2-2011

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As submitted to:

Neuropharmacology

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Volume 60, Issue 2-3, February 2011, Pages 423-431

DOI: 10.1016/j.neuropharm.2010.10.015

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Abstract

The protective effect of iron chelators in experimental models of intracerebral hemorrhage suggests that nonheme iron may contribute to injury to perihematomal cells. Therapy with high affinity iron chelators is limited by their toxicity, which may be due in part to sequestration of metals in an inaccessible complex. Transferrin is unique in chelating iron with very high affinity while delivering it to cells as needed via receptor-mediated endocytosis. However, its efficacy against iron-mediated neuronal injury has never been described, and was therefore evaluated in this study using an established cell culture model of hemoglobin neurotoxicity. At concentrations similar to that of CSF transferrin (50-100 micrograms/ml), both iron-saturated holotransferrin and apotransferrin were nontoxic per se. Overnight exposure to 3 μM purified human hemoglobin in serum-free culture medium resulted in death, as measured by lactate dehydrogenase release assay, of about three-quarters of neurons. Significant increases in culture iron, malondialdehyde, protein carbonyls, ferritin and heme oxygenase-1 were also observed. Holotransferrin had no effect on these parameters, but all were attenuated by 50-100 micrograms/ml apotransferrin. The effect of apotransferrin was very similar to that of deferoxamine at a concentration that provided equivalent iron binding capacity, and was not antagonized by concomitant treatment with holotransferrin. Transferrin receptor-1 expression was localized to neurons and was not altered by hemoglobin or transferrin treatment. These results suggest that apotransferrin may mitigate the neurotoxicity of hemoglobin after intracerebral hemorrhage. Increasing its concentration in perihematomal tissue may be beneficial.

Key Words: intracerebral hemorrhage; iron; oxidative

1. Introduction

Hemoglobin is present in millimolar concentrations in an intracerebral hematoma. Although most of this hemoglobin appears to remain within intact erythrocytes until they are phagocytosed by microglia and macrophages (Wagner and Dwyer, 2004), increased nonheme iron staining in perihematomal tissue indicates that at least some is released and metabolized locally (Hua et al., 2006; Wu et al., 2003). Attenuation of tissue edema, oxidation, cell death, and behavioral deficits by deferoxamine and other metal chelators supports the hypothesis that this iron contributes to the pathogenesis of intracerebral hemorrhage (Masuda et al., 2007; Nakamura et al., 2004).

The primary limitation of effective iron chelator therapy in a clinical setting is toxicity, which may be due in part to metal deficiency (Porter and Huehns, 1989). High affinity chelators such as deferoxamine sequester iron and other essential metals in a complex that is largely inaccessible to cells. Although tolerated in patients with systemic iron overload, deferoxamine must be given at relatively low doses by continuous infusion, and the dose must be rapidly reduced as iron concentrations decline (Kushner et al., 2001; Porter and Huehns, 1989). Since tissue iron excess after ICH is highly localized, systemic administration may produce toxicity more rapidly than observed when treating iron poisoning or transfusional iron overload.

An alternative approach to exogenous chelator therapy is to enhance endogenous iron binding capacity. Prior studies have demonstrated that hyperexpression of ferritin, the primary intracellular iron storage protein, markedly reduces cellular vulnerability to hemoglobin and hemin (Balla et al., 1992; Regan et al., 2008). Sequestration of extracellular iron is primarily accomplished by transferrin, a glycoprotein that binds two Fe^{3+} ions with extraordinary affinity (stability constant 10^{22} at neutral pH), thereby preventing nonspecific iron transfer (Halliwell and Gutteridge, 1999). Compared with exogenous chelators, transferrin may be less likely to induce toxicity by cell iron deprivation. Iron-saturated holotransferrin is taken up by cells as needed via receptor-mediated endocytosis (Huebers et al., 1985), with upregulation of its receptor in iron-deficient cells (Pantopoulos, 2004). Subsequent acidification of the endosome releases

iron, which is then transported into the cytosol by divalent metal transporter-1 (Garrick and Garrick, 2009).

Despite its unique and potentially beneficial iron-chelating properties, the efficacy of transferrin in models relevant to hemorrhagic CNS injury has not been reported. Binding of iron to transferrin increases the affinity of the complex for transferrin receptor-1 (TfR1), which is expressed by most central neurons (Huebers et al., 1985; Moos et al., 2007). If transferrin-bound iron is then transported into neural cells in sufficient quantities, any therapeutic benefit of increasing transferrin iron binding capacity after intracerebral hemorrhage may be negated. Consistent with this hypothesis, Nakamura et al.(Nakamura et al., 2005) have observed that striatal injection of holotransferrin at a concentration exceeding that in serum contributed to striatal edema and DNA oxidation, and increased cell iron content. Moreover, despite an increase in nonheme iron, transferrin receptor-1 (TfR1) expression was paradoxically increased after experimental ICH (Wu et al., 2003), although the cellular source of this expression has not yet been characterized. In order to further investigate the effect of transferrin on iron-mediated toxicity, in the present study we tested the hypothesis that physiologic concentrations of transferrin would protect neurons from the iron-dependent neurotoxicity of hemoglobin.

2. Methods

2.1 Cell cultures

All procedures on animals were conducted in accordance with a protocol approved by the Thomas Jefferson University Institutional Animal Care and Use Committee. Mixed cortical cell cultures containing both neurons and glia were prepared from B6129 fetal mice at gestational age 14-16 days as previously described (Regan and Choi, 1994). Two-thirds of the culture medium was replaced on days 5 and 8-9 in vitro with minimal essential medium (Invitrogen, Carlsbad, CA, USA) containing 10% equine serum (Hyclone, Logan, UT, USA), 2 mM glutamine, and 23 mM glucose. After ten days

in vitro this procedure was performed daily. Cultures were used for experiments at 12-16 days in vitro.

2.2. Hemoglobin and transferrin exposure

On the day prior to the experiment, the serum concentration of all cultures was reduced to 3.3% by replacing two-thirds of the medium with serum-free MEM containing 10 mM glucose (MEM10). Immediately prior to hemoglobin and transferrin exposure, remaining serum was washed out of cultures with MEM10. They were then treated with purified, endotoxin-free hemoglobin (Hemosol, Inc, Etobicoke, CA), holotransferrin, and/or apotransferrin (both purchased from Calbiochem/EMD, Gibbstown, NJ, USA) in MEM10, and were incubated at 37^{o}C in a 5% CO2 atmosphere.

