice were inoculated i.p. with 10\textsuperscript{7} half-maximal tissue culture infective dose (TCID\textsubscript{50}) of a T cell-tropic HIV-1 isolate. Five days after infection, hemin (4 mg/kg) was administered daily i.p. to a group of 10 mice. Ten mice were injected daily with 0.2 ml of PBS i.p. and served as the controls. On day 7, mice were reinjected with 10\textsuperscript{7} TCID\textsubscript{50} HIV-1 to ensure infectivity. Two weeks after the first hemin or PBS injection, mice were killed, and sera were collected and stored at −70°C until assayed for HIV-1 p24 by ELISA.

**Statistics**

The statistical values were calculated by one-way ANOVA with Dunnett’s multiple comparison test.

**Results**

Chemically known as chloro-[7,12-diethenyl-3,8,13,17-tetramethyl-21H,23H-porphine-2,18-dipropanoato(2-)-N21,N22,N23,N24\textsuperscript{+}]iron, hemin represents a critical component of the hemoglobin molecule, the structure of which is shown in Fig. 1A. Hemin-mediated signaling pathways involving HO-1 activation have been implicated in a variety of inflammatory disorders. However, its function in regulating HIV infection has remained unknown. To examine the role of HO-1 activation in HIV infection, monocytes were exposed to hemin, infected with HIV-1, and examined for virus replication.
As shown in Fig. 1B, treatment of cultured monocytes with hemin efficiently inhibited viral RNA expression in a dose-dependent manner. Consequently, HIV-1 replication was completely inhibited at a 100-μM optimal concentration (Fig. 1C). HIV-1 replication remained suppressed even when monocytes were infected with high MOI, as evident by undetectable cell-free HIV-1 p24 production measured on day 7 after infection (Fig. 1D).

Hemin treatment of monocytes 24 h before HIV infection significantly suppressed viral DNA expression (Fig. 2A). The levels of HIV DNA in cells treated with hemin at the time of infection were also substantially lower compared with untreated controls. To determine the ability of hemin to suppress postentry viral replication, cells were incubated with hemin 4 h after viral exposure, and genomic DNA was analyzed for the presence of HIV env-specific sequences by PCR amplification at 24 h. The results of these experiments show that hemin treatment 4 h after HIV inoculation, which allows sufficient time for viral entry, also significantly reduced viral gene expression. The set of primers used for PCR amplification was specific to highly conserved HIV-1 envelope sequences. The PCR products from cellular DNA or cDNA from uninfected cells using the same primers were negative for HIV bands, suggesting that the amplified PCR products from HIV-infected cells were highly specific. Treatment of monocytes with hemin 24 h before infection, at the time of infection, or even 24 h after infection substantially suppressed HIV replication (Fig. 2B). These results were consistent with a marked reduction of HIV-associated cytopathic effects in monocytes treated with hemin 24 h before infection (−24 h), at the time of infection (0 h), or 24 h after infection (+24 h) as shown in Fig. 2C.

To determine whether the hemin-induced cellular response was a host defense mechanism for the refractoriness of cells to HIV infection and, hence, was independent of viral tropism, hemin-treated monocytes were challenged with X4, R5, and dual tropic R5X4 viruses, and culture supernatants were examined for p24 on day 7 after infection. As expected, in the absence of hemin, monocytes were infectable with R5 (92US714 HIV-1 env subtype B, and 93IN101 HIV-1 env subtype C) and R5X4 (92RW009 HIV-1 env subtype A, and 92HT596 HIV-1 env subtype B) virus strains. However, surprisingly, except for HIV-1 92UG046 (HIV-1 env subtype D), HIV-p24 was practically undetectable in culture supernatants infected with the other two known X4 tropic viral strains CMU02 (HIV-1 env subtype EA) and 98IN017 (HIV-1 env subtype C). Nevertheless, regardless of viral tropism, hemin treatment inhibited infection of monocytes with all tested HIV-1 isolates (Fig. 3A). Similarly, in addition to the laboratory-adapted virus isolates, hemin treatment inhibited HIV replication in PBL infected with an azidothymidine-resistant virus containing a mutation in RT amino acid residue 215Y (HIV-1 RTMF/MT-2), a mutation in RT amino acid residue 215V (HIV-1 RTMF/MT-2) rendering resistance to 2′,3′-dideoxyinosine and 2′,3′-dideoxycytidine, a nevirapine-resistant virus (N119) (21–23), as well as a virus isolated from patients infected with X4 tropic 92UG029 (HIV-1 env subtype A) and R5X4 tropic 93BR020 (HIV-1 env subtype F) viral strains (Fig. 3B).

