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Hemin Uptake and Release by Neurons and Glia

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Abstract

Hemin accumulates in intracerebral hematomas and may contribute to cell injury in adjacent tissue. Despite its relevance to hemorrhagic CNS insults, very little is known about hemin trafficking by neural cells. In the present study, hemin uptake and release were quantified in primary murine cortical cultures, and the effect of the hemin-binding compound deferoxamine (DFO) was assessed. Net uptake of ^{55}Fe-hemin was similar in mixed neuron-glia, neuron, and glia cultures, but was 2.6–3.6-fold greater in microglia cultures. After washout, 40–60% of the isotope signal was released by mixed neuron-glia cultures into albumin-containing medium within 24 hours. Inhibiting hemin breakdown with tin protoporphyrin IX (SnPPIX) had minimal effect, while release of the fluorescent hemin analog zinc mesoporphyrin was quantitatively similar to that of ^{55}Fe-hemin. Isotope was released most rapidly by neurons (52.2±7.2% at 2 hours), compared with glia (15.6±1.3%) and microglia (17.6±0.54%). DFO did not alter ^{55}Fe-hemin uptake, but significantly increased its release. Mixed cultures treated with 10 μM hemin for 24 hours sustained widespread neuronal loss that was attenuated by DFO. Concomitant treatment with SnPPIX had no effect on either enhancement of isotope release by DFO or neuroprotection. These results suggest that in the presence of a physiologic albumin concentration, hemin uptake by neural cells is followed by considerable extracellular release. Enhancement of this release by DFO may contribute to its protective effect against hemin toxicity.

Keywords

heme; intracerebral hemorrhage; iron; ischemia; stroke; subarachnoid hemorrhage

Introduction

Hemin is released from methemoglobin after an intracerebral hemorrhage, and reaches micromolar concentrations in the hematoma [1]. Although it is a lipophilic compound that can directly intercalate into the membranes of adjacent cells, recent evidence indicates that its uptake is regulated at least in part by the action of one or more transport proteins [2–4]. In excess, hemin is a potent neurotoxin that directly catalyzes free radical chain reactions [5]. Its breakdown by the heme oxygenase (HO) enzymes releases iron, which may further increase oxidative stress if not rapidly sequestered or exported [6].

Despite its likely relevance to hemorrhagic CNS injuries, hemin trafficking by neural cells has not been intensively investigated. In vitro experiments using non-neural cell lines indicate that hemin loading is followed by substantial export over the following few hours

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Declaration of Interest

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[7,8]. This process is facilitated by extracellular albumin or hemopexin, which are acceptor proteins for the putative cell membrane hemin exporters that have been identified to date [8,9]. Loss of this export capacity markedly increases cellular vulnerability to exogenous hemin [10], suggesting that it is an important defense against hemin toxicity. This key observation raises the possibility that enhancing hemin export may also be beneficial after CNS hemorrhage, provided that hemin trafficking is similar in cells of neural origin. However, quantitative data about hemin export in neurons, astrocytes, and microglia are completely lacking. Furthermore, no low molecular weight pharmaceuticals that accelerate hemin loss from neural cells have been identified to date.

In the present study, we first tested the hypothesis that primary cultured neural cells release hemin into the extracellular space within hours of uptake. We subsequently tested the hypothesis that hemin release could be enhanced by deferoxamine (DFO), a chelator with well-characterized hemin-binding properties that increases its release from erythrocytes [11,12].

Methods

Cell cultures

Primary cultures containing neurons only or mixed neurons and glia were prepared in 24-well plates from fetal mice (gestational age 14–16 days) following previously published methods [13,14]. Glial cultures (predominantly astrocytes, >90% GFAP+) were prepared from 2–3 day postnatal mice using a similar protocol [13]. Microglial cultures were prepared by harvesting microglial cells from confluent mixed glial cultures growing in 80 cm^2 flasks (Nunc 153732) by shaking, also as previously described [15]. All procedures using animals for culture preparation were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee.

