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Heme Oxygenase Activity and Hemoglobin Neurotoxicity Are Attenuated by Inhibitors of the MEK/ERK Pathway

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Summary

Hemoglobin breakdown produces an iron-dependent neuronal injury after experimental CNS hemorrhage that may be attenuated by heme oxygenase (HO) inhibitors. The HO enzymes are phosphoproteins that are activated by phosphorylation in vitro. While testing the effect of kinase inhibitors in cortical cell cultures, we observed that HO activity was consistently decreased by the MEK inhibitor U0126. The present study tested the hypothesis that MEK/ERK pathway inhibitors reduce HO activity and neuronal vulnerability to hemoglobin. The MEK inhibitors U0126 and SL327 and the ERK inhibitor FR180204 reduced baseline culture HO activity by 35–50%, without altering the activity of recombinant HO-1 or HO-2; negative control compounds U0124 and FR180289 had no effect. Hemoglobin exposure for 16 hours produced widespread neuronal injury, manifested by release of 59.2±7.8% of neuronal lactate dehydrogenase and a twelve-fold increase in malondialdehyde; kinase inhibitors were highly protective. HO-1 induction after hemoglobin treatment was also decreased by U0126, SL327, and FR180204. These results suggest that reduction in HO activity may contribute to the protective effect of MEK and ERK inhibitors against heme-mediated neuronal injury.

Keywords

cell culture; free radical; hemoglobin toxicity; intracerebral hemorrhage; mouse; oxidative stress

Introduction

A considerable body of experimental and clinical evidence suggests that toxins released from an intracerebral hematoma may contribute to cell injury in adjacent tissue (Xi, et al., 2006). One putative neurotoxin is hemoglobin, the most abundant protein in blood, which is released from lysed erythrocytes in the days after hemorrhage and contributes to peri-hematomal edema and oxidative stress (Huang, et al., 2002). Investigation of hemoglobin neurotoxicity in cell culture models and in vivo suggests that the hemoglobin molecule per se is not the primary toxin (Sadrzadeh, et al., 1987, Regan, et al., 1993). However, at least under some experimental conditions, the quantity of iron released as a consequence of the breakdown of its heme moieties apparently exceeds the sequestration or export capacity of CNS cells. The result is an injury that is largely selective for neurons, which are highly sensitive to low molecular weight iron (Kress, et al., 2002).

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Heme degradation to equimolar quantities of iron, biliverdin, and carbon monoxide is catalyzed by the heme oxygenase (HO) enzymes (Abraham, et al., 2008). Two isoforms have been identified to date in the mammalian CNS (Schipper, 2004). Heme oxygenase-1 is expressed primarily by glial cells and is induced by heat shock, heme, and a variety of oxidants. Heme oxygenase-2 is constitutively expressed by neurons and endothelial cells. The effect of heme oxygenase activity on acute CNS injury has been extensively investigated in studies using either HO inhibitors or genetically modified mice. A protective effect has been consistently observed in models that are relevant to ischemia or trauma (Takizawa, et al., 1998, Panahian, et al., 1999, Chang, et al., 2003), which has been attributed to the antioxidant and anti-inflammatory effects of biliverdin/bilirubin and carbon monoxide (Abraham, et al., 2008, Parfenova, et al., 2008). In contrast, HO activity increased or accelerated injury in most (Wagner, et al., 2000, Koeppen, et al., 2002, Koeppen, et al., 2004, Gong, et al., 2006, Wang, et al., 2006a, Qu, et al., 2007) but not all (Wang, et al., 2006b) experimental models of intracerebral hemorrhage (ICH), presumably due to iron toxicity that negated any benefit of the other breakdown products.

Clinical ICH is a complex injury that may include varying degrees of compressive ischemia, mechanical injury from hematoma expansion or retraction, inflammation, and the toxicity of blood components (Xi, et al., 2006). The disparate effect of HO on heme-mediated and other CNS injuries suggests that it may be a challenging therapeutic target, since any benefit of direct HO inhibitors against hemoglobin neurotoxicity may be negated by their deleterious effects on other injury cascades. An alternative approach to direct enzyme inhibition is to prevent the increase in HO activity produced by hemorrhage, which may be due to HO activation and/or HO-1 induction. Both HO-1 and HO-2 are phosphoproteins, and in vitro are activated by the phosphatidylinositol-3-kinase and protein kinase C/CK2 pathways, respectively (Boehning, et al., 2003, Salinas, et al., 2004). However, we have recently observed that selective inhibitors of these pathways had no effect on HO activity in murine cortical cell cultures (Chen-Roetling, et al., 2008). In the course of these kinase inhibitor experiments, we noted that the MEK 1/2 inhibitor U0126 surprisingly reduced baseline culture HO activity. In the present study, we tested the effect of MEK and ERK inhibitors on HO activity and hemoglobin neurotoxicity in this culture system.

Methods

Cortical 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 (IACUC). Mixed neuron–glia cortical cell cultures were prepared from fetal B6129 mice (gestational age 13- to 15-days), using a previously described protocol (Rogers, et al., 2003). After cell dissociation by trituration, cultures were plated on confluent glial feeder cultures in 24-well plates (Falcon, Becton Dickinson, Franklin Lakes, NJ), at a density of three hemispheres/plate. Plating medium contained Minimal Essential Medium (MEM, Invitrogen, Carlsbad, CA), 5% equine serum (Hyclone, Logan, UT), 5% fetal bovine serum (Hyclone), 23 mM glucose, and 2 mM glutamine. On day 5 in vitro, two-thirds of the culture medium was aspirated and replaced with feeding medium, which was similar to plating medium except that it contained 10% equine serum and no fetal bovine serum. This procedure was repeated on day 9 or 10 and then daily beginning on day 11. Glial feeder cultures were prepared from postnatal day 1–3 mice, using plating medium similar to that described above, except that it was supplemented with 10 ng/ml epidermal growth factor (Sigma, St. Louis, MO), 10% equine serum and 10% fetal bovine serum. Glial culture medium was partially changed twice weekly.

