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Mechanism of Metallothionein Gene Regulation by Heme-Hemopexin

ROLES OF PROTEIN KINASE C, REACTIVE OXYGEN SPECIES, AND *cis*-ACTING ELEMENTS*

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Yafei Ren and Ann Smith‡

From the Division of Molecular Biology and Biochemistry, School of Biological Sciences, University of Missouri, Kansas City, Missouri 64110

Heme-hemopexin or cobalt protoporphyrin (CoPP)-hemopexin (a model ligand for hemopexin receptor occupancy) is shown to increase transcription of the metallothionein-1 (MT-1) gene by activation of a signaling pathway. Promoter deletion analysis followed by transient transfection assays show that 110 base pairs (−153 to −43) of 5′-flanking region of the murine MT-1 promoter are sufficient for increasing transcription in response to heme-hemopexin or to CoPP-hemopexin in mouse hepatoma cells. The protein kinase C inhibitor, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7), prevented the increase in MT-1 transcription by heme-hemopexin, CoPP-hemopexin, or phorbol 12-myristate 13-acetate, but the protein kinase A inhibitor, HA1004, was without effect. *N*-Acetylcysteine (NAC) and glutathione, as well as superoxide dismutase and catalase, inhibited both the increase in endogenous MT-1 mRNA and the activation of reporter gene activity by heme-hemopexin, CoPP-hemopexin, and phorbol 12-myristate 13-acetate. In sum, these data suggest that reactive oxygen intermediates are generated by heme-hemopexin via events associated with receptor binding, including protein kinase C activation. Induction of heme oxygenase-1 expression, in contrast to MT-1, is significantly less sensitive to NAC. Deletion and mutation analyses of the MT-1 proximal promoter revealed that the sequence 5′-GTGACTATGC-3′ (from −98 to −89 base pairs) is, in part, responsible for the hemopexin-mediated regulation of MT-1 which is inhibited by H7. Regulation via this element is also induced by H_2O_2 showing that it is an antioxidant response element. Heme itself acts via more distal elements on the MT-1 promoter. In contrast to NAC and glutathione, diethyl dithiocarbamate and pyrrolidine dithiocarbamate, which inactivate reactive oxygen intermediates and chelate Zn(II), synergistically augment the induction of MT-1 mRNA levels and reporter gene activity in response to heme-hemopexin via the antioxidant response element by both metal-responsive element-dependent and -independent mechanisms.

Hemopexin and transferrin are unique among known endocytic transport systems since both the transport glycoprotein and its receptor recycle (2–5). The hepatic reclamation of heme[1] by hemopexin (K_d <1 pM) (6) sequesters heme from invading organisms (7) and conserves heme-iron for reutilization, storage on ferritin (8), or gene regulation (9, 10). Hemopexin also acts as an extracellular antioxidant (11–13), as do transferrin (14), haptoglobin (15), and ceruloplasmin (16), by coordinating and thus inactivating the reactive heme-iron (12).

Heme is a pleiotropic regulator of gene expression in *Escherichia coli*, yeast, and mammalian cells, although the mechanisms remain to be elucidated in each case. Work in this laboratory has established that the expression of heme oxygenase-1 (HO-1) (9), the transferrin receptor (9), and metallothionein-1 (MT-1) (10) is specifically and differentially regulated in response to heme-hemopexin in cultured mammalian cells. We have proposed (10) that the coordinate induction of MT-1 and HO-1 gene transcription by heme-hemopexin is an adaptive response of cells to maintain homeostasis by minimizing oxidative damage from heme and iron to survive under stress conditions.

