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## Mechanisms of neuroprotection by hemopexin: modeling the control of heme and iron homeostasis in brain neurons in inflammatory states

Peter Hahl<sup>\*</sup>, Taron Davis<sup>\*</sup>, Cecilia Washburn<sup>†</sup>, Jack T. Rogers<sup>†</sup>, and Ann Smith<sup>\*</sup>

<sup>\*</sup>School of Biological Sciences, University of Missouri - Kansas City, Kansas City, Missouri, USA

<sup>†</sup>Neurochemistry Laboratory, Department of Psychiatry-Neuroscience, Massachusetts General Hospital (East), Harvard Medical School, Charlestown, Massachusetts, USA

### Abstract

Hemopexin provides neuroprotection in mouse models of stroke and intracerebral hemorrhage and protects neurons *in vitro* against heme or reactive oxygen species (ROS) toxicity via heme oxygenase-1 (HO1) activity. To model human brain neurons experiencing hemorrhages and inflammation, we used human neuroblastoma cells, heme–hemopexin complexes, and physiologically relevant ROS, for example, H<sub>2</sub>O<sub>2</sub> and HOCl, to provide novel insights into the underlying mechanism whereby hemopexin safely maintains heme and iron homeostasis. Human amyloid precursor protein (hAPP), needed for iron export from neurons, is induced ~twofold after heme–hemopexin endocytosis by iron from heme catabolism via the iron-regulatory element of hAPP mRNA. Heme–hemopexin is relatively resistant to damage by ROS and retains its ability to induce the cytoprotective HO1 after exposure to tert-butylhydroperoxide, although induction is impaired, but not eliminated, by exposure to high concentrations of H<sub>2</sub>O<sub>2</sub> *in vitro*. Apo-hemopexin, which predominates in non-hemolytic states, resists damage by H<sub>2</sub>O<sub>2</sub> and HOCl, except for the highest concentrations likely *in vivo*. Heme–albumin and albumin are preferential targets for ROS; thus, albumin protects hemopexin in biological fluids like CSF and plasma where it is abundant. These observations provide strong evidence that hemopexin will be neuroprotective after traumatic brain injury, with heme release in the CNS, and during the ensuing inflammation. Hemopexin sequesters heme, thus preventing unregulated heme uptake that leads to toxicity; it safely delivers heme to neuronal cells; and it activates the induction of proteins including HO1 and hAPP that keep heme and iron at safe levels in neurons.

### Keywords

APP; heme; hemopexin; iron; neuroprotection; stroke

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Iron metabolism is altered in cerebrovascular disease and elevated brain iron is associated with the pathogenesis of the aging brain, Alzheimer's disease [AD (Crapper McLachlan *et al.* 1991; Bartzokis *et al.* 2000)], and neurodegeneration in stroke and intracerebral hemorrhage. The incidence of brain microhemorrhages that release heme increases with age (Takahashi *et al.* 2000; Qureshi *et al.* 2001) and contributes to AD pathology (Tong *et al.*

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Address correspondence and reprint requests to Dr. Ann Smith, PhD, Division of Molecular Biology & Biochemistry, School of Biological Sciences, University of Missouri-K.C., 5007 Rockhill Road, Kansas City, MO 64110, USA. smithan@umkc.edu. The first two authors contributed equally to this work; A. Smith conceived and designed the research, helped by J. T. Rogers; P. Hahl carried out the spectral analyses, data analyses, and designed the figures; T. Davies carried out the HO1 and APP induction experiments; C. Washburn & J. T. Rogers carried out the APP–IRE luciferase experiments. A. Smith wrote the paper.

2005; Brenner 2008; Cordonnier and van der Flier 2011) probably by heme-induced oxidative stress via lipid peroxidation (Gutteridge and Smith 1988; Timmins *et al.* 1995) and release of redox-active ferrous iron from heme catabolism by heme oxygenases. Iron toxicity from ferrous iron, produced within the reducing environment of cells, generates the highly reactive hydroxyl radical (Hershko 2007). Normally, iron storage, use in biochemical and regulatory purposes, or export together minimize levels of reactive 'free' iron. The human amyloid precursor protein (hAPP), whose expression and processing is dysregulated in AD leading to toxic amyloid burden, acts in neurons as a ferroxidase for iron export (Duce *et al.* 2010). Ferroxidases prevent oxidative stress by converting ferrous ions to ferric. In human neuroblastoma SH-SY5Y cells, considered good models of human neurons, iron levels are controlled in part by hAPP as high levels promote iron export, whereas hAPP ablation increases iron retention (Zheng *et al.* 2009). Furthermore, hAPP is induced by iron in SH-SY5Y cells, and iron influx induces APP (ferroxidase-II) translational up-regulation mediated by the release of Iron-regulatory protein repressor from iron-responsive element sequences (IRE) in the 5' untranslated region of the precursor transcript (Cho *et al.* 2010).

Inflammation rapidly follows brain damage from stroke, hemorrhage, and trauma. Oxidative stress from reactive oxygen species (ROS) generated by neutrophils and other phagocytic cells is exacerbated by heme from hemoglobin. Heme, free or bound to globin, reacts with hydroperoxides generated *in vivo* from lipid peroxides during oxidative stress or normal metabolism to produce ROS, including highly reactive radical species. This reactivity of heme that destroys biomolecules is prevented when it is bound to hemopexin because access of ROS to the heme is then restricted (Gutteridge and Smith 1988; Timmins *et al.* 1995). Neuroprotection by hemopexin has been documented in mouse models of stroke (Li *et al.* 2009) and intracerebral hemorrhage (Chen *et al.* 2010) implicating heme as a toxic molecule. Cytoprotection of neurons by hemopexin against damage by heme and ROS in part requires active heme oxygenase-1 [HO1 (Li *et al.* 2009)]. Numerous published studies show that raising HO1 levels is protective (Morse and Choi 2002). This occurs using carbon monoxide released from heme catabolism to activate the ataxia telangiectasia mutated (ATM) protein needed for double-stranded DNA repair (Otterbein *et al.* 2011). However, whether the inducible HO1 and the constitutively expressed HO2, whose basal levels are high in brain cells, protects neurons in brain injury is not clear (Chen and Regan 2004; Schipper 2004; Song *et al.* 2006), and there may be cell-type-specific differences, for example, astrocytes may differ from neurons (Schipper *et al.* 2009a,b; Song *et al.* 2012). Therefore, it is important to characterize the mechanistic details of iron and heme metabolism in brain cells and the role in this of neuroprotective hemopexin.

Under *in vitro* conditions designed to mimic brain damage after bleeding and oxidative stress from inflammation, we here address whether hemopexin binds heme and regulates HO1 and hAPP in models of human neurons, the SH-SY5Y neuroblastoma cells. Our observations support that hemopexin protects brain neurons immediately after such damage and the ensuing inflammatory conditions, by heme sequestration, safe heme delivery, and induction of HO1 and hAPP. While the extracellular antioxidant role of hemopexin (Gutteridge and Smith 1988) is maintained, the intracellular free heme and iron are kept at safe levels in neurons. Thus, protection by hemopexin is proposed to be important in stroke, hemorrhage, and traumatic brain injury. Such protection may be progressively lost in neurodegenerative conditions like AD in which redox-active metals and oxidative stress contribute to the pathology.

## Methods

### Chemicals and reagents

Ham's F12 Nutrient Mixture, Dulbecco's Modified Eagle Medium (DMEM), Penicillin-Streptomycin, and sodium pyruvate were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Eagle's Minimum Essential Medium (EMEM) and fetal bovine serum (FBS) were purchased from the American Type Culture Collection (Manassas, VA, USA). Iron-protoporphyrin IX (heme) was obtained from Frontier Scientific (Logan, UT, USA), dissolved in dimethyl sulfoxide (DMSO) and the concentration determined spectrophotometrically as described previously (Eskew *et al.* 1999) (Flaherty *et al.* 2008). All other chemicals were purchased from Sigma Aldrich Chemical Company (St. Louis, MO, USA).

### Preparation of heme–hemopexin complexes

Apo-hemopexin was isolated and characterized as described (Eskew *et al.* 1999). Stoichiometric iron-protoporphyrin IX (heme)–HPX complexes (> 90–95% saturated) were prepared, characterized and quantified following published procedures and using published extinction coefficients at 414 nm (Eskew *et al.* 1999). Heme–hemopexin complexes (15 and 25  $\mu\text{M}$ ) were prepared for tissue culture experiments at a molar ratio of 0.9 : 1 (heme : protein) in phosphate-buffered saline (PBS), exposed to ROS or solvent as described, dialyzed to remove ROS, then filter sterilized (Millex-GV Syringe-Driven Filter Unit, Hydrophilic Durapore, 0.22  $\mu\text{m}$ , Millipore, Cork, Ireland) before addition to the incubation medium. Throughout this work, the concentrations of apo-hemopexin and heme–hemopexin complexes were determined from their absorbance spectra using published extinction coefficients [ $\epsilon_{\text{mM}} = 110/\text{mM}/\text{cm}$  at 280 nm and  $\epsilon_{\text{mM}} = 120/\text{mM}/\text{cm}$  at 414 nm (Morgan and Muller-Eberhard 1972; Morgan and Smith 2001; Flaherty *et al.* 2008)].