Quantification of hemin and zinc mesoporphyrin uptake and release

Hemin trafficking was studied using ^{55}Fe-hemin (Perkin Elmer, Waltham, MA, USA) or zinc mesoporphyrin (ZnMP, Frontier Scientific, Logan, UT, USA). The latter is a fluorescent hemin analog that has previously been validated as a surrogate for hemin in uptake and export studies [8,16]. Cultures were washed free of serum and were then placed into uptake medium containing Minimal Essential Medium (MEM) with 10 mM glucose (MEM10), 0.67 mg/ml bovine serum albumin (BSA), and 100 μg/ml apotransferrin. The albumin concentration used was similar to that observed in cerebrospinal fluid after hemorrhagic stroke [17]; apotransferrin was added to prevent iron-mediated injury [18]. Loading concentrations and exposure durations for ^{55}Fe-hemin (5 μM x 2h, 0.38μCi/ml) and ZnMP (1 μM x 0.5h) were determined from preliminary experiments that demonstrated that they were nontoxic in this culture medium. After incubation at 37°C for the defined interval, cultures were washed once with 500 μl MEM10 containing 1mg/ml BSA, followed by three additional washes with 500 μl MEM10 alone. Cells were then either immediately lysed by treatment with 0.1% Triton X-100 or were placed into release medium that was identical to uptake medium except for the absence of ^{55}Fe-hemin and ZnMP. Medium was collected at defined intervals; cells were then washed and lysed as described above. In some experiments, DFO (Sigma-Aldrich, St. Louis, MO, USA), 2,2′-dipyridyl (DP, Sigma-Aldrich) or tin protoporphyrin IX (SnPPIX, Frontier Scientific) were present in the uptake or release medium. After quantification of medium and lysate radioactivity by liquid scintillation counting or fluorescence measurement (Ex 410 nm, Em 580 nm), the ^{55}Fe-hemin or ZnMP content was calculated, and was then normalized to culture protein (BCA assay). Net uptake is defined as uptake minus release (i.e. export or extraction from

membranes) during the incubation period. Net release is defined as export or extraction after washout minus re-uptake prior to cell lysis.

Neurotoxicity assay

Mixed neuron-glia cultures were washed free of serum and then were treated with unlabeled hemin (Frontier Scientific) alone or with study drugs in MEM10 containing 0.67 mg/ml BSA and 100 μg/ml apotransferrin. After exposure for 24 hours, culture medium (25 μl) was sampled; cell death was then quantified by lactate dehydrogenase (LDH) assay [19]. In other experiments, cultures were treated with unlabeled hemin for 2 hours using the same protocol described above for uptake and release experiments; study drugs were then added after hemin washout, and medium was sampled for LDH activity 24 h later. The mean LDH value in the medium of sister cultures exposed to washes only was subtracted from all values to yield the signal that was due to hemin toxicity, following the protocol of Koh and Choi [20]. In order to facilitate comparison of cell death in cultures with varying neuronal densities, all LDH values were normalized to those in sister cultures treated with 300 μM N-methyl-D-aspartate (NMDA); this treatment kills all neurons.

Statistical analysis

Data were analyzed by one-way ANOVA, followed by the Bonferroni multiple comparisons test to assess differences between three or more experimental groups. Differences between two groups were assessed with an unpaired t-test.