2.3. Cell injury assessment

At the end of the exposure interval, cultures were examined with phase contrast microscopy. Cell death was then quantified by measurement of lactate dehydrogenase (LDH) activity in the culture medium, as previously described (Regan and Rogers, 2003). The low LDH activity in sister cultures subjected to medium exchange only was subtracted from all values to yield the signal specific for the neurotoxic insult, according to the protocol of Koh and Choi (Koh and Choi, 1988). To facilitate comparisons of experiments conducted on cultures from different platings, LDH values were scaled to the mean value in sister cultures exposed to NMDA 300 μM for the duration of the experiment. This treatment releases all neuronal LDH in these cultures without injuring astrocytes. Since the low micromolar concentrations of hemoglobin that were used in this study do not injure glial cells (Chen-Roetling and Regan, 2006), the contribution of astrocyte LDH to the total signal is negligible.

After sampling medium for LDH assay, proteins were precipitated with 4.5% trichloroacetic acid. Lipid peroxidation was quantified by malondialdehyde assay, as previously described (Regan et al., 1998). Protein content was assayed by the BCA method (Pierce, Biotechnology, Rockford, IL, USA); malondialdehyde values were expressed as nanomoles/milligram protein.

Culture protein oxidation was quantified on additional cultures using the Oxyblot™ kit (Millipore, Inc., Billerica, MA, USA). Cultures were washed free of hemoglobin with MEM10, and then were lysed in an ice-cold buffer containing 210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EDTA, 0.1 % sodium dodecyl sulfate, and 0.1 % Triton X-100. Lysate proteins were protected from in vitro oxidation by adding 2-mercaptoethanol to a final concentration of 1%. Carbonyl groups were derivatized to 2, 4-dintrophenylhydrazone (DNP-hydrazone) by reaction with 2, 4-dinitrophenylhydrazine, following the manufacturer's instructions. Proteins were then separated on a 12% polyacrylamide gel (Ready Gel, Bio-Rad, Hercules, CA, USA) and were transferred onto a polyvinylidene difluoride (PVDF) transfer membrane filter (Millipore). Carbonyl groups were detected with rabbit anti-DNP (1:150) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:300). Immunoreactive proteins were visualized using Super Signal West Femto Reagent (Pierce) and Kodak Gel Logic 2200.

2.4. Immunoblotting

Cultures were washed and lysed as described for protein oxidation assay. After sonication, centrifugation, and BCA protein assay, samples (30 µg in 30 µl) were diluted with 10 µl 4X loading buffer (Tris-Cl 240 mmol/L, β-mercaptoethanol 20%, sodium dodecyl sulfate (SDS) 8%, glycerol 40%, and bromophenol blue 0.2%). Samples for heme oxygenase-1, ferritin, and actin immunoblotting were then heated to 95°C for 3 minutes; transferrin receptor-1 (TfR1) samples were incubated for 10 minutes at 37°C. Proteins were separated on 12% or 7.5% gels and transferred to PVDF membranes as described above. Membranes to be probed with anti-TfR1 were then treated with 100 mM β-mercaptoethanol, 2% SDS in 62.5 mM tris buffer (pH 6.7) according to the protocol of Kaur and Bachhawat to enhance the signal of membrane-bound proteins (Kaur and Bachhawat, 2009). After washing, nonspecific sites of all membranes were blocked with 5% non-fat dry milk in a buffer containing 20 mM Tris, 500 mM NaCl, and 0.1% Tween 20 (pH 7.5) for 1 h at room temperature. Membranes were then incubated at 4°C overnight with primary antibodies (rabbit anti-horse spleen ferritin 1:250 dilution, Sigma-Aldrich Cat No. F5762, St Louis MO, USA; rabbit anti-heme oxygenase-1, 1:

4000 dilution, Assay Designs Cat. No. SPA-895, Ann Arbor, MI, USA; mouse monoclonal anti-TfR1, 1:500, Invitrogen; rabbit anti-actin, 1: 1000, Sigma-Aldrich Cat. No. A2066). After washing, membranes were treated with horseradish peroxidase-conjugated secondary antibody (Pierce, 1:3000). Immunoreactive proteins were visualized as described in Section 2.3.

2.5. Immunostaining. Cultures were washed with MEM10 (750 ml x 3) and then were fixed in ice-cold 4% paraformaldehyde for one hour. After TBS wash, cultures immunostained with anti-transferrin receptor-1 antibody were placed into antigen retrieval buffer (10mM sodium citrate, 0.05% Tween 20, pH 6.0) and heated to 95^{o}C for 30 min in a water bath. They were then treated serially, at room temperature unless otherwise noted, with: 0.3% Tween 20 in TBS for 10 min, 10% normal goat serum in TBS with 0.05% Tween 20 for 1 hour, avidin/biotin blocking solutions for 30 min (Vector Laboratories, Burlingame, CA), monoclonal anti-transferrin receptor-1 antibody (Invitrogen, 1:100 dilution) overnight at 4ºC, biotinylated anti-mouse IgG (1:200, Vector Laboratories) for 30 min, and NeutrAvidin Rhodamine Red-X conjugate (1:400 dilution, Invitrogen) for 30 min. NeuN immunostaining was performed via the same method but with omission of the antigen retrieval step, using a 1:100 dilution of anti-NeuN (Millipore).

2.6. Enhanced Perl's Staining. Cultures were washed with PBS (750 ml x 3) and then were fixed with 4% paraformaldehyde for one hour. After another PBS wash, they were treated with Perl's solution (1:1, 2% HCl and 2% potassium ferrocyanide) for 30 minutes at room temperature. After washout, cultures were then treated with 0.05% 3,3'-diaminobenzidine (DAB, Sigma-Aldrich) in PBS for 10min, followed by 0.033% H_2O_2 in 0.05% DAB in PBS, following a modification of the procedure of Smith et al.(Smith et al., 1997). Staining of 200X images was then quantified with Kodak molecular Imaging software, Version 4.0.