Equimolar heme–hemopexin complexes (10  $\mu\text{M}$ ) in PBS were incubated with either  $\text{H}_2\text{O}_2$  or *tert*-butyl hydroperoxide (tBuOOH) at molar ratios of 1 : 2.5 to 1 : 15 at 37°C for between 2 min and 2 h as noted in the figure legends. The concentration of  $\text{H}_2\text{O}_2$  (Sigma-Aldrich) was determined spectrophotometrically [ $\epsilon_{\text{M}} = 43.6/\text{M}/\text{cm}$  at 240 nm (Jiang *et al.* 1990)]; and  $\text{H}_2\text{O}_2$  was also used as the standard to determine the concentration of tBuOOH (MP Biomedicals, Solon, OH, USA) using the PeroXoquant Quantitative Peroxide Assay Kit (Thermo Scientific, Rockford, IL, USA). The stability to peroxides of heme bound to human serum albumin (HSA; purchased from Sigma-Aldrich) was similarly assessed [ $\epsilon_{\text{mM}} = 36.6/\text{mM}/\text{cm}$  at 280 nm (Hunter and McDuffie 1959; Wallevik 1973)]. In all cases, the concentration of hemopexin protein and heme-protein complex was determined from their absorbance spectra (Morgan *et al.* 1976). The extent of heme binding was assessed from the Soret absorbance, that is, 414 nm for heme–HPX and 402 nm for heme–HSA. In a second series of experiments, apo-hemopexin solutions were treated with either  $\text{H}_2\text{O}_2$  or tBuOOH by incubation for 15 min at 37°C followed by subsequent measurement of heme binding following addition of equimolar amounts of heme/DMSO (2  $\mu\text{L}$ ). The effect of the peroxides on hemopexin or HSA was assessed by comparison of the absorbance spectra of proteins exposed to equal volumes of water. The extent of heme binding was determined by subtracting baseline absorbance at 650 nm (absorbance at this wavelength remained constant under experiment conditions) from the Soret absorbance. In a third series, to model ROS derived from  $\text{H}_2\text{O}_2$  in the respiratory burst of neutrophils, both heme–hemopexin and apo-hemopexin were similarly treated, as described above for peroxides, with hypochlorous acid (HOCl) at molar ratios of 1 : 1 to 1 : 500 and 1 : 1 to 1 : 40, respectively. The stability of albumin and heme–albumin complexes to HOCl was also assessed as described above for peroxide treatments. HOCl was prepared by diluting sodium hypochlorite (NaOCl, Sigma-

Aldrich) in PBS. The concentration of NaOCl was determined by dilution in 1 M NaOH and measurement of absorbance [ $M = 350/M/cm$  at 292 nm, pH 11 (Morris 1966)].

### Tissue culture, cell lysis, immunoblot, and IRE regulation analyses

Neuroblastoma cells, SH-SY5Y, were purchased from ATCC (Manassas, VA, USA) and grown in 50:50 EMEM (Eagles Minimal Essential Medium): Ham's F12 medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. Cells were seeded ( $5.0 \times 10^5$ – $1.0 \times 10^6$  cells/well in six-well plates) until nearly confluent. The cells were rinsed with warm, equilibrated growth medium and incubated with heme–hemopexin or PBS as described in the figure legends. For assessment of the ability of peroxide-exposed heme–hemopexin complexes to induce HO1, the protein complexes in PBS were treated with either  $H_2O_2$  or  $tBuOOH$  at 1 : 1 or 1 : 10 molar ratio for 10 min at 24°C followed by dialysis for 1 h at 4°C. The solution was then sterile filtered before addition to the cell incubation medium (25  $\mu$ M) followed by incubation for 6–17 h before preparation of cell lysates as described in figure legends.

Whole-cell extracts were prepared by the addition of Mammalian Protein Extraction Buffer (M-PER, 100  $\mu$ L; Pierce Biotechnology, Rockford, IL, USA) and centrifugation (3000  $g$  for 10 min). The protein concentration of the cell extracts was determined using the Bicinchoninic acid assay (BCA; Pierce Biotechnology) with bovine serum albumin as standard. Proteins in samples of whole-cell extracts (15  $\mu$ g) were resolved on SDS–PAGE gradient gels (4–20% acrylamide gels, Bio-Rad, Hercules, CA, USA) under reducing conditions, and the target proteins detected by western immunoblotting using polyvinylidene difluoride membranes. The primary antibodies used were anti-HO1 (rabbit polyclonal raised against a synthetic peptide 1–30 amino acid residues: SPA-896, Assay Designs, Ann Arbor, MI, USA; 1 : 5000 dilution); anti-HO2 (rabbit polyclonal raised against a synthetic peptide specific to an N-terminal sequence: SPA-897, Assay Designs; 1 : 1000) anti-glyceraldehyde 3-phosphate dehydrogenase, GAPDH, (mouse monoclonal anti-GAPDH: sc-47724, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; 1 : 5000 dilution), anti-actin (mouse monoclonal: sc-8432, Santa Cruz Biotechnology Inc.; 1 : 1000) and anti-C-terminal APP (rabbit polyclonal antibody raised against a synthetic peptide 676–695 amino acid residues: A8717, Sigma Aldrich; 1 : 1000 dilution). The secondary antibodies used (both at 1 : 10 000 dilution) were goat anti-mouse IgG–HRP and goat anti-rabbit IgG–HRP (Santa Cruz Biotechnology Inc.). The ECL Western Blot Detection reagent (GE Healthcare Life Sciences, Piscataway, NJ, USA) was used as instructed by the manufacturer. The blots were scanned with a Typhoon 9400 Variable Mode Imager (GE Healthcare Life Sciences) and the signals quantified by segment analysis using UN-SCAN-IT gel digitizing software (Silk Scientific, Orem, UT, USA) as previously published (Flaherty *et al.* 2008). Neuroblastoma SH-SY5Y cells were stably transfected with the pIRES (APP 5 UTR) construct as previously described (Bandyopadhyay *et al.* 2006) and, following published procedures, incubated with heme–hemopexin, apo-hemopexin or PBS at the concentration and times indicated in the text.

### Statistical analyses

Statistical analyses were conducted using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA) as previously (Rish *et al.* 2007) and details are presented in the figure legends.

## Results

### Heme–hemopexin increases the endogenous levels of holo-APP in human neuroblastoma SH-SY5Y cells in part via iron from heme catabolism

To determine how heme delivery to human neuronal cells by hemopexin affects heme and iron metabolism, we first established that human neuroblastoma SH-SY5Y cells respond to heme–hemopexin in a manner similar to primary cultures of mouse neurons (Li *et al.* 2009), that is, via heme delivery for heme oxygenase-1 (HO1) induction. We used 10–25  $\mu\text{M}$  heme–hemopexin as a model of heme load as previously published (Li *et al.* 2009). HO1 is readily induced by heme–hemopexin and remains elevated for at least 17 h (Fig. 1a and Fig. 6); whereas HO2, which is constitutively expressed at high levels in neurons does not change, as expected.

Heme delivered to cells by hemopexin is rapidly catabolized and the iron released is stored on ferritin (Davies *et al.* 1979; Smith and Morgan 1979) and ferritin protein is induced (Hunt *et al.* 1996b; Sung *et al.* 2000). Because iron induces hAPP (Rogers *et al.* 2002; Schobel *et al.* 2006; Cho *et al.* 2010) at the translational level via IRE/IRP analogous to ferritin, the APP–IRE is a strong candidate site for translational regulation by heme–hemopexin. APP protein levels are increased ~twofold when neuroblastoma cells are incubated with heme–hemopexin for 17 h (Fig. 1c), and iron is required as the induction is reduced significantly by an iron chelator, desferrioxamine (DF, Fig. 1d). To determine the role of the IRE in the 5' UTR of human APP mRNA in hAPP regulation by heme–hemopexin, we utilized SH-SY5Y neuroblastoma cells stably transfected with a construct comprising the APP 5' UTR with a luciferase reporter gene. After incubation with heme–hemopexin (10–25  $\mu\text{M}$ ), luciferase activity was significantly higher in these cells than in those with apo-hemopexin or the PBS solvent control (Fig. 1f).