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MEK/ERK pathway inhibitors and negative controls

U0126 (Promega, Madison, WI) is a potent inhibitor of both MEK1 and MEK2 (Favata, et al., 1998). It differs from the commonly-used MEK inhibitor PD98059 by directly inhibiting MEK enzyme activity, rather than by preventing its activation by Raf, and also by having much greater activity against MEK2 (Alessi, et al., 1995). U0124 (Tocris Bioscience, Ellisville, Missouri) is a similar compound that does not inhibit MEK, and is marketed as a negative control. SL327 (Tocris) also selectively inhibits both MEK1 and MEK 2 (Scherle, et al., 2000). FR180204 (EMD/Calbiochem, San Diego, CA) is inactive against MEK, but directly inhibits ERK1 (MAPK3) and ERK2 (MAPK3(Ohori, et al., 2005). FR180289 (EMD) differs from FR180204 only by substitution of a hydroxyl group for the 3′ amine (Ohori, et al., 2005), but does not inhibit ERK1 or ERK2 and is marketed as its negative control.

HO activity assay

HO activity was quantified using a modification of the method of Vreman and Stevenson (Vreman, et al., 1988). Concentrated inhibitor stock solutions were freshly prepared in dimethylsulfoxide (DMSO). Dilutions were adjusted so that all conditions including sham-wash controls and hemoglobin without inhibitors had the same DMSO concentration (0.25%). Cultures were treated for either 30 minutes or 4 hours with enzyme inhibitors, hemoglobin, or both in MEM containing 10 mM glucose (MEM10) at 37°C in a 5% CO_2 incubator; control cultures received MEM10 with DMSO vehicle only. At the end of the exposure interval, cultures were washed and harvested in ice-cold Dulbecco's Phosphate Buffered Saline (DPBS) with 3X concentration of inhibitors or DMSO vehicle. Samples of the cell suspension (40 μl) were placed in amber septum-sealed glass vials on ice, and were diluted with equal volumes of freshly prepared 75 μM hemin and 4.5 mM NADPH (final reactant concentrations 25 μM hemin, 1.5 mM NADPH, total volume 120 μl); control vials lacked NADPH. Vials were purged for 4 sec with CO-free air at a flow rate of 250 ml/min. Reactions were then run for 15 minutes at 37°C in a water bath under reduced light, and were terminated by quick-freezing vials on dry ice. CO was quantified in the vial head space by gas chromatography (Peak Laboratories, Mountain View, CA). HO activity was expressed as nanomoles CO produced per hour per milligram protein. Protein concentrations were determined by the BCA method (Pierce, Rockford, IL).

Immunoblotting

Cells were lysed in ice-cold lysis buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EDTA, 0.1 % sodium dodecyl sulfate, 0.1 % Triton X-100). After sonication, debris was removed by centrifugation, and the protein concentration of the supernatant was quantified (BCA method, Pierce, Rockford, IL). Samples (20 μg protein each) were then boiled in sample buffer (Tris-Cl 60 mM, β-mercaptoethanol 5%, sodium dodecyl sulfate 2%, glycerol 10%, and bromophenol blue 0.05%) for 3 min. Proteins were separated on 12 % SDS polyacrylamide gels at 80–100 V. The gels were then soaked in transfer buffer (glycine 39mM, Tris-CL 48mM, SDS 0.037% and methanol 20%), and were transferred to polyvinylidene difluoride membranes with a semidry transfer apparatus for 40 minutes at 20V. Completion of transfer was assessed by observing the transfer of the pre-stained protein marker (Bio-Rad Laboratories, Hercules, CA, Cat. No. 161–0375). After blocking with 5% nonfat dry milk, membranes were exposed overnight at 4 °C to rabbit anti-HO-1 or anti-HO-2 antibodies (Assay Designs, Ann Arbor, MI, 1:5000 and 1:2000 dilutions, respectively) combined with rabbit anti-actin antibody (1/1500, Sigma, St. Louis, MO) as a gel loading control. After washing, they were then exposed to HRP-conjugated goat anti-rabbit IgG antibody (1:3500) for 1h at room temperature. Immunoreactive proteins were visualized using Super Signal West Femto Reagent (Pierce) and Kodak Gel Logic 2200.

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Immunocytochemistry

Cultures were washed with MEM10 and then fixed with ice-cold 4% paraformaldehyde for one hour. After washing with tris-buffered saline (TBS), cultures were serially treated with: 0.25% Triton X-100 for 10 min, 10% normal goat serum for 15 min, 1:100 dilutions of primary antibodies (anti-NeuN, clone A60, Alexa Fluor®488 conjugated, Millipore, Billerica, MA or rabbit anti-glial fibrillary acidic protein (GFAP), Invitrogen, Carlsbad, CA) overnight. Cultures treated with anti-NeuN were then washed and imaged. Cultures treated with anti-GFAP were treated for 30 min with biotinylated anti-rabbit IgG (1:200, Vector Laboratories, Burlingame, CA) followed by NeutrAvidin Rhodamine Red-X conjugate (Invitrogen) 5 μg/ml for 30 min.