2.7. Statistical analysis. Data were analyzed with one-way analysis of variance. Differences between groups were assessed with the Bonferroni multiple comparisons test. A statistically significant difference was denoted by a P value < 0.05.

3. Results

3.1. Apotransferrin attenuates hemoglobin neurotoxicity. Neurons in this highly-characterized culture system are easily identified under phase contrast microscopy by their large, phase bright cell bodies that form groups and send out extensive processes (Fig 1A). The neuronal identity of these cells has been demonstrated in prior studies by their immunoreactivity to both anti-neuron specific enolase (Regan and Panter, 1993) and anti-NeuN (Chen-Roetling et al., 2009). Consistent with prior results, overnight treatment with 3 μM hemoglobin produced injury to neurons, without morphologic change to the glial monolayer (Fig. 1B). In initial experiments, the effects of apotransferrin and holotransferrin were determined and directly compared with that of the iron chelator deferoxamine, which provides robust protection in this model (Regan and Rogers, 2003). A transferrin concentration of 50 μg/ml (0.625 μM) was chosen since it approximates that found in rodent cerebrospinal fluid (Moos et al., 1999), which has a transferrin concentration 1-2% of that in serum. Near complete protection was provided by apotransferrin, while holotransferrin had no effect (Figs. 1 C-D, 2A). Deferoxamine 1.25 μM, which has the same iron binding capacity as 0.625 μM apotransferrin, provided very similar neuroprotection. Apotransferrin and holotransferrin alone were not neurotoxic at this concentration, as measured by LDH release assay (Table 1).

3.2. Oxidative injury markers are reduced by apotransferrin. Malondialdehyde (MDA) is a sensitive marker of lipid oxidation by hemoglobin (Regan et al., 1998; Sadrzadeh et al., 1987). The approximately seven-fold increase in MDA produced by hemoglobin treatment was largely prevented by apotransferrin or deferoxamine (Fig 2B), while holotransferrin had no significant effect.

Iron catalyzes the oxidation of lysine, arginine, proline and threonine on vulnerable proteins to carbonyl groups (Stadtman and Levine, 2003), which can be derivatized and then detected using specific antibodies. Consistent with the iron-dependence of hemoglobin neurotoxicity (Sadrzadeh et al., 1987), overnight incubation with hemoglobin increased protein carbonyls in these cultures by 2.4-fold (Fig.3). This increase was completely prevented by either apotransferrin or deferoxamine, but holotransferrin had no effect.

Heme oxygenase (HO)-1 has an antioxidant response element in its promoter region that mediates its induction in an oxidative cellular environment (Tyrrell and Basu-Modak, 1994); its expression is rapidly increased in glial cells in these mixed cultures by heme or hemoglobin (Benvenisti-Zarom et al., 2006). HO-1 was minimally expressed in control cultures subject to medium exchange only (sham), but was increased 14-fold by hemoglobin treatment. About half of this increase was prevented by either apotransferrin or deferoxamine (Fig. 4). The relative increase in ferritin expression after hemoglobin treatment was quantitatively similar to that of heme oxygenase. The latter was also reduced by deferoxamine and aprotransferrin, but not by holotransferrin.

3.3. Apotransferrin reduces iron deposition after hemoglobin incubation. Perl's staining demonstrated significantly increased culture iron after overnight exposure to 3 μM hemoglobin, which was most intense in the vicinity of degenerating neuronal cell bodies (Fig. 5). Staining was reduced by 50 μg/ml (0.625 μM) apotransferrin or 1.25 μM deferoxamine, but not by 50 μg/ml of holotransferrin.

3.4. Holotransferrin does not antagonize the protective effect of apotransferrin. CSF transferrin is iron-saturated (Moos et al., 1999), so any therapeutic administration of apotransferrin would inevitably be accompanied by endogenous holotransferrin. In order to determine if the presence of holotransferrin would alter the beneficial effect of apotransferrin, cultures were treated with hemoglobin plus various combinations of apotransferrin and holotransferrin, producing total transferrin concentrations of 50 μg/ml or 100 μg/ml (Fig. 6). Neuroprotection was a direct function of the apotransferrin concentration and was not influenced by the presence of holotransferrin. For example,

cell death was very similar in cultures treated with hemoglobin plus either 50 µg/ml apotransferrin alone or 50 µg/ml apotransferrin plus 50 µg/ml holotransferrin. Likewise, near-identical cell death was observed in cultures receiving 25 µg/ml apotransferrin plus 25 µg/ml holotransferrin or 25 µg/ml apotransferrin plus 75 µg/ml holotransferrin. Significant protection was observed when cultures were treated with as little as 12.5 µg/ml apotransferrin, combined with 37.5 µg/ml holotransferrin.

3.5. Treatment with hemoglobin and/or transferrin has no effect on transferrin receptor-1 (TfR1) expression. TfR1 expression was demonstrated by immunostaining (Fig. 7A-D) and Western blot analysis (Fig. 7E). Consistent with prior observations that central neurons but not astrocytes express transferrin receptor-1 (Dickinson and Connor, 1998; Moos et al., 1999), immunoreactive cells had the characteristic appearance of neurons in this primary culture system (Chen-Roetling et al., 2009), as described in Section 3.1. Expression was not significantly altered by treatment with hemoglobin alone, transferrins alone, hemoglobin plus transferrins, or hemoglobin plus deferoxamine.

4. Discussion

Although the high affinity of apotransferrin for iron is well-established (Halliwell and Gutteridge, 1999), neuronal expression of transferrin receptor-1 (TfR1) suggested that any chelated iron might enter these cells and participate in free radical reactions. The present study is the first to demonstrate that apotransferrin protects central neurons from an iron-dependent injury. This protection was as robust as that of deferoxamine, and was not antagonized by iron-saturated holotransferrin. Moreover, despite persistent TfR1 expression, apotransferrin significantly attenuated the increase in culture nonheme iron observed after hemoglobin treatment. These results suggest that increasing perihematomal apotransferrin may be beneficial after hemorrhagic CNS injuries. Testing of apotransferrin in models of neurodegenerative diseases associated with disruption in iron homeostasis, such as Alzheimer's and Parkinson's disease, may also be indicated (Connor et al., 1992; Sayre et al., 2000; Weinreb et al., 2010).