Results

Hemin is released from cortical cells

In initial experiments, hemin release was quantified at defined intervals after loading, using mixed cultures containing both neurons and glia. Approximately one quarter of the isotope signal was present in the medium at two hours after ^{55}Fe-hemin washout, and 40–60% by 24 hours (Fig 1 A, B). In order to determine if blocking cell hemin breakdown would alter the extracellular signal, additional experiments were conducted in the presence of the HO inhibitor tin protoporphyrin IX (SnPPIX) in both the uptake and release media. At concentrations that inhibit hemin breakdown in neural cells [21], SnPPIX had no effect on the extracellular ^{55}Fe signal at 4 hours, but did reduce it slightly (~10%) at 24 hours at the highest concentration tested (Fig. 1B). The release of the fluorescent hemin analog ZnMP [8,16] from these cultures at 24 hours was quantitatively similar to that of ^{55}Fe-hemin, although ZnMP release proceeded somewhat more rapidly on a percentage basis (Fig. 1C).

^{55}Fe-hemin uptake and release were next compared in different neural cell populations (Fig. 2). Uptake was similar in cultures containing neurons, glia (>90% GFAP+), or a mixture of neurons and glial cells. However, it was 2.6–3.6-fold greater in cultures containing only microglia ($P < 0.001$ vs. other conditions). After ^{55}Fe-hemin washout, its release from cells proceeded most rapidly in cultures containing only neurons, with over half detected in the medium at 2 hours, and over 70% at 24 hours. In contrast, pure microglial cultures released only about one-sixth of ^{55}Fe-hemin at 2 hours, and only ~40% by 24 hours.

Effect of DFO on hemin uptake and release

DFO binds hemin by interacting with its iron moiety, and is the only pharmaceutical described to date that increases cellular hemin release [11,12]. We hypothesized that this compound, which is protective in models of CNS hemorrhage [22,23] would reduce hemin uptake by neural cells and increase its release. These experiments were conducted in mixed neuron/glia cultures, which may be more relevant to conditions in the intact CNS than cultures containing only a single cell population. Contrary to our hypothesis, DFO had no

effect on net hemin uptake, but it increased release into the culture medium by approximately 60% in the presence of BSA (Fig. 3A). 2,2′-dipyridyl, an iron chelator that increases export of nonheme iron in this culture system [24], had no effect on either hemin uptake or release. ^{55}Fe-hemin release was diminished in medium lacking BSA (58.8±4.4 pmol/mg protein vs. 129.5± 7.8 pmol/mg protein, $P < 0.001$, Figs. 3A,B), consistent with prior reports that albumin enhances hemin export [8,9]. DFO increased hemin release in a concentration-dependent fashion between 1.0 and 100 μM in BSA-free medium, with a 2.5-fold increase at the latter concentration (Fig. 3B). Enhancement of isotope release into the medium was not significantly altered by concomitant treatment with SnPPIX to inhibit hemin breakdown by the heme oxygenases (Fig. 3C).

Deferoxamine protects neurons from hemin toxicity

In mixed cultures treated continuously with hemin 10 μM for 24 hours, widespread neuronal death was observed without injury to the glial monolayer, consistent with our prior observation that neurons are selectively vulnerable to hemin in these cultures [25]. Cell loss was decreased by over 60% by concomitant DFO treatment (Fig. 4A), and was not reversed by the addition of SnPPIX to inhibit hemin breakdown and iron release. When cultures were treated with higher concentrations of hemin without DFO for only 2 hours, cell death was observed over the following 24 hours that was also attenuated when DFO was added to the release medium only (Fig. 4B).

Discussion

This study provides the first quantitative data about hemin trafficking by primary cells of CNS origin. In the presence of albumin as a physiological carrier protein, net hemin uptake in neuron, glia (primarily astrocyte), and neuron-glia cultures was similar, but it was significantly greater in pure microglia cultures. After washout, neurons rapidly released hemin, while microglia retained more hemin than other cell types. These observations suggest that hemin scavenging may be a key protective function of microglia after CNS hemorrhage that complements their established role in erythrophagocytosis [26].