ERK activity assay

The effect of inhibitors and negative controls was confirmed using the p44/42 MAP Kinase assay kit (Cell Signaling Technology, Danvers, MA), following the manufacturer's instructions. Cell lysates were incubated overnight at 4°C with immobilized phospho-p44/42 ERK monoclonal antibody. Immunoprecipitation pellets were then collected by centrifugation and were incubated in kinase buffer containing substrate (Elk-1 fusion protein) and 200 μM ATP at 30°C for 30 minutes. Phosphorylated Elk-1 was detected by immunoblotting as described above, using a 1:1000 dilution of rabbit anti-phospho-Elk (Ser 383) primary antibody.

Cytotoxicity experiments

Cultures were used for experiments on 12–16 days in vitro. Neurons are easily distinguished from glial cells during this interval by their phase-bright cell bodies and extensive network of processes. All exposures were conducted in MEM10 at 37°C in a 5% CO_2 atmosphere. As described above, the dilution of all inhibitors was adjusted so that all conditions contained 0.25% DMSO vehicle. Control cultures in each experiment were subjected to medium exchange only, following which they were treated with an equal concentration of DMSO vehicle. The hemoglobin exposure concentration was determined from prior studies using this model, which demonstrated that 10 μM hemoglobin (tetramer concentration) produced widespread neuronal injury with overnight treatment (Rogers, et al., 2003), without injuring astrocytes (Chen-Roetling, et al., 2006).

Assessment of injury

Neuronal death was quantified by assaying lactate dehydrogenase (LDH) activity in the culture medium, a method that correlates well with cell counts after trypan blue staining in mixed cortical cultures (Koh, et al., 1988), as previously described (Regan, et al., 1998). In order to compare results from experiments using cultures prepared in different platings, all LDH values were normalized to those in sister cultures from the same plating treated with 300 μM NMDA, which releases virtually all neuronal LDH without injuring glial cells. The mean baseline LDH activity of sister cultures subjected to medium exchange and vehicle treatment only was subtracted from all values to quantify the signal that was specific to the cytotoxic exposure, following the protocol of Koh and Choi (Koh, et al., 1988).

Malondialdehyde is a sensitive marker of oxidative injury in this model (Regan, et al., 1998). After sampling for LDH assay, cultures were harvested, proteins were precipitated with 4.5% trichloroacetic acid, and malondialdehyde was quantified as previously detailed (Regan, et al., 1998). Protein content of each sample was estimated by the BCA method (Pierce, Rockford, IL); all malondialdehyde values were expressed as nanomoles/milligram protein.

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Statistical Analysis

Data were analyzed with one-way analysis of variance. Differences between groups were then assessed with the Bonferroni Multiple Comparisons test.

Results

MEK and ERK inhibitors attenuate culture HO activity

In initial experiments, the effect of 30 min incubation with MEK or ERK inhibitors on culture HO activity was assessed. HO activity in control cultures treated with DMSO vehicle only for this interval was 1.01±0.07 nmol CO/h/mg protein (Fig. 1A). In cultures treated with the MEK inhibitors U0126 or SL327 or the ERK inhibitor FR180204, a significant reduction in HO activity was observed. U0124 and FR180289, structural analogs of U0126 and FR180204 that are marketed as negative controls for these compounds, had no effect on HO activity at the same concentrations. Kinase activity assays confirmed that, at the concentrations used, U0126, SL327, and FR180204 inhibited phosphorylation of Elk-1 by ERK, while U0124 and FR180289 had no such effect (Fig. 1B, C).

In order to determine if these MEK and ERK inhibitors were direct HO inhibitors, their effect on the activity of recombinant HO-1 and HO-2 (both purchased from Assay Designs, Ann Arbor, MI) was determined. In the presence of DMSO vehicle only, recombinant HO-1 and HO-2 had activities of 297.0±39.0 nmol CO/h/mg protein and 504.2±44.1 nmol CO/h/mg protein, respectively (Fig. 2). These activities were not significantly altered by U0126, SL327, or FR180204. Not surprisingly, the activity of both recombinant isoenzymes was strongly inhibited by the HO inhibitor zinc protoporphyrin IX (50 μM).

MEK and ERK inhibitors protect neurons from hemoglobin

We have previously reported that reducing HO activity by either HO-2 gene knockout or treatment with an HO inhibitor protects neurons in this culture system from hemoglobin (Rogers, et al., 2003). Since MEK and ERK inhibitors rapidly reduced HO activity, their effect on hemoglobin neurotoxicity was assessed. Consistent with prior observations, widespread neuronal injury was observed in cultures treated for 16h with 10 μM hemoglobin plus DMSO vehicle only (Fig. 3); the glial monolayer remained intact and morphologically normal throughout the course of the experiment, also as previously reported (Chen-Roetling, et al., 2006). Cell death, as quantified by LDH release into the culture medium, was 59.2±7.8% of that in sister cultures treated with 300 μM NMDA, which releases essentially all neuronal LDH (Fig. 4A). Concomitant treatment with U0126, SL327, or FR180204 consistently had a robust protective effect (Fig. 3, 4A).

MEK and ERK inhibitors reduce expression of oxidative injury markers after hemoglobin treatment

Malondialdehyde is a sensitive marker of the oxidative injury produced by hemoglobin in CNS cells (Sadrzadeh, et al., 1987). In cultures treated with hemoglobin plus vehicle only, it was increased by 12.5-fold compared with control cultures subjected to medium exchange and vehicle treatment only (Fig. 4B). Malondialdehyde levels in cultures treated with hemoglobin plus kinase inhibitors were similar to those in vehicle-treated controls.