Native CSF transferrin is fully saturated with iron (Moos et al., 1999), and therefore is unlikely to provide any protection against iron neurotoxicity produced by intracranial hemorrhage or other processes. Serum transferrin in contrast is normally only 20-50% iron-saturated. Immediately after hemorrhage, the iron binding capacity of the hematoma should be substantial, and may be sufficient to attenuate if not prevent any iron-mediated injury. However, in contrast with hemoglobin, which is released from erythrocytes in a delayed fashion beginning at least a few days after intracerebral hemorrhage (Xi et al., 1998), the transferrin concentration would likely decline rapidly as it diffuses away from the site of hemorrhage and re-enters the circulation. Since no published data exist on the transferrin concentration in an experimental or clinical hematoma over time, a neuroprotective effect of residual serum transferrin cannot be excluded. Iron deposition in tissue surrounding a hematoma in rodents and humans nevertheless suggests that it is insufficient per se after ICH (Hardy et al., 1990; Hua et al., 2006; Wu et al., 2003).

Extracellular hemoglobin is preferentially taken up by neurons (Lara et al., 2009), where its heme moieties are metabolized in a reaction catalyzed by constitutively-expressed heme oxygenase-2 (Robinson et al., 2009; Wagner et al., 2003). Compared with glial cells, neurons express little ferritin at baseline or in response to iron loading (Hansen et al., 1999; Regan et al., 2008; Wu et al., 2003). These observations, combined with the widespread expression of ferroportin, has led to the hypothesis that any excess neuronal iron is rapidly exported (Moos and Morgan, 2004). Iron chelated to either deferoxamine or transferrin is detected in vitro by the Prussian Blue reaction that is the basis of enhanced Perl's staining (unpublished observations). If deferoxamine or apotransferrin were sequestering iron within neurons or glial cells, it is unlikely that they would have any effect on the intensity of iron staining. The significant reduction in Perl's staining intensity produced by both agents in this study is therefore consistent with extracellular sequestration of iron in this model, and the iron export hypothesis.

In addition to iron scavenging and detoxification, iron chelators may protect cells from oxidative injury by increasing expression of hypoxia inducible factor (HIF)-1α via inhibition of iron-dependent prolyl hydroxylases, which target it for proteasomal degradation (Weinreb et al., 2010). The HIF-1 transcription factor increases the

expression of several proteins that may enhance cellular resistance to oxidative stress (Harten et al., 2010). Although HIF-1 activation by apotransferrin has not been described, a contribution of this regulatory mechanism to the neuroprotection observed in the present series of experiments cannot be excluded. However, it is noteworthy that both the HO-1 and TfR1 genes are well-characterized HIF-1 targets, but their expression was not increased in cultures treated with either apotransferrin or deferoxamine.

TfR1 expression in most cell types is an inverse function of cell iron, with regulation primarily at the post-transcriptional level (Pantopoulos, 2004). Binding of iron regulatory proteins (IRP's) to iron responsive elements in the 3' untranslated region of TfR1 mRNA increases its stability. In iron-replete cells, IRP binding activity is attenuated and receptor synthesis is thereby reduced. IRP binding to the 5' untranslated region of ferritin mRNA has the opposite effect, inhibiting translation. We have previously demonstrated that iron regulatory proteins are expressed by these cultures and inhibit baseline ferritin expression, which increases rapidly with hemoglobin treatment (Regan et al., 2008). In contrast, the present results demonstrate that neuronal TfR1 expression is surprisingly unresponsive to rising iron levels, at least in the 16 hour time frame of these experiments. The effect of iron on neuronal TfR1 expression has not been intensively investigated. It is noteworthy that in a rat striatal hemorrhage model, tissue iron and TfR1 expression, as assessed by Western blot analysis, were both increased at three days (Wu et al., 2003). Conversely, in iron deficient rats, TfR1 mRNA was not increased in central neurons despite reduced brain iron (Moos et al., 1999). These and the present results suggest that standard TfR1 regulation based on feedback from cell iron levels may not predominate in rodent central neurons. Regulation by other mechanisms would not be without precedent, having been previously described for differentiating erythroid cells (Lok and Ponka, 2000) and monocytes-macrophages (Testa et al., 1989).

Apotransferrin therapy has never been tested in models of acute ischemic, hemorrhagic, or traumatic CNS injury. In vivo use has been reported in models of renal and intestinal ischemic-reperfusion, and significant protection has been demonstrated in both (de Vries et al., 2004; Hernandez et al., 1987). The efficacy of systemic therapy after hemorrhagic stroke may be limited by an intact blood-brain barrier. Although transferrin transport through brain capillary endothelial cells has been convincingly

demonstrated in vitro (Burdo et al., 2003), in vivo evidence for transcytosis in significant quantities is conflicting to date (Moos et al., 2007). However, the relatively high concentration of unsaturated transferrin in serum (1-4 mg/dl) but its absence in CSF (Moos et al., 1999) suggests that intravenous administration of apotransferrin would be unlikely to increase the iron binding capacity of brain interstitial fluid. A growing body of evidence suggests that minimally invasive aspiration or endoscopic removal of intracranial hematomas improves outcome after ICH (Rincon and Mayer, 2010). Since these methods do not completely remove the hematoma, infusion of apotransferrin into the cavity may be a useful adjunct that may be less toxic than systemic therapy with exogenous chelators. The feasibility of delivery of a radiolabeled apotransferrin conjugate directly to the primate brain via high flow interstitial infusion has already been demonstrated (Laske et al., 1997). Further investigation of this approach in experimental models of ICH is warranted.

5. Acknowledgement

This study was supported by a grant from the National Institutes of Health (NS42273) to RFR.