![FIGURE 2](http://www.jimmunol.org/Downloaded_from http://www.jimmunol.org)
To evaluate whether hemin-induced anti-HIV activity is preserved in vivo, NOD-SCID mice implanted with human PBMC were inoculated with HIV-1 (10^3 TCID_{50}/mouse) and then 5 days later administered a 4 mg/kg daily dose. On day 7, mice were reinjected with 10^4 TCID_{50}/mouse HIV-1 to ensure productive infection. As shown in Fig. 4, the HIV-1 p24 level in the sera of infected mice was detectable on day 4, 1 day before hemin administration. On day 14 after the first inoculation, the level of p24 was 6-fold lower than that in the untreated controls (Fig. 4). The suppression of HIV replication by hemin treatment of mice was consistent with the in vitro inhibition of HIV replication and the reduced viral gene expression.

Hemin is known to induce HO-1 expression in a variety of cell types. Treatment of monocytes with hemin induced HO-1 expression in a dose- and time-dependent manner (Fig. 5A). The maximum induction was observed when cells were incubated with 100 μM hemin for 18 h at 37°C. These data were consistent with the suppression of HIV replication, as described above. No cytotoxic effects were observed at these concentrations (data not shown). To determine the specificity of hemin-induced HO-1 activity in mediating anti-HIV-1 activity, monocytes were incubated with various concentrations of SnPP IX, an inhibitor of HO-1 function, 1 h before hemin treatment at 25 μM for 1 h, infected with HIV-1, and then examined for cell-free HIV-1 p24 5 days after infection. The results from this experiment shown in Fig. 5B demonstrate that treatment with SnPP IX at an optimal concentration of 6.25 μM attenuated hemin-induced inhibition of HIV infection, suggesting that the hemin-mediated suppression of HIV infection was mediated by HO-1 induction. A low concentration of hemin (25 μM)

was used in these experiments due to the cytotoxic effects of SnPP at high concentrations. In another set of experiments, 12.5 μM SnPP attenuated suppression of HIV replication by hemin at 50 μM (data not shown). SnPP is a competitive inhibitor for HO-1 enzyme activity (24). Therefore, it attenuated the HO-1 enzyme activity without affecting protein expression (data not shown).

GHOST(3) cells express CD4, R5 as well as X4 coreceptors, and a Tat-dependent GFP reporter cassette. These cells, therefore, represent an ideal model system to visualize infection with viruses using one or more coreceptors. Similar to monocytes, treatment of GHOST(3) cells with hemin induced HO-1 expression in a dose-dependent manner (Fig. 6A). These cells were infected with an R5X4-tropic HIV-1 strain and examined for GFP protein by fluorescence microscopy. As shown in Fig. 6B, GHOST(3) cells were productively infected, as evidenced by the HIV-long terminal repeat (LTR)-driven GFP expression and associated cytopathic effects. Hemin treatment significantly suppressed the induction of GFP expression, indicating a low level of HIV replication. No GFP expression was observed in the uninfected cells. Consistent with these observations, hemin treatment substantially reduced HIV-associated cytopathic effects, and as expected, no such effects were
observed in uninfected cells (Fig. 6B). GFP expression in GHOST cells depends on intracellular Tat produced by productive HIV infection. To confirm that GFP induction in productively infected cells was driven by Tat protein, uninfected GHOST(3) cells were pretreated with hemin for 24 h, electroporated with exogenous recombinant HIV-1 Tat protein, and then examined for GFP expression. As shown in Fig. 6C, the expression of GFP was induced by Tat protein even in the absence of HIV infection. As observed with HIV-infected cells, treatment with hemin significantly inhibited GFP expression in cells electroporated with exogenous Tat protein. Heat-inactivated Tat protein (∆Tat) or buffer alone did not cause GFP induction. The cell viability, determined by trypan blue exclusion test, after Tat transfection was in >90%. GFP expression was observed in >80% of cells, suggesting the transfection efficiency to be >80%. The background fluorescence in cells transfected with either heat-inactivated Tat or buffer alone was substantially less than that in cells transfected with active Tat protein, providing additional support for these observations. These results provide evidence for the inhibition of Tat-dependent activation of the LTR promoter, leading to reduction of GFP expression and, hence, reduced HIV replication in hemin-treated GHOST(3) cells.

Discussion

The present study demonstrates a function of HO-1 activity as a potent host defense factor for HIV-1 infection. Our results clearly show that activation of HO-1 by its substrate hemin protected them against HIV infection with various clinical HIV isolates, including some of those that developed resistance to conventional antiretroviral drugs. In vivo, hemin administration in humanized NOD-SCID mice substantially suppressed HIV replication. These findings suggest that the HO-1 inducer, hemin, is a potentially effective endogenous biologic in inducing a host defense response against HIV infection. A critical component of a variety of proteins, including hemoglobin, hemin has been shown to exert numerous beneficial physiological functions (25). After being approved by the Food and Drug Administration, several formulations of hemin have been used since the 1970s to successfully treat acute porphyries, to control liver allograft failure due to recurrence of erythropoietic protoporphoria, and in patients with thalassemia intermedia (10–12) with minimal side effects. Therefore, subject to further studies, hemin could serve as a novel therapeutic biologic for the treatment of HIV infection.