Direct interaction of DFO with hemin was first reported by Baysal et al. [12] in studies investigating hemin-induced hemolysis. They reported that the DFO binding affinity for hemin was sufficient to remove it from erythrocyte ghost membranes. Hemolysis was reduced in a concentration-dependent fashion at a DFO:hemin molar ratio exceeding 2:1, while other iron chelators and ferrioxamine (DFO-Fe^{3+} complexes) were ineffective. Binding of hemin by DFO was recently characterized in detail by Lu et al. [11], who calculated a binding constant of 10^4-$10^5 M^{-1}$. Analysis of various thermodynamic parameters indicated that the interaction was likely to be spontaneous and exothermic under physiologic conditions. The present results suggest that this binding affinity may allow DFO to function like the hemin binding proteins albumin and hemopexin in facilitating hemin release from neural cells [8].

Although enhancement of hemin release may account in part for the neuroprotection provided by DFO against hemin in this study, lysosomal hemin binding by DFO may also contribute. DFO is a very hydrophilic compound that has little ability to permeate biological membranes. However, it does enter cells by fluid phase endocytosis, and subsequently concentrates in lysosomes without significantly entering the cytosol [32]. Its protective effect in these cultures could also be mediated by increasing the transit of hemin into lysosomes, as has previously been reported for ferritin-bound iron [33].

Hemin neurotoxicity may be due to direct oxidative injury and membrane destabilization [5,28] or iron release after its HO-catalyzed breakdown [29]. In a prior study, we reported

that the latter mechanism predominated in murine cortical cultures when hemin exposure was conducted in MEM10 without any added proteins [25]. This conclusion was based on the consistent protection provided by HO-2 gene knockout and iron chelation. In the present study, the iron-mediated component of injury was mitigated by the addition of BSA and apotransferrin to the culture medium [18,30]. Under these conditions, a higher hemin concentration was required to produce neurotoxicity, and HO inhibition with SnPPIX was not protective, but rather tended to worsen injury. These results suggest that the effect of HO activity on hemin neurotoxicity is dependent in part on cellular vulnerability to iron. If the cellular milieu favors chelation of iron in a manner that prevents its participation in free radical reactions, then any beneficial effect HO inhibition is likely to be lost. It is noteworthy that Wang et al. reported that HO-2 gene knockout increased the vulnerability of rat cortical neurons to hemin in culture medium containing BSA and iron-poor transferrin [31], which was attributed to reduced hemin catabolism.

Since ^{55}Fe-hemin was used in these experiments, some release of the isotope as nonheme iron cannot be excluded. However, four lines of evidence suggest that this accounted at most for a small part of the total signal appearing in the culture medium after washout. First, the HO inhibitor SnPPIX at concentrations sufficient to block hemin breakdown [21] had a minimal effect on isotope release alone or in the presence of DFO. Second, the iron chelator 2,2′-dipyridyl, which enhances the cellular export of non-heme iron in this culture system [24], had no effect. Third, albumin increased isotope release, consistent with observations that it facilitates hemin export by serving as an extracellular acceptor protein [8,9]. Fourth, release of the fluorescent hemin analog zinc mesoporphyrin, which is not a substrate for HO but rather a potent HO inhibitor [27], was quantitatively similar to that of ^{55}Fe-hemin.

The molecular mechanisms that mediate hemin uptake and release in neural and other cell populations remains undefined, and are worthy topics for future investigation. Although hemin intercalates directly into cell and organelle membranes, its anionic carboxylate side chains limit cell entry by passive diffusion [34], indicating a need for transporters to meet cellular needs and prevent toxicity [35]. Several membrane proteins that bind hemin in vitro and may serve as importers have recently been identified, including protein coupled folate transporter/heme carrier protein-1, HRG-1, and FLVCR2 [2,3]. However, due to their relatively low affinity for hemin and interaction with other ligands, hemin import activity under normal or pathological conditions has not yet been confirmed. ABCG2 and FLVCR1 are cell membrane proteins with a putative hemin export function [8,9]. Mutation of the latter has been associated with posterior column ataxia, retinitis pigmentosa, and Diamond-Blackfan anemia, suggesting that it may have a primary role in regulating heme/hemin levels in some neuronal populations and erythroid progenitor cells. Delineation of hemin transport mechanisms in neural cells may lead to the identification of new therapeutic targets to prevent hemin toxicity after CNS hemorrhage.