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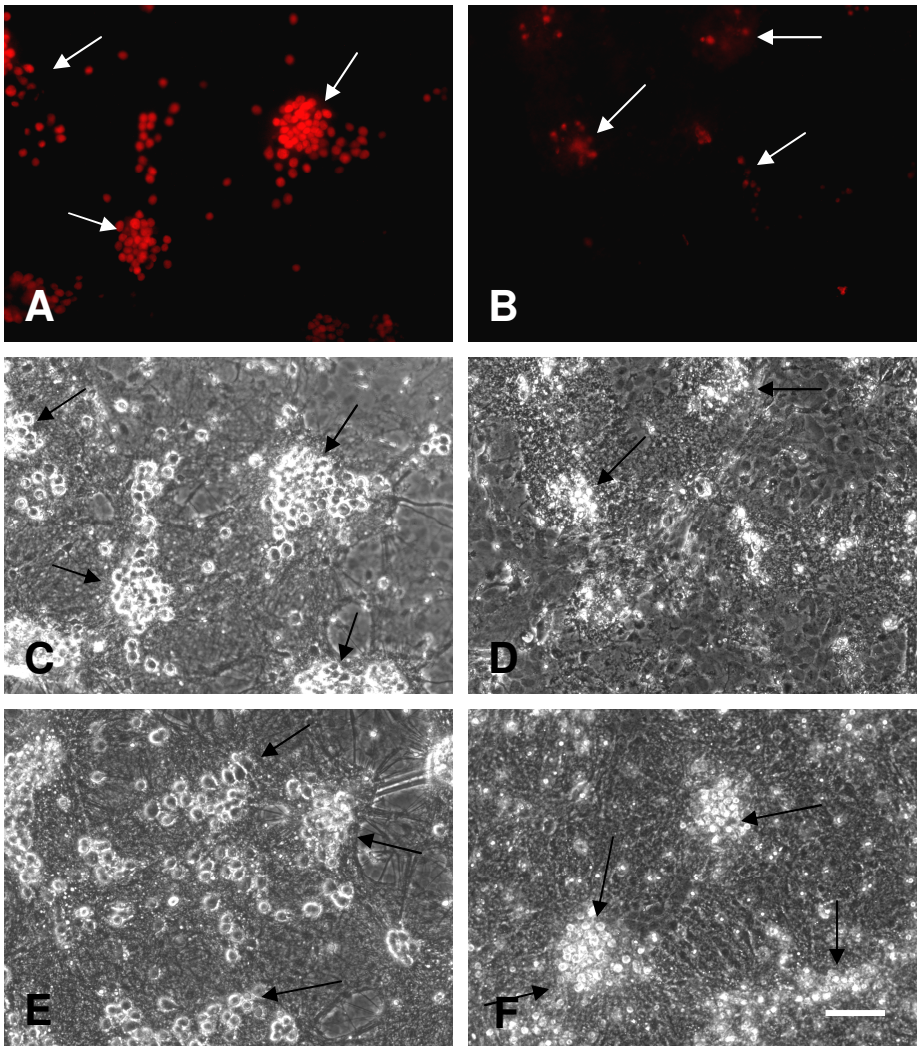

Fig. 1. Morphologic appearance of cultures treated with hemoglobin alone or with transferrins. Immunofluorescence (A,B) and phase contrast (C-F) photomicrographs of cultures 16 hours after the following treatments: A,C) sham medium exchange only; neurons (arrows) are easily distinguished from the background glial monolayer by their prominent phase-bright cell bodies, which are immunoreactive for NeuN; B,D) 3 µM hemoglobin (Hb); most neurons have degenerated to debris, reducing NeuN immunoreactivity; E) Hb 3 µM plus apotransferrin 50 µg/ml; morphology of neurons is preserved; F) Hb 3 µM plus holotransferrin 50 µg/ml; no protection is apparent. Scale bar = 100µm.

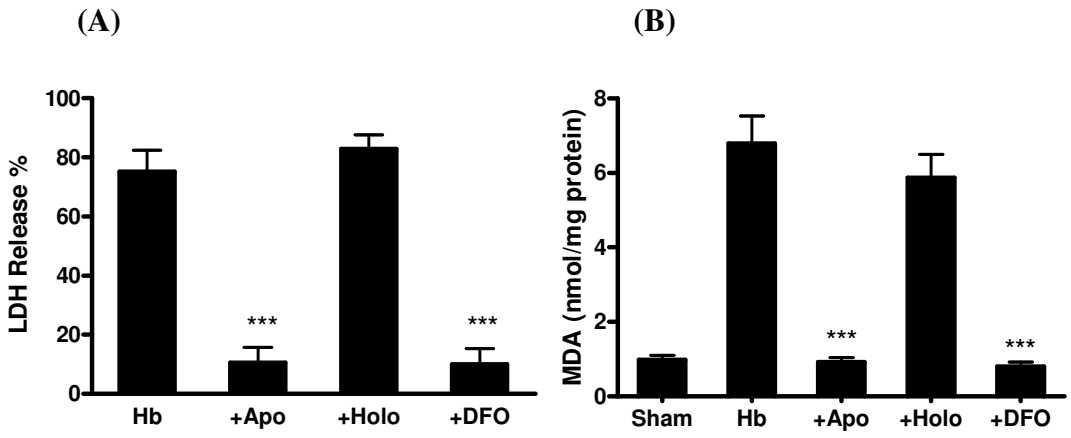

Fig. 2. Apotransferrin but not holotransferrin protects cortical neurons from hemoglobin. A) Culture medium LDH activity (± S.E.M, n = 21-25/condition) after 16 hour treatment with hemoglobin (Hb) 3 μM alone, with 50 μg/ml (0.625 μM) apotransferrin (Apo) or holotransferrin (Holo), or with 1.25 μM deferoxamine (DFO), which has the same iron binding capacity as 0.625 μM apotransferrin. Medium LDH values are scaled to those in sister cultures treated with NMDA 300 μM (=100), which releases all neuronal LDH without injuring glial cells. The weak signal in sister cultures subjected to medium exchange only (sham) was subtracted from all values to yield the LDH activity associated with neurotoxicity. B) Cultures (10-14/condition) were treated as in A, and were assayed for malondialdehyde (MDA) at the end of the exposure period. ***$P < 0.001$ v. Hb-treated cultures, Bonferroni multiple comparisons test.