Here, we address the mechanism of heme-hemopexin-mediated transcriptional activation of the MT-1 gene. MTs are small, cysteine-rich, metal (*e.g.* Cu(II), Zn(II), and Cd(II))-binding proteins. The mRNA levels of mouse metallothionein (MT-1) (10) have been shown to increase in response to heme-hemopexin. Notably, when heme or the heme analogs, tin-protoporphyrin (SnPP) and cobalt-protoporphyrin (CoPP), are bound to hemopexin, they are far more effective inducers of MT-1 gene transcription than when unbound (10, 17). Interestingly, while the uptake of SnPP is facilitated by hemopexin, CoPP-hemopexin binds to the hemopexin receptor without intracellular transport of CoPP (17) yet is an effective regulator. This indicates that occupation of the hemopexin receptor itself produces intracellular events, postulated to be activation of a signaling pathway (17), that enhances MT-1 gene transcription.

Protein kinase C (PKC) activation has been linked to the regulation of MT expression. First, phorbol 12-myristate 13-acetate (PMA), which activates PKC, induces MT in rats and cultured cells (18). Second, the human MT-2A gene promoter has a functional AP-1 site (5′-TGAGTCA-3′) to which members of the AP-1 family of transcription factors, *i.e.* Jun/Fos, bind, and this DNA binding is induced by phorbol ester activation of

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‡ To whom correspondence should be addressed. Tel.: 816-235-2579; Fax: 816-235-5158; E-mail: annsmith@cctr.umkc.edu.

[1] The abbreviations used are: heme, iron-protoporphyrin IX; HO, heme oxygenase; MT, metallothionein; MT-1, metallothionein-1 isozyme form; ARE, antioxidant response element; MRE, metal-responsive element; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; HA1004, *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride; PMA, phorbol 12-myristate 13-acetate; CoPP, cobalt-protoporphyrin; SnPP, tin-protoporphyrin; GSH, glutathione; DDC, diethyl dithiocarbamate; PDC, pyrrolidine dithiocarbonate; NAC, *N*-acetylcysteine; ROI, reactive oxygen intermediate; PKC, protein kinase C; PKA, protein kinase A; CAT, chloramphenicol acetyltransferase; bp, base pair(s).

TABLE I

Effects of inhibitors of PKC and PKA on heme-hemopexin (H-HPX)- and CoPP-HPX-mediated MT-1 gene expression

The left columns show the results of Northern analyses of endogenous MT-1 gene transcription. Mouse Hepa cells were incubated for 3 h with either PBS, 10 μM heme-hemopexin CoPP-hemopexin, or 50 ng/ml PMA, in the presence and absence of the PKC inhibitor H7 (10 μM) or the PKA inhibitor HA1004 (10 μM) as indicated. Total cellular RNA was extracted and the steady state MT and tubulin mRNAs quantitated by densitometry. The "fold-increase" of MT-1 mRNA levels were calculated with respect to the solvent-treated control cells and normalized to tubulin mRNA and the values are mean ± S.D. from two experiments. The right columns show the results of transient transfection studies. Hepa cells were co-transfected with plasmid −750MTβGeo (10 μg) and pCAT-control (2 μg) as described under "Materials and Methods." After 24 h of incubation with either PBS, heme-hemopexin, CoPP-hemopexin, or PMA in the presence or absence of H7 or HA1004, the fold increase in expression of −750MTβGeo was calculated from the β-galactosidase activity of the fusion gene and normalized to CAT activity. The mean values ± S.D. from six independent transfections are shown. The extent of induction by heme-hemopexin (5–6 fold) was consistently higher than with PMA (~3–4-fold) for −750MTβGeo, pMTLacF (containing 600 bp of proximal promoter (Table II) and −150MTβGeo (Fig. 1). The effects of PMA shown here may not be maximal for the Hepa cells, but this amount of PMA has previously been shown to activate PKC in several cell types. H7 prevented the increase in expression of −150MTβGeo by heme-hemopexin by 38, 59, and 74% at 5, 10, and 15 μM, respectively (data not shown).

