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Forum Review Article

Links Between Cell-Surface Events Involving Redox-Active Copper and Gene Regulation in the Hemopexin Heme Transport System

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ABSTRACT

Heme is considered to play an instrumental role in the pathology of hemolysis, trauma, and reperfusion following ischemia. However, data are sparse and experimental models are required. The transport of heme by hemopexin to tissues is a specific, membrane receptor-mediated process. Hemopexin recycles after endocytosis like transferrin. Heme oxygenase-1 (HO-1), transferrin, the transferrin receptor, and ferritin are regulated by heme-hemopexin. Genes that encode proteins important for cellular defenses against oxidative stress, such as the cysteine-rich metallothioneins (MTs), are also activated by hemopexin, as are proteins that regulate cell cycle control including $p21^{WAF1}$ and the tumor suppressor p53. The hemopexin system is being investigated to establish how intracellular events are affected by signal(s) from the plasma membrane due to hemopexin receptor occupancy and heme transport. A transient oxidative modification of proteins, shown by carbonyl production, takes place. Redox processes at the cell surface, which generate cuprous ions, are involved in the regulation of the MT-1 and HO-1 genes by heme-hemopexin before heme catabolism and intracellular release of iron. The "redox-sensitive" transcription factors activated by the hemopexin system include c- Jun, RelA/NFκB and MTF-1. The specific copper chelator bathocuproine disulfonate prevents carbonyl production, the nuclear translocation of MTF-1, and the induction of MT-1 revealing a novel, pivotal role for copper in the hemopexin system. In addition, surface redox-active copper is the first link shown for the concomitant regulation of HO-1 and MT-1 and is required for the activation of the amino-terminal c-Jun kinase (JNK) by heme- hemopexin. Antiox. Redox Signal. 2, 157–175.

INTRODUCTION

Environmental stimuli requiring adaptation of cells

IT IS VITAL for cells to monitor changes in their environment and then to respond appropriately. Proteins in the plasma membrane detect shear stress, nutrients, growth factors, hormones, minerals (calcium, magnesium), nucleosides (adenosine), metal ions (copper, nickel, and zinc), and heavy metals (cadmium, lead). Hemolysis produces hemoglobin-haptoglobin and heme (iron-protoporphyrin IX)-hemopexin complexes. In addition, upon injury and inflammation, the level of circulating cytokines rises, including interleukins and interferons such as tumor necrosis factor. Specific receptor systems and ion channels at the plasma membrane are the sensors of these stimuli and transduce each in a manner whereby one or more signaling cascades are activated. Consequently, there is regulation of metabolism (phosphorylase kinase is a substrate for the cAMP-dependent kinase), cell cycle control (phosphorylation of cyclin-dependent kinases), and gene expression (c-Jun and NFκB). Oxygen tension is also sensed, and the transcription factor hypoxia inducible factor (HIF-1α) is activated at

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low oxygen tension, possibly *via* a b-type heme-flavoprotein (Zhu *et al.*, 1999). Cross talk between signaling pathways takes place, and one current major challenge in understanding cell adaptation is to determine the mechanism(s) whereby the various pathways are integrated.

Cell-surface stimuli in vivo *involving heme*

The molecular events involving heme at the plasma membrane of cells when there is hemolysis, and trauma and upon perfusion following ischemia require defining. Redox active metals (heme-iron, iron, and copper) are known to contribute to the production of reactive oxygen species (ROS). Oxidative damage from heme is currently considered to contribute to the pathology of these conditions and potentially also to neurodegeneration and aging. Free heme is toxic to cells. However, data on the mechanism(s) whereby heme might mediate or might be prevented from causing oxidative damage *in vivo* is limited. Experimental model systems are required and the focus here is on the hemopexin heme transport system. Endocytosis of heme-hemopexin is analogous to that of iron transport by transferrin (Smith and Hunt, 1990). Cell-surface events in response to binding of heme-hemopexin to the hemopexin receptor and, subsequently, upon heme transport followed by heme catabolism are being delineated. These include: determination of any oxidative effects of free heme and heme-HPX complexes; investigation of the pathway from the cell surface hemopexin receptor to the nucleus for gene regulation; delineation of events from the receptor alone in the absence of heme uptake using the heme analog cobalt-protoporphyrin IX (CoPP)-hemopexin complex, and delineation of cellular responses as heme moves through the cell to heme oxygenase.

Inflammation follows hemolysis and accompanies injury and infection. These are conditions in which hemopexin plays a protective role by sequestering heme. However, some oxidative stress occurs at the same time *via* events that do not involve heme. For example, neutrophils migrate to sites in the vasculature (tissue capillary beds) and stimulation of their "respiratory burst" produces ROS extracellularly, *e.g.*, hydrogen peroxide. Oxidative stresses *in vivo* also associated with production of hydrogen peroxide take place when epidermal growth factor and cytokines bind to their respective receptors (Lander *et al.*, 1995; Deora *et al.*, 1998). The type, extent, and duration of the oxidative stimulus together with the level of defense molecules and enzymes determine whether cells survive or undergo apoptosis. Survival may require a period of cell arrest to repair damaged DNA. Hydrogen peroxide directly affects intracellular GTP-binding proteins (oxidation of Cys 118 of the G-protein, Ras; Deora *et al.*, 1998). A form of NAD(P)H-oxidase comprised of several protein subunits exists in nonphagocytic cells to oxidize the GTP-exchange protein Rac (Irani and Goldschmidt-Clermont, 1998; Kim *et al.*, 1998). This is likely to be one means *in vivo* whereby signaling cascades for the activation of transcription factors (*e.g.*, AP-1 family members, NFκB) are controlled. Rac activates the c-Jun amino-terminal kinase (JNK), raising the levels of phospho-c-Jun. Consequently, cell processes, including cell cycle control, growth, and gene expression, are regulated.

Oxidative stress at the cell surface and intracellularly

Any redox process (electron transfer alone or with proton translocation) is a potential source of oxidative stress. A new electron transfer process at the cell plasma membrane that occurs when iron is reduced before uptake has recently been recognized. This occurs in mammalian, yeast (see Fig. 1), and plant cells and has been implicated in the regulation of cell growth (Alcain *et al.*, 1994). During hypoxia, due to decreased blood supply or a clot, there is a switch from aerobic metabolism to glycolysis and lactate production that results in organelle and cell swelling. This is due to the inactivation of ATP-dependent ion pumps because of inadequate amounts of ATP. The cell cytosol becomes acidic and the ratio of glutathione (GSH): oxidized glutathione (GSSG) changes dramatically from 1:1,000 to 1:0.1. But oxygen is required for cell survival. Thus, upon reoxygenation, the intracellular oxidative state increases when more ROS are produced (*e.g.*,