### Heme–hemopexin complexes are relatively resistant to damage by reactive oxygen species *in vitro* compared with the apo-protein

Many different types of brain injury are thought to be because of toxicity from heme released from hemoglobin, as recently shown for stroke (Li *et al.* 2009); and brain damage is rapidly followed by activation of the inflammatory cascade generating ROS. For hemopexin to protect brain cells, including neurons, under these conditions, it needs to be stable in the presence of ROS. Therefore, we next tested the hypothesis that heme–hemopexin complexes and apo-hemopexin would be resistant to damage by representative ROS known to be generated *in vivo* during the inflammatory response. In addition to the *tert*-butylhydroperoxide (*t*Bu-OOH) used previously (Timmins *et al.* 1995; Li *et al.* 2009), we investigated the effects of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). Also, because  $\text{H}_2\text{O}_2$  is a substrate for myeloperoxidase (MPO) in the respiratory burst of neutrophils and macrophages to generate hypochlorous acid (HOCl) at sites of inflammation and MPO is also released into biological fluids, we then assessed whether HOCl impairs the ability of hemopexin to bind heme. The concentration ranges of ROS investigated here are based on published studies of levels that are likely *in vivo*.

To model hemolysis followed by inflammation, as occurs in stroke with ischemia reperfusion injury and intracerebral hemorrhage, we first determined whether heme–hemopexin complexes were stable in the presence of  $\text{H}_2\text{O}_2$  *t*BuOOH and then compared with HOCl. Characteristic absorbance spectra reveal that heme–hemopexin complexes (10  $\mu\text{M}$ ) were essentially completely stable in the presence of up to a 10-fold molar excess of *t*BuOOH at 37°C (Fig. 2b and d). These complexes were also unaffected by up to 2.5-fold molar excess of  $\text{H}_2\text{O}_2$ , which is well within the 5–15  $\mu\text{M}$   $\text{H}_2\text{O}_2$  physiological range of production by human neutrophils (Test and Weiss 1984; Liu and Zweier 2001); but there

was a 20% loss of heme binding after 10-min exposure to supraphysiological levels of H<sub>2</sub>O<sub>2</sub> at 37°C (indicated by the decreased absorbance at the Soret region 414 nm; see Fig. 2c and d), which occurred within a few minutes (Fig. 2e). Nevertheless, there appears to be no gross denaturation of the protein by H<sub>2</sub>O<sub>2</sub>.

Normally, only minor amounts of heme–hemopexin are expected from the wear and tear of red blood cells in capillary beds in the plasma and other biological fluids including cerebrospinal fluid. Heme levels in normal volunteers is ~0.2 μM (Reiter *et al* 2002) and haptoglobin levels range from 2–23 μM (Gupta *et al.* 2011; Vermeulen Wind-sant *et al.* 2012). Thus, apo-hemopexin (at 10–25 μM) often predominates in biological fluids. Heme binds to hemopexin quickly [K<sub>d</sub> less than 1 pM (Hrkal *et al.* 1974)]. Here, we have shown that at physiological temperature of 37°C, stoichiometric complexes are formed completely within seconds and are cleared by the liver from the circulation within 1–5 min (Smith and Morgan 1979). Both tBuOOH (Fig. 3a – e) and H<sub>2</sub>O<sub>2</sub> (Fig. 3f – j) appeared to have little effect on the apo-protein; and supraphysiological levels caused only a ~10% and 25% loss, respectively, in its ability to bind heme (Fig. 3e and j).

In contrast to these *in vitro* conditions where hemopexin is the sole protein present, *in vivo* hemopexin would be in an environment surrounded by many other proteins, all of which are potential ROS targets. In human plasma, albumin is present in a 70-fold molar excess over hemopexin (Morgan *et al.* 1976) and provides a potential reservoir of two heme-binding sites per molecule in hemolytic states (Beaven *et al.* 1974). However, the 5-coordination state of the heme and low affinity binding do not protect the heme effectively (Grinberg *et al.* 1999). These published studies were carried out at 22°C with a 40-fold molar excess of peroxides. Using more physiologically relevant conditions, the absorbance data in Fig. 4 reveal that heme–albumin complexes (1 : 1– 1 : 10 molar ratio) are more susceptible to damage by tBuOOH (Fig. 4d) and H<sub>2</sub>O<sub>2</sub> (Fig. 4c) than are heme– hemopexin complexes (Fig. 2e).

Hypochlorous acid (HOCl), a potent bactericidal and viricidal agent, is considered one of the most physiologically relevant ROS/oxidizing agents *in vivo*. The concentrations of HOCl are estimated to be 12.5–250 μM (McCall *et al.* 2001), although significantly higher levels (~3–8 mM) may occur in diseased lungs including in cystic fibrosis (Guo *et al.* 1995). Heme–hemopexin complexes are essentially unaffected by HOCl even at molar ratios of 1 : 40, that is, 400 μM (Fig. 5a). However, 1.6–5 mM HOCl cause a small dose-dependent loss of heme binding (~10–25%), which occurs almost instantaneously (i.e., within 10–20 s). The heme-binding site appears to remain intact because there is no change in the Soret maximum (414 nm). While heme binding to hemopexin protects the heme and stabilizes the protein, damage to heme–albumin complexes by HOCl is more extensive, especially at concentrations above 400 μM (Fig. 5b). Heme loss, indicated by a decrease in absorbance in the Soret region with a concomitant increase at 370– 390 nm, and denaturation of albumin, indicated by an increase in absorbance at 250 nm (data not shown), occurs at a 40- to 320-fold molar ratio of albumin to HOCl. In addition, the maximum Soret absorbance of albumin-bound heme becomes red shifted from 402 nm to 404/406 nm with increasing concentrations of HOCl (starting at 100 μM) and with longer exposure times. This indicates that there are changes in the environment of the heme.

The initial essentially instantaneous heme-binding ability of apo-hemopexin is not greatly affected by HOCl at concentrations less than or equal to 200 μM (Fig. 5c); however, higher concentrations do begin to decrease its ability to bind heme. Above 200 μM, there are small increases in absorbance at 250 nm of apo-hemopexin after exposure to HOCl, consistent with direct effects on the protein; although, there are only very small decreases in absorbance at 280 nm but no aggregation (data not shown). Because of these changes in the

protein spectra, we normalized the total heme binding by subtracting the absorbance at 650 nm, which does not change, from the absorbance at the Soret maximum. After heme addition to the HOCl-treated hemopexin, the decreased absorbance in the Soret region accompanied by increased absorbance at 370–390 nm with time is consistent with a loss of heme binding (data not shown) compared with the untreated hemopexin. There is a 25–40% loss in total heme binding by hemopexin at concentrations of HOCl above 200  $\mu\text{M}$  (see data summary, Fig. 5c).

In the presence of HOCl, there are small incremental changes in the albumin spectrum at 250 nm that are apparent at 100  $\mu\text{M}$  and increase dose dependently with HOCl concentrations (data not shown). The total heme binding by albumin, normalized as described above (Fig. 1d), appears to be retained, but damage to albumin that impairs heme binding starts to occur at 100–200  $\mu\text{M}$  HOCl. Furthermore, total heme binding is significantly decreased by 200–400  $\mu\text{M}$  HOCl (see Fig. 5d). Although albumin maintains its ability to quickly bind heme after exposure to 400  $\mu\text{M}$  HOCl, this binding per se is not protective as the heme–albumin complex is very susceptible to damage by HOCl as described above. Overall, these observations support that heme binding to albumin does not protect the heme. Also, *in vivo* hemopexin will be protected in biological fluids by albumin, which is a preferential target of ROS.

### **Peroxide-exposed heme–hemopexin induces heme oxygenase-1 in human neuroblastoma cells**

As expected, the human neuroblastoma cells, like primary neurons, normally express high basal levels of the HO2 isozyme and low levels of the inducible HO1 isozyme, which is increased by heme–hemopexin (see Fig. 1). Tert-butyl hydroperoxide-treated heme–hemopexin induced HO1 ~40–60% compared with the untreated complexes. Exposure to equimolar  $\text{H}_2\text{O}_2$  reduced induction by heme–hemopexin to about half that seen with the water-treated complex and higher peroxide concentrations caused further impairment of this ability, but did not prevent induction (Fig. 6). Because of the high concentrations of protein targets more susceptible to  $\text{H}_2\text{O}_2$ , including albumin (as shown in Fig. 4) in biological fluids, which will limit damage to hemopexin, we conclude that heme–hemopexin complexes formed *in vivo* would remain effective inducers of the HO1 enzyme under most inflammatory conditions.