Inducer		Steady state endogenous MT-1 mRNA levels, MT-1 mRNA	
		−H7	+H7
		-fold increase	
H-HPX		6.1 ± 0.03	1.5 ± 0.5
	+HA1004	5.9 ± 0.02	1.9 ± 0.3
CoPP-HPX		5.1 ± 0.04	1.2 ± 0.13
	+HA1004	5.0 ± 0.15	1.1 ± 0.06
PMA		3.0 ± 0.12	1.5 ± 0.1

Inducer		Transient transfection with −750MTβGeo, β-galactosidase activity	
		−H7	+H7
		-fold increase	
H-HPX		6.05 ± 0.3	2.2 ± 0.25
	+HA1004	5.9 ± 0.2	1.9 ± 0.15
CoPP-HPX		5.1 ± 0.4	1.48 ± 0.45
	+HA1004	4.8 ± 0.65	1.67 ± 0.06
PMA		3.7 ± 0.04	1.5 ± 0.2

PKC (19). We also note that a putative AP-1 site (5′-TGAGTGA-3′) lies at −538 to −545 bp of the mouse MT-1 promoter. Since PMA also increases the rate of endocytosis of hemopexin (20), the possibility of involvement of PKC in hemopexin-mediated MT-1 gene regulation is raised.

MT-1 is also readily induced in response to a variety of stimuli including metals, glucocorticoids, cytokines (22–25), and ultraviolet radiation (26). MTs have also been proposed (27, 28) to act as intracellular antioxidants by sequestering reactive metals and inactivating hydroxyl radicals and superoxide. Reactive oxygen intermediates (ROIs)[2] have been implicated in the regulation of MT gene expression since MT levels are increased when cells are incubated with chemicals which undergo redox cycling (*e.g.* paraquat) (29) or decrease glutathione (GSH) concentration (*e.g.* diamide) (30). ROIs are not only produced as cytotoxic agents under pathological conditions, *e.g.* by granulocytes in inflammation, but are also generated as side products of electron transfer reactions, both in mitochondria and the endoplasmic reticulum, during normal cellular metabolism. Since heme (iron-protoporphyrin IX) participates in oxygen-radical reactions that can lead to the degradation of proteins, lipids, carbohydrates, and DNA (1, 31, 32), hemopexin-mediated heme transport by increasing intracellular levels of heme and iron may concomitantly raise ROI levels.

Several genes encoding proteins which function to protect against oxidative stress, including the rat GSH *S*-transferase Ya subunit and human quinone reductase genes, contain within their respective promoters an antioxidant response element (ARE, 5′-GTGACNNNGC-3′) which confers transcriptional activation in response to β-napthaquinone (33) or H_2O_2 (34, 35). Interestingly, there is a putative ARE sequence (5′-GTGACTATGC-3′ from −98 to −89 bp) in the mouse MT-1 promoter. The present study[3] was undertaken to define the regions of the MT-1 promoter required for heme-hemopexin-mediated regulation and to assess the roles of PKC, ROIs, and *cis*-acting elements, such as the putative ARE, in this regulation.

MATERIALS AND METHODS

Hemopexin and Heme and Heme Analog Complexes of Hemopexin—Hemopexin was isolated from rabbit serum; complexes of hemopexin with mesoheme, CoPP, or SnPP (Porphyrin Products, Logan, UT) were prepared, characterized, and quantitated using their characteristic absorbance spectra as described previously (36, 37). Mesoheme (iron-mesoporphyrin IX) was employed here rather than the less stable protoheme, since mesoheme-hemopexin is chemically and biologically equivalent to protoheme-hemopexin (36).