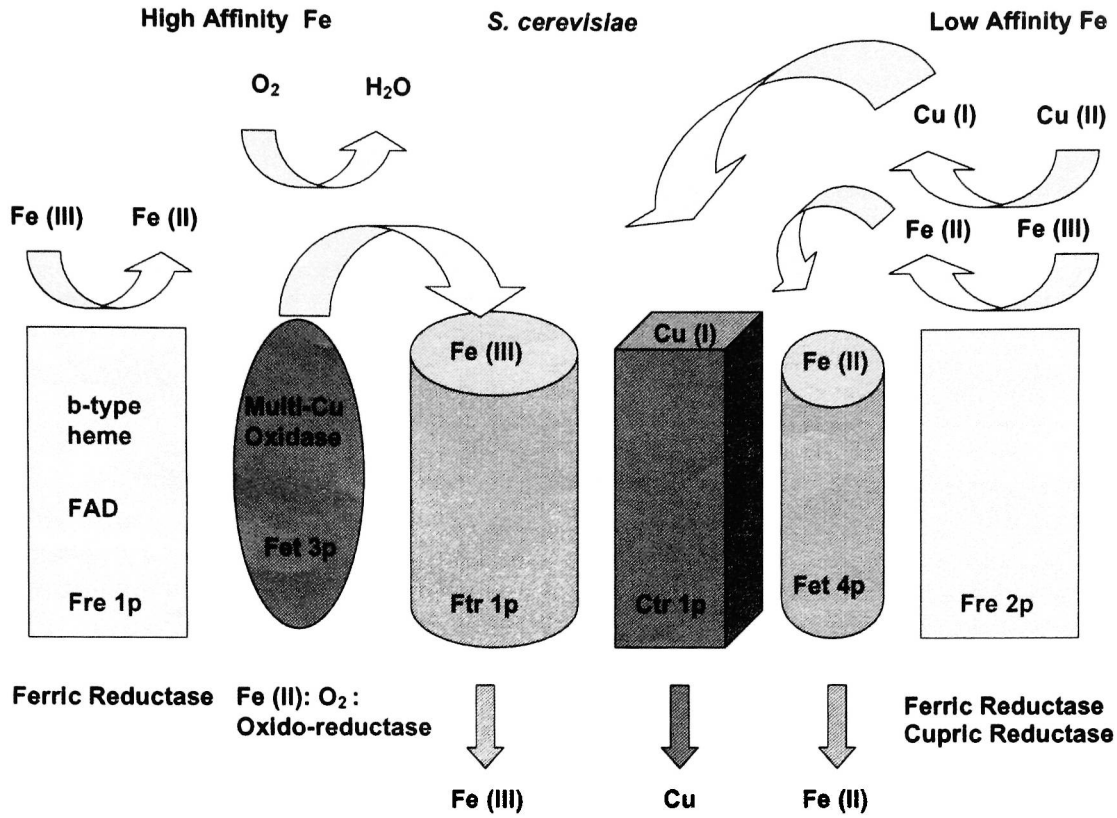


FIG. 1. Iron uptake in *Saccharomyces cerevisiae*. This model for iron uptake with an unexpected requirement for copper is derived from elegant genetic studies in yeast. Briefly, yeast surface reductases, including Fre1p with a b-type heme, provide ferrous iron for low- and high-affinity transporters. An additional protein, Fet3p, with an extracellular copper multi-copper oxidase domain acts as a ferroxidase generating ferric iron for cellular uptake by the Fe (III)-specific permease/transporter Ftr1p. Fet3p is inhibited by BCDS. Disruption of surface or intracellular copper transporters affects the copper loading of Fet3p and concomitantly iron uptake by yeast. How the Fe(III) substrate for Fre1p differs from that for Fet3p, or is distinguished by these two proteins, remains to be established.

from normal metabolism and mitochondrial electron transport required for oxidative phosphorylation) than can be inactivated by the cell's defenses (enzymes and sulfhydryl-rich molecules). Apoptosis or necrosis then ensues. Deterioration of cell functions due to the accumulation of oxidatively damaged proteins is the major cause of aging in invertebrates and mammals (Stadman, 1990). Such intracellular oxidation is minimized under conditions of caloric restriction. Resistance to oxidative stress increases life span, as has been shown unambiguously in *Caenorhabditis elegans* and *Drosophila*. These are fundamental metabolic events, so there is every indication that mammalian cells are similarly affected. The cellular defenses include Cu, Zn superoxide dismutase (CuZnSOD), mitochondrial Mn SOD, and catalase; GSH peroxidase together with GSH reductase; the sulfhydryl-rich tripeptide GSH and the metallothioneins; as well as the iron-binding ferritin and copper-storing proteins. SOD together with catalase, or other peroxidases some of which are heme-proteins, are needed to protect the cells from superoxide and the hydrogen peroxide generated from it.

THE HEMOPEXIN HEME TRANSPORT SYSTEM

Hemopexin protects cells from heme and hemopexin receptors regulate cellular events

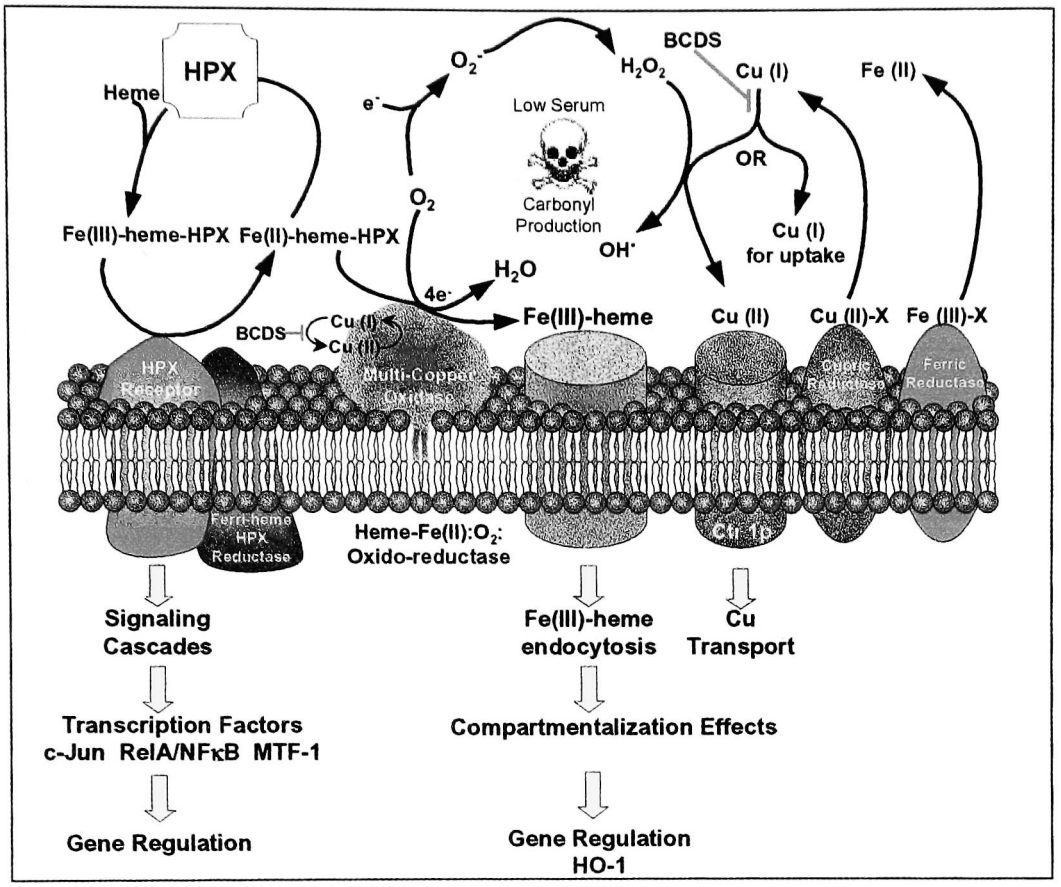
The highly reactive hydroxyl radical is generated from hydrogen peroxide in the presence of redox-active metals, including copper, iron, and heme-iron. Proteins that bind copper, iron, heme, and hemoglobin in the circulation, namely ceruloplasmin, transferrin, hemopexin, and haptoglobin, respectively, act as extracel-

FIG. 2. Surface processes involving copper in the hemopexin heme transport system. Surface redox processes, detectable in low serum, generate Cu(I), lead to carbonyl production and participate in the concomitant regulation of heme oxygenase-1 and MT-1 genes by heme-hemopexin. This model is based on that of yeast iron uptake and is described in the text. Hemopexin-bound ferric heme acts an electron acceptor and heme brought to the surface by hemopexin upon binding to the hemopexin receptor is reduced to facilitate heme release. Subsequently, there is oxidation by a copper-dependent protein for uptake during endocytosis of heme-hemopexin complexes. The sites of copper accessible to bathocuproine disulfonate (BCDS) are indicated. A mammalian counterpart for FET3 (*i.e.*, Fet3p), the Fe(II):O_2:oxido-reductase Hephaestin, has been identified and plays a role in iron transport from the enterocyte across the basalateral surface into plasma. The copper status of the cell appears to affect the expression of the P-type copper transporter, the Wilson's disease gene product that has been located in the plasma membrane. Others have evidence that it is expressed only on the canalicular membrane of hepatocytes. The transporter for iron associated with the ferric reductase is not shown. The redox potential for heme-hemopexin, like Frelp, is low, suggesting that heme-hemopexin can be a substrate for an analogous system for heme uptake.

lular antioxidants. Numerous *in vitro* studies support this concept, as do recent data from haptoglobin-null and hemopexin-null mice. The kidney in particular is damaged by phenylhydrazine-induced hemolysis in both normal and Hp knockout mice (Lim *et al.*, 1998). The clearance of hemoglobin from the plasma is normal in these mice, presumably due to functional hemopexin. Lack of hemopexin or haptoglobin is not a lethal condition. Hemopexin-null mice have confirmed (Tolosano *et al.*, 1999) the importance of hemopexin for the clearance of heme in hemolytic conditions (Smith and Morgan, 1978, 1979).