HO-1 is rapidly induced by oxidants in CNS cells (Schipper, 2004). Since MEK and ERK inhibitors appeared to be acting as antioxidants in this model, their effect on HO-1 expression was determined. In cultures treated with hemoglobin plus DMSO vehicle for 4h, which is a time point prior to the onset of neuronal lysis, HO-1 expression was increased 2.2-fold over control cultures treated with vehicle only (Fig. 5A). Consistent with prior observations (Benvenisti-Zarom, et al., 2006), this increase was reduced by about half by U0126 and SL327,

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and was completely prevented by the direct ERK inhibitor FR180204. HO-2 expression was not altered by hemoglobin or kinase inhibitor treatment. HO activity was increased approximately 2.5-fold by hemoglobin (Fig. 5B). This increase was completely prevented by concomitant treatment with U0126, SL327, or FR180204.

Discussion

These results suggest the following conclusions. First, baseline HO activity is attenuated by inhibitors of the MEK/ERK pathway in this mixed neuron/glia cell culture system. Second, this decrease is associated with reduced neuronal vulnerability to hemoglobin, in agreement with prior observations that HO activity accelerates hemoglobin neurotoxicity in cell culture and in vivo (Huang, et al., 2002, Rogers, et al., 2003). Third, HO-1 induction in this hemoglobin neurotoxicity model is also reduced by these inhibitors, suggesting that it is mediated at least in part by the MEK/ERK signal transduction pathway.

The inhibition in HO activity after brief incubation with ERK or MEK inhibitors was unexpected, since activation of HO-1 or HO-2 by ERK-catalyzed phosphorylation has not been reported. HO-1 has possible ERK phosphorylation sites at T108, S174, and T252, and an ERK1 binding site at P170, while HO2 has several ERK docking domain sites (Obenauer, et al., 2003). The effect of ERK-catalyzed phosphorylation on HO activity has not been defined and seems worthy of further investigation. It is unlikely that the inhibitors acted directly on HO catalysis, since they had no significant effect on the activity of recombinant HO-1 or HO-2. In assessing these compounds in oxidative injury models, the potentially confounding effect of reduced HO activity should be considered.

The protective effect of MEK and ERK inhibitors provides an additional line of evidence that excessive HO activity may be detrimental to neurons exposed to hemoglobin, and complements prior and more specific observations using HO-2 knockout neurons or HO inhibitors (Huang, et al., 2002, Rogers, et al., 2003). A growing body of experimental evidence suggests that HO activity may have either antioxidant or pro-oxidant effects, with the net effect varying with the cell population and the type of injury. Two factors may account for a pro-oxidant effect in this hemoglobin neurotoxicity model. First, HO substrate was added to cultures at a relatively high concentration (10 μM hemoglobin, containing 40 μM heme). While this concentration is considerably lower than that adjacent to an intracerebral hemorrhage (Letarte, et al., 1993), it is markedly higher than the endogenous heme that is available to cells in models more relevant to CNS ischemia (~ 1 nM, Taketani, 2005), in which HO activity is protective (Takizawa, et al., 1998, Panahian, et al., 1999). Second, neurons are very vulnerable to low molecular weight iron (Kress, et al., 2002), a product of heme breakdown by HO, which likely reflects their limited capacity to sequester it in ferritin (Moos, et al., 2004). Release of supraphysiologic quantities of iron may overwhelm any benefit provided by HO activity (Dennery, et al., 2003), which includes bilirubin and carbon monoxide production (Abraham, et al., 2008, Parfenova, et al., 2008).

In addition to reducing HO activity, several other mechanisms may contribute to the robust neuroprotection provided by U0126, SL327, and FR180204 in this model. Activation of the MEK/ERK pathway may mediate neuronal injury in an oxidative environment by directly activating calpain (Glading, et al., 2000), phospholipase A2 (Geijsen, et al., 2000), and NADPH oxidase (Dewas, et al., 2000), and also by increasing production of inflammatory cytokines (Wang, et al., 2004). In addition, ERK signaling mediates apoptosis in CNS cells by upregulating Bax and p53 while downregulating Akt (Zhuang, et al., 2006). Although the association of reduced HO activity and neuronal resistance to hemoglobin is consistent with prior observations using more specific approaches (Huang, et al., 2002, Rogers, et al., 2003),

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the downstream mechanistic information provided by inhibiting the MEK/ERK pathway is limited by the myriad effects of ERK signaling.

We have previously reported that HO-1 is expressed at a low level in primary cultured neurons and glia, and that it is rapidly induced in glia by hemoglobin or hemin treatment (Regan, et al., 2000, Benvenisti-Zarom, et al., 2006). In contrast to the effect of HO on neurons, HO-1 expression protects glial cells from hemoglobin (Regan, et al., 2000, Chen-Roetling, et al., 2006). Prolonged hemoglobin treatment produces no injury in glial cultures prepared from wild-type mice, but widespread cell death in HO-1 knockout cultures (Chen-Roetling, et al., 2006), and also in wild-type cultures treated concomitantly with HO inhibitors (Regan, et al., 2000). In the present study, the glial feeder monolayer remained morphologically normal after treatment with hemoglobin plus MEK or ERK inhibitors despite a reduction in culture HO-1 expression and HO activity. Immunostaining demonstrated that most of these glial cells were astrocytes; however, a low level of microglial contamination cannot be excluded (Saura, 2007), and may have contributed to neuronal injury in this model. The disparate effect of direct HO inhibitors and MEK/ERK inhibitors on astrocyte vulnerability to hemoglobin suggests that astrocytes treated with the latter have sufficient residual HO activity to prevent hemoglobin-mediated injury. Alternatively, MEK and ERK inhibitors may antagonize other ERK-activated injury cascades in astrocytes, and thereby compensate for the deleterious effect of reduced HO activity in this cell population.