### **Discussion**

Our basic premise is that characterization of the natural protective action of hemopexin against heme toxicity in stroke, intracerebral hemorrhage and other brain trauma, including the regulatory consequences of uptake of heme–hemopexin by brain cells, will provide a window to understand events that normally maximize defense against neurodegeneration. Our first goal was to help further define the changes in iron metabolism in neurons after endocytosis of heme–hemopexin. We show that HO1 and hAPP protein levels are induced in human neuroblastoma cells that are considered to be good models of human neurons. Human APP induction by heme–hemopexin requires iron and the IRE of APP mRNA as expected as the APP–IRE is 75% homologous with the canonical ferritin IRE and heme–hemopexin induces ferritin (Hunt et al. 1996b). We conclude that hAPP is induced by iron derived from the breakdown of heme after delivery by hemopexin because: (i) hAPP induction by heme–hemopexin is decreased by simultaneous incubation of cells with the iron chelator, desferrioxamine; and (ii) heme–hemopexin increases luciferase activity in hAPP–IRE constructs stably transfected in SH-SY5Y cells, more than apo-hemopexin. The induction of HO1, known to occur at the transcriptional level, is detectable before the translational regulation of hAPP by heme–hemopexin. By inducing the expression of proteins that control iron homeostasis in addition to those controlling heme levels, heme

delivery by hemopexin ensures that intracellular concentrations of heme and also iron from heme breakdown are kept at a safe level. Our second goal was to model human neurons experiencing microhemorrhages and inflammation in the brain. We used human neuroblastoma cells, heme–hemopexin complexes, and physiologically relevant levels of H<sub>2</sub>O<sub>2</sub> and HOCl. The effects of these two ROS were compared with that of *tert*-butyl hydroperoxide, another model ROS. Our principal findings from these experiments are that heme–hemopexin complexes and apo-hemopexin are essentially stable to the levels of H<sub>2</sub>O<sub>2</sub> and HOCl generated from the respiratory burst and that hemopexin can still bind heme and induce HO1 in models of human neurons. *In vivo*, other proteins (e.g., albumin) present in plasma and cerebrospinal fluid are expected to provide preferential targets for ROS and thus ameliorate the damage to apo-hemopexin seen here at the supraphysiological levels of H<sub>2</sub>O<sub>2</sub> *in vitro*. Levels of H<sub>2</sub>O<sub>2</sub> at 100 μM or higher have been recorded in various disease states (Schroder and Eaton 2008). Thus, our observations provide strong evidence that hemopexin will remain protective in the brain as an extracellular antioxidant (i.e., heme binder) and HO1 inducer both for normal metabolism of neurons and after stroke, trauma, and intracerebral hemorrhage. Nevertheless, the potential for damage to apo-hemopexin *in vivo* by localized high concentrations of H<sub>2</sub>O<sub>2</sub> and HOCl certainly exists, for example, at the neutrophil surface as the respiratory burst is stimulated and ROS are released from the cell. Any damaging effects of HOCl to circulating plasma proteins including hemopexin and albumin may be tempered by dietary flavonoids, as shown for hemoglobin (Gebicka and Banasiak 2012).

In the context of neuroprotection by the hemopexin system, hAPP induction, whose ferroxidase activity is needed for iron export and transferrin iron loading in neurons (Duce *et al.* 2010), helps to maintain intracellular free iron concentrations at a safe level by facilitating iron release to transferrin. Neurons express ferritin for iron storage. In addition, hemopexin delivers heme safely to primary neurons, in a controlled and regulated manner that is needed for HO1 gene transcription (Alam and Smith 1989) via Nrf2 (Alam *et al.* 1999; Smith 2011) and a regulatory soluble domain of HO1 (Lin *et al.* 2007). In fact, not only does hemopexin induce HO1 and ferritin (Hunt *et al.* 1996b), but it also provides cells with the heme substrate for this enzyme. Thus, neuroprotection by hemopexin will also come from HO1 activity releasing CO from heme, which has recently been shown to activate double-stranded DNA repair via the actions of the ATM protein (Otterbein *et al.* 2011). Consistent with this model, HO1 activity is needed for the protection by heme–hemopexin against heme toxicity and oxidative stress in primary neurons (Li *et al.* 2009).

In ischemia, neuroprotection by HO1 would be attenuated by a limitation of substrates such as oxygen for HOs and NADPH for the obligatory NADPH-cytochrome P-450 reductase needed for both HO1 and HO2 enzymatic activity. NADPH is central to the enzymes that are needed for cellular defense against ROS, including GSH and thioredoxin reductases. Insufficient NADPH generation may become critical when glucose availability is compromised in neurons and when there is hypoxia because the major source for NADPH is the oxidative phase of the pentose phosphate pathway. The retina is an isolated part of the brain and, in fact, a coordinated balance of glycolysis with heme catabolism in liver and retinal pigment epithelial cells has recently been proposed (Li *et al.* 2012).

Another potential mechanism that might negatively impact neuroprotection within cells, involves the observation that HOs are inactivated by binding to APP (Takahashi *et al.* 2000). This protein–protein interaction involves the catalytic domain of HO and the zinc-binding site of APP (Takahashi *et al.* 2000). In APP, the HO1-binding domain (amino acid residues 190–288) and neighboring zinc-binding site (residues 181–188) are N-terminal to a ferroxidase-1 (FD-1) homology domain (residues 401–417, Duce *et al.* 2010). The FD-1 domain and other relevant ferroxidase catalytic sites are thought to aid in APP-dependent



iron export via ferroportin. While the APP domain with the zinc-binding site faces the ER lumen (Takahashi *et al.* 2000), there is evidence from two groups that the catalytic site of HO1 faces the cytosol: fluorescent protein protection assays after expression of GFP-tagged HO1 constructs in macrophages (Gottlieb *et al.* 2012); and complex formation between HO1, its obligatory P-450 reductase, and the enzyme next in the heme catabolic pathway—the cytosolic protein, biliverdin reductase (Yoshinaga *et al.* 1982). Furthermore, there is no apparent toxicity when primary neurons (Li *et al.* 2009) or neuroblastoma cells are incubated with high concentrations of heme–hemopexin to mimic a hemolytic event, as might be expected if intracellular heme or iron levels rise uncontrollably because of inactive HO and/or APP. Thus, after heme delivery by hemopexin, an HO–APP interaction that inhibits their function is not supported by experimental evidence.

Given that brain injury rapidly leads to the activation of inflammatory processes, it is important that we here provide evidence that hemopexin protects neurons and potentially other brain cells during inflammation because it continues to bind heme and induce HO1 after exposure to three types of ROS. In inflammation, ROS including HOCl are released into plasma and other biological fluids principally from polymorphonuclear neutrophils, but also by some monocytes and tissue macrophages. HOCl production requires the combined action of plasma membrane NADPH oxidase, which generates superoxide that dismutates to H<sub>2</sub>O<sub>2</sub>, and myeloperoxidase, which converts H<sub>2</sub>O<sub>2</sub> to hypochlorous acid by oxidizing chloride in plasma (Fliss 1988; Weiss 1989; Kettle and Winterbourn 1994). The damage from heme comes from intermediates generated during the two-electron reduction by heme of hydroperoxides, for example, peroxy radicals and a high-energy oxidized state of heme (Timmins *et al.* 1995). Although heme is ultimately regenerated, the intermediates produced react with, and thus damage, many biological substrates. *Tert*-butyl hydroperoxide is one model for such peroxides. On the basis of a comparison of the effects of cumene hydroperoxide and *t*- and *n*-BuOOH, Timmins and colleagues (Timmins *et al.* 1995) concluded that the ability to access heme in the heme-binding site of hemopexin varies depending upon the size, reactivity, and hydrophobicity of the peroxides, and the steric hindrance of the individual peroxide functional group. The carbohydrate chains of hemopexin were also considered protective. Consistent with this, we found that there were differences in the effects of the three ROS investigated on hemopexin. In general, heme–hemopexin complexes were stable to peroxides. Access of *t*BuOOH to hemopexin, compared with H<sub>2</sub>O<sub>2</sub>, may be because of limited access as *t*BuOOH is larger than H<sub>2</sub>O<sub>2</sub>. Even greater steric hindrance effects are likely for the types of ROS that hemopexin would encounter *in vivo* because they will be non-solvated, membrane-bound lipid hydroperoxides or protein hydroperoxide species (Timmins *et al.* 1995). Only the very highest physiological concentrations of HOCl (i.e., > 200 μM) slightly damaged apo-hemopexin with some impairment of heme binding, whereas the heme–hemopexin complex was essentially stable in the presence of this ROS. Thus, *in vivo*, the extent of any damage to hemopexin will depend upon the type of ROS (molecular size and reactivity) as well as its concentration and where hemopexin is located in the body.

Nevertheless, some potential for damage to apo-hemopexin especially by the highest concentrations of H<sub>2</sub>O<sub>2</sub> and HOCl that might occur *in vivo* exists. Such damage in severe and prolonged inflammatory states as in sepsis may be one means that leads to a hemopexin deficiency if the oxidized protein is recognized as non-native, removed from the circulation, and catabolized, instead of being recycled after endocytosis (Smith and Morgan 1979; Hunt *et al.* 1996a). This is relevant because while local inflammation in the brain contributes to neurodegeneration, systemic infection also leads to poorer outcomes in stroke and neurodegenerative conditions like AD with neurovascular alterations (McColl et al. 2008). Also, hemopexin supplementation protects mice with severe sepsis from death (Larsen *et al.* 2010).