Hemopexin is one principal means of defense against the potentially oxidizing toxicity of heme. The source of all plasma proteins is the liver, and hemopexin is also expressed, potentially as a tissue-specific form, in certain cells of barrier tissues including the eye (Hunt *et al.*, 1996; Chen *et al.*, 1998) and the central (Morris *et al.*, 1993) and peripheral nervous systems (PNS) (Madore *et al.*, 1994; Camborieux *et al.*, 1998). The constitutive expression of hemopexin in brain is not extensive (Tolosano *et al.*, 1996) and was not detected by Camborieux and colleagues. The induction of expression of hemopexin upon injury in the PNS is more clear-cut. Endocytosis of heme-hemopexin complexes has been shown using both morphological and biochemical techniques (Smith and Hunt, 1990) in a variety of cell types and is retained in several transformed cell lines. Catabolism of the heme by heme oxygenase takes place after heme reaches the smooth endoplasmic reticulum. There are many similarities between the hemopexin and transferrin systems. Hemopexin, like transferrin, recycles intact after endocytosis (Taketani *et al.*, 1986; Smith and Hunt, 1990). We proposed (Smith, 1999) that heme uptake from heme-hemopexin complexes requires reduction of heme-iron and reoxidation by a heme oxidoreductase copper-enzyme (Fig. 2), just as iron uptake requires reduction of the iron and an associated copper-dependent iron oxido-reductase analogous to the yeast Fet 3 (Askwith *et al.*, 1994; Askwith and Kaplan, 1998). We wished to determine whether and, if so, how the regulation of heme oxygenase-1 (HO-1) and metallothionein-1 (MT-1) gene expression by heme-HPX was associated with the activation of surface redox processes. We have also investigated whether extracellular or surface copper, rather than intracellular iron from heme catabolism, was the first redox active metal required. Metallothionein, induced by heme-hemopexin, binds the transition metal zinc, which, unlike iron and copper, is not redox active.

The hemopexin receptor permits cellular access of heme in a controlled manner and transmits signals that regulate cellular responses to heme preventing oxidant damage. As reviewed here, the emerging picture for the hemopexin system is that the principal stimulus for MT-1 transcription is activation of signaling cascades mediated by the hemopexin receptor (Fig. 4). A role for compartmentalization in gene expression in response to heme-hemopexin is also evident. The transcription of HO-1 requires heme transport. The model ligand for investigating events mediated by the receptor in the absence of tetrapyrrole uptake is CoPP bound to hemopexin. This complex does not regulate HO-1, but it does elevate phospho-c-Jun levels. These



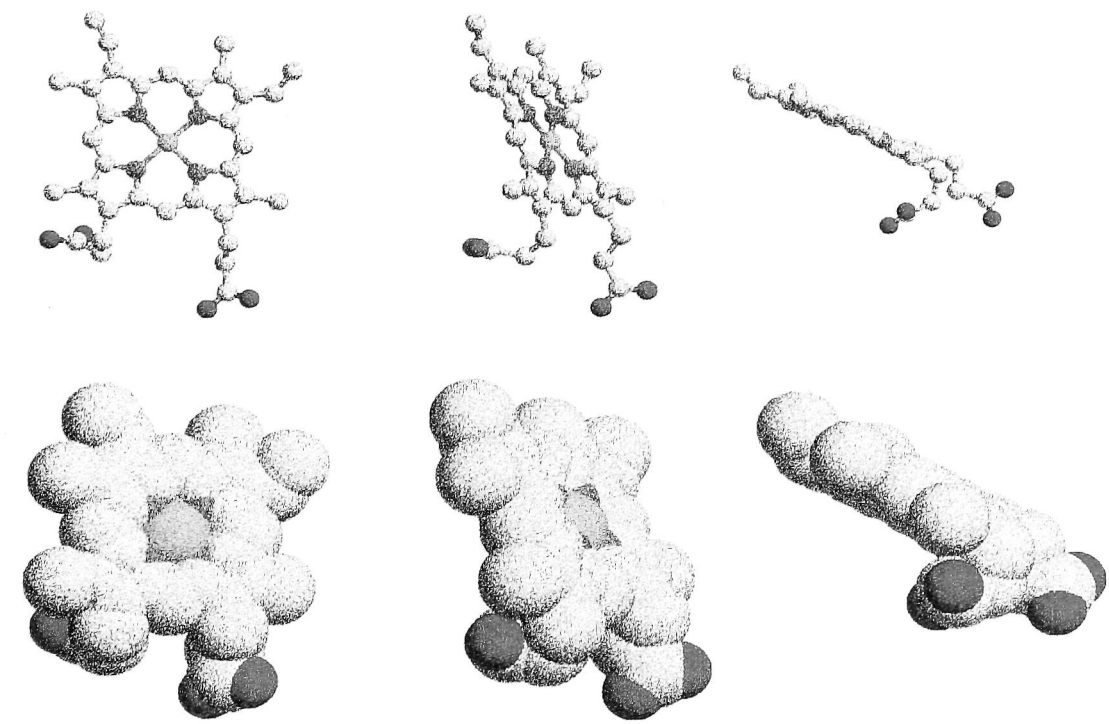


FIG. 3. Heme on the go! "Stick-and-ball" representations of the amphipathic heme molecule, iron-protoporphyrin IX, are shown in the upper panels with space-filling models below. The central iron atom is orange, the tetrapyrrole nitrogen atoms are blue, and the oxygen atoms of the propionates on rings C and D are red. Heme is a stable molecule requiring the enzyme heme oxygenase for release of iron.

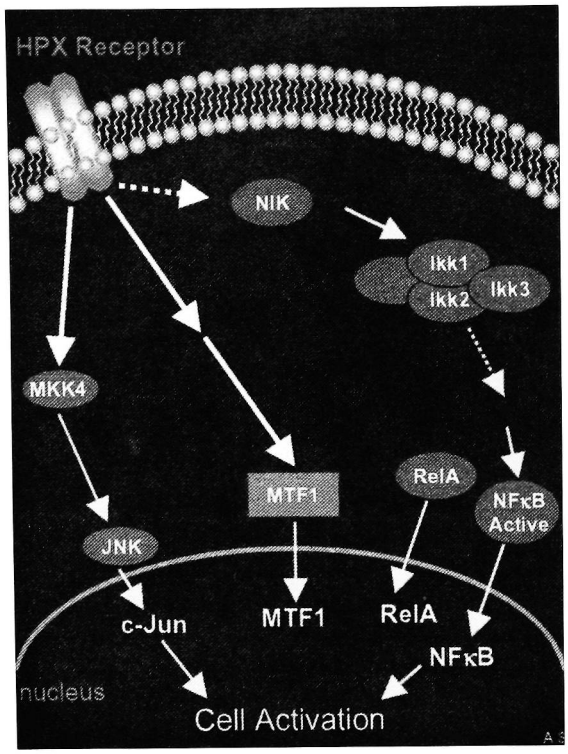


FIG. 4. Representation of signal mechanism(s) for transcription factors activated by the hemopexin receptor. The solid arrows indicate steps for which there is experimental evidence in the hemopexin system. JNK is one member of the family of MAPKs and is the substrate for the JNK kinase termed SEK1 or MKK4. SEK1 is activated by hemopexin (Eskew & Smith, unpublished observations). The metal response element binding protein is MTF-1. MTF-1 is thought to be retained in the cytosol analogously to NFκB (Palmiter, 1994). Most inducers of NFκB activate the NFκB-inducing kinase (NIK), but the mechanism for the nuclear translocation of RelA/NFκB family members by hemopexin is not yet established. Models for the hemopexin receptor were previously based on the trimeric tumor necrosis factor receptor. The current model is based on a dimeric growth factor receptor.

observations alone argue against a simple direct activation of HO-1 via c-Jun. Several lines of evidence support that CoPP binds to hemopexin and like heme binding causes conformational changes necessary for receptor recognition (Smith *et al.*, 1993). CoPP-hemopexin binds to the hemopexin receptor almost as tightly as heme-hemopexin and activates MT-1 gene transcription although there is no cell uptake of CoPP. CoPP itself is a potent inhibitor of HO yet is an extremely effective inducer of HO-1 transcription (Smith *et al.*, 1993) more so than heme itself (Alam and Smith, 1992). Changes in intracellular redox state are reflected in increases in the amount of oxidized GSH decreasing the ratio of GSH/GSSG. The mechanism whereby CoPP activates HO-1 gene expression is likely to be due in part to the rapid depletion of GSH by CoPP, a known stimulus for HO-1 induction (Tyrrell, 1997), whereas heme does not deplete GSH (Tomaro *et al.*, 1991). The effects of heme-hemopexin are therefore not considered to be due to extensive changes in GSSG levels, although there may be subtle changes or effects on GSH metabolism.