MEK/ERK pathway inhibitors have been extensively tested in small animal models of CNS ischemia and trauma, and have been found to reduce lesion volume and neurological deficits (Namura, et al., 2001, Wang, et al., 2003, Clausen, et al., 2004, Wang, et al., 2004, Otani, et al., 2007). However, comparable studies have not yet been reported in intracerebral hemorrhage models. The results of the present study suggest that U0126, SL 327, and FR180204 may ameliorate the component of injury produced by the breakdown of extracellular hemoglobin in tissue surrounding an intracerebral hematoma. Investigation of these compounds in vivo therefore seems warranted.

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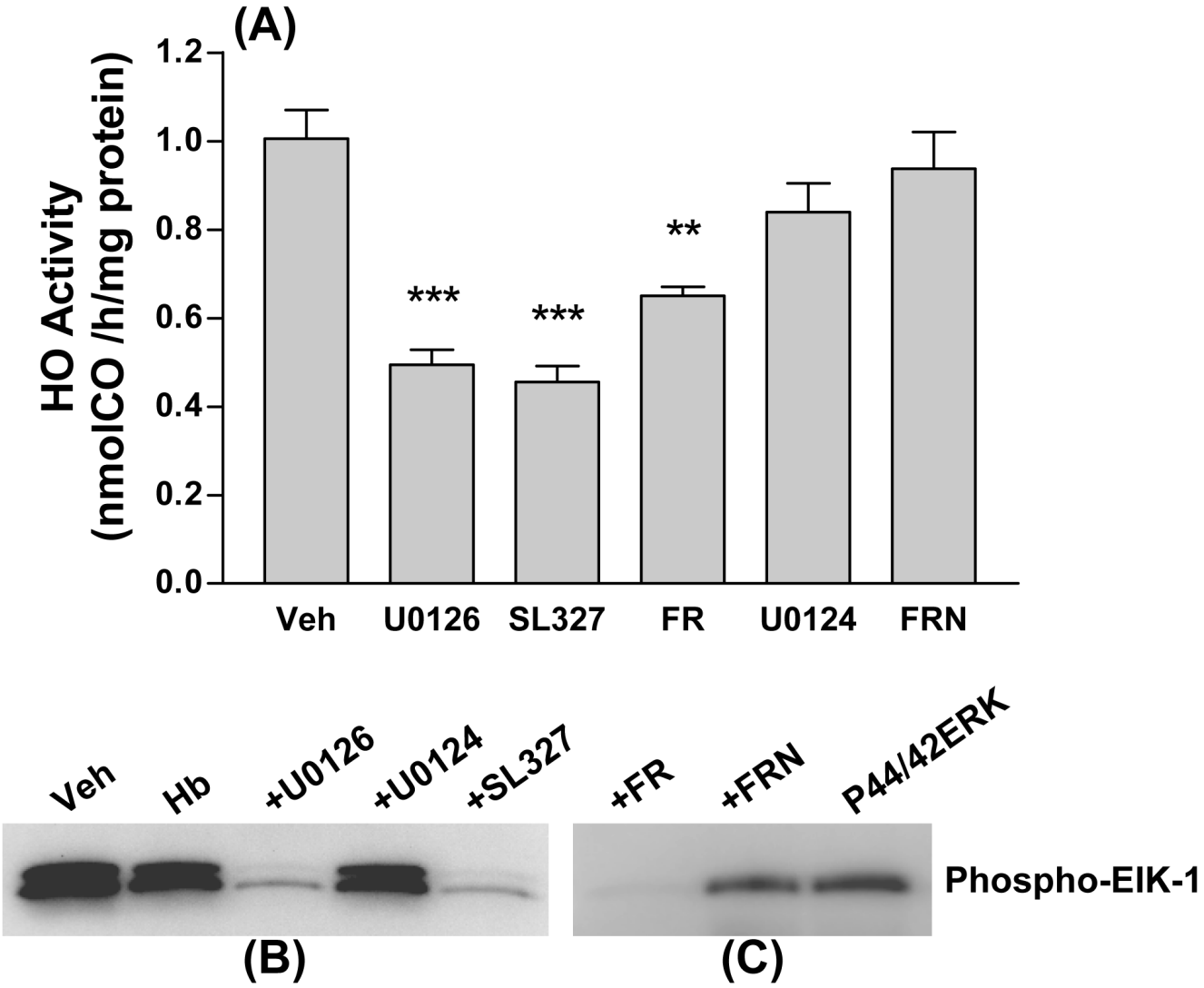

Figure 1.

MEK and ERK inhibitors reduce culture heme oxygenase (HO) activity. A) Mean HO activity (nmol CO/h/mg protein ±SEM, n = 5–12/condition) in cultures washed and then treated with DMSO vehicle only (Veh) or with U0126 (30 μM), SL327 (30 μM), FR180204 (FR, 50 μM), U0124 (negative control for U0126, 30 μM), or FR180289 (FRN, negative control for FR180204, 50 μM) for 30 minutes. ***P<0.001 and **P<0.01 v. activity in vehicle-treated cultures, Bonferroni multiple comparisons test. B) Immunoblot demonstrating effect of MEK inhibitors and negative control U0124 on kinase activity at these concentrations, as assessed by phosphorylation of ERK substrate Elk-1. Cultures were treated with hemoglobin (Hb) 10 μM alone or with inhibitors or control for 4 hours before lysis and immunoprecipitation of active ERK for kinase assay. C) Immunoblot demonstrating inhibition of active ERK by FR180204 (FR, 50 μM) but not by FR180289 (FRN, 50 μM).

