

Effects of hemoglobin on heme oxygenase gene expression and viability of cultured smooth muscle cells

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Marton, Linda S., Xiaoyu Wang, Andrew Kowalczyk, Zhen-du Zhang, Emily Windmeyer, and R. Loch Macdonald. Effects of hemoglobin on heme oxygenase gene expression and viability of cultured smooth muscle cells. *Am J Physiol Heart Circ Physiol* 279: H2405–H2413, 2000.—Ferrous Hb contributes to cerebral vasospasm after subarachnoid hemorrhage, although the mechanisms involved are uncertain. The hypothesis that cytotoxic effects of ferrous Hb on smooth muscle cells contribute to vasospasm was assessed. Cultured rat basilar artery smooth muscle cells were exposed to pure Hb, dog erythrocyte hemolysate, or Hb breakdown products; and heme oxygenase (HO-1 and HO-2) and ferritin mRNA and protein were measured. Cytotoxicity was assessed by lactate dehydrogenase release and fluorescence assays. Pure Hb or hemolysate caused dose- and time-dependent increases in HO-1 mRNA and protein. Hemin was the component of Hb that increased HO-1 mRNA. Cycloheximide inhibited the increase in HO-1 mRNA in response to hemin. Ferritin protein heavy chain but not mRNA increased upon exposure of cells to Hb. Hemin and ferric but not ferrous Hb were toxic to smooth muscle cells. Toxicity was increased by exposure to Hb plus tin protoporphyrin IX. In conclusion, exposure of smooth muscle cells to Hb induces HO-1 mRNA and protein through pathways that involve new protein synthesis. Hemin is the component of Hb that induces HO-1. Hemin and ferric but not ferrous Hb are toxic.

ferritin; subarachnoid hemorrhage; vasospasm

CEREBRAL VASOSPASM is constriction of the cerebral arteries that starts 3 days after subarachnoid hemorrhage (SAH) and persists for up to 14 days (22). The development of vasospasm is closely correlated with the volume and location of subarachnoid blood. Furthermore, the delay in onset of vasospasm after SAH has been suggested to be related to the time taken for hemolysis to release the contents of the erythrocytes into the cerebrospinal fluid around the cerebral arteries. Hb, a major constituent of the erythrocyte cytosol, is believed to play an important role in the pathogenesis of vasospasm. Mechanisms by which Hb may contract cerebral arteries include removal of vasodilating nitric oxide, increased synthesis of endothelins, damage to vasodilating perivascular nerves, and catalysis

of oxidation reactions that may be directly cytotoxic or that may produce other vasoactive products (14, 23). Ferrous Hb increases intracellular calcium in smooth muscle cells (3, 48, 50) perhaps by free radical-mediated reactions that are toxic to the cell and that result in loss of calcium homeostasis (45). The increased intracellular calcium could cause vasoconstriction and therefore may be important in the pathogenesis of vasospasm (25, 32, 45).

The suggestion that toxic effects of ferrous Hb on smooth muscle cells are involved in vasospasm led us to become interested in the mechanisms by which these cells metabolize Hb. Ferrous Hb undergoes spontaneous oxidation to ferric iron-containing methemoglobin, which releases its heme groups more readily than ferrous Hb (7). Heme is enzymatically broken down by heme oxygenases (HO) to biliverdin, iron, and carbon monoxide. Three isoforms of HO have been identified. HO-1, also known as heat shock protein 32, is inducible in many types of cells by numerous physiological and pathological stimuli, including heme, heavy metals, some hormones, oxidative stress, and ultraviolet light, whereas HO-2 is constitutively expressed and induced only by glucocorticoids (26). HO-3 was identified in several rat tissues, including neurons (31). In some cells, exposure to Hb or heme increases HO-1 protein, and this is followed by an increase in ferritin (2, 4, 13, 34). Ferritin sequesters the iron released by metabolism of heme, possibly rendering it nontoxic to the cells. This response occurs in the brain after SAH or injection of Hb-containing solutions into the subarachnoid space of rats (20, 29, 30, 47, 53). Our preliminary data suggest that it occurs in brain tissue and cerebral arteries after SAH in monkeys (38). The induction of HO-1 and in some cases the subsequent increase in ferritin has been suggested to prevent cell and tissue injury in other diseases mediated by Hb and oxidative stress (1, 21, 36, 39, 54, 59).

These experiments therefore tested the hypothesis that exposure of cerebrovascular smooth muscle cells to Hb would increase HO-1 and ferritin proteins and that increased HO-1 would prevent or decrease Hb toxicity.

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MATERIALS AND METHODS

Protocols. Growth-arrested rat basilar artery smooth muscle cells were exposed to the following solutions: 1) pure ferrous human HbA₀; 2) pure ferric human HbA₀; 3) impure bovine Hb; 4) dog erythrocyte hemolysate; or 5) Hb breakdown products, including hemin, protoporphyrin IX, or globin chains. After exposure, changes in HO-1, HO-2, and ferritin mRNA and protein were measured by competitive RT-PCR and Western blotting. In parallel experiments, cytotoxicity was assessed by lactate dehydrogenase (LDH) release and fluorescence assays with propidium iodide and fluorescein diacetate. The effect of tin protoporphyrin IX (SnPP, an inhibitor of HO) on cytotoxicity in response to the above solutions was assessed. All procedures that involved animals were approved by the Institutional Animal Care and Use Committee.