Consistent with its role in defending against heme toxicity even under inflammatory conditions, peroxide-exposed heme–hemopexin was still able to induce HO1 in human neuroblastoma cells. Nevertheless, our studies reveal that depending on the type of peroxide and its concentration there is a potential for impairment *in vivo*. The induction levels were less (40–60%) than those seen with the control heme–hemopexin complexes and there were differences: the effects of tBuOOH, which is a larger molecule than H<sub>2</sub>O<sub>2</sub>, were less than those of H<sub>2</sub>O<sub>2</sub>. Furthermore, receptor-mediated delivery of heme by hemopexin activates the expression of certain protective proteins in addition to HO1 that heme does not (Eskew et al. 1999; Vanacore et al. 2000). Although haptoglobin protects hemoglobin from H<sub>2</sub>O<sub>2</sub>, exposure of haptoglobin–hemoglobin complexes to 100 μM H<sub>2</sub>O<sub>2</sub> causes heme to become covalently attached to protein. This completely prevents HO1 induction, although the complexes are recognized by the receptor CD163 and taken up into CD163-expressing human embryonic kidney cells *in vitro* (Buehler et al. 2009).

Several studies (Gutteridge and Smith 1988; Vincent et al. 1988; Timmins et al. 1995) have shown that when heme is bound to hemopexin, its chemical reactivity is dampened much more than when bound to albumin. For example, heme-catalyzed peroxidation of linoleic acid micelles was inhibited ~90% by hemopexin, but only ~50% by albumin (Gutteridge and Smith 1988). The susceptibility of heme–albumin complexes to oxidative damage is considered to be because of the high spin, 5-coordinated heme (Pasternack et al. 1983) and the far lower affinity for heme [K<sub>d</sub>s of 10 nM and 1 μM, (Beaven et al. 1974)] than hemopexin [K<sub>d</sub> less than pM, (Hrkal et al. 1974)]. We have shown here that albumin, present in ~100-fold molar excess over hemopexin in plasma, is also susceptible to ROS-mediated damage and thus provides an ROS ‘sink’ *in vivo*.

In hemopexin deficiency states, heme (from methemoglobin or heme-albumin) readily enters cells and is toxic. The mechanism(s) are not defined, but probably involve diffusion across the plasma membrane lipid bilayer and possibly diffusion-limited passage through channels, both of which would be unregulated rapidly raising intracellular heme levels to where they are toxic. Also, although not addressed directly here, hemopexin is expected to bind heme (Morgan and Muller-Eberhard, 1972; Smith 2011) even when the extracellular pH at nerve junctions decreases from 7.4 to 6.5 after ischemic stroke (Siesjo et al. 1996). Thus, neuroprotection by hemopexin when hemoglobin is released comes initially from heme sequestration and then from regulatory events on heme–hemopexin receptor binding followed by endocytosis and heme release within the cell. The extracellular antioxidant activity of hemopexin not only protects surface molecules and those in biological fluids from damage by heme and the activation by heme of immune system cells that secrete pro-inflammatory cytokines (Figueiredo et al. 2007; Liang et al. 2009; Lin et al. 2012) but also prevents the uncontrolled uptake of heme that leads to toxicity.

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## Abbreviations used

<b>APP</b>	amyloid precursor protein
<b>BCA</b>	bicinchoninic acid
<b>CNS</b>	central nervous system
<b>DF</b>	desferrioxamine
<b>DMEM</b>	Dulbecco's Modified Eagle Medium
<b>DMSO</b>	dimethyl sulfoxide
<b>EMEM</b>	Eagle's Minimum Essential Medium
<b>FBS</b>	fetal bovine serum
<b>GAPDH</b>	glyceraldehyde-3-phosphate dehydrogenase
<b>hAPP</b>	human amyloid precursor protein
<b>HO1</b>	heme oxygenase-1
<b>HOCl</b>	hypochlorous acid
<b>MPO</b>	myeloperoxidase
<b>NaOCl</b>	sodium hypochlorite
<b>PBS</b>	phosphate-buffered saline
<b>ROS</b>	reactive oxygen species
<b><i>t</i>BuOOH</b>	<i>tert</i> -butyl hydroperoxide

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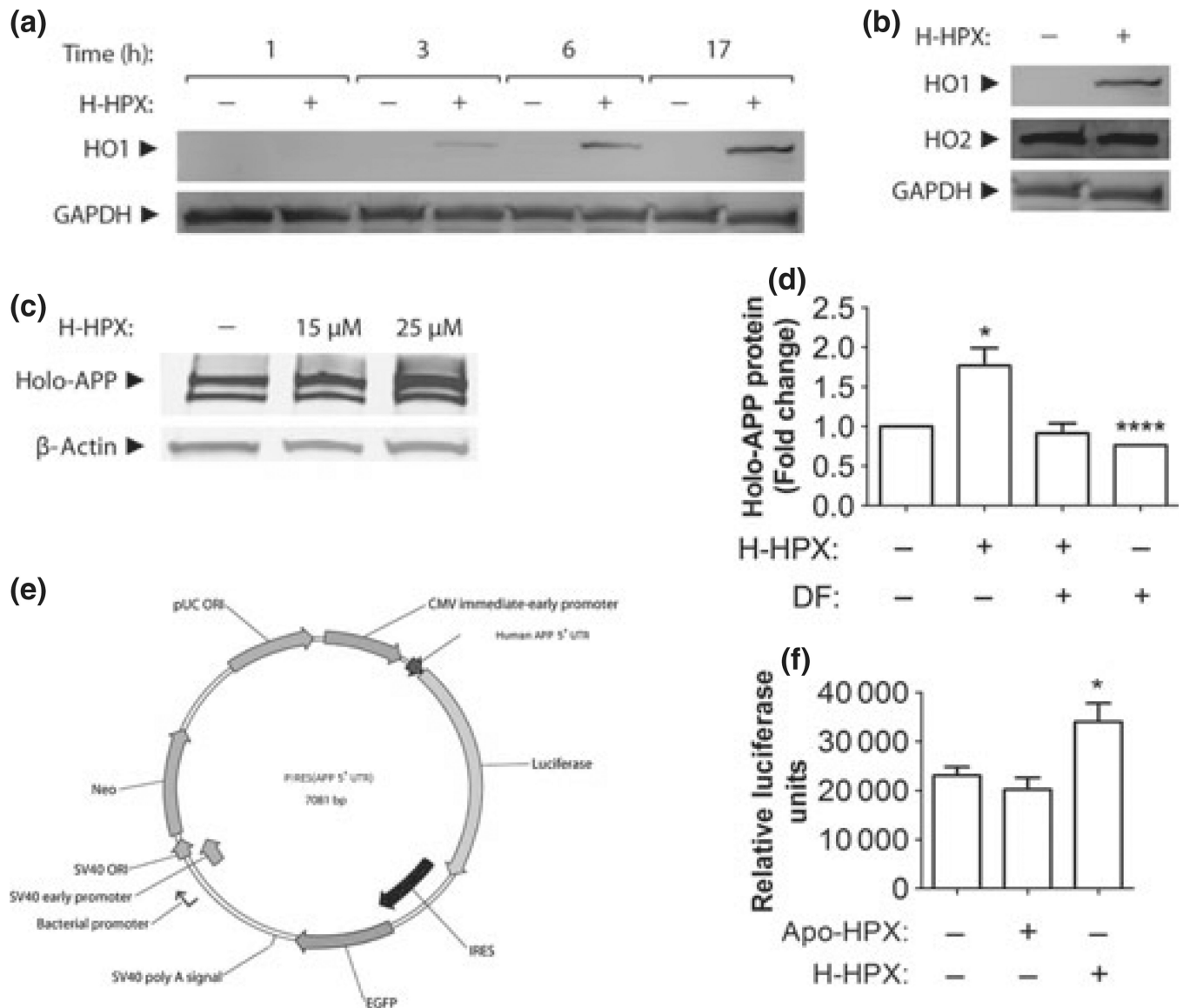
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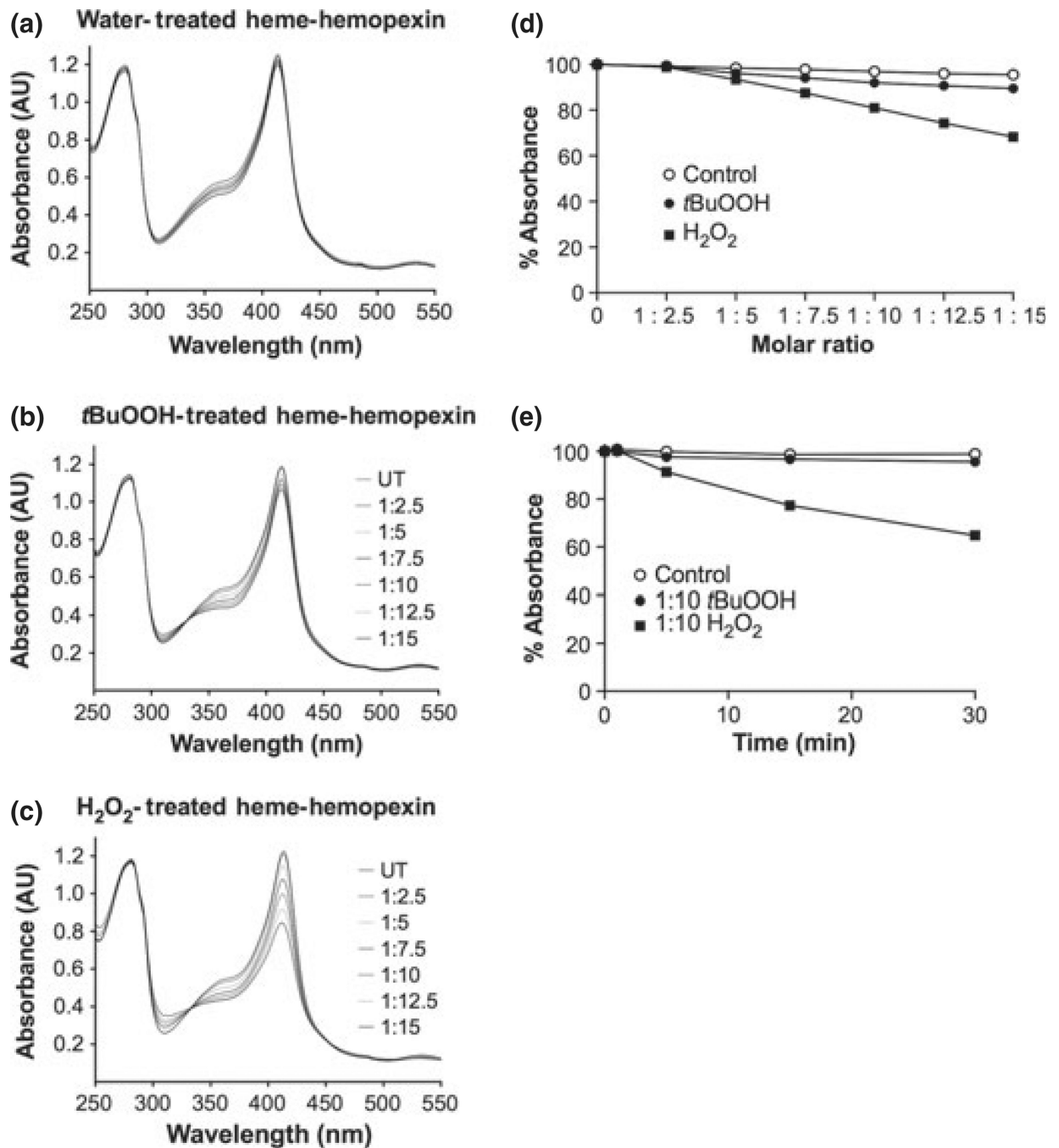
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**Fig. 1.** Heme-hemopexin induces amyloid precursor protein in an iron-dependent manner in human neuroblastoma cells. Human SH-SY5Y cells growing in normal culture medium that contains 10% fetal bovine serum (FBS) were incubated for up to 17 h with heme-hemopexin (H-HPX) or an equivalent volume of phosphate-buffered saline (PBS) as indicated. In (a), HO1 protein induction by 25  $\mu$ M heme-hemopexin is apparent within 3 h and continues to increase over 17 h using immunoblots of whole-cell extracts (15  $\mu$ g protein/lane) with GAPDH as the loading control. In (b), while HO1 is induced, the high basal levels of HO2 are unchanged at 17 h after heme-hemopexin. The induction of amyloid precursor protein (APP) following 17-h incubation with heme-hemopexin (c) is prevented by the iron chelator, desferri-oxamine [100  $\mu$ M DF, (d)]. Holo-APP levels (Mr ca. 100 kDa) were increased ~1.8-fold by 25  $\mu$ M H-HPX (d). A one-tailed, unpaired Welch's t test between the control and treated samples gave  $p = 0.0204$  and  $p < 0.0001$ . The data shown are the mean  $\pm$  SEM values from four independent experiments. One set of error bars is so small they are not visible on this scale. A higher molecular weight form of hAPP detected in these blots after cells are incubated with H-HPX is an intracellular precursor of hAPP. In (f),