Conclusion

Neural cells respond to hemin loading by exporting a significant portion over the subsequent 24 hours. This process is most rapid and complete for neurons, and is increased by the presence of albumin and deferoxamine in the culture medium. Enhancement of hemin export may be a novel therapeutic target after hemorrhagic CNS injuries.

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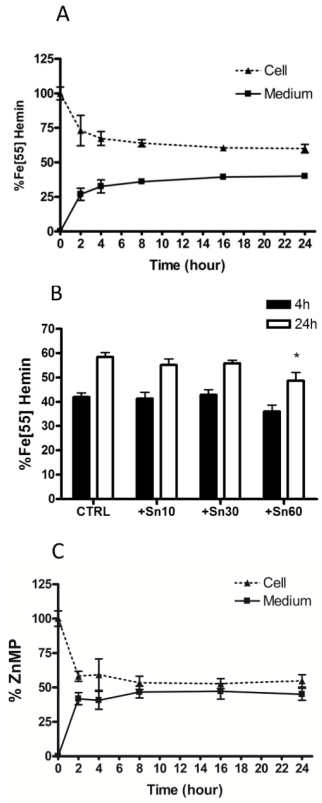

Figure 1.

Time course of hemin release from neuron-glia cultures, and effect of HO inhibition. A) Percentage ^{55}Fe signal in cell lysate and culture medium at indicated time points after ^{55}Fe-hemin loading; B) Effect of indicated concentrations (μM) of the HO inhibitor tin protoporphyrin IX (Sn) on percentage isotope release into the culture medium at 4 and 24 hours after ^{55}Fe-hemin loading; C) Percentage of zinc mesoporphyrin fluorescence signal in cell lysate and culture medium at indicated time points after loading. All values represent mean ± S.E.M. (*P < 0.05 vs. mean value in corresponding control cultures (CTRL) lacking tin protoporphyrin IX, Bonferroni multiple comparisons test, n = 6 per condition).