Materials. Hemin, globin chains, impure bovine Hb, and protoporphyrin IX were purchased from Sigma Chemical (St. Louis, MO). SnPP was obtained from Porphyrin Products (Logan, UT). Pure human HbA₀ was obtained from Hemosol (Toronto, Canada). This was ultrapure, uncross-linked Hb containing 99% ferrous Hb. Smooth muscle cell proliferation media was purchased from Becton-Dickinson (p-Stim media, Bedford, MA). Chemiluminescence reagents for Western blotting were obtained from NEN Life Science Products (Boston, MA). All other tissue-culture reagents and chemicals were obtained from Sigma. Rabbit polyclonal antibodies to HO-1 and HO-2 were from StressGen Biotechnologies (Victoria, Canada), ferritin was from Sigma, and anti-rabbit immunoglobulin was purchased from DAKO (Denmark).

Cell culture. Rat basilar artery smooth muscle cells were cultured by an explant method (58). Sprague-Dawley rats were anesthetized with pentobarbital sodium (100 mg/kg ip) and then decapitated. The basilar artery was isolated under sterile conditions and cut into small pieces. The pieces were placed in culture plates initially in media (p-Stim) supplemented with 5% FCS, 0.5 ng/ml epidermal growth factor, 2 ng/ml basic fibroblast growth factor, and 5 µg/ml insulin. When the plates were confluent, usually after 7–14 days, the cells were treated with trypsin and replated in the same medium. The growth of the cells was arrested in serum-free DMEM for 24 h before use in experiments. They were characterized as smooth muscle cells by the presence of positive immunohistochemical staining for α-actin and by a characteristic hills-and-valleys appearance (45). Cells from passages 2–4 were used.

Preparation of solutions. Erythrocyte hemolysate was prepared from fresh blood obtained from dogs that were exsanguinated under general anesthesia maintained with isoflurane and oxygen (33). The blood was drawn into tubes containing 10 mM EDTA to prevent clotting (62). The erythrocytes were isolated by centrifugation and washed six times with cold buffer (0.15 mM NaCl, 0.05 mM Tris·HCl, pH 7.6, and EDTA at 4°C), removing the buffy coat and platelets each time. The erythrocytes were lysed in five volumes of cold phosphate buffer (5 mM, pH 7.2). Membranes were removed by centrifugation at 31,000 g for 15 min. The supernatant fluid was centrifuged again, and the resulting erythrocyte hemolysate was filtered and stored at –80°C until use. Concentrations of oxyhemoglobin, methemoglobin, and total Hb in the hemolysate were determined by spectrophotometry (60).

Pure, sterile ferrous human HbA₀ was stored at –20°C and thawed and diluted in phosphate buffer immediately before use. Pure ferric human HbA₀ was prepared by allowing the pure, sterile ferrous Hb to oxidize at 37°C for 7 days.

Impure bovine Hb was dissolved at the desired concentration in phosphate buffer immediately before use. Hemin solution was prepared in the dark as a 1 mM solution in 20 mM NaOH. It was diluted to the appropriate concentration by addition of serum-free DMEM or phosphate buffer containing 4 mM NaHCO₃. All work with hemin and Hb solutions was carried out in glass. Protoporphyrin solutions were also prepared in glass containers in the dark.

RNA extraction and competitive RT-PCR. Total RNA was extracted from smooth muscle cells using TriZOL reagent (GIBCO-BRL Life Technologies, Gaithersburg, MD) and reverse-transcribed into cDNA (18). Specific primers for PCR for each target cDNA were selected using rat cDNA sequences published in the GenBank (Table 1). Competitors were prepared for HO-1, HO-2, and ferritin heavy (H) and light (L) chains by constructing eight overlapping 40-mer primers (18). Each competitor contained two 20-mer primers that are for different target cDNAs. PCR was performed by using a pair of 40-mer primers to add 20 nucleotides to each end of the template. PCR was carried out in the presence of 1 µCi [³²P]dCTP (3,000 Ci/mmol, Amersham, Arlington Heights, IL). The PCR conditions were 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 32 cycles. The PCR products were separated by electrophoresis on a 5% polyacrylamide gel. The intensity of the bands corresponding to each specific PCR product were analyzed using a computerized phosphor-imager (PhosphorImager, Molecular Dynamics, Sunnyvale, CA).

Western blotting. Smooth muscle cells were harvested with 400 µl lysate buffer consisting of 1% SDS and 40% urea. Proteins were denatured by boiling in lysate buffer containing 1% 2-mercaptoethanol and then separated by 12% SDS-PAGE. They were electrotransferred onto nitrocellulose membranes. HO-1 was detected with rabbit polyclonal anti-rat HO-1 antibody (1:500 dilution). HO-2 was detected with rabbit polyclonal anti-rat HO-2 antibody (1:500 dilution). Ferritin H and L chains were detected with rabbit polyclonal anti-horse spleen ferritin antibody (1:2,000 dilution). The membranes were then incubated with peroxidase-conjugated pig anti-rabbit immunoglobulin (1:2,000 dilution) and reaction products were visualized by chemiluminescence. Densities of specific protein bands were quantified by densitometry of exposed films (National Institutes of Health Gel Scan program).

Cytotoxicity assays. Cell toxicity was assessed by LDH release from the cells and by fluorescence assays with fluorescein diacetate and propidium iodide. For the LDH assay, the culture media was collected after exposure of cells to the

Table 1. Sequences of PCR primers

Gene	Forward Primer (5'–3') Reverse Primer (5'–3')	PCR Size*	GenBank Access No.
HO-1	CTGCTAGCCTGGTTCAAGATA CATCTCCTTCCATTCCAGAG	316 361	J-02722
HO-2	AGTGAAGTGGACCAGGCTG TGGAAGAGGTCACCTGGGAT	295 268	J-05405
Heavy-chain ferritin	CCAGTAAAGTCACATGGCCT GGCTACTGACAAGAATGATC	221 271	M-18053
Light-chain ferritin	AGAAGCCATCTCAAGATGAG CTAGTCGTGCTTCAGAGTGA	304 358	K-01930
β-actin	CGTAAAGACCTCTATGCCAA AGCCATGCCAAATGTGTCTAT	349 473	J-00691