Plasmids—Plasmid pMT-lacF contains 600 bp of 5′-flanking DNA (−600 to +64) of the mouse MT-1 promoter linked to the *E. coli* β-galactosidase (*lacZ*) reporter gene. Additional constructs, −750MTβGeo and −150MTβGeo, contain ~750 and 150 bp, respectively, of the promoter linked upstream to the basal MT-1 promoter (−42 to +60; −42MTβGeo) driving expression of a selectable reporter gene encoding a β-galactosidase-neomycin phosphotransferase (*lacZ-neo*) fusion gene (38). Plasmid −750(Δ110)MTβGeo, was derived from −750MTβGeo and contains a 110-bp deletion from positions −153 to −43. MREβGeo contains five copies of the metal-responsive element d′ (MREd′). The vector used in constructing these plasmids was derived from Stratagene's Bluescript vector. Constructs AREMTβGeo and ARE_2MTβGeo were prepared in this laboratory and contain one and two copies, respectively, of the putative ARE (5′-GATCGTGACTATGCA-3′) of the MT-1 promoter ligated into the *Bgl*II cloning site of −42MTβGeo, and plasmids ARE_MMTβGeo and ARE_{M2}MTβGeo contain one and two copies, respectively, of a mutated ARE oligonucleotide (5′-GATCGaGACTATGCA-3′). Using a polymerase chain reaction method, the additional constructs −153(−67)MTβGeo, −124(−67)MTβGeo and −124(−43) MTβGeo were made. They contain −153 to −67 bp (MREs c and d and MT-ARE), −124 to −67 bp (MT-ARE), and −124 to −43 bp (MT-ARE and MREs a and b), respectively, of the MT-1 proximal promoter cloned into the *Bgl*II cloning site of −42MTβGeo. Plasmids pSp64MT-1, containing mouse MT-1 cDNA; pSp64Tu, containing chicken β-tubulin cDNA; and pGEMHO, containing an 883-bp cDNA fragment of mouse HO-1 cloned into the pGEMEX vector (Promega, Madison, WS) were used as templates for the synthesis of [^{32}P]cRNAs (specific activities of about 8×10^7 cpm/μg) as described elsewhere (39).

Cell Culture and Transient Transfections—Minimal deviation hepatoma cells (Hepa), derived from the mouse solid tumor BW 7756, were grown in Dulbecco's modified Eagle's medium supplemented with 0.35% glucose and 2% fetal bovine serum as previously described (40). Hepa cells were seeded (~2.5×10^5 cells/well in 6-well plates) 48 h before co-transfection with the specified MT-1 fusion gene plasmid DNA (10 μg) and pCAT-control (2 μg, from Promega) using a calcium phosphate/DNA precipitation method (41). For each experimental series, two independent transfections were carried out, and each series was repeated several times with more than one plasmid preparation. Four h

[2] The term ROI encompasses singlet oxygen, hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and the hydroxyl radical ($OH^\cdot$), all of which can be interconverted in reactions that depend in part on redox metals such as iron (1).

[3] Preliminary accounts of portions of this work were presented at the Scientific Education Partnership symposium on "Molecular Mechanisms of Aging," Kansas City, MO, October 1992; at the American Society for Biochemistry and Molecular Biology meeting, San Diego, CA, May 1993 (*FASEB J.* **7,** A1235 (Abstr. 1054); and at the Keystone Symposium on Molecular and Cellular Biology, Keystone, CO, January 1993 (*J. Cell. Biochem.*, Suppl. 17A, 124a (Abstr. B410).

later, the cells were exposed to 15% glycerol for 30 s, fresh culture medium was then added, and incubation was continued for 20 h. Various inducers (heme-hemopexin, CoPP-hemopexin, SnPP-hemopexin, heme, CoPP, $ZnCl_2$, or PMA) or the inhibitors H7 and HA1004 (Seikagaku America Inc., Rockville, MD) were then added to the cell culture medium. After 24 h the cells were harvested and lysed by three cycles of freeze-thawing in 0.25 M Tris-HCl, pH 7.5, and cell extracts were prepared by centrifugation for 10 min at 12,000 × *g* and 4 °C. Protein concentrations of cell extracts were measured using the BCA protein assay (Pierce). The β-galactosidase activity of the cell extracts (units/μg of protein, determined from duplicate 50-μl aliquots/well) was determined using an assay kit (Promega Biotech Inc.) and CAT activity (dpm/μg of protein) quantitated by radiometry of the solvent-extracted acetylated ^{14}C-radiolabeled product. The change in β-galactosidase reporter gene activity of the MT-1-promoter constructs was normalized using the activity in solvent-treated control cells and the CAT activity.