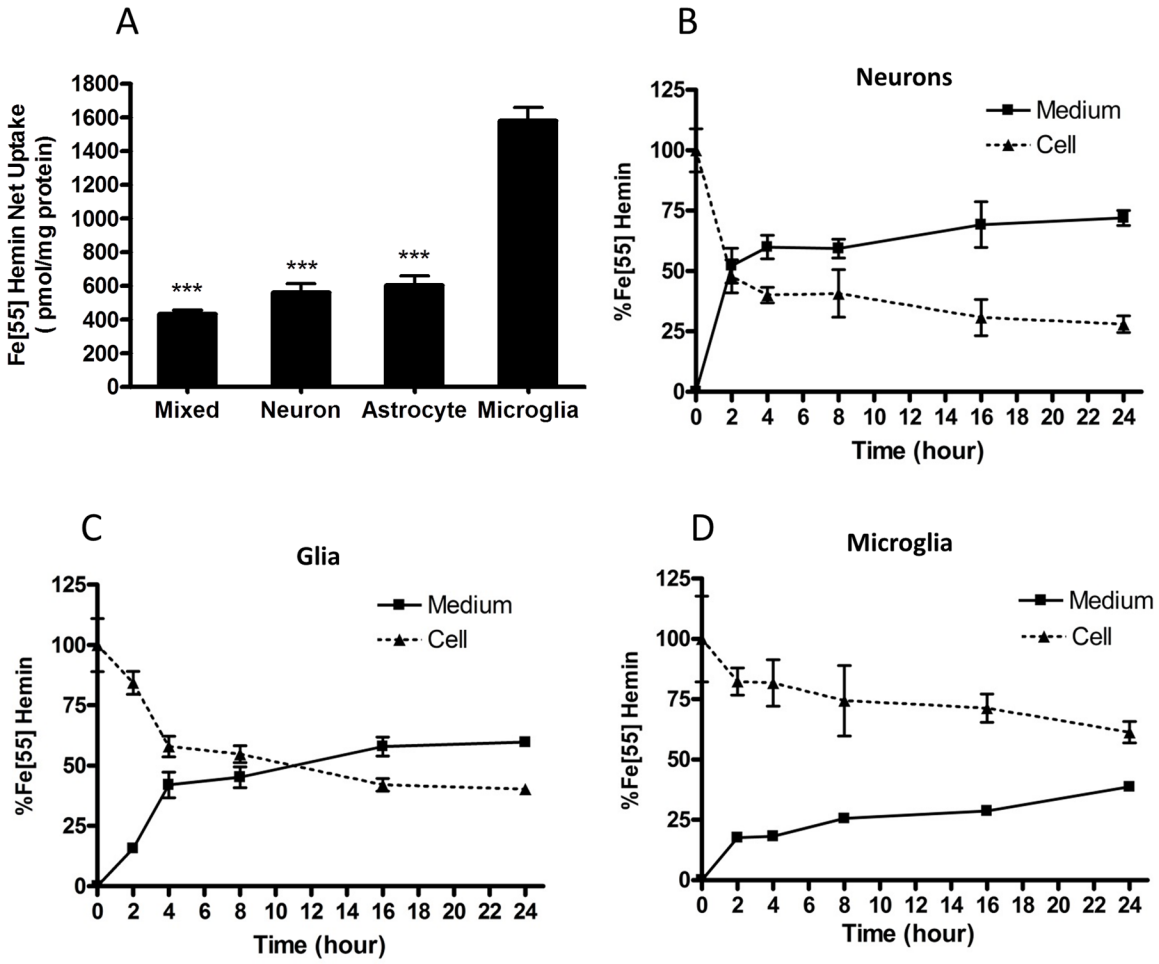

Figure 2.

Comparison of ^{55}Fe-hemin uptake and release in neurons, glia, and microglia. A) ^{55}Fe-hemin signal (± S.E.M.) in cell lysates of indicated culture types after 2 hour treatment with 5 μM ^{55}Fe-hemin. B–D) % ^{55}Fe signal in cell lysate and culture medium at indicated time points after ^{55}Fe-hemin loading. ***P < 0.001 vs. mean value in microglia cultures, n = 8–10/condition).

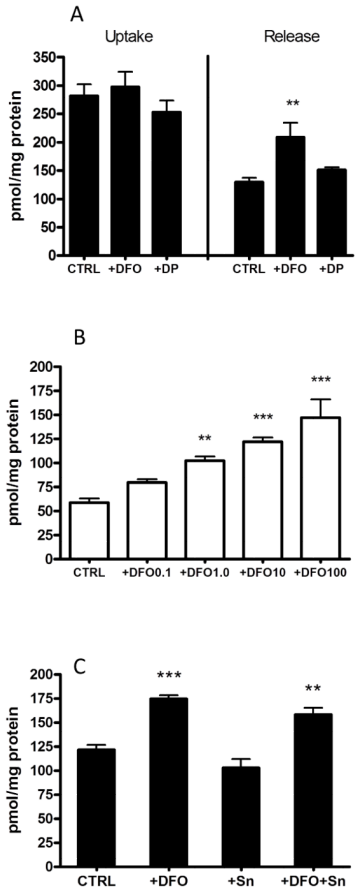

Figure 3.