*The top number is the size of the cDNA in base pairs; the bottom number refers to the size of the competitor.

test solution. Smooth muscle cells were lysed with 1% Triton X-100 solution for 30 min and centrifuged at 14,000 *g* for 2 min. The supernatant fluid was collected (cell lysate). LDH activity was estimated spectrophotometrically at 366 nm by measuring conversion of pyruvate to lactate in a reaction coupled to the oxidation of NADH (34). Samples were mixed with a reaction solution containing sodium pyruvate (50 μ l of 0.8 mg/ml sodium pyruvate in Tris buffer), culture medium, or cell lysate (200 μ l) and NADH (50 μ l of 3 mg/ml NADH in Tris buffer) in 400 μ l Tris buffer. Cytotoxicity was expressed as the percent LDH activity in the supernatant fluid according to the formula: (LDH activity in culture media)/[(LDH activity in culture media) + (LDH activity in cell lysate)].

Cell fluorescence assays were carried out by exposing cells to fluorescein diacetate (50 μ g/ml) and propidium iodide (50 μ g/ml) for 30 s. Cells were then washed with phosphate buffer, counted under a fluorescence microscope, and photographed. Normal viable cells stain uniformly green, whereas dead cells show deep red nuclear staining.

Data analysis. All experiments were performed at least three times on cells obtained from different rats. To calculate changes in mRNA levels, the ratio of intensity of bands of products of cDNA and corresponding internal standard DNA (cDNA/standard DNA) were determined. Relative mRNA increases are given as comparisons to the level of mRNA at baseline. For Western blotting, changes in protein levels were expressed as a ratio to the amount of protein in control untreated cells. For fluorescence assays, 100 cells were counted per plate from experiments conducted in triplicate. Values are expressed as means \pm SD. Statistical analysis between groups was by ANOVA for multiple comparisons, followed by pairwise comparisons using Tukey's test if significant variance was found. Student's *t*-test was used for comparisons between two measurements. Significance was taken at $P < 0.05$.

RESULTS

HO-1 mRNA expression. Dog erythrocyte hemolysate and purified Hb caused time- and dose-dependent increases in HO-1 mRNA (Figs. 1 and 2). Time-dependent effects were measured after exposure to 10 μ M Hb or hemolysate containing 10 μ M total Hb. Dose dependence was measured after 6 h of exposure to the solution. The increase in HO-1 mRNA began within 2 h of exposure to Hb and peaked after 6 h with a maximal 6.0 ± 0.9 -fold increase at 6 h. Hemolysate caused a smaller, more delayed response that started at 4 h and was maximal at 8 h (maximal 4.8 ± 1.0 -fold increase at 8 h). Hemoglobin or hemolysate (8.5 nM) did not increase HO-1 mRNA after 6 h, whereas a significant increase was observed at concentrations over 0.14 μ M ($P < 0.05$, ANOVA). HO-1 mRNA in response to both Hb and hemolysate remained elevated for at least 24 h, the latest time examined. In contrast to HO-1 mRNA, there was no significant increase in HO-2 mRNA at any dose of Hb or hemolysate or at any time after exposure (Figs. 1 and 2).

To determine the nature of the substance in Hb that was increasing HO-1 expression, cells were exposed to Hb breakdown products: heme, globin chains, and protoporphyrin IX (all at 1 μ M). Heme was the most potent inducer of HO-1 mRNA, producing a 4.2 ± 0.2 -fold increase in HO-1 mRNA after 6 h (Fig. 3). This was significantly more potent than pure Hb (2 μ M),