Extracellular free heme and heme-hemopexin cause a transient increase in cellular oxidation, in contrast to CoPP-hemopexin

Heme (iron-protoporphyrin) is vital to aerobic life; however, redox-active heme is potentially toxic because of the chemical reactions that occur in the presence of oxygen. Heme is a reactive amphipathic molecule (see Fig. 3) with limited water solubility and readily inserts into lipid bilayers. Therefore, heme binding-proteins including hemopexin have evolved to control the redox reactions and for heme transport. The forms of heme in the circulation that predominate after an injury are hemoglobin, heme-hemopexin, and hemoglobin-haptoglobin. If hemolysis is extensive, then heme-albumin and heme-histidine-proline-rich glycoprotein are formed. At 37°C, heme rapidly transfers to hemopexin from deoxyhemoglobin, and from heme-albumin in the presence of low (micromolar) concentrations of hydrogen peroxide.

When cells are incubated with "free" heme *in vitro*, heme diffuses across the plasma membrane. Heme can cause radical-mediated lipid peroxidation as well as the oxidation of proteins, carbohydrate, and DNA. Heme is not soluble in aqueous solutions at neutral pH and even at concentrations of 1–2 μM forms dimers. With time, and at higher concentrations of heme, some covalent oxo-μ dimers and aggregates form. The sources of redox active metal that could be involved in generating ROS in the hemopexin heme transport system include: the heme-iron *en route* from receptor-bound hemopexin into the cell; extracellular iron or copper;

surface iron, or copper bound to a membrane protein; intracellular heme or iron released from heme catabolism moving through cellular sites.

Cultured mouse hepatoma cells were incubated for 5 min to 2 hr at 37°C in serum-free Dulbecco's modified essential medium (DMEM) in the presence and absence of 10μM heme or 0.5–10 μM heme-hemopexin. Metal-catalyzed oxidative modification of proteins was assessed by protein carbonyl production in whole-cell extracts using published techniques (Levine *et al.*, 1994; Eskew *et al.*, 1999). A rapid but transient oxidation occurs within minutes, reaching a maximum at 15 min and returning to control levels within 1–1.5 hr. The effect of free heme is similar to the extent and duration of carbonyl production in response to heme-hemopexin previously published (Eskew *et al.*, 1999). Furthermore, these levels are equivalent to those produced by addition of 400 μM hydrogen peroxide to cells in media with 0.5% fetal bovine serum (FBS) or generated in brain *in vivo* upon perfusion after ischemia. Thus, free heme causes an increase in the oxidation of cellular proteins, but the effect is short-lived. These data support that the transient protein oxidation when heme-hemopexin binds to cells expressing the hemopexin receptor is due to processes associated with heme release from hemopexin and cellular heme uptake. Oxidized proteins are rapidly ubiquitinated and degraded. The rapid restoration of initial carbonyl levels after exposure to heme or to heme-hemopexin is probably due to such degradation. Cobalt is not inert but it is far less redox-active than iron. Thus, as expected and in contrast to heme and heme-HPX complexes, carbonyl production upon incubation with CoPP-hemopexin was similar to the control cells (Eskew *et al.*, 1999).

Micromolar heme-hemopexin complexes (2–10 μM) are neither toxic nor apoptotic, and transient cell arrest follows with increased levels of $p21^{WAF1}$ and p53 (Eskew *et al.*, 1999). ROS generation is one means whereby cytosolic components of signaling cascades are activated, *e.g.*, Ras- and Rac-activated mitogen-activated protein kinases (Irani and Goldschmidt-Clermont, 1998). The hemopexin system may use ROS as signaling molecules in its role as an early warning signal for danger from hemolysis or cell damage (see below for further discussion). The carbonyl production clearly contrasts with the prevention of the oxidative effects of heme on macromolecules *in vitro* when tightly bound to hemopexin *via* bis-histidyl coordination of the heme-iron (Gutteridge and Smith, 1988). Consistent with the decreased redox activity of cobalt compared with iron, CoPP-hemopexin, which binds to the hemopexin receptor analogously to hemopexin, does not increase carbonyl production. Hydrogen peroxide (10 μM) produces superoxide in large amounts in solution in phosphate-buffered saline (PBS) from heme (1 μM), a very small amount from heme-hemopexin (10 μM), and none from CoPP-hemopexin (10 μM) (Eskew and Smith, unpublished observations). This observation is consistent with the inability of cobalt to undergo Fenton-like chemistry. Superoxide anion formation was detected using luminol, which is a specific oxidant for superoxide.

The increase in protein carbonyl production by heme-hemopexin complexes is likely to be due principally to events set in motion by the redox-active heme-iron rather than to conformational changes in the hemopexin receptor induced by ligand binding *per se*. These data, together with other data presented below also support a proposed model (Smith, 1999) in which ROS are generated as part of the participation of cell-surface processes needed to reduce heme in heme-hemopexin complexes bound to the hemopexin facilitating heme release for cell uptake.

The effect of increasing concentrations of serum on carbonyl production by heme-hemopexin was also investigated to mimic physiological conditions further. Supplementation of culture medium with serum would provide protein targets for oxidation instead of the cellular proteins. Mouse hepatoma cells are routinely incubated in DMEM containing 0.5% serum and cultured in medium supplemented with 2–5% serum. Carbonyl production decreased when FBS levels were raised to 1% and were eliminated in the presence of 2% and 5% FBS (Eskew and Smith, unpublished observations). This shows that plasma proteins and those in the interstitial fluid of barrier tissues would therefore provide protection for cells from oxidation at the cell surface *in vivo*.

Serum supplementation was also used to determine which cellular responses to heme-HPX take place in the absence of carbonyl production. As described below, activation of JNK by 2–10 μM heme-HPX takes place in the presence of 5% FBS. The ratio of heme-HPX complex concentration to serum levels is key. Hemopexin still affects cells incubated with high concentrations of serum *in vitro* when the concentration of heme-HPX is increased to 15–30 μM. The concentrations of 2–10 μM heme-hemopexin are used as model levels for trauma and cellular "heme load" since plasma concentrations of hemopexin in most mammalian species range from 20 to 30 μM. Extracellular concentrations ranging from nanomolar levels during red blood cell lysis in capillary beds to at least 30 μM heme-hemopexin would probably be generated locally *in vivo*.

Gene regulation by heme-HPX

In this part of the review, some background information will be presented for each section first, followed by an overview of relevant studies on the hemopexin system in intact rats. Finally, the more recent work on the molecular aspects of regulation by heme-and CoPP-hemopexin complexes in cultured mammalian cells is discussed.

Induction of heme oxygenase-1. HO-1 catalyzes the rate-limiting step in heme catabolism and is therefore normally present in all cells at extremely low levels. Its physiological substrate is heme, which is cleaved at the α-meso carbon with the release of iron from a peroxy-intermediate (Wilks *et al.*, 1994). The products of heme catabolism are biliverdin, which is rapidly converted to bilirubin by the NADPH-dependent biliverdin reductase and carbon monoxide (CO). Interestingly, biliverdin reductase also acts as a ferric reductase. CO, like nitric oxide (NO), activates the soluble guanylate cyclase that generates cGMP. CO may also act as a neurotransmitter, inhibits platelet aggregation, and is a vascular smooth muscle relaxant. These biological effects may be the consequences of pathological levels of heme. NO is generally the expected signal, and the affinity for CO, when measured, has been found to be lower than for NO. Most recently, CO has been suggested to regulate trophoblast invasion and spiral artery transformation in human placenta (Lyall *et al.*, 2000).

The role for hemopexin in heme transport was shown in rats using intravenous injection of small amounts (700 pmol per rat) of well-characterized [^{59}Fe]heme-rat ^{125}I-hemopexin complexes (Smith and Morgan, 1979). These associate with the liver rapidly, within 1–2.5 min, and in a saturable manner. The recipient patterns of accumulation of both isotopes in the liver were compared with those in the circulation. Heme enters the cells and hemopexin recycles intact after endocytosis. Importantly, and as a rigorous control, the pattern and extent of hepatic radioactivity from apo-^{125}I-hemopexin is quite different from that of heme-^{125}I-hemopexin. The liver retains very little radioactivity when ^{125}I-hemopexin is injected. The pattern of accumulation is similar to that of ^{125}I-albumin, which is used to determine any remaining trapped plasma proteins in the saline-perfused liver. There is no catabolism of hemopexin, indicating that the protein remained in its native conformation after iodination and was acting like the endogenous hemopexin. The turnover is slow like most constitutive plasma proteins synthesized by the liver. Hepatic uptake of heme from hemopexin is 54 pmol/min, per gram of liver (*i.e.*, calculated as V_{max} from saturation data; Smith and Morgan, 1979).