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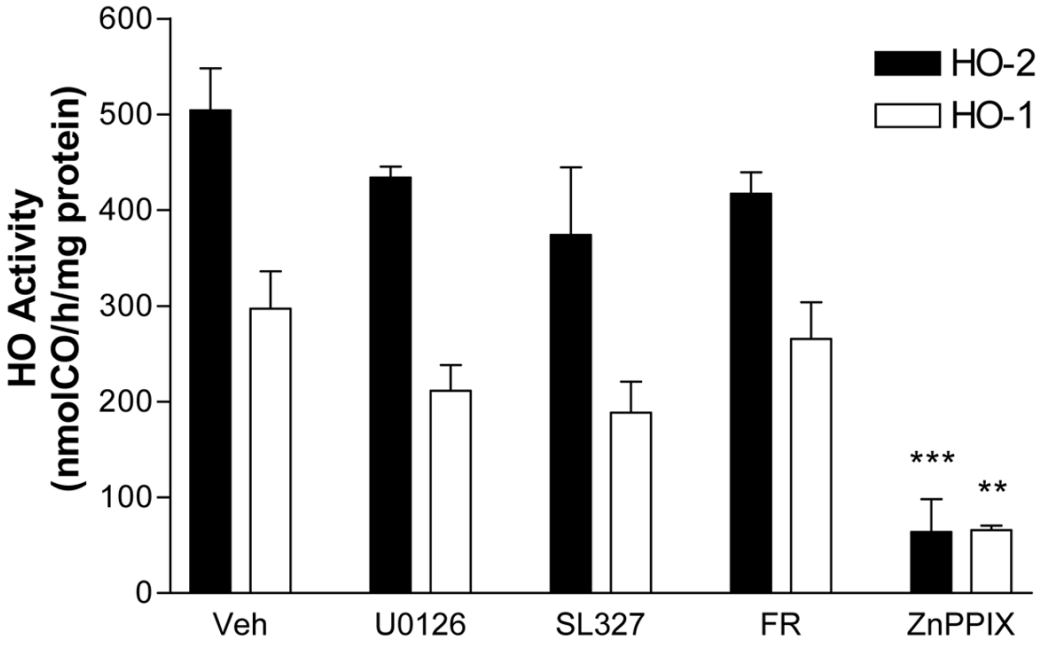

Figure 2.

Effect of MEK and ERK inhibitors on activity of recombinant HO-1 and HO-2. HO activity of recombinant rat HO-1 and HO-2 with DMSO vehicle alone (Veh) or with U0126 (30 μM), SL327 (30 μM), FR180204 (FR, 50 μM), or zinc protoporphyrin IX (ZnPPIX, 50 μM). Reaction vial contained 0.25μg cytochrome P450 reductase with 2.5 μg HO-1 or 1 μg HO-2. **P<0.01, **P<0.001 v. corresponding vehicle condition, Bonferroni multiple comparisons test.

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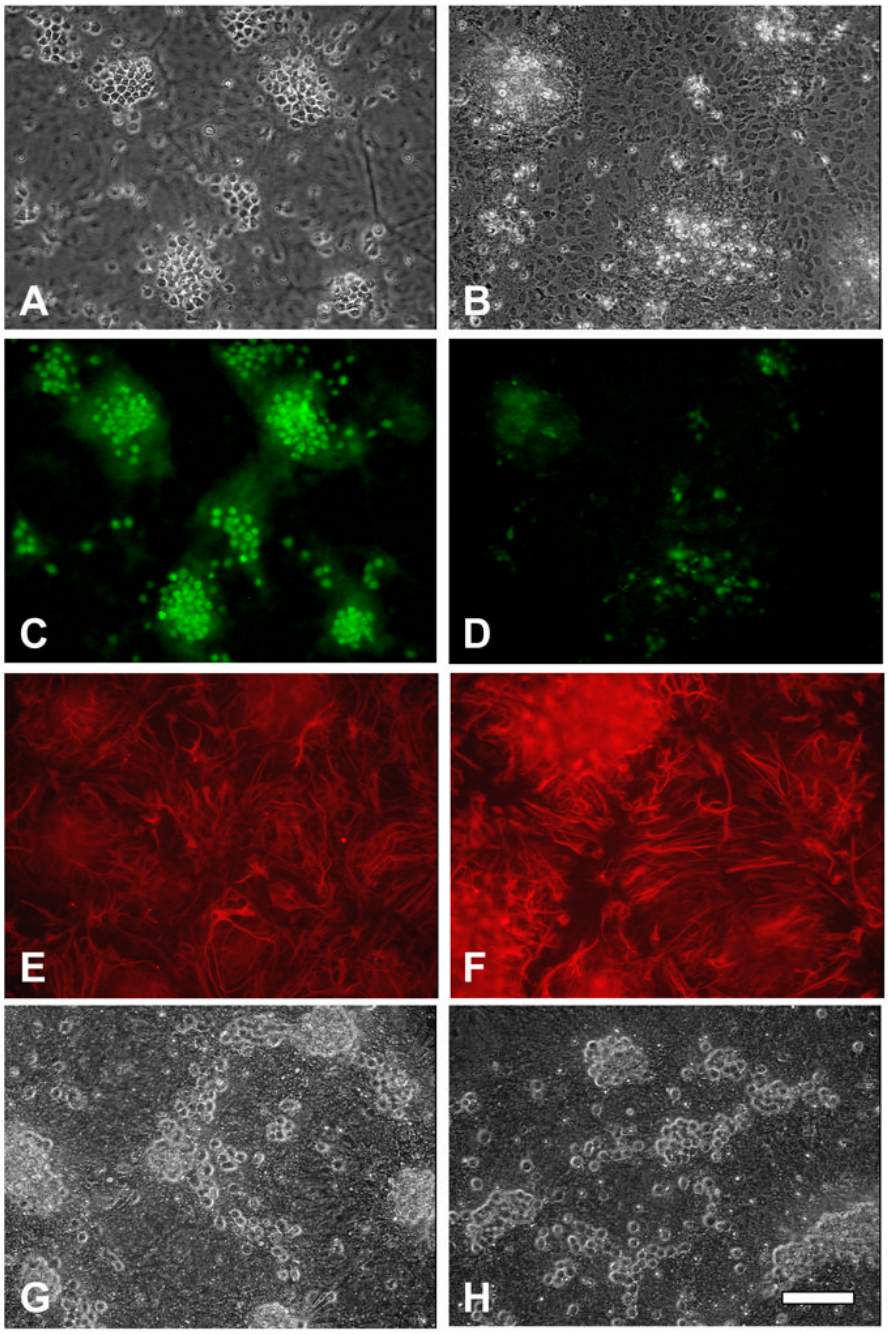