Isolation of Total Cellular RNA and Northern Blot Analysis—Exponentially growing Hepa cells (~10^7 cells/75-cm^2 culture flask) were rinsed three times with 10 ml of prewarmed, serum-free Dulbecco's modified Eagle's medium buffered with 10 mM Hepes, pH 7.2, and equilibrated in 5% CO_2 air prior to use. The cells were subsequently incubated with 10 ml of the same medium containing the specified inducer or inhibitor for the indicated time period. Total cellular RNA was isolated using guanidinium isothiocyanate and cesium chloride centrifugation, and after separation of RNA species on a 1% agarose gel, the RNA was transferred to a Zeta-Probe nylon membrane which was then baked. Northern blots were prehybridized, hybridized, washed (42), and then exposed to Kodak X-Omat PR film using an intensifying screen at −70 °C for up to 16 h. Autoradiographic signals were quantitated by densitometry using a PDQUEST™ system (Protein Data Bases, Inc., Huntington Station, NY), and the extent of induction of HO-1 and MT-1 mRNA levels was compared after normalization relative to tubulin mRNA.

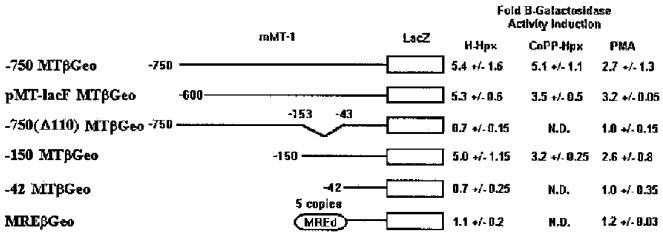


FIG. 1. **Effects of heme-hemopexin and CoPP-hemopexin on the transcriptional activities of the MT-1 proximal promoter.** The *left panel* shows a schematic representation of the MT-1 fusion genes investigated, and the numbers shown delineate the 5′ end of the regulatory region covered by each fusion gene. In the case of −750(Δ110)MTβGeo, the 3′ and 5′ sites of the deletion are indicated. The *right panel* shows the results of the transient transfection assays carried out as described in the legend to Table I. Twenty-four hours after transfection heme-hemopexin (*H-HPX*), CoPP-hemopexin (10 μM), or PMA (50 ng/ml) were added. The β-galactosidase activity of the fusion gene in cell extracts was measured 24 h later and normalized to the CAT activity. Each data point represents the mean value ± S.E. of four (*pMT-lacF*) or mean ± S.D. of six or more (*−750MTβGeo*, *−750(Δ110)MTβGeo*, *−150MTβGeo*, *−42MTβGeo*, and *MREβGeo*) independent transfections. *N.D.*, not determined. Two and 5 μM heme-hemopexin induces −150MTβGeo reporter gene activity 76 ± 3 and 87 ± 4% as effectively as 10 μM complex (data not shown).

RESULTS AND DISCUSSION

Involvement of PKC in Hemopexin-mediated MT-1 Expression—Heme-hemopexin and CoPP-hemopexin elevate MT-1 steady state mRNA levels in Hepa cells as does PMA (Table I, left-hand column). The PKC inhibitor H7 (K_i = 6 μM) prevented this induction. A PKA inhibitor, HA1004 (K_i = 2.3 μM for PKA; K_i = 40 μM for PKC) has neither a direct effect nor an indirect augmenting effect when MT induction is inhibited by H7 (Table I, left-hand column). These observations indicate that induction of MT-1 mRNA by heme-hemopexin and CoPP-hemopexin involves an H7-sensitive pathway, likely a PKC-dependent signal transduction.