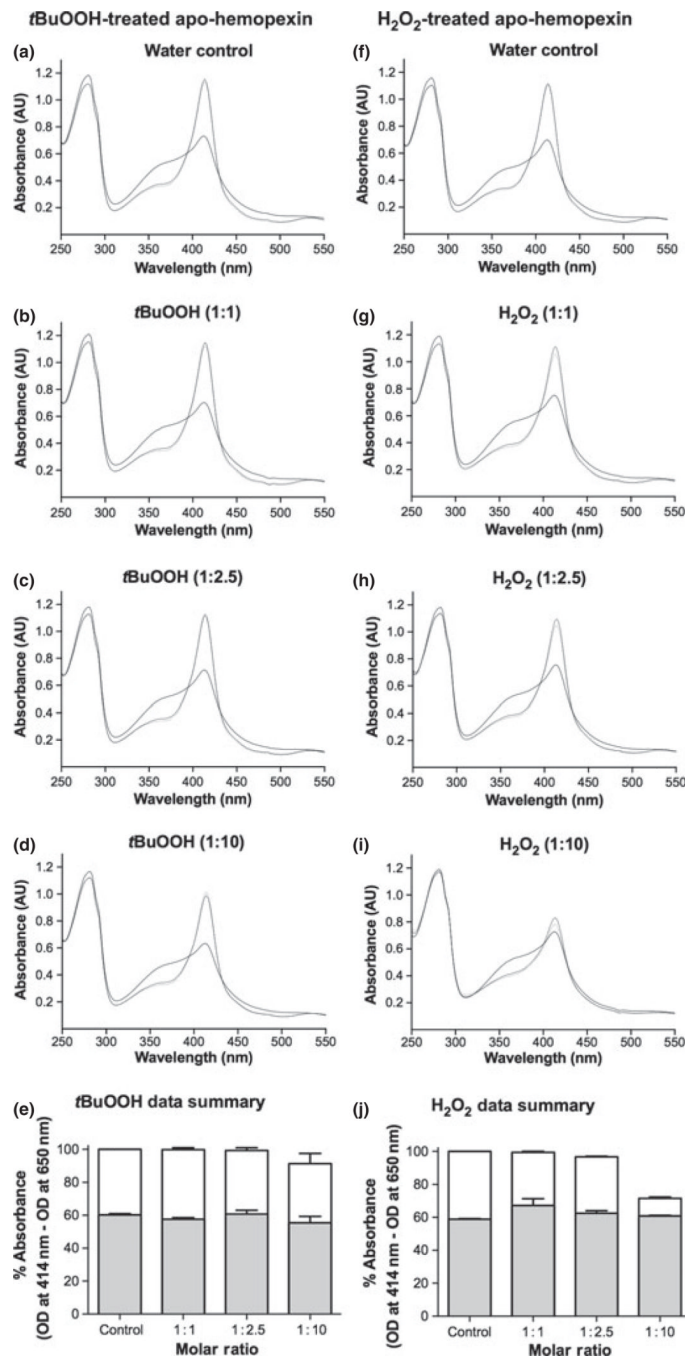
25  $\mu\text{M}$  heme–hemopexin is a more effective inducer than 25  $\mu\text{M}$  apo-hemopexin of luciferase activity from a reporter gene construct, pIRES(APP 5'UTR) in (e), containing the APP 5'UTR with its IRE stably transfected in SH-SY5Y neuroblastoma cells. PBS is the solvent control. The data shown in (f) are from a representative experiment ( $n = 6$  each set), of three independent experiments, using 25  $\mu\text{M}$  apo-hemopexin and heme–hemopexin with PBS as solvent control. A two-sided, Dunnett- $t$  test gave  $*p = 0.0260$  between the control and H-HPX treatments. Induction of luciferase activity by heme–hemopexin was also apparent at 10- $\mu\text{M}$  concentrations (data not shown).



**Fig. 2.**

The heme-hemopexin complex (H-HPX) is resistant to damage by *tert*-butyl hydroperoxide and to physiological amounts of H<sub>2</sub>O<sub>2</sub>. Shown are the characteristic absorbance spectra (a–c) after heme-hemopexin (10 μM) was incubated at 37°C with (a) the solvent control, water, (b) *t*BuOOH, or (c) H<sub>2</sub>O<sub>2</sub>. The peroxide concentration was increased in the sample by the sequential addition of stock peroxide (1.5 μL) over a range of 1 : 2.5–1 : 15 molar ratios, and the controls received an equivalent volume of water. The absorbance spectra were recorded 2 min after each addition of peroxide. A summary of these absorbance data as percent absorbance at 414 nm of the untreated sample is shown in (d). In both titrations, an isosbestic point was apparent [at 328 and 330 nm in (b and c), respectively], which is