Heme-HPX rapidly induces HO-1 mRNA and protein levels within 30–60 min in several cell types such as retinal pigment epithelial (RPE) cells, mouse hepatoma, and rat phaeochromocytoma (PC 12) (Eskew and Smith, unpublished data) cells. Consistent with a function for hemopexin as an extracellular antioxidant and in protecting hemopexin receptor-null cells from heme, heme-hemopexin does not induce HO-1 in cells that do not express hemopexin receptors, *e.g.*, cultured mouse L fibroblasts (Alam and Smith, 1992). Avian embryonic liver cells have been reported to become refractory to induction of HO-1 by exogenous free heme (Srivastava *et al.*, 1993). But 24 hr after Hepa cells are exposed to 10 μM heme-hemopexin HO-1 remains responsive and is induced once more

by heme-hemopexin (Eskew *et al.*, 1999). The cells do become refractory to the growth stimulation seen with 0.1–0.75 μM heme-hemopexin.

After intravenous injection of [^{59}Fe]heme-rat ^{125}I-hemopexin *in vivo*, the radioactive heme-iron is detected incorporated into cytosolic ferritin within 10 min (Davies *et al.*, 1979). When cultured cells are incubated with heme-hemopexin ferritin induction is detectable by Western analysis within 3–5 hr as shown in mouse Hepa (Sung *et al.*, 2000) and human retinal pigment epithelial cells (Hunt *et al.*, 1996). Induction is not extensive due to the high capacity for iron of each ferritin shell.

The synthesis of heme is highly regulated, like heme catabolism, and is coordinated with the synthesis of the protein moiety of heme-proteins. Newly synthesized heme is released initially from the active site of ferrochelatase, an inner mitochondrial membrane protein. Most cellular heme-proteins are long-lived. Therefore, constitutive heme turnover is expected to be slow and "free" heme would not be expected to build up intracellularly. Intracellular pools of heme may only exist perhaps after incubation of cells with heme *in vitro* and are likely to be membrane-associated. Heme exists "free" in transit from one protein-binding site to another within the cell. Perturbations in the distribution of heme molecules within the cell may take place when HO-1, which contains a heme-binding site of reasonable affinity, is highly induced or overexpressed because it might compete effectively for heme noncovalently bound to other proteins. Furthermore, depending on heme availability, high levels of HO-1 would require significant amounts of NADPH and oxygen for catalysis. Restoration of oxygen levels after a period of anaerobiosis (*e.g.*, in perfusion after ischemia) would enable both HO-1 and HO-2 isozymes to catabolize heme bound at their active site after release from cellular proteins only while sufficient NADPH is available. HO-1 deficiency leads to increased sensitivity to oxidative stress and to low serum iron, yet with hepatic and renal iron overload (Poss and Tonegawa, 1997a,b). These data indicate potential effects of HO-1 on iron reutilization (Maines *et al.*, 1992; Morris *et al.*, 1993). A second isozyme, HO-2, appears to function to augment iron turnover in the lungs where cells are exposed to high oxygen tension (Dennery *et al.*, 1998).

Regulation of the HO-1 gene at the level of transcription is rapidly increased in response to its substrate heme, transported by hemopexin, or added to cells *in vitro*. In addition, a surprising number of diverse stimuli also induce this enzyme via transcription. Hypoxia as well as hyperoxia, hydrogen peroxide, protein kinase C-activating phorbol esters like PMA, bacterial lipopolysaccharide, cadmium and cobalt (but not copper or iron), and chemical depletion of intracellular GSH, and the poison, sodium arsenite. Conditions such as perfusion after ischemia result in induction of HO-1 in heart, kidney (Maines *et al.*, 1992; Raju and Maines, 1996), and brain (Dalton *et al.*, 1995).

Heme-hemopexin induces activation of transcription of both the HO-1 and MT-1 genes (Alam and Smith, 1992). However, as mentioned above CoPP-hemopexin does not induce HO-1 (Smith *et al.*, 1993). The CoPP-hemopexin complex binds to the hemopexin receptor analogously to heme-hemopexin, resulting in activation of signaling cascades (see below) and MT-1 gene transcription but without uptake of CoPP (Smith *et al.*, 1993). The stability of the CoPP-hemopexin complex is attested to by the fact that CoPP is a more extensive inducer of HO-1 than free heme.

Our original proposal that transcriptional activation of the HO-1 gene by heme-hemopexin occurs largely by de-repression (Alam and Smith, 1989) rather than regulation by heavy metals differs markedly from that of MT genes where proximal metal-responsive elements (MREs) function have been described as positive regulatory elements. The murine HO-1 promoter comprises an 11-kb 5'- flanking region in which two enhancers, designated SX2 and AB1, have been identified. These are 268- and 161-bp fragments located 4 kb and 10 kb upstream of the transcription initiation site, respectively. SX2 contains two AP-1 binding sites and two CCAAT-enhancer-binding protein (C/EBP) binding sites that are involved in regulation by heme. Heme requires only the enhancer to activate transcription. An extended AP-1 site resembling an antioxidant-responsive element (ARE) in the SX2 enhancer responds to

antioxidants such as *tert*-butylhydroquinone and drug-metabolizing phase II-inducing chemicals like β-naphoflavone (Prestera *et al.*, 1995). Only regulation by lipopolysaccharide (LPS) involves the AP-1 sites located on both enhancers (Camhi *et al.*, 1998). Chromosomal integration is required for heme activation of HO-1, suggesting a role for chromatin. Stable transfectants are required for HO-1 regulation by heme via the enhancer elements. Another important aspect is the regulation of HO-1 by proximal promoter elements but space constraints preclude discussion here.

Our current view on the mechanism of HO-1 gene regulation by heme-hemopexin is that it is partly related to the oxidation shown by carbonyl production because it is inhibited by bathocuproine disulfonate (BCDS) (see below), but heme uptake is required. Regulation by hemopexin is due to activation of transcription of the HO-1 gene. The fold increases in mRNA levels are similar to the increases in transcription in nuclear run-on assays. In contrast, HO-1 mRNA levels induced by cadmium are maintained, suggesting that there may be additional regulation at the level of HO-1 mRNA stabilization (Alam *et al.*, 1989), as shown in response to NO (Demple, 1999). HO-1 gene transcription by heme-hemopexin is, at least in part, in response to heme uptake because receptor-occupancy alone with CoPP-hemopexin is without effect. Significantly, copper is required for HO-1 mRNA induction by heme-hemopexin (see below). Transcription factors implicated by others in HO-1 regulation, including c-Jun, are activated by heme-hemopexin. In addition, the hemopexin receptor does play a role in a copper-dependent step for JNK activation but only when heme-hemopexin is bound. How this is linked to HO-1 regulation is the subject of current research. Studies of stable transfectants (to be reported elsewhere) revealed that the regulation of HO-1 by heme-hemopexin does involve the SX2 enhancer, which responds to heme.

Induction of metallothionein 1: Within cells, copper and zinc are reversibly bound to the sulfhydryl-rich family of MTs. The physiological role of MTs seemed elusive, but protection against oxidative stress is now apparent. MTs function in metal metabolism, for example, in zinc and copper homeostasis and detoxification of heavy metals such as cadmium. MTs may have a specific function in zinc-responsive neurons. Redox-active copper is bound to MT more tightly than zinc. MT-1 and MT-2 isoforms, present in most organs, increase after ischemia. A cerebral edema develops when transient focal cerebral ischemia is induced by occlusion of the right middle cerebral artery, and this is followed by impaired motor performance in control mice. In mice expressing MT-1 at levels above normal, the infarct size is smaller and the sensorimotor defects are less severe (van Lookeren Campagne *et al.*, 1999). Cerebral ischemia results in induction of HO-1 and the essentially brain-specific MT-3 (Dalton *et al.*, 1995). Many inducers, including hydrogen peroxide, cytokines, PKC-activating phorbol esters (PMA), certain heavy metals, and LPS, raise both HO-1 and MT mRNA levels. Whether there is a primary cellular response of coordinated regulation of HO-1 and MT-1 genes by any of these inducers remains to be established. The hemopexin receptor system is providing some clues to such a process. The data summarized here provide evidence that redox cycling of copper at the cell surface is one initial signal for both MT-1 and HO-1 regulation by heme-hemopexin.