Figure 3.

Morphologic appearance of mixed neuron-astrocyte cultures 24h after: A, C, E) Treatment with vehicle only. Neurons with phase-bright cell bodies appear primarily in clusters on the glial feeder layer monolayer, which is in a different plane and therefore slightly out of focus. Phase-bright cells stain with Alexa Fluor®488 conjugated anti-NeuN (C), confirming neuronal identity, while background glial monolayer stains with anti-GFAP (E). B, D, F) Treatment with hemoglobin 10 μM with vehicle. Most neurons have degenerated to debris, astrocyte monolayer is intact (B), NeuN immunoreactivity is diminished and limited to areas of degenerating cells (D), GFAP immunoreactivity persists and is increased, particularly near degenerating neurons (E). G, H) Unfixed cultures treated with hemoglobin 10 μM plus U0126

(30 μM) or FR180204 (50 μM), respectively; neuronal morphology is preserved. Scale bar = 100 μm.

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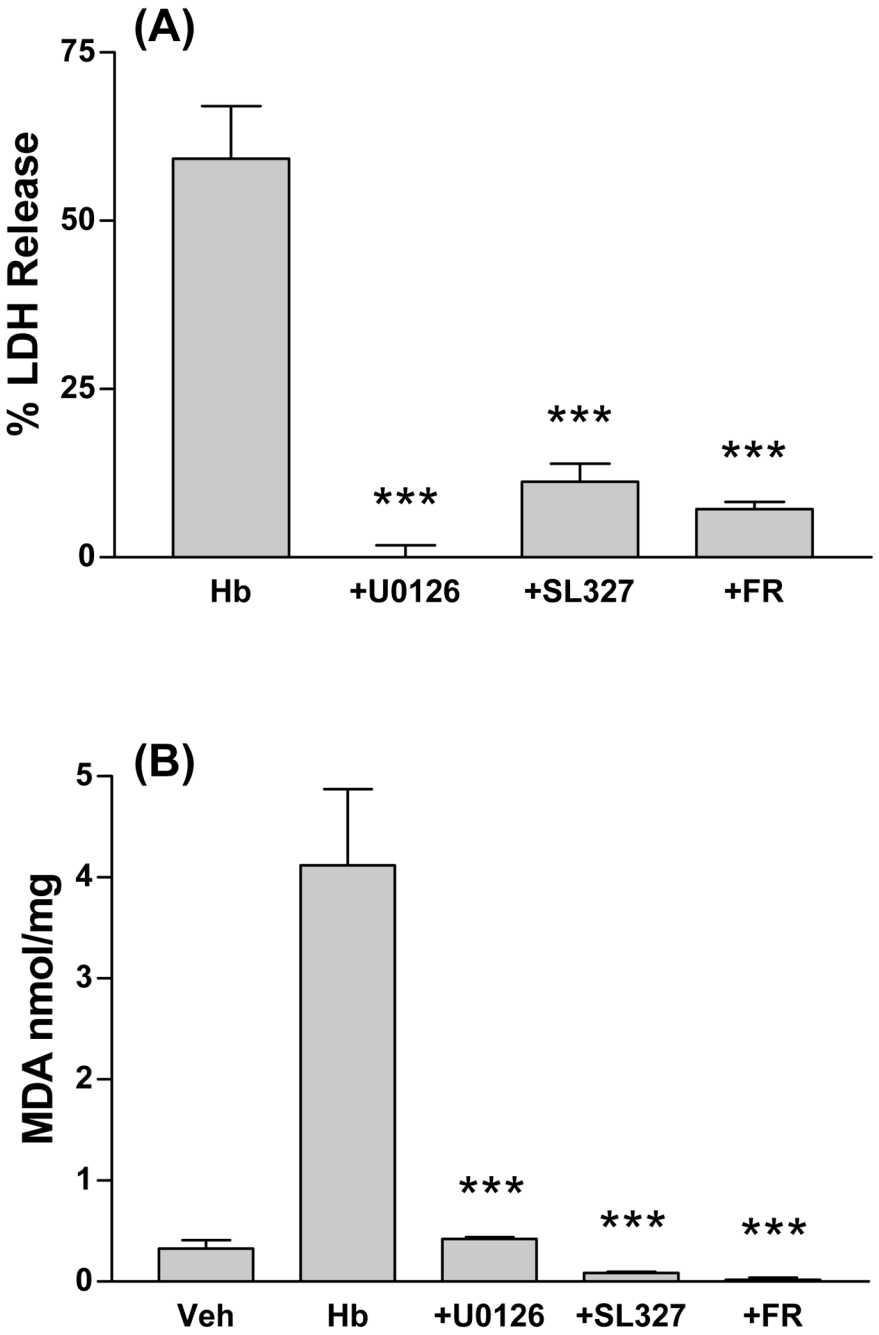

Figure 4.