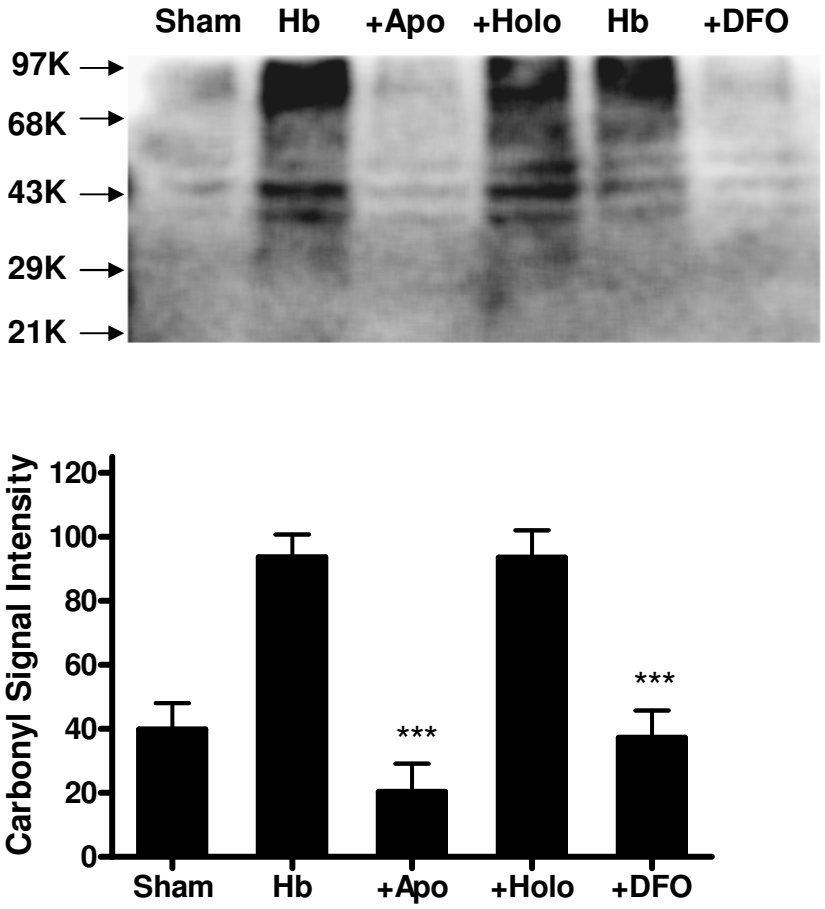

Fig. 3. Apotransferrin attenuates protein oxidation by hemoglobin. Immunoblot of proteins from cortical cultures 16 hours after treatment with 3 μM hemoglobin (Hb) alone, with 50 μg/ml (0.625 μM) apotransferrin or holotransferrin, with 1.25 μM deferoxamine (DFO), or medium exchange only (sham), stained with antibody to derivatized carbonyl groups. Bars represent mean lane densities (± S.E.M.) after background subtraction. ***$P < 0.001$ v. mean signal in Hb alone group, Bonferroni multiple comparisons test, n = 3-6 cultures/condition.

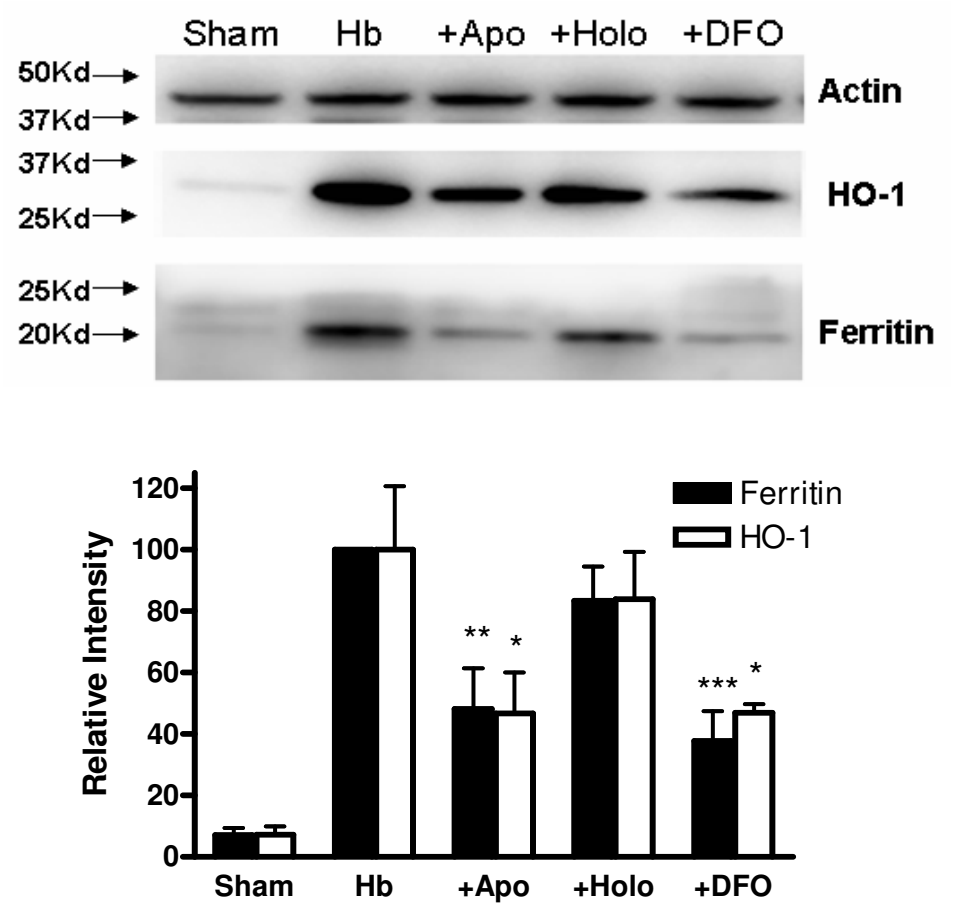

Fig. 4. Apotransferrin attenuates ferritin and heme oxygenase-1 expression after hemoglobin treatment. Bars represent mean band density (± S.E.M.) after 16 h treatment with 3 μM hemoglobin (Hb) alone or with 50 μg/ml (0.625 μM) apotransferrin or holotransferrin, with 1.25 μM deferoxamine (DFO), or medium exchange only (sham). Lane order of representative immunoblots stained with antibodies to actin (gel loading control), ferritin, or heme oxygenase-1 is the same as bar order. *$P < 0.05$, ***$P < 0.01$, ***$P < 0.001$ v. mean signal in corresponding Hb alone group, Bonferroni multiple comparisons test, n = 3-5 cultures/condition.