Hemopexin activates MT-1 gene transcription via one or more signaling cascades and formation of superoxide and hydrogen peroxide (Ren and Smith, 1995). An increase in oxidation of the intracellular redox state has recently been linked to MT. Elevation of cytoplasmic Ca^{2+} due to NO generated endogenously causes a rapid change of the fluorescence energy transfer of a chimeric MT-green fluorescent protein molecule consistent with a conformational change in MT considered to release bound metal (Pearce *et al.*, 2000). In aerobiosis, cytosolic GSH levels are normally high and thus GSH and GSH-dependent peroxidase and reductase would be considered to form the primary line of defenses, followed by MT. However, depletion of NADPH would not permit reduction of GSSG and the defenses of MT may then prove more critical for maintaining cell viability. HO-1 also requires NADPH. Thus, we propose that elevated HO-1 would be part of

this switch from defense by GSH to that by MT, explaining why so many conditions in which HO-1 is induced also cause increased expression of MT. In most cells, the principal source of NADPH is the pentose phosphate pathway, which has clear links with nucleotide metabolism and DNA synthesis. Ribose-5 phosphate is a principal product, as are certain intermediates of glycolysis.

Nuclear run-on assays and primer extension analysis showed that heme-HPX controls MT-1 gene regulation primarily at the level of initiation of transcription (Alam and Smith, 1992). Free heme causes a much smaller induction of MT-1 transcription than heme-HPX, a threefold increase compared with 11-fold by heme-hemopexin. A heme-responsive element or mode of regulation was predicted, and heme acts on distal elements rather than the proximal promoter region defined for regulation by heme-hemopexin. Promoter deletion analysis followed by transient transfection assays revealed that the region −153 to −43 of 5′-flanking region of the murine MT-1 promoter is sufficient for increasing transcription in response to heme-hemopexin or CoPP-hemopexin in mouse Hepa cells (Ren and Smith, 1995). The activation by heme-hemopexin of the fusion gene construct −150 and −750 MT βGeo was prevented by *N*-acetyl cysteine (NAC), a precursor of GSH, as well as by GSH itself. These are both sulfhydryl-rich molecules, which act as antioxidants by quenching radicals or by chelating Cu(I) or Fe(II) preventing Fenton chemistry. Addition of Cu Zn superoxide dismutase significantly inhibited MT fusion gene activation by heme-hemopexin, more so than by catalase alone. As expected, the combination of SOD and catalase completely inhibited fusion gene expression providing evidence for the generation of superoxide at the cell surface by heme-hemopexin. Xanthine oxidase together with its substrate xanthine also induced MT-1 (Ren and Smith, 1995). Thus, the inhibitory effects of NAC, GSH, and SOD, together with catalase, are consistent with the increased carbonyl production by heme-hemopexin and with a role for ROS in MT regulation by hemopexin.

Originally, we suggested that heme-hemopexin induces MT because of the need to sequester zinc, which otherwise could compete with iron and occupy sites on regulatory proteins, as well as the proposed role of MT as an intracellular antioxidant (Ren and Smith, 1995). During infection and inflammation circulating zinc levels decline as Zn-MT levels increase in cells. Redox-active iron, copper, and heme are also sequestered within cells as part of the host defense response to limit nutrient availability. Infection causes a dramatic decrease in the level of saturation of transferrin with iron from 30 to 5%. Although MTs can bind iron *in vitro* (Ding *et al.*, 1994), this family of proteins has not been shown to be directly involved in iron or heme metabolism. The induction of MT by intraperitoneal injection of iron was deemed to be due to an indirect effect of the iron (Fleet *et al.*, 1990). Iron availability is normally limiting for heme synthesis. Intracellular heme levels would rise upon an influx of iron, especially if ALA-synthase is induced due to barbiturate intake. This might generate sufficient heme intracellularly in excess of available apo-proteins to induce HO-1.

Heme-hemopexin readily and extensively induces MT transcription and on a molar basis, heme-hemopexin appears to be a far more effective inducer of MT-1 than free heme, CoPP, tin-protoporphyrin (SnPP), or even Zn(II). Induction of MT-1 mRNA by heme-hemopexin is rapid reaching maximal levels within 3 hr. When heme or SnPP and CoPP are bound to hemopexin, the induction of MT-1 transcription is far more extensive. SnPP but not CoPP is transported into cells by hemopexin. These data point to a mechanism for MT regulation via the hemopexin receptor itself. Thus, activation of one or more signaling pathways seemed likely and this was the first evidence for a receptor-mediated signaling pathway from the plasma membrane for MT-1 gene regulation by an inducer (Smith *et al.*, 1992). Protein kinase C (PKC) had earlier been implicated in MT regulation because phorbol esters induce MTs although less effectively than hemopexin complexes (Ren and Smith, 1995). Significantly, however, several fusion gene constructs as well as the endogenous MT-1 gene were also activated by CoPP-hemopexin, which does not generate protein carbonyl production. Furthermore, CoPP-hemopexin induces MT somewhat

more readily than heme-hemopexin. In spite of the proposed role for MT induction to protect against oxidative stress, in the hemopexin system, there is no obvious link between the cell oxidation measured by protein carbonyl oxidation and MT induction. Serum-free conditions *in vitro* allow for the detection and delineation of certain processes but may not be ideal models for the situation *in vivo*. These observations are consistent with a mechanism whereby ligand binding to the hemopexin receptor activates a signaling cascade for MT regulation. As described below, one candidate transcription factor is MTF-1, which binds to MREs in the proximal promoter of the MT-1 gene. MTF-1 has been proposed to be retained in the cytosol analogously to NFκB (Palmiter, 1994).

CuZnSOD protects cells against superoxide, and metal chelators such as dithiocarbamates inactivate this enzyme *in vitro*. The cause of progressive neuromotor degeneration in 10% of cases of amylotrophic lateral sclerosis is directly linked with mutations inactivating CuZnSOD. We addressed whether DDCs increase the basal expression of MT-1 or augment induction by heme-hemopexin using transient transfection assays. The region (−153 to −42) in the proximal promoter of the mouse MT-1 gene responds to heme-and CoPP-hemopexin in transient transfection assays. This DNA contains an Sp1 site, six metal-responsive elements that bind the metallothionein transcription factor, MTF-1, and an ARE overlaps a GC-rich E-box to which USF-1 and USF-2 bind. Both diethyl dithio carbonate (DDC) and pyrrolidine dithio carbamate (PDTC) synergistically augment the induction by heme-hemopexin of MT-1 mRNA and reporter gene activity (Ren and Smith, 1995). Furthermore, PDTC or DDC alone are effective inducers of MT-1 mRNA and transcription of −150 MT β-Geo. Therefore, these data are consistent with an inhibition of SOD by DDCs, but more definitive proof is required. DDC and PDTC are also considered effective antioxidants because metal chelation prevents the production of ROS. Significantly, the activation by DDC and PDTC contrasts with the inhibition by NAC and GSH of the induction of fusion gene activity by heme-hemopexin. There is also evidence that the MREs contribute to MT-1 gene regulation by heme-hemopexin and that ARE-binding proteins are required (Ren and Smith, 1995). Deletion of MREs 5′ or 3′ to the E box/ARE composite element drastically diminishes the transcriptional activation by heme-hemopexin as well as that augmented by dithiocarbamates, which also act via the MREs (Palmiter, 1994). Regulation in response to hemopexin in the presence of PDTC takes place via the region containing an overlapping E box and antioxidant response element by both metal-responsive element-dependent and -independent mechanisms (Ren and Smith, 1995). There are, however, two observations that are discrepant with a simple activation process mediated by MTF-1 via the MRE and with the reported transport of copper by PDTC into cells. The extent of activation of a fusion gene construct comprised of five MREs by Zn(II) is far higher than by PDTC (Ren and Smith, 1995). Heme-hemopexin is without effect on this construct, leading to the tentative conclusion that chromatin structure is involved. MTF-1 is a DNA-binding protein but no partners have yet been identified.

Identification of signaling cascades upstream of the hemopexin receptor underlying transcription factor activation: The signal transduction mechanism resulting in the activation of transcription factors by hemopexin is under investigation. The data show that MT-1 transcription in response to heme-hemopexin requires primarily activation of signaling pathways by the hemopexin receptor and that oxidation *per se* plays a minor role. This reinforces the physiological importance of the hemopexin receptor (Fig. 3). Free heme, which causes extensive carbonyl production, is a poor inducer of MT in comparison with heme-, CoPP-, or SnPP-hemopexin. There are several potential targets of signaling cascades for activation via the hemopexin receptor leading to MT regulation. As mentioned above, MT is induced by phorbol myristate acetate (PMA) (Arizono *et al.*, 1993; Garrett *et al.*, 1992), thus protein kinase C is one kinase pathway identified; however, the MT mRNA induction and effects on fusion gene constructs are not as extensive as with other MT inducers. Hydrogen peroxide, superoxide,

and cytokines, which may induce ROS production, all induce MTs. The mechanism of induction may be due to activation of MAP kinase cascades such as JNK and p38 via known oxidation of Ras/GTP-exchange proteins downstream in the pathways. There is evidence for a role for AP-1 family members (*e.g.*, c-Jun, c-Fos) in MT and HO-1 gene regulation, but whether this is due to a straightforward activation by binding to *cis*-acting elements remains to be determined.