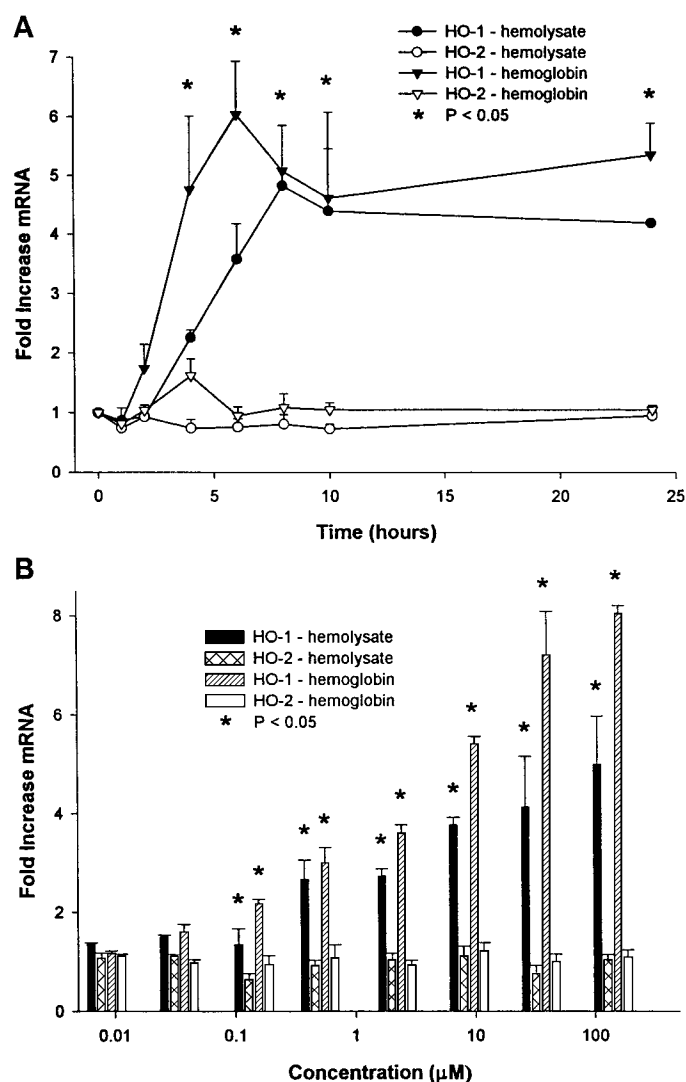


Fig. 1. Time- (A) and dose-dependent (B) effects of pure human HbA₀ and dog erythrocyte hemolysate on heme oxygenase (HO) mRNA expression in cultured rat basilar artery smooth muscle cells as assessed by competitive RT-PCR. HO-1 and HO-2 mRNA levels are expressed as the ratio compared with untreated control cells. Both Hb and hemolysate cause significant ($P < 0.05$, ANOVA; $n = 3$ per measurement) increases in HO-1 mRNA, whereas there is no change in HO-2 mRNA. Time-dependent effects are after exposure to 10 μ M Hb or hemolysate containing 10 μ M total Hb. Dose dependence was measured after 6 h of exposure to the solution.

which produced a 3.6 ± 0.2 -fold increase after 6 h ($P < 0.05$, unpaired *t*-test). Heme also produced a dose-dependent increase in HO-1 mRNA (Fig. 4). Heme did not change HO-2 mRNA expression (Fig. 4).

To determine whether heme-induced HO-1 mRNA expression required synthesis of new protein, the effect of cycloheximide was assessed. Cycloheximide (10 μ M) completely blocked the increase in HO-1 mRNA in response to 1 μ M heme, showing that new protein synthesis is required for induction of HO-1 mRNA (Fig. 3).

HO-1 protein expression. To determine whether the HO-1 mRNA increases were translated into increases in protein and how long the increases lasted, we ex-

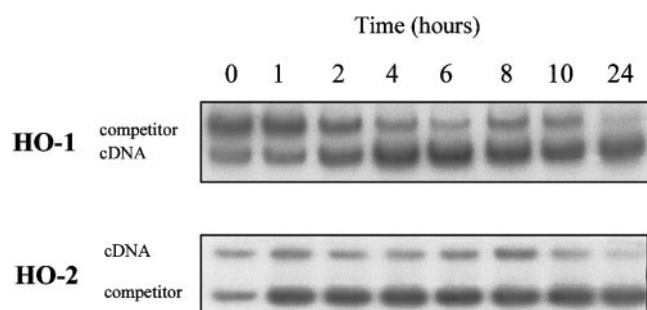


Fig. 2. Images of gels of competitive RT-PCR for HO-1 (top) and HO-2 (bottom) mRNA after exposure of rat basilar artery smooth muscle cells to 10 μ M pure Hb for 0–24 h. There is an increase in HO-1 cDNA in relation to the competitor, whereas there is no change in HO-2 mRNA. Sizes of PCR products are given in Table 1.

posed cultured rat basilar artery smooth muscle cells to pure Hb (8.8 μ M) and measured HO-1 protein by Western blotting (Figs. 5 and 6). The polyclonal antibody to HO-1 reacted with one protein corresponding to the molecular weight of HO-1. There was no observable HO-2 protein detectable by this antibody. A polyclonal antibody to HO-2 failed to detect HO-2 in these cells. The increase in HO-1 protein appeared later than that of mRNA. The increase of HO-1 protein was significant after 6 h and reached a peak at 36 h. The expression of HO-1 protein decreased at 48–72 h but remained significantly higher than control.

Ferritin expression. Ferritin mRNA and protein expression were examined by RT-PCR and Western blotting of smooth muscle cells exposed to pure Hb in increasing doses and for increasing times (Figs. 5 and 6). Purified apoferritin from the horse spleen was used as a positive control and was found to contain only ferritin L chain mRNA. In the cultured rat basilar artery smooth muscle cells, ferritin H chain mRNA was

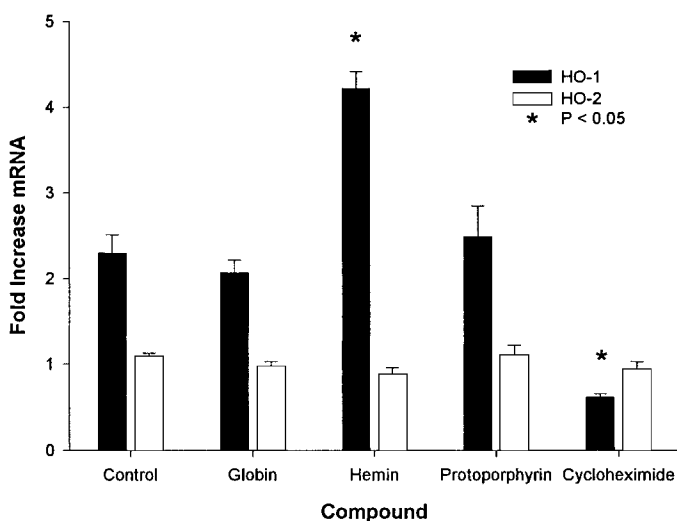


Fig. 3. Effect of 1 μ M each of globin chains, hemin, protoporphyrin IX, or hemin and 10 μ M cycloheximide on HO-1 mRNA in rat basilar artery smooth muscle cells after 6 h. Only hemin significantly increased HO-1 mRNA ($P < 0.05$, ANOVA; $n = 3$ per measurement). Cycloheximide completely inhibited the hemin-induced increase in HO-1 mRNA ($P < 0.05$, unpaired t -test).