MEK and ERK inhibitors protect neurons from hemoglobin. A) Cultures were treated for 16h with hemoglobin (Hb) 10 μM plus DMSO vehicle alone or with U0126 (30 μM), SL327 (30 μM), or FR180204 (FR, 50 μM). LDH values are scaled to mean values in sister cultures treated with 300 μM N-methyl-D-aspartate (NMDA), which produces near-100% neuronal death, after subtraction of mean LDH in sister cultures subjected to wash and vehicle treatment only, in order to yield the LDH signal specific for hemoglobin neurotoxicity. B) Culture malondialdehyde (MDA) after treatment as in A. ***P<0.001. v. Hb, Bonferroni multiple comparisons test, n = 8–23/condition.

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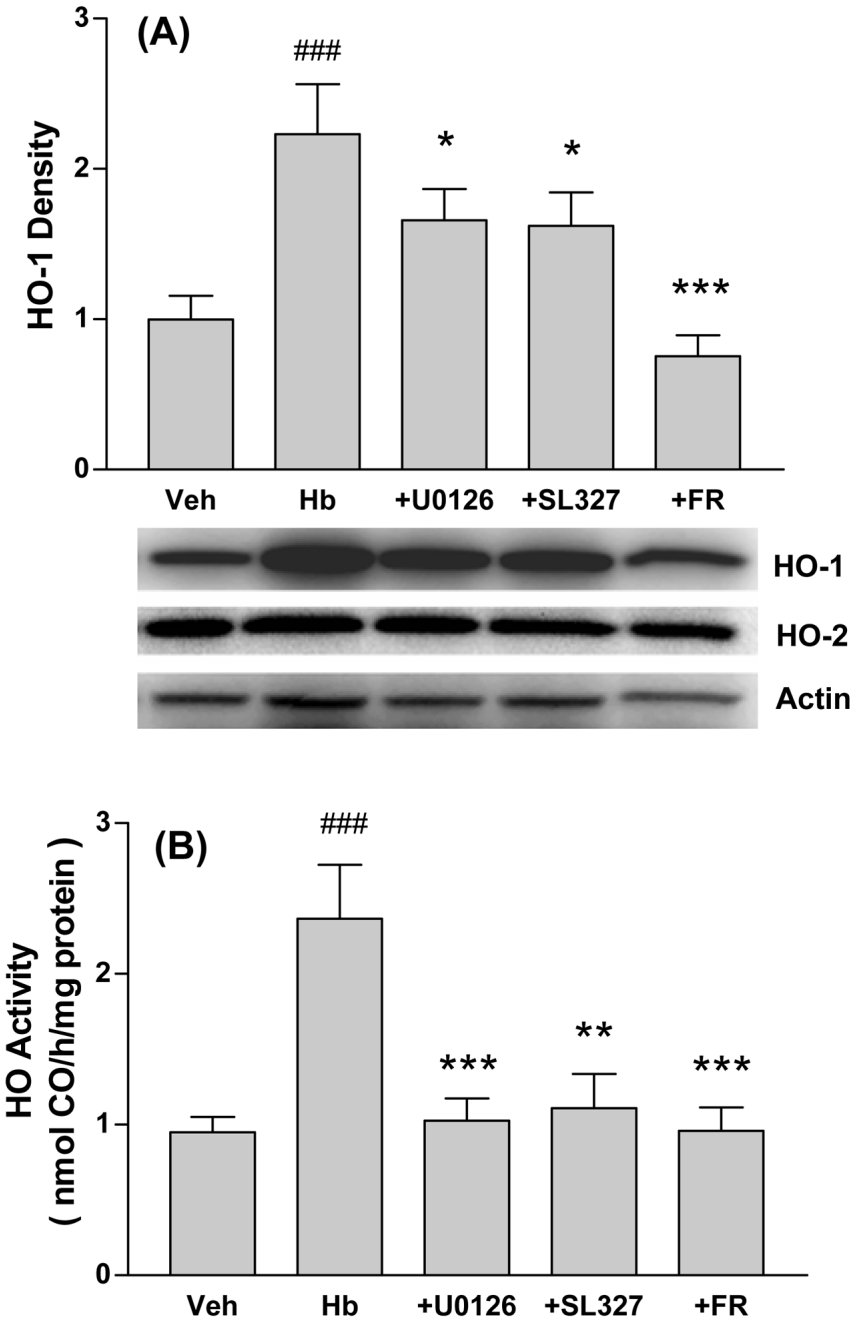

Figure 5.

MEK and ERK inhibitors prevent HO-1 induction after hemoglobin treatment. A) Expression of HO-1, HO-2 and actin (gel loading control) in cultures treated with vehicle (Veh) only for 4h, with hemoglobin (Hb) 10 μM plus vehicle, or with Hb plus U0126 (30 μM), SL327 (30 μM), or FR180204 (FR, 50 μM). B) HO activity in cultures treated as in A. ###P<0.001. v. vehicle, *P<0.05, **P<0.01, ***P<0.001 v. Hb, Bonferroni multiple comparisons test, n = 5–13/condition.