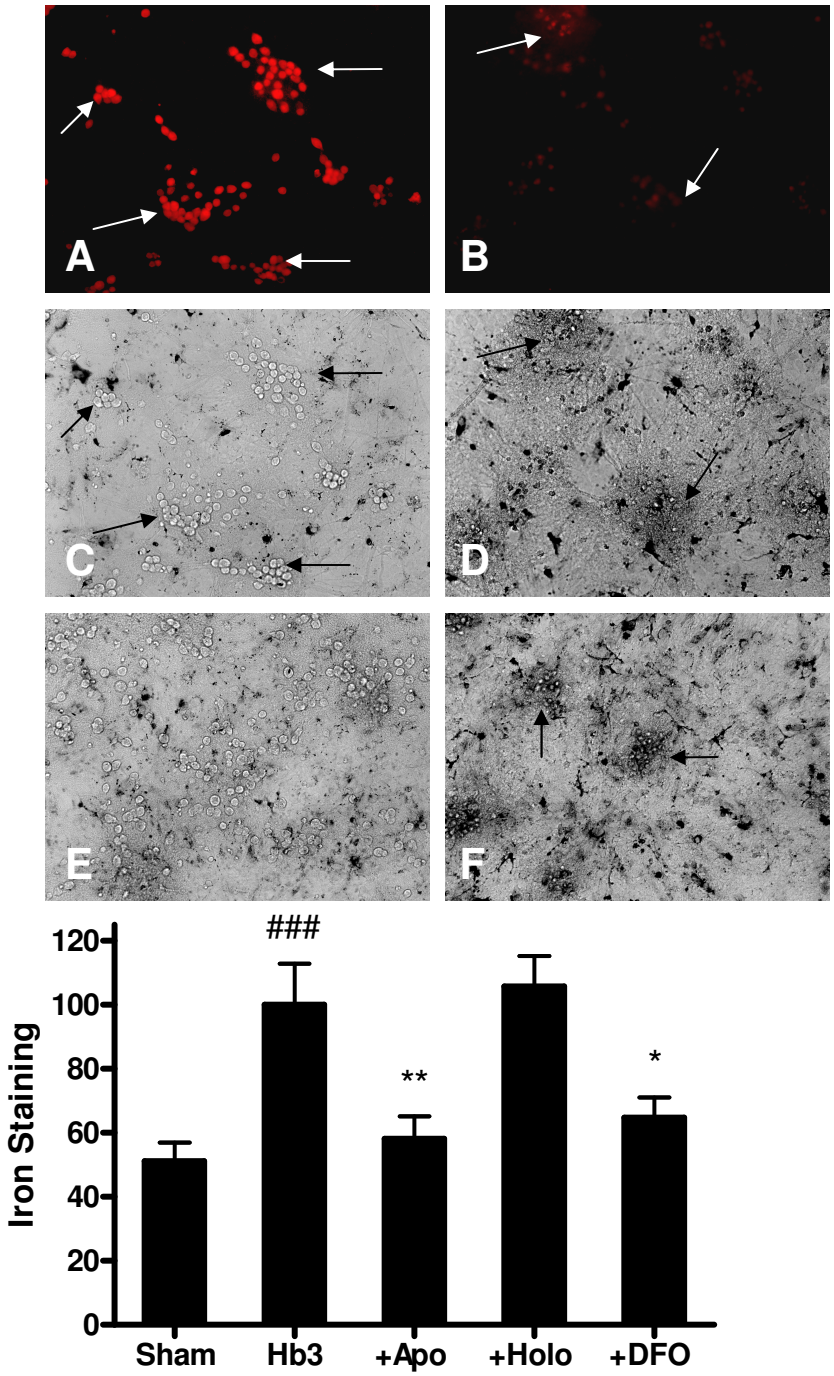

Fig. 5. Apotransferrin attenuates iron deposition in hemoglobin-treated cultures. Immunofluorescence and bright field photomicrographs of cultures fixed 16 hours after the following treatments: A,C) sham medium exchange only; iron staining is limited to the glial monolayer, and NeuN-positive neuronal cell bodies (arrows) are Perl's negative; B,D) Hb 3 µM; iron staining in both glial monolayer and degenerating neuronal cell bodies is apparent; NeuN immunoreactivity is diminished; E) Hb 3 µM plus 50 µg/ml apotransferrin; F) Hb 3 µM plus 50 µg/ml holotransferrin. Bars represent mean iron staining (± S.E.M.), normalized to that in Hb-alone condition (= 100). Deferoxamine

(DFO) concentration was 1.25 µM. *P < 0.05, **P < 0.01, v. mean signal in corresponding Hb alone group, ###P < 0.001 v. signal in sham group, Bonferroni multiple comparisons test, n = 12-14/condition. Scale bar = 100µm.