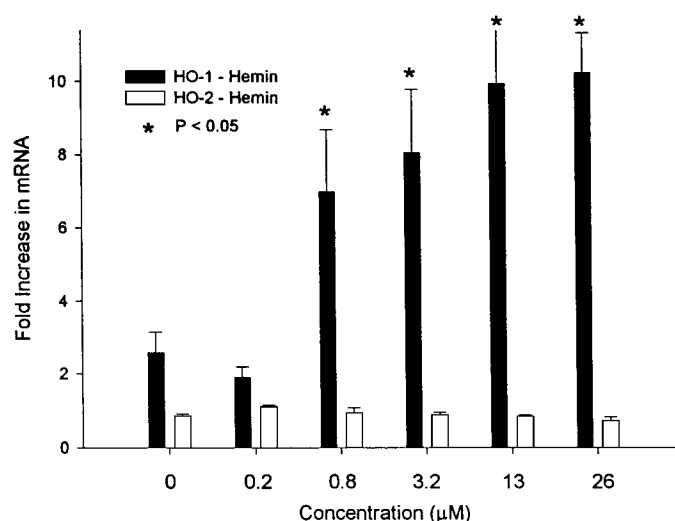


Fig. 4. Dose-dependent effects of hemin on HO-1 and HO-2 mRNA expression in cultured rat basilar artery smooth muscle cells. Cells were exposed to different concentrations of hemin, and mRNA was measured by competitive RT-PCR after 6 h. HO-1 mRNA was increased significantly after exposure to concentrations of hemin ≥ 0.8 μ M ($P < 0.05$, ANOVA; $n = 3$ per measurement). HO-2 mRNA levels were not affected.

the major form detected by Western blotting (Fig. 6). Ferritin protein increased significantly 6 h after exposure of cells to 8.8 μ M Hb and continued to increase for up to 72 h after treatment ($P < 0.05$, ANOVA, Fig. 5). Ferritin H and L chain mRNA were detected in the smooth muscle cells and were not changed as assessed by competitive RT-PCR after exposure to pure Hb or hemolysate in concentrations up to 140 μ M (data not shown).

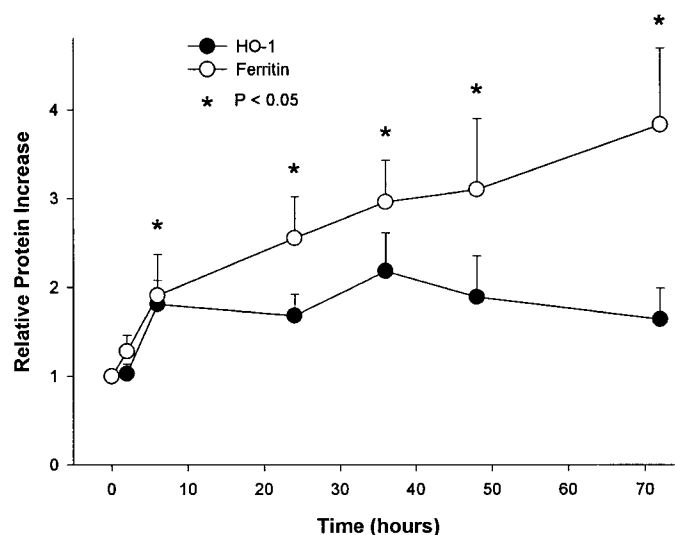


Fig. 5. Effect of 8.8 μ M Hb on HO-1 and ferritin proteins in cultured rat basilar artery smooth muscle cells over time. Hb causes a significant increase in HO-1 protein starting at 6 h, peaking at 36 h (2.2 ± 0.4 -fold increase), and declining but remaining significantly elevated at 72 h ($P < 0.05$, ANOVA compared with 0 h). Ferritin protein also was significantly increased by 6 h after exposure and continued to increase with time to a maximum at 72 h (3.8 ± 0.9 -fold increase; $P < 0.05$, ANOVA compared with 0 h; $n = 3$ per measurement).

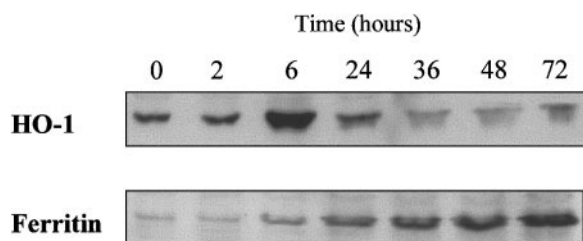


Fig. 6. Western blotting of HO-1 (32 kDa, top) and ferritin (21 kDa, bottom) proteins over time. A 10- μ g protein was loaded per lane. There is an increase in HO-1 protein peaking after 6 h, whereas ferritin protein continues to increase for up to 72 h.

Effect of HO-1 induction on cytotoxicity. Pure ferrous Hb (50 μ M) was not significantly toxic, as assessed by LDH release after exposure of cultured smooth muscle cells for 72 h (Fig. 7). Pure ferric methemoglobin at the same concentration, however, was significantly toxic. Coincubation of ferrous or ferric Hb with SnPP (100 μ M) significantly increased the toxicity of both Hbs. Hemin (50 μ M) was much more toxic than pure ferrous or ferric Hb, causing more LDH release after only 24 h of exposure (Fig. 7); in addition, it caused $73 \pm 3\%$ LDH release after 72 h compared with $16 \pm 5\%$ for ferrous and $37 \pm 4\%$ for ferric Hb at 72 h. Coincubation of hemin with SnPP significantly increased toxicity (Fig. 7, $P < 0.05$, unpaired t -test). Virtually identical results were obtained when toxicity was assessed by fluorescence assays (Fig. 8).