Direct movement of PKC from cytosol to plasma membrane in response to heme-hemopexin was detected in both hepatoma (P.V. Escriba, P. Morales, M. Pendrak, and A. Smith, unpublished manuscript) and MOLT-3 cells (Smith *et al.*, 1997). As expected for endogenous lipid metabolites that bind less tightly than phorbol esters, the extent of PKC activation was variable. The PKC isozyme has not yet been identified. Both heme-hemopexin and CoPP-hemopexin rapidly and extensively activate JNK, whereas free heme has no effect (Eskew *et al.*, 1999). When Hepa cells are incubated with heme-hemopexin, essentially all of the constitutive c-Jun is phosphorylated at Ser-63 and -73 within 30 min incubation of cells with heme-hemopexin.

Heme-hemopexin causes the nuclear translocation of the RelA/NFκB family of transcription factors involved in the innate immune response (Eskew *et al.*, 1999). Electrophoretic mobility shift assays (EMSA) showed that the DNA binding of RelA/NFκB is increased by both heme-hemopexin and CoPP-hemopexin, demonstrating a role for the hemopexin receptor in RelA/NFκB activation. Free heme (0.5–10 μM) has minimal effects on RelA/NFκB in mouse hepatoma cells but has been reported to induce NFκB (p65/p50) in cells of the immune system. PKC phosphorylates IκB *in vitro*. Whether the PKC or additional kinases such as NFκB-inducing kinase (NIK) are involved in release of the p65 and p50 proteins after hemopexin binds to its receptor is not yet known.

JNK is activated by 2–10 μM heme-hemopexin, yet the increased intracellular heme levels are neither toxic nor apoptotic. JNK activation occurs within 5 min in several different types of cultured cells, including rat pheochromocytoma (PC12) cells, which differentiate into neurons upon incubation with nerve growth factor (NGF) (Eskew and Smith, unpublished data). In mouse Hepa cells, activation continues for up to 1 hr, and within 30–60 min essentially all of the constitutive c-Jun has been phosphorylated. The JNK activation response is biphasic. The initial activation declines by 3 hr, but levels are increased again by 6 hr to levels sustained for at least 10 hr. During this time, there is no apparent change in total c-Jun levels determined by Western analysis. The hemopexin receptor is a key player in JNK activation because CoPP-hemopexin also activates JNK and with essentially same time course as heme-hemopexin and to a similar extent (Eskew *et al.*, 1999). Because free heme does not induce JNK, the signaling events generated by heme-hemopexin appear to be of paramount importance in cellular protection by heme-hemopexin.

A role for copper in HO-1 and MT-1 regulation by hemopexin: A model (Fig. 2) for heme uptake from hemopexin with reduction of heme as part of the process of heme release has been proposed. Thermal unfolding studies monitored by absorbance in the Soret region show that upon reduction the heme-hemopexin complex is less stable with a decrease in T_m from 55.5°C to 48°C (Morgan and Smith, 2000). In the hemopexin heme transport system, a copper-dependent oxido-reductase (Smith, 1999) similar to that required by the yeast high-affinity transporter Ftr1 (Askwith and Kaplan, 1998) is proposed to be required. Although no such protein has yet been identified or cloned from in mammalian cells, copper is needed for iron uptake (Yu and Wessling-Resnick, 1998), but its function remains undefined. We anticipated that the surface redox active processes involving copper would be part of the pathway from the hemopexin receptor for gene regulation.

Therefore, we investigated whether chelation of copper at the cell surface inhibited HO-1 and MT-1 regulation. Bathocuproine disulfonate (BCDS), a chelator specific for copper, which does not move through the plasma membrane, prevented induction of HO-1 mRNA by heme-hemopexin shown using Northern analyses (Sung *et al.*, 2000). MT-1 mRNA induction is significantly more sensitive to inhibition by BCDS

(6.25–25 μM) than HO-1. Basal MT-1 mRNA levels were decreased by 50 μM BCDS within 30–60 min but were unaffected at levels below 25 μM. There were no detectable effects of BCDS on actin or tubulin mRNA. In contrast to BCDS, the nonpermeable ferrous iron chelator bathophenanthroline sulfonate (BPS) had only a small inhibitory effect. Inhibition by 50 μM BPS was detectable when the heme-hemopexin concentrations were decreased to 2 and 4 μM. These levels of chelators are low, but are of the order of the expected iron and copper. We chose to decrease hemopexin levels, rather than raising chelator concentrations to minimize possible pharmacological and toxicological side effects of high concentrations of chelators. The chelator diethylene triaminepentaacetic acid (DTPA) caused rapid and extensive inhibition of HO-1 and MT-1 mRNA induction. DTPA is known to bind metals other than ferric ions. No inhibition was observed with deferroxamine (DF), which effectively decreases basal ferritin concentrations and ferritin induction, by heme-hemopexin (Sung *et al.*, 2000).

Copper is required for carbonyl production by heme-hemopexin

Carbonyl production may take place when superoxide is dismutated to hydrogen peroxide during the incomplete reduction of oxygen to water. Hydrogen peroxide generates hydroxyl radicals in the presence of ferrous or cuprous ions. If this takes place at the cell surface, hydrogen peroxide can diffuse across the plasma membrane. Alternatively, its site of generation may be into the cytosol from the inner lipid leaflet. Potential sources for copper available for chelation by BCDS include copper in an extracellular domain of a copper-dependent oxido-reductase or copper en route for cell uptake (Fig. 2). The former would be part of the group of proteins for heme uptake after reduction of hemopexin bound heme by a heme reductase. The latter would be, by analogy with yeast, a ligand for a copper transporter/permease associated with a cupric reductase. If the copper is involved in carbonyl production, then BCDS should inhibit the production of protein carbonyls.

Incubation of cells, growing in medium with 0.5% FBS, with heme-hemopexin (2–10 μM) together with BCDS (25 μM) significantly decreased carbonyl production, which was maximal at 15 min. The nonpermeable ferrous ion chelator BPS was without effect on carbonyl production demonstrating that cuprous, rather than ferrous, ions were required. The carbonyl production at 15 min by free heme (5 μM) of 10–12 nmol dinitrophenyl hydrazine (DNPH)/mg protein was decreased to 8.9 and 6.6 nmol DNPH/mg protein by 25 and 50 μM BCDS, respectively (Eskew and Smith, unpublished observations).

Evidence for a role for copper in the activation of the transcription factors c-Jun and MTF-1 by heme-hemopexin

A further point of interest is that copper is needed for both HO-1 and MT-1 regulation by heme-hemopexin because BCDS prevents induction of both these mRNAs (Sung *et al.*, unpublished manuscript). BCDS caused a 50% decrease in the induction of MT-1 mRNA by CoPP-hemopexin (Sung *et al.*, 2000), the hemopexin receptor ligand, which does not generate carbonyls. Thus, for MT-1 regulation, BCDS is considered to affect a step mediated by the hemopexin receptor itself. Surface copper, probably Cu(I), may be part of the requirement whereby signaling cascades for transcription factors are activated by the hemopexin receptor. This indicates again that heme uptake from hemopexin triggers events requiring surface, rather than intracellular, copper due to the nonpermeability of BCDS.

By analogy with the activation of mitogen-activated protein kinase (MAPK) external receptor kinase (ERK) by growth factors binding to their cognate tyrosine kinase receptors, JNK activation by heme-hemopexin will result from a conformational change in the hemopexin receptor triggered by binding of hemopexin complexes. The GTP exchange proteins Ras and Rac are oxidized by hydrogen peroxide generated intracellularly, or after diffusion across the plasma membrane, leading to activation of the MAPK cascades, respectively.

BCDS decreases by about 50% the extent of JNK activation by heme-hemopexin but is without any effect on the amount of phospho-

c-Jun formed when CoPP-hemopexin binds to the receptor (Eskew and Smith, unpublished data). On the basis of this observation, we conclude that copper is needed for carbonyl formation when a redox-active metal lies within the protoporphyrin ring of the heme analog bound to hemopexin since BCDS prevented carbonyl formation by heme-hemopexin. Therefore, one copper-sensitive step is proposed to take place after receptor occupancy, in one or more of the steps needed for heme release and heme uptake. These data suggest, but do not prove, that the total amount of JNK activation, reflected by the levels of phosphorylation of c-Jun, is derived partly from a surface or extracellular copper-dependent step and partly from the hemopexin receptor itself.