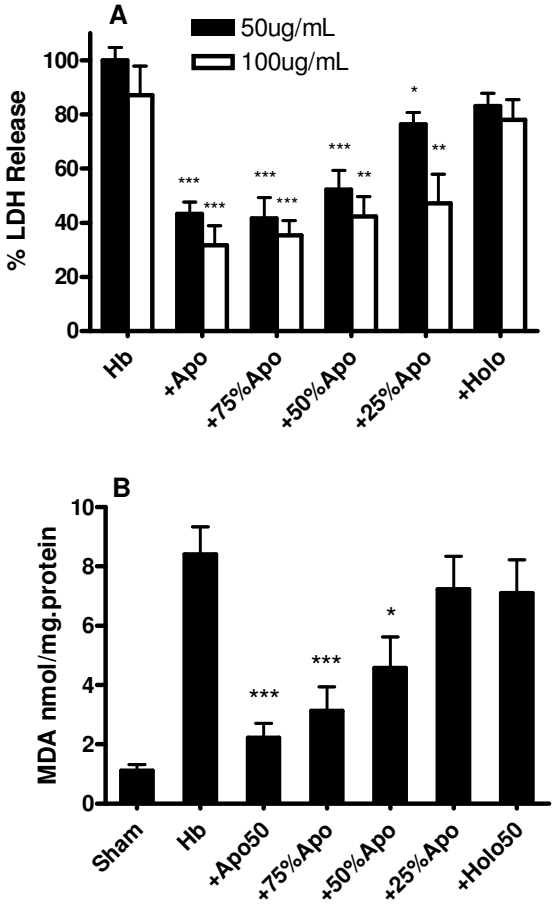

Fig. 6. Holotransferrin does not antagonize the protective effect of apotransferrin. A) Culture medium LDH activity (± S.E.M.) after 16-hour treatment with hemoglobin (Hb) 3 µM alone, with 50 µg/ml or 100 µg/ml apotransferrin (+ apo) or holotransferrin (+holo), or with 50 µg/ml or 100 µg/ml total transferrin containing indicated percentage of apotransferrin and balance holotransferrin. Medium LDH values are scaled to those in sister cultures treated with NMDA 300 µM (=100), which releases all neuronal LDH without injuring glial cells. The weak signal in sister cultures subjected to medium exchange only was subtracted from all values to yield the LDH activity associated with neurotoxicity. B) Cultures were treated as in A, using 50 µg/ml total transferrin, and were assayed for malondialdehyde (MDA) at the end of the exposure period. Sham cultures were subjected to medium exchange only. *P < 0.05, **P < 0.01, ***P < 0.001 v. mean signal in corresponding Hb alone group, Bonferroni multiple comparisons test, n = 7-12 cultures/condition.

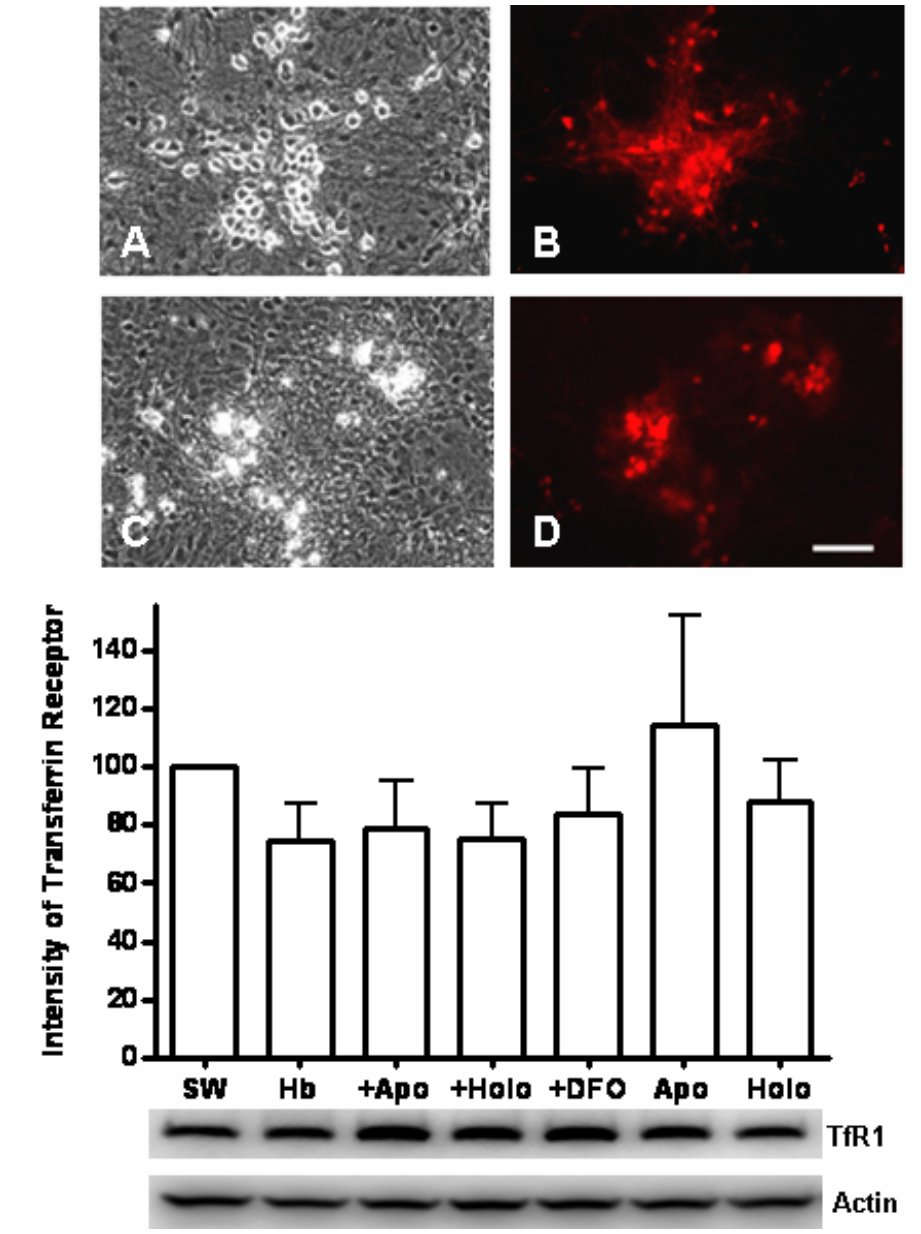

Fig. 7. Transferrin receptor-1 (TfR1) expression is not altered by hemoglobin, transferrin or deferoxamine. Phase contrast (A,C) and fluorescent photomicrographs after anti-TfR1 immunostaining (B,D) of sister cultures treated with: A,B) medium exchange (sham) only; prominent phase-bright neuronal cell bodies and adjacent processes express TfR1; C,D) hemoglobin 3 µM for 16h; degenerating neurons continue to express TfR1. E) Bar graph represents mean TfR1 immunoblot band densities (± S.E.M., n = 6-8/condition) from cultures treated for 16h with hemoglobin (Hb) alone or with apotransferrin (+Apo) 50 µg/ml, holotransferrin (+Holo) 50 µg/ml, deferoxamine (+DFO) 1.25 µM, or with same concentrations of apotransferrin (Apo) or holotransferrin (Holo) alone. Band order of representative immunoblot is the same as bar order. $P > 0.05$ for all conditions v. sham or Hb alone, Bonferroni multiple comparisons test. Scale bar = 100µm.