Impure Hb and hemolysate appeared to be markedly toxic to smooth muscle cells after a 24- to 72-h exposure, as assessed by the LDH assay ($63 \pm 22\%$ to $78 \pm 13\%$ LDH release). This was not born out, however, by results of fluorescence cytotoxicity assays (Fig. 8). Fluorescence assays showed that impure Hb (50 μ M) was more toxic than control ($P < 0.05$) but not to the degree suggested by the LDH assay. Furthermore, there was no significant difference in the toxicity of impure Hb, which contained 98% ferric Hb, and pure ferric Hb; nor was there a difference between hemolysate, which contained 99% ferrous Hb, and pure ferrous Hb. The toxicity of impure Hb was significantly greater when incubated with SnPP (100 μ M, $P < 0.05$, Fig. 8).

DISCUSSION

These experiments show that Hb and its breakdown products that contain heme induce expression of HO-1 and ferritin proteins in cerebral arterial smooth muscle cells. The increase in HO-1 and ferritin are time and dose dependent. Hemin was the active compound of Hb that induced HO-1, whereas two Hb breakdown products, globin chains and protoporphyrin IX, did not increase HO-1 expression. Hemolysate resulted in less induction of HO-1 compared with pure Hb. The smaller effect of hemolysate may be due to decreased release of heme from the Hb in hemolysate (5). Balla and colleagues (5) reported that ferric but not ferrous Hb increased HO-1 mRNA and protein in cultured endothelial cells. This was related to the increased rate of release of heme from ferric Hb. If this were true, then

the rate of oxidation of Hb should be higher in pure Hb solution than in hemolysate. However, the oxidation rate of Hb in hemolysate was faster than oxidation of pure Hb solutions (R. L. Macdonald, unpublished observations). Another possibility is that substances in hemolysate inhibit HO-1 induction. The marked difference in the pattern of immediate early gene induction in smooth muscle cells in response to Hb and hemolysate is consistent with this possibility (58). Finally, Motterlini and co-workers (34) noted that pure ferrous Hb did not release heme to endothelial cells but it did increase HO activity. They therefore postulated that there were additional mechanisms for induction of HO-1 in response to ferrous Hb that are independent of the release of heme when ferrous Hb is oxidized to

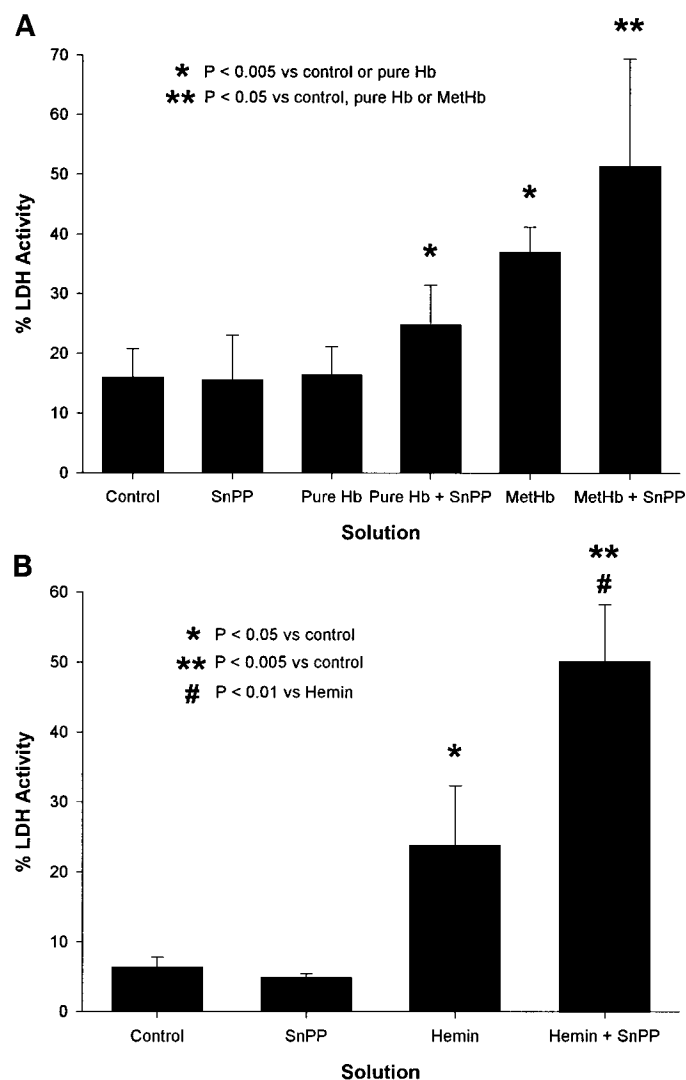


Fig. 7. Cytotoxicity of various 50 μ M solutions as measured by lactate dehydrogenase (LDH) release ($n = 12$ per measurement). Pure ferrous Hb was not toxic after 72 h (A), whereas ferric Hb (MetHb) was toxic. Addition of 100 μ M tin protoporphyrin IX (SnPP) significantly increased the toxicity of both forms of Hb. Hemin (50 μ M) was more toxic, showing greater LDH release than that occurring with ferrous Hb but after only 24 h of exposure (B). Hemin toxicity was also increased by coincubation with 100 μ M SnPP.