The DNA binding of MTF-1 is induced within 1 hr by heme-hemopexin approximately eight-fold in EMSA (Vanacore *et al.*, unpublished manuscript). MTF-1 is relatively insensitive to diamide oxidation, consistent with a role in activating gene expression when the cells are in a more oxidized state intracellularly. Furthermore, BCDS prevents the nuclear translocation of MTF-1 by both heme-and CoPP-hemopexin complexes. BCDS also decreases MT-1 mRNA induction by CoPP-hemopexin. We conclude that Cu(I) is not only needed for the surface oxidation events but also for the nuclear translocation of MTF-1. MT-1 gene transcription in response to hemopexin occurs principally via Cu(I)-dependent signaling cascades from the hemopexin receptor rather than the oxidation per se. Although it is well established that MTF-1 can regulate MT via the MREs, a role for MTF-1 in MT transcription by hemopexin has not yet been shown directly. The nuclear translocation of MTF-1 in response to heme-hemopexin may be linked with GSH metabolism. MTF-1 is also required for synthesis of the heavy subunit of the γ-glutamylcysteine synthetase gene that encodes a key enzyme in glutathione synthesis (Gunes *et al.*, 1998).

PERSPECTIVES AND CONCLUSIONS

The models used as a foundation for interpreting the data from the hemopexin system have two sites for the location of ROS formation. In one, based essentially on the data from yeast, with reduction of heme iron for release from hemopexin followed by oxidation, the ROS are depicted generated at the cell surface. This is consistent with the lack of permeability of BCDS. The reactivity of the superoxide anion, hydrogen peroxide, and hydroxyl radical vary and affect the range of their sites of action compared with their sites of formation. The hydroxyl radical attacks molecules essentially at its site of formation, whereas hydrogen peroxide can diffuse across the lipid bilayer. In the model for Rac activation by GTP-GDP, exchange of the NADPH oxidase multiprotein complex in nonphagocytic cells assembled at the inner leaflet of the plasma membrane bilayer leads to ROS generation in the cytosol. Similarly, a plasma membrane flavo-heme protein functioning as an NADPH oxidase provides an elegant means to regulated hypoxia inducible factor-1α (HIF-1α) by generating ROS intracellularly (Fig. 5), which oxidatively modify HIF-1α 12 causing degradation. HIF-1α is stable in hypoxia (Zhu and Bunn, 1999).

Normally, cytosol contains GSH, MTs, GSH peroxidase, and reductase, CuZnSOD as pri-

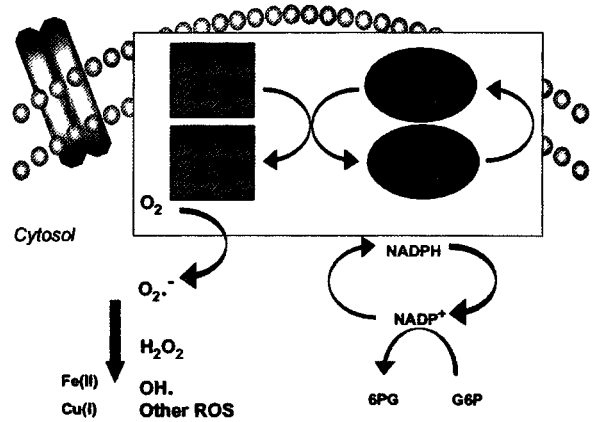


FIG. 5. Schematic model for oxygen sensing and ROS signaling based on that of Zhu and Bunn (1999) for hypoxia inducible factor-α (HIF-α). A flavo-heme-protein in the plasma membrane functions as an NADPH oxidase, transferring electrons through the flavin and heme to molecular oxygen. This generates superoxide, which can in the presence of redox-active metals such as iron be converted to hydroxyl radicals and other ROS. Oxidative modification of HIF-α by the ROS leads to degradation. Stable expression of this transcription factor requires hypoxia. In this model, the ROS are shown generated in the cytosol.

mary defenses against ROS. It is also replete in the iron-binding ferritin and copper-binding MTs. The conditions used *in vitro* for cell incubations can cause significant global changes in cell biology known to affect, or be affected by, increases in intracellular iron, heme, and oxidative stress. For example, many published protocols include a "preincubation" period with iron chelators including DF. An incubation of 10 hr of more with either DTPA or with DF will completely deplete cells of ferritin. This would facilitate a stress response. The extent to which cells are deficient in ferritin, perhaps in anemia, is not yet apparent. Another consideration is whether cells are growing exponentially or are in stationary phase at the moment of stimulation by iron, heme, or oxidative stress. Iron must be available for DNA synthesis, surface transferrin receptors are downregulated during mitosis, and cell cycle regulation takes place in response to DNA damage. The consequences to cells of high HO-1 levels may represent a significant perturbation of the normal physiological situation. Overexpression of HO-1 leads to cell cycle arrest as can high levels of ferritin. When levels of the H-ferritin subunit with ferroxidase activity are high, there are no effects on cell growth but the multidrug resistance transporter is induced (Epsztejn *et al.*, 1999). This is an interesting observation and impressive cellular adaptation. Attempts to establish whether, and to what extent, cell responses are normal in either physiological or pathological terms or when the cells are struggling to survive an artificial stimulus require consideration and evaluation. Otherwise, for example, effects on cellular processes including ribonucleotide reductase and hence on DNA synthesis or conversely on DNA damage may be obscured, confounded, or induced!

In the hemopexin system, the effect of the impermeable copper chelator supports a surface copper-protein in which the copper accepts and donates electrons, as BCDS binds Cu(I) preferentially. A more "dynamic" role for copper undergoing transport into cells remains a possibility. When antibodies to a surface protein, which cross-react with cytochrome b_5, are added to cells heme uptake from hemopexin is inhibited in Hepa cells (Smith, unpublished information). This reinforces the concept that an integral membrane protein homologous to the family of b-type proteins is involved in surface events associated with the hemopexin system. Probably several novel proteins are involved. Interestingly, a cytochrome *b*-type NAD(P)H oxidoreductase, a flavohemoprotein containing unusually both cytochrome b_5 and b_5 reductase domains, has recently been cloned and shown to be present in the cytosol of mammalian cells (Zhu *et al.*, 1999).

In conclusion, the hemopexin system is an important means whereby the body can respond to the potential dangers of hemolysis, tissue damage, iron deprivation, inflammation, and infection. It may also protect the body against the adverse effects of oxidative damage during aging. Furthermore, the mechanism of gene expression in response to heme-hemopexin is the first example of a physiological pathway in which intracellular changes in molecules with redox potential—heme, iron, and copper—are clearly associated with consensus regulatory sequences and transcription factors associated with oxidative stress for both HO-1 and MT-1 genes. Surface copper, as Cu(I), is the first link established for the concomitant regulation of HO-1 and MT-1 gene regulation by heme-hemopexin, and copper is required for the nuclear translocation of MTF-1 and for the activation of the JNK signaling cascade by hemopexin.

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ABBREVIATIONS

AP-1, activator protein; ARE, antioxidant-responsive element; BCDS, bathocuproine disulfonate; BPS, bathophenanthroline sulfonate; C/EBP, CCAAT-enhancer-binding protein; CO, carbon monoxide; CoPP, cobalt-protoporphyrin; DDC, diethyl dithio carbonate; DF, deferroxamine; DMEM, Dulbecco's modified essential medium; DNPH, 2,4-dinitrophenyl hydrazine; DTPA, diethylene triamine pentaacetic acid; DTT, dithiothreitol; EGF, epider-

mal growth factor; ERK, external receptor kinase; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; GSH, glutathione; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; heme, iron-protoporphyrin IX; HIF-1α, hypoxia inducible factor-1α; HO-1, heme oxygenase-1; IFκB, inhibitory factor κB; JNK, c-Jun amino-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MRE, metal-responsive element; β-MSH, β-mercaptoethanol; MT, metallothionein; MTF-1, metallothionein transcription factor-1; NAC, *N*-acetyl cysteine; NFκB, nuclear factor κB; NGF, nerve growth factor; NIK, NFκB inducing kinase; NO, nitric oxide; PBS, phosphate-buffered saline; PDTC, pyrrolidine dithiocarbamate; PKC, protein kinase C; PMA, phorbol myristate acetate (also known as TPA); PNS, peripheral nervous system; ROS, reactive oxygen species; RPE, retinal pigment cells; SnPP, tin-protoporphyrin; SOD, superoxide dismutase; t-BHQ, *tert*-butylhydroxyquinone; TNF, tumor necrosis factor; TPA (also known as PMA), 12-O-tetradecanoylphorbol-13-acetate; TRE, TPA response element; USF, upstream stimulating factor.

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