Essentially identical results are found with reporter gene constructs (Fig. 1, *right-hand column*) transiently transfected into Hepa cells. H7, but not HA1004, prevented the increases in β-galactosidase reporter gene activity of −750MTβGeo (Table I) and −150MTβGeo (Fig. 1) in response to heme-hemopexin, CoPP-hemopexin, or PMA (Table I). Thus, the responses monitored by β-galactosidase activity of the reporter-gene constructs accurately reflect the cellular responses of the endogenous MT-1 gene. Since CoPP-hemopexin which binds to the receptor without tetrapyrrole transport is as effective as heme-hemopexin, the results suggest that occupancy of the receptor plays a role in transducing signals which result in PKC activation as part of the signaling pathway which regulates MT-1 gene transcription.

Elements of the Mouse MT-1 Proximal Promoter Involved in Transcriptional Activation by Hemopexin—In Hepa cells transiently transfected with up to 600 or 750 bp of 5′-flanking region of the promoters in plasmids pMT-lacF or −750MTβGeo, respectively, heme-hemopexin increases reporter gene expression (Fig. 1 and Table I) similar to the extent of MT-1 mRNA induction (Table I). In addition, heme-hemopexin, CoPP-hemopexin, or SnPP-hemopexin (10 μM) induced pMT-lacF 4.9 ± 0.8-, 3.4 ± 0.3-, and 3.4 ± 0.3-fold, respectively. Somewhat more than free heme or CoPP which induced 3.0 ± 0.2- and 2.0 ± 0.2-fold, respectively, or SnPP which has no significant effect (data not shown), consistent with the reported relative effects of these inducers on endogenous MT mRNA levels (17). Thus, the −600 to +64 bp of the 5′-flanking region of the MT-1 promoter is sufficient for regulation of MT-1 gene expression by metalloporphyrin-hemopexin complexes and by free metalloporphyrins.

The induction of the β-galactosidase reporter gene by heme-hemopexin was abolished when the −153 to −43 bp region was deleted (−750(Δ110)MTβGeo in Fig. 1). Moreover, the β-galactosidase activity of −150MTβGeo, which contains up to −153 to −43 bp of the promoter, is induced by heme-hemopexin, while −42MTβGeo containing only the basal promoter does not respond (Fig. 1). Furthermore, the extent of induction of −150MTβGeo by heme-hemopexin is similar to that seen with either −750MTβGeo or pMTLacF (Fig. 1). Importantly, CoPP-hemopexin also induces the reporter gene activity of

TABLE II

Effects of extracellular superoxide dismutase (SOD) and catalase on heme-hemopexin-mediated induction of MT-1 mRNA

Hepa cells were incubated with 10 μM heme-hemopexin (H-HPX) either alone or with superoxide dismutase (150 units/ml) or catalase (130 units/ml) or with both enzymes for 3 h in serum-free medium. When superoxide dismutase or catalase alone were incubated with the cells, MT-1 mRNA levels were essentially unchanged (1.1 ± 0.2 and 1.2 ± 0.1 times control levels, respectively). The left columns show the changes in MT-1 mRNA levels using Northern blot analysis from two independent experiments; the right columns show the data from six transient transfection experiments with −150MTβGeo carried out as described under "Materials and Methods" and the increases β-galactosidase activity calculated as described in Table I.

Inducer		Steady state MT-1 mRNA		Inducer	Transient transfection with −150MTβGeo		
		−SOD	+SOD			+SOD	+Catalase
		-fold change				*-fold change*	
H-HPX		3.9 ± 0.09	2.95 ± 0.25	H-HPX	4.7 ± 0.85	3.2 ± 0.9	1.8 ± 0.5
H-HPX	+catalase	2.4 ± 0.03	1.5 ± 0.04				