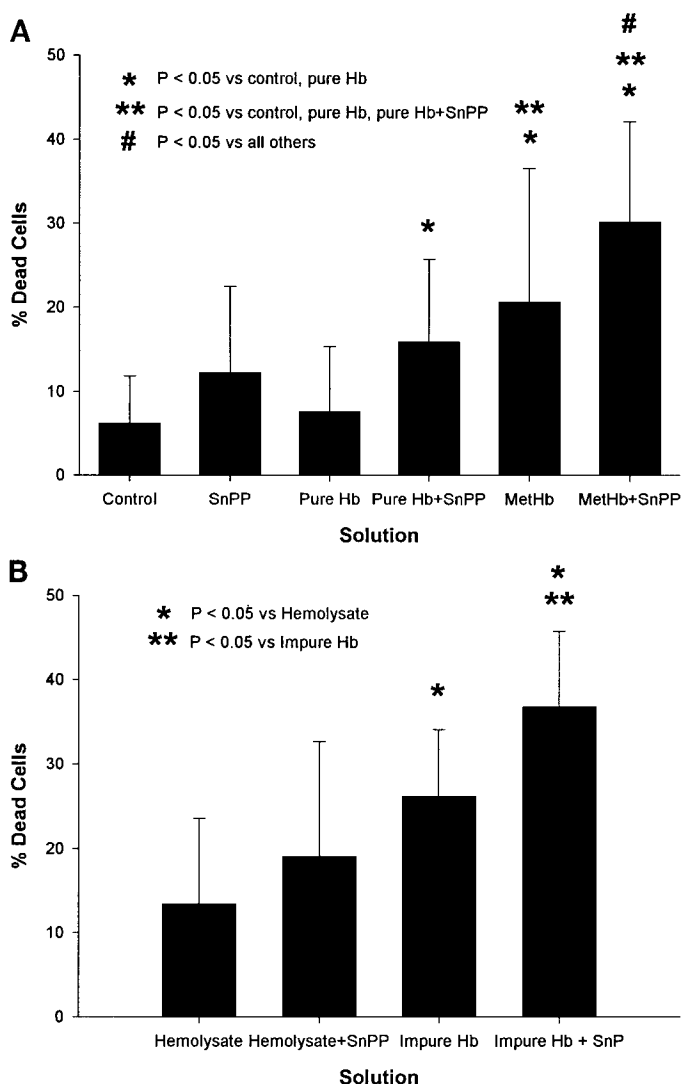


Fig. 8. Cytotoxicity of various 50 μ M solutions as measured by fluorescence assay. Pure ferrous Hb (pure Hb) was not toxic after 72 h (A), whereas MetHb was toxic. Addition of 100 μ M SnPP significantly increased the toxicity of both forms of Hb. Hemolysate and impure Hb (both containing 50 μ M Hb) were more toxic than control ($P < 0.05$) and the toxicity of impure Hb was significantly greater when incubated with 100 μ M SnPP (B). Data for counts of 100 cells per plate from three separate experiments.

ferric Hb. Iron-mediated oxygen free-radical generation may be involved (34).

Previous studies showed that ferrous Hb did not increase HO-1 mRNA in lung 16 h after treatment in vivo (6) or in endothelial cells 8 h after exposure in vitro (5). In contrast to these reports, our results suggest that HO-1 can be induced rapidly by ferrous Hb in cultured cerebral artery smooth muscle cells. The Hb used in our study contained 99% ferrous Hb. This pure Hb oxidized to methemoglobin slowly over time starting immediately (R. L. Macdonald, unpublished observations). Because ferric Hb is known to release its heme groups more readily than ferrous Hb, because this process occurs rapidly in our culture system, and because heme is a potent inducer of HO-1, it seems

likely that heme release accounts for the increase. Other mechanisms, however, cannot be ruled out (34, 58).

The induction of HO-1 mRNA and protein, followed a short time later by an increase in ferritin, has been reported in human skin fibroblasts in response to ultraviolet A radiation (54, 55). Oxidant stress increased HO-1 protein in cultured porcine aortic smooth muscle cells (44). Yet and colleagues (61) reported induction of HO-1 mRNA and enzyme activity in aortic smooth muscle cells from rats injected with endotoxin. We report that Hbs and hemin cause the same response in cerebral smooth muscle cells. This suggests that induction of HO-1 in mammalian cells is a general response to heme-containing compounds and probably to oxidant stress which results from the oxidation of ferrous to ferric Hb (2, 14, 34). HO-1 expression was regulated at the level of transcription, whereas ferritin expression was regulated posttranslationally. This is consistent with known mechanisms of regulation of these proteins (19). In keeping with studies in other types of cells, the level of HO-2 mRNA and protein did not change after exposure to Hb or Hb-containing solutions (26).

The mechanism of HO-1 mRNA induction requires new protein synthesis because cycloheximide suppressed this induction. Durante and colleagues (12) reported that HO-1 induction in response to nitric oxide exposure in vascular smooth muscle cells was inhibited by cycloheximide. Initiation of HO-1 expression may be mediated by activation of protein kinases. Tyrosine phosphorylation was involved in HO-1 expression induced by hemin in HeLa cells (28). We have shown that hemolysate induces tyrosine phosphorylation in endothelial cells (27). The HO-1 promoter contains numerous transcription-factor binding sites, including sites for nuclear transcription factor- κ B (NF- κ B) and AP-1 (9, 26) and Hb has been shown to activate NF- κ B in endothelial cells (43).

Hb also increased ferritin protein in cerebrovascular smooth muscle cells, a response not previously described in this cell type. Ferritin is an iron-binding protein; its expression may increase in a delayed fashion by stimuli that first induce HO-1 (4, 54, 55). The regulation of ferritin expression at a posttranscriptional level in cerebrovascular smooth muscle appears to be similar to that in most cells (17, 19), although the mechanism may depend on the stimulus and cell type. Pang and co-workers (41) showed that interleukin-1 and tumor necrosis factor but not oxidized low-density lipoprotein increased ferritin H-chain mRNA in human aortic smooth muscle cells. Hb could be considered as a source of oxidative stress and our results are therefore consistent with those of Pang and colleagues in that their source of oxidative stress, oxidized low-density lipoprotein, did not increase ferritin mRNA. We also found that Hb induced only ferritin H-chain expression in cerebral artery smooth muscle. The proportion of ferritin H and L chains varies in different cells and tissues (17). Ferritin L chain is expressed predominantly in the spleen and liver. The L chain is involved

primarily in iron storage. Ferritin H chain is important in intracellular iron transport and possesses ferroxidase activity that converts ferrous to ferric iron, a critical step in the sequestration of iron in ferritin. Ferritins rich in H chains predominate in tissues like heart and pancreas. The marked increase in H chain in cerebrovascular smooth muscle suggests that an important role of ferritin is preventing oxidative damage due to iron in this type of cell.