Deferoxamine (DFO) increases release of cell hemin. A) Uptake: bars represent mean cell ^{55}Fe signal in mixed cortical cultures after two hour incubation with 5 μM ^{55}Fe-hemin alone (CTRL) or with 100 μM DFO or 2,2′-dipyridyl (DP); Release: mean medium ^{55}Fe signal at 24 hours after ^{55}Fe-hemin washout followed by incubation in medium containing 0.67 mg/ml bovine serum albumin alone (CTRL) or with 100 μM DFO or DP. B) Medium ^{55}Fe signal at 24 hours after ^{55}Fe-hemin washout followed by incubation in BSA-free medium alone (CTRL) or with indicated concentrations of DFO. C) As in A, testing the effect of concomitant treatment with the HO inhibitor tin protoporphyrin IX (Sn, 60 μM) on enhancement of ^{55}Fe-hemin release by DFO, in medium containing BSA. **P < 0.01, ***P < 0.001 vs. mean value in corresponding CTRL condition.

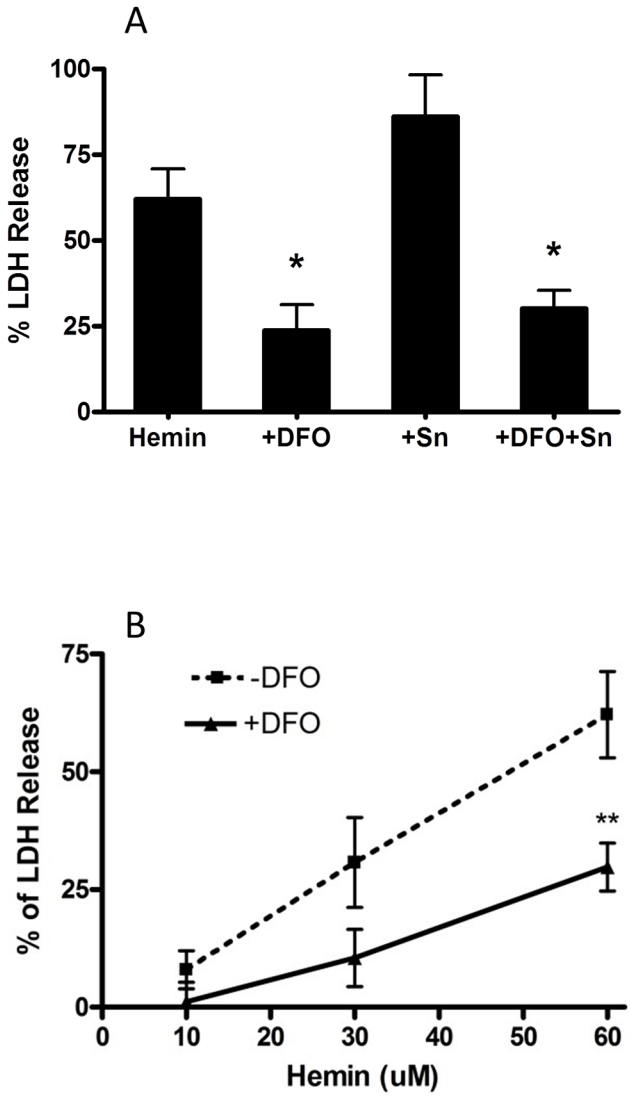

Figure 4.

Deferoxamine attenuates hemin neurotoxicity. A) Bars represent mean LDH release (± S.E.M., n = 10–12/condition) in cultures treated for 24 hours with 10 μM hemin or with 10 μM hemin plus 100 μM DFO, 60 μM tin protoporphyrin IX (Sn) or both DFO and tin protoporphyrin IX. B) Cultures (n= 6/condition) were treated with indicated concentrations of hemin for 2 hours without DFO; after hemin washout, cultures were placed into medium containing or lacking 100 μM DFO. *P < 0.05, **P < 0.01 vs. corresponding condition treated with hemin without DFO. LDH values are normalized to those in sister cultures treated with NMDA for 24 hours (=100), which kills all neurons in these cultures. The LDH signal in controls exposed to washes only was subtracted from all values to calculate the signal due to hemin toxicity.