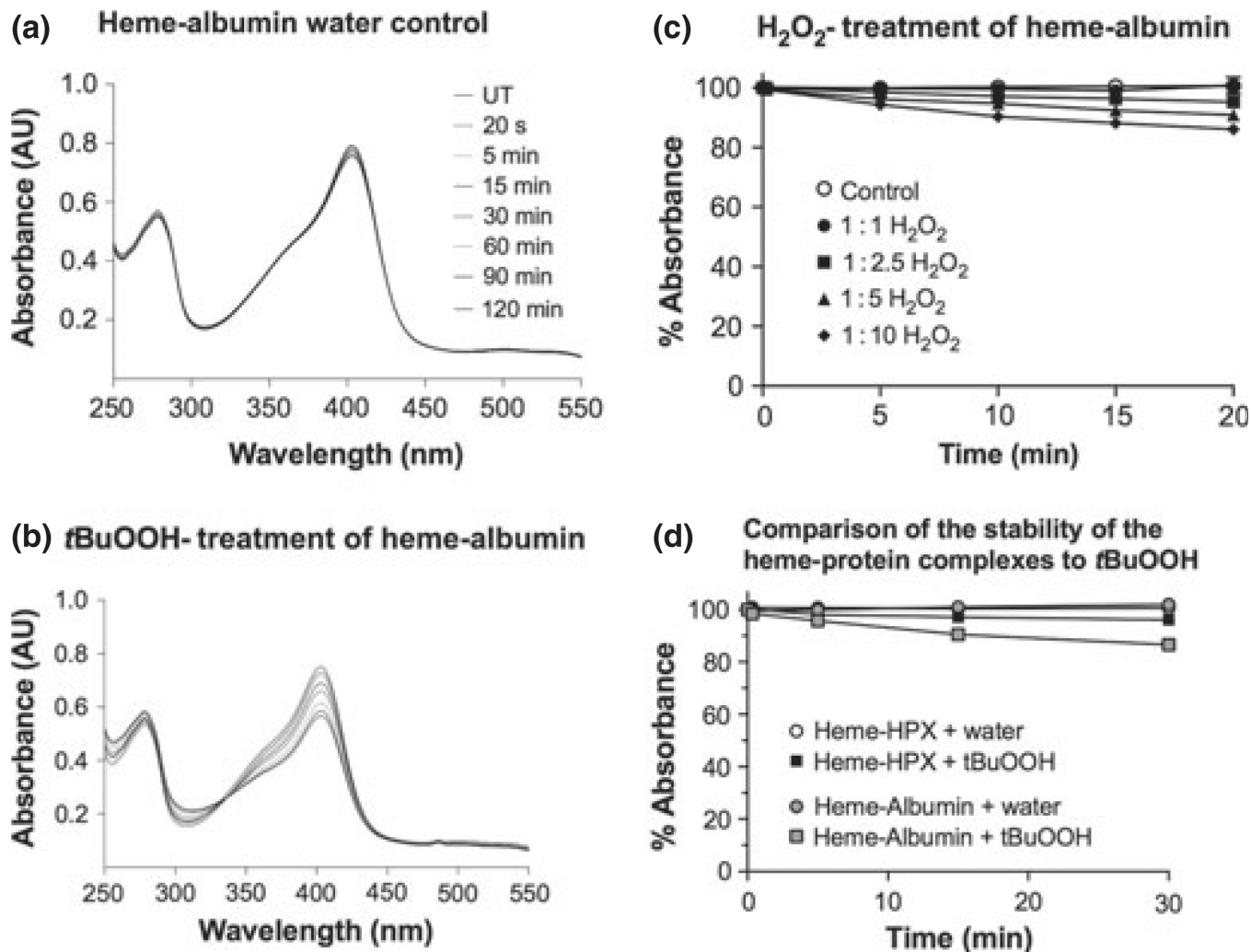
evidence for two species of heme. Heme–hemopexin complexes were incubated at 37°C with either *t*BuOOH or H<sub>2</sub>O<sub>2</sub> at a molar ratio of 1 : 10 and the absorbance spectra recorded at 1, 5, 15, and 30 min after addition of peroxide or an equivalent volume of water as control (e). At these supraphysiological concentrations of H<sub>2</sub>O<sub>2</sub>, some heme binding loss is detected by a decrease in absorbance at 414 nm over 30 min (and less than 10% more over the next 90 min). However, this is a relatively slow process compared with the rate that the complex is cleared from the circulation, which occurs within a few minutes (Smith and Morgan 1979). Data shown in panels a–d are from one representative experiment of two to three independent experiments. Data in panel e are the mean ± SEM values from two independent experiments.



**Fig. 3.**

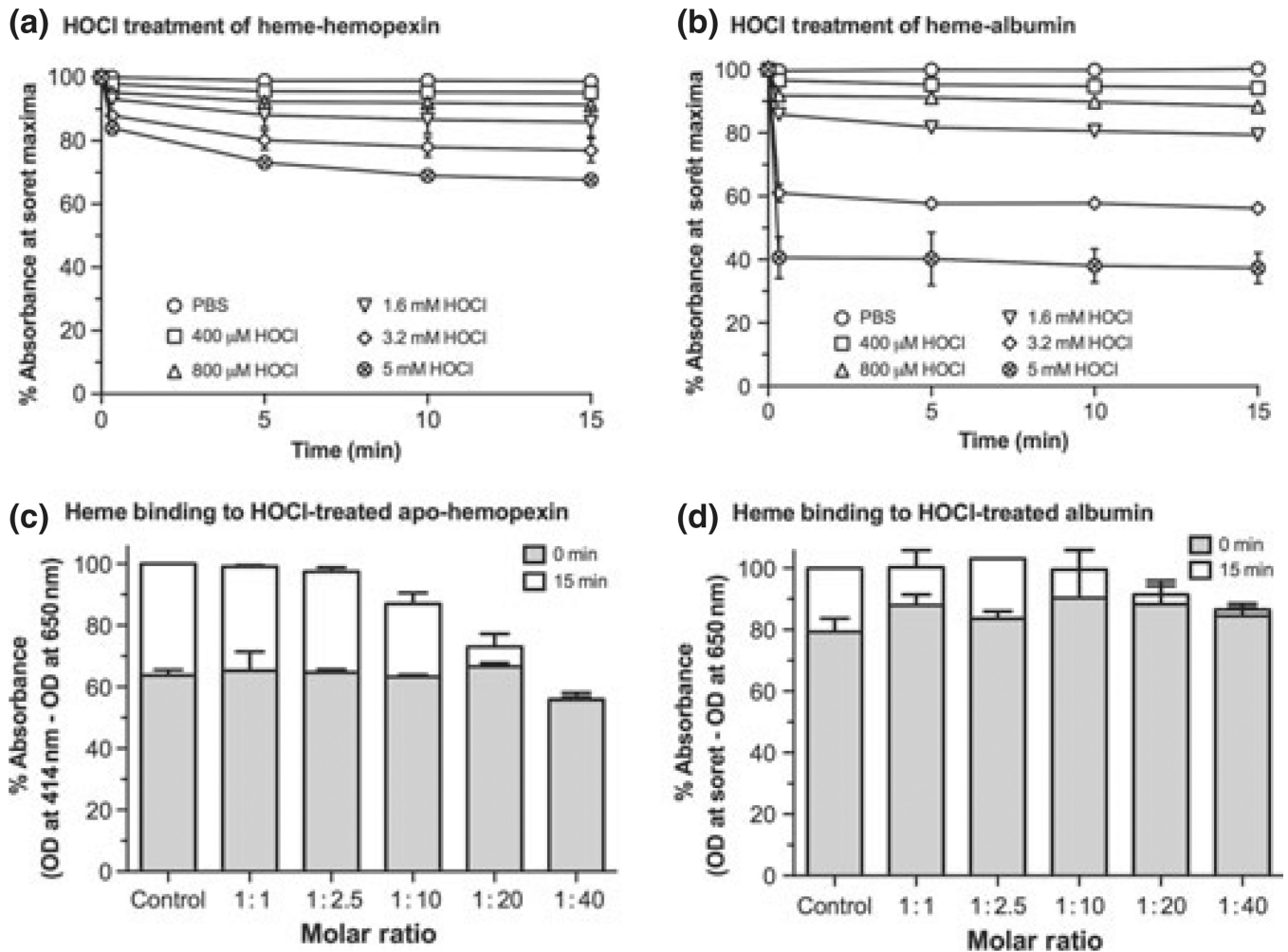
Apo-hemopexin is stable to peroxides and can bind heme under *in vitro* conditions designed to mimic inflammation. Apo-hemopexin (10  $\mu$ M) was incubated at 37°C with either (b–d) *tert*-butyl hydroperoxide (*t*BuOOH) or (g–i) H<sub>2</sub>O<sub>2</sub> at 1 : 1, 1 : 2.5, and 1 : 10 molar ratios for 15 min or water as solvent control (a and f, respectively). A stoichiometric amount of heme was then added and the absorbance spectra were recorded immediately (solid line) and at 15 (dashed line) and 30 min (dotted line) showing maximum heme binding. The OD at 414 nm is a measure of the amount of heme bound to hem-opexin. Spectra shown are from one of two independent experiments for each set. Absorbance data (e and j) are the mean  $\pm$  SEM from two independent experiments for each set. Gray and white bars represent the initial

heme binding and maximum heme binding (after 15 min.), respectively. While the initial rapid heme binding is unaffected by *t*BuOOH or H<sub>2</sub>O<sub>2</sub>, apo-hemopexin is damaged only by supraphysiological levels of H<sub>2</sub>O<sub>2</sub> [i.e., 100 μM, (j)], which decreased total heme binding by ~25% compared with 8% by 100 μM *t*BuOOH. The heme binding has been normalized by subtracting the absorbance at 650 nm, which is identical in all spectra, from the absorbance at the Soret maximum.