Inhibition of HO-1 with SnPP enhanced cell death caused by Hb or hemin, suggesting that HO-1 protects cerebrovascular smooth muscle cells from injury due to these compounds. These acute responses may represent a defense mechanism in smooth muscle cells against Hb and heme. This may be mediated by removal of heme, which may be toxic to cells, and by generation of bilirubin, which may be an antioxidant (46). In addition, ferritin was increased and will take up iron released from heme, possibly protecting the cell against oxidation reactions that could be catalyzed by the iron if it remained free in the cytosol. We did not investigate whether or not ferritin mediates cytoprotection in these cells. There is controversy about whether HO-1 protects cells from toxicity due to heme and various oxidative agents, whether ferritin is the important protectant, or whether both are involved (13, 21, 37, 54). Manipulation of HO-1 may or may not affect ferritin (8, 11). Blocking the induction of HO-1 with antisense oligonucleotides in human skin fibroblasts abrogated increases in HO-1 and ferritin and cytoprotection (54). Blockade of HO-1 enzyme activity with SnPP prevented human pulmonary epithelial cells from becoming resistant to hyperoxia *in vitro* (21). Abraham and colleagues (1) transfected rabbit coronary endothelial cells with HO-1 and showed that this protected the cells from heme and Hb toxicity. Blockade of HO-1 with SnPP increased, whereas preinsult induction of HO-1 with ferriprotoporphyrin IX decreased, inflammation after injection of carrageenin into the rat pleural cavity (59). Blockade of HO-1 does not protect adenocarcinoma cells from oxidative DNA damage *in vitro* (37) or the lung from hyperoxia (52). In these studies and other studies suggesting a protective role of HO-1 against various cell insults (1, 21, 39, 51, 59), ferritin was not measured, so it is possible that ferritin also was increased and that this contributed to cytoprotection (56). On the other hand, induction of HO-1 is associated with oxidative stress, whereas ferritin is not altered in hamster fibroblasts (11), and adenoviral-mediated transfection of lung tissue *in vivo* with HO-1 protects from hyperoxia without altering ferritin (40). Therefore, both HO-1 and ferritin may mediate cell resistance to oxidative stress, hemin, and Hb (36).

The suggestion that HO-1 is cytoprotective is based on inhibition of HO-1 activity with SnPP. SnPP is not a specific HO-1 inhibitor, and at high doses it may inhibit other heme-containing enzymes such as guanylate cyclase, nitric oxide synthase, and biliverdin reductase (10, 16, 57). At the doses we employed, however, SnPP is believed to be a relatively specific

inhibitor of HO isozymes. The possibility that cytotoxicity was increased because of inhibition of other heme-containing enzymes cannot be ruled out.

The toxicity of Hb varies depending on the type of cell that is exposed to Hb. Regan and Panter (42) reported that Hb was toxic to neurons but not glia. There was 100% LDH release when neurons were exposed to 25 μ M pure, probably ferrous, Hb for only 24 h. Pure ferrous Hb was more toxic to endothelial cells than to smooth muscle cells (35, 49). We found that pure ferrous Hb was not toxic to smooth muscle cells. This is at variance with other reports (15, 45). Fujii and Fujitsu (15) reported that rat aortic smooth muscle cells exposed to erythrocyte hemolysate for 7 days became progressively contracted and developed vacuoles. We did not observe cell vacuoles or contractile effects of Hb, although we did not specifically measure these end points. Hb was reported to kill rat cerebrovascular smooth muscle cells (45). We show here that pure ferrous Hb is not toxic, but that the solution used by other investigators, which was equivalent to the impure Hb solution that we used, was toxic to the same type of cell. Furthermore, our results of calcium imaging studies (62) suggest that pure Hb does not reliably increase intracellular calcium in smooth muscle cells as reported by others (3, 50). We suggest that toxicity and increases in intracellular calcium are due to contaminants and breakdown products in the Hb solutions. This finding is of importance to investigation of cerebral vasospasm that complicates subarachnoid hemorrhage because studies continue to be published that erroneously conclude that the intact Hb molecule has direct effects on smooth muscle cells (3).

Our results suggest that the predominant component of Hb that causes cell injury is hemin. Hemin and ferric but not ferrous Hb solutions were toxic to smooth muscle cells. The toxicity of ferric Hb solutions is probably due to the more rapid release of hemin from ferric compared with ferrous Hb (5). This may overwhelm cellular defenses associated with increased HO-1 and ferritin. Hemin was also the component of Hb that induced HO-1. Because HO-1 prevented toxicity from Hb, the results are consistent with the idea that HO-1 protects against hemin toxicity. This response, however, could be overwhelmed by, for example, ferric Hb or hemin, which may release more hemin and/or iron than can be detoxified by HO-1 and ferritin. These effects of extracellular Hb and hemin on HO-1 and ferritin may be important in diseases in which vascular smooth muscle cells are exposed to Hb and/or hemin, such as atherosclerosis and vasospasm after subarachnoid hemorrhage. Changes in arterial HO-1 and ferritin after subarachnoid hemorrhage, however, have not been studied in detail. We showed that HO-1 and ferritin proteins were increased in cerebral arteries after subarachnoid hemorrhage in monkeys (24, 38). Our results *in vitro* showing a protective effect of HO-1 suggest that modulation of the induction of HO-1 and ferritin may be useful for altering cerebral vasospasm after subarachnoid hemorrhage.

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