Although there was substantially lower HIV infectivity relative to the untreated controls, detectable HIV DNA in the infected cells pretreated with hemin suggests that viral entry was substantially inhibited, but not completely blocked. Similarly, hemin exposure to cells preinoculated with HIV also suppressed virus replication by >90%. Therefore, reduced viral entry into the target cells may not be the sole factor responsible for the suppression of HIV infection. Since entry, there are multiple steps in the HIV life cycle (9), such as viral integration, entry, or suppression of HIV-LTR activation, where intracellular hemin or hemin-induced HO-1 could interfere and retard virus replication. Despite the presence of viral DNA in the infected cells, the inability of HIV to productively replicate strongly suggests a putative role of hemin in inhibiting postentry events. Our data provide a direct correlation between HO-1 induction and inhibition of HIV replication in hemin-activated cells. Attenuation of hemin-suppressed HIV replication by SnPP IX, an inhibitor of HO-1 activity, further supports this endogenous inducible enzyme as an important factor in modulating HIV infection.

Infections with dual tropic viruses that use both R5 and X4 coreceptors for their entry also have been associated with T cell depletion and progression to AIDS (26). We have shown that hemin treatment inhibited infection of cells not only with laboratory viral isolates, but also with various clinical isolates, using multiple coreceptors for their entry into target cells. In addition, cells treated with hemin were refractory to infection with azidothymidine-resistant and other drug-resistant or mutant HIV strains. These results indicate that regardless of viral tropism, hemin-activated cells remained protected from infection by all HIV-1 strains tested in this study. Most drugs currently used for the treatment of HIV infection are synthetic compounds and elicit highly undesirable side effects in HIV-infected individuals (27–29). Highly activated antiretroviral therapy, although beneficial in suppressing viremia in infected individuals, may contribute to drug mutants after prolonged use and induce metabolic adverse effects, such as lipodystrophy, hypertension, diabetes mellitus, osteopenia, and hyperlipidemia (30–32). The lipodystrophic syndrome affects up to 60% of HIV-infected patients treated with highly activated antiretroviral therapy and, for this reason, is emerging as a serious medical concern. Induction of host resistance to HIV infection by hemin, an endogenous host factor reported in the present study, could provide an attractive alternative strategy for the development of novel therapeutic interventions for the treatment of HIV infection. This approach could be potentially very advantageous, especially for treating infections with multiple viral isolates, recombinant strains, and drug-resistant HIV strains. These significant findings should be further explored in other drug-resistant viral strains.

Hu-NOD-SCID mice have been widely accepted as a valuable model to study HIV pathogenesis and testing of antiretroviral drugs (20). Our data demonstrate that administration of hemin significantly suppressed HIV infection in hu-NOD-SCID mice with no behavioral changes, weight loss, or other apparent toxicity. These results were consistent with the in vitro findings. The dose of hemin used in this study is typically used to treat patients with a variety of clinical symptoms. Therefore, hemin treatment may provide a potentially safe therapeutic biologic for the treatment of HIV infection. Other pathogenic effects caused by HIV infection of hu-NOD-SCID mice and possible attenuation or reconstitution of the immune system by hemin treatment are being explored and will be the subject of future studies.

Patients with AIDS commonly develop various hematological abnormalities, such as anemia, leucopenia, thrombocytopenia, and alterations of stem cell plasticity in the bone marrow microenvironment (33–36). These clinical conditions suggest that HIV-1 infection may affect processes involved in the early stages of hemopoiesis or stem cell differentiation. The latter could be the result of high levels of circulating proinflammatory cytokines in HIV-infected individuals (33–35). Recently, induction of HO-1 has been shown to mediate the anti-inflammatory response in vivo (37). Thus, it is likely that hemin-mediated HO-1 induction may suppress the deleterious effects of proinflammatory cytokines in HIV infection. Currently, hemin is successfully used for the treatment of acute porphyries (38). Therefore, subject to future studies, the limited use of this biologic component could provide potentially beneficial clinical outcome with controlled adverse effects.

In conclusion, the findings of the present study provide strong evidence for HO-1 as a novel endogenous biologic component with anti-HIV activity. The in vivo inhibition together with the in vitro inhibition activity warrant that HO-1-inducing substrate hemin be developed as a potential therapeutic agent for the treatment of HIV infection.

Acknowledgments

We thank Drs. Zhaohua Zhou and Dennis Kopecko for critical review of the manuscript, and Dr. Indira Hewlett for support. We also thank Faith Williams for excellent artwork. The HIV-1 Tat protein and virus
strains used in this study were contributed by Drs. J. N. Brady, B. A. Larder, M. H. St. Clair, and D. Richman and by other unlisted investigators and were obtained through the National Institutes of Health AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Disclosures

The authors have no financial conflict of interest.

References