**Fig. 4.**

Heme–albumin is more sensitive to damage by peroxides than heme–hemopexin. Human serum albumin (10  $\mu$ M) was incubated with an equimolar amount of heme for 30 min at 37°C followed by addition of 100  $\mu$ M *t*BuOOH (b) at a 1 : 10 molar ratio or equivalent volumes of water as control (a). Absorbance spectra were recorded prior to treatment and after treatment at 20 s, 5, 15, 30, 60, 90, and 120 min. The heme–albumin complex was not stable in the presence of either peroxide as shown by the rapid decrease in the absorbance at 402 nm (within 5–10 min.) indicating loss of bound heme. The isosbestic point at 330 nm is evidence for two species of heme. (c) shows the rapid decrease in heme bound to albumin on exposure to H<sub>2</sub>O<sub>2</sub>. Heme-albumin complexes (10  $\mu$ M) were prepared (30-min binding at 37°C), incubated with 10, 25, 50, or 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>, and spectra recorded after 5, 10, 15, and 20 min. Absorbance spectra were normalized relative to untreated heme–albumin sample. Data shown are the mean  $\pm$  SEM from two independent experiments. The data in (d) show that heme-albumin (grey circle and square) was significantly more sensitive to damage by 100  $\mu$ M *t*BuOOH than heme–hemopexin (open circle, closed square). Data shown are the mean  $\pm$  SEM from two independent time courses for each protein complex.

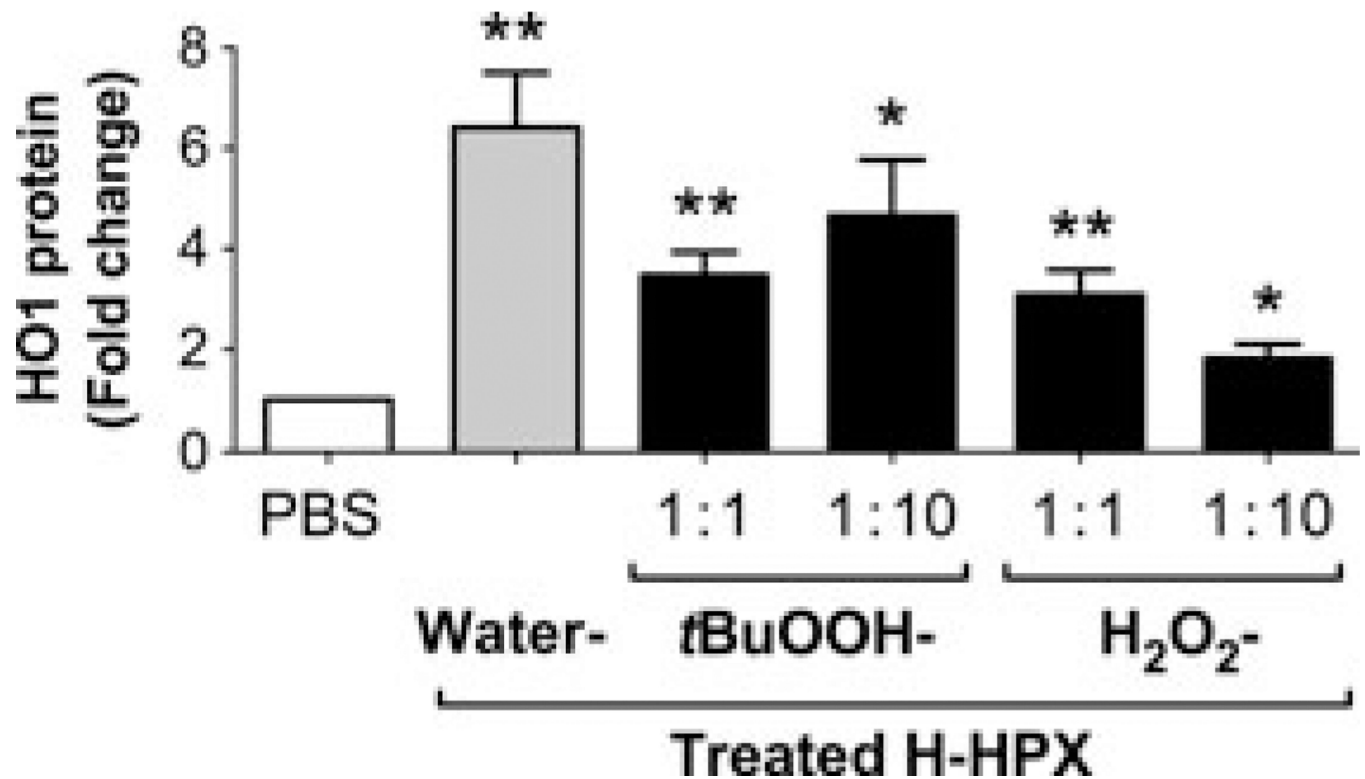


**Fig. 5.**

Heme-hemopexin is more resistant to damage by hypochlorous acid than heme-albumin, whereas albumin appears initially more resistant to this reactive oxygen species (ROS) than apo-hemopexin. Panels a and b: Heme-hemopexin or heme-albumin complexes, respectively, at 10  $\mu$ M in phosphate-buffered saline (PBS) were incubated for up to 15 min at 37°C with increasing concentrations of hypochlorous acid (HOCl) at 1:40–1:500 molar ratios. The absorbance spectra were recorded from 250 to 700 nm at the times indicated (~10 s to 15 min). Heme-hemopexin was more resistant to damage than heme-albumin, retaining ~90% heme-binding capacity in the presence of 800  $\mu$ M HOCl; and even at 5 mM HOCl there was only ~25% loss of heme binding. Any damage to heme-hemopexin was rapid and increased slowly over 10 min; whereas heme-albumin was damaged almost instantaneously, especially at millimolar levels of HOCl (1.6–5 mM). Panels c and d: apo-hemopexin (10  $\mu$ M) or albumin were incubated for 15 min at 37°C with HOCl at 1:1–1:40 molar ratios as indicated. A stoichiometric amount of heme was then added and the absorbance spectra were recorded immediately (i.e., ~10–20 s, gray bars) and after 15 min (white bars) for maximum heme binding (414 nm for heme bound to hemopexin; and Soret maximum for heme-albumin). Heme binding was normalized by subtracting baseline absorbance at 650 nm from these absorbances. Data shown are the mean  $\pm$  SEM of two independent experiments. The initial very rapid binding of heme to hemopexin was not affected even by the highest physiological levels of HOCl (up to 400  $\mu$ M). Total heme

binding (white bars) was decreased by ~12, 24, and 45% by 100, 200, and 400  $\mu\text{M}$  HOCl, respectively, but the heme-binding site remains intact (i.e., the Soret maximum, 414 nm, did not change significantly). The rapid heme binding to albumin was also essentially unaffected by HOCl (up to 400  $\mu\text{M}$ ), but total binding was decreased after exposure of albumin to 200  $\mu\text{M}$  HOCl. Damage that affected heme binding, at concentrations greater than 100  $\mu\text{M}$ , was revealed by decreased absorbance in the Soret region with a gradual red shift in the absorbance maximum from 402 to 406 nm that developed with time and increasing HOCl concentration (data not shown). Because of these changes in the heme environment, the data have been normalized and heme binding is calculated as the percent change in absorbance at the Soret maximum minus that at 650 nm as described in the legend to Fig. 3. There was no apparent change in the absorbance spectra of heme at the concentrations of HOCl used (data not shown).





**Fig. 6.**

Heme–hemopexin exposed to physiologically relevant concentrations of peroxides still induces the cytoprotective heme oxygenase-1 (HO1). Comparison of the levels of HO1 after 6-h incubation of human SH-SY5Y cells with either phosphate-buffered saline (PBS) or 25  $\mu$ M heme–hemopexin complexes (H-HPX) exposed to either H<sub>2</sub>O<sub>2</sub> or *tert*-butyl hydroperoxide at 1 : 1 or 1 : 10 molar ratios or with an equivalent volume of water as indicated in the figure (further details are presented in the Materials and Methods section). The levels of HO1 were assessed by immunoblotting as described above in the legend for Fig. 1 and the data shown are the mean and SEM from three independent experiments with GAPDH as the loading control. The figure is composed of the pairwise comparisons using a one-tailed, unpaired Student's *t*-test between the PBS control and each H–HPX incubation (significance levels are \**p* < 0.05 and \*\**p* < 0.01). Individual *p* values from left to right are *p* = 0.0038, *p* = 0.0029, *p* = 0.0151, *p* = 0.0067, and *p* = 0.0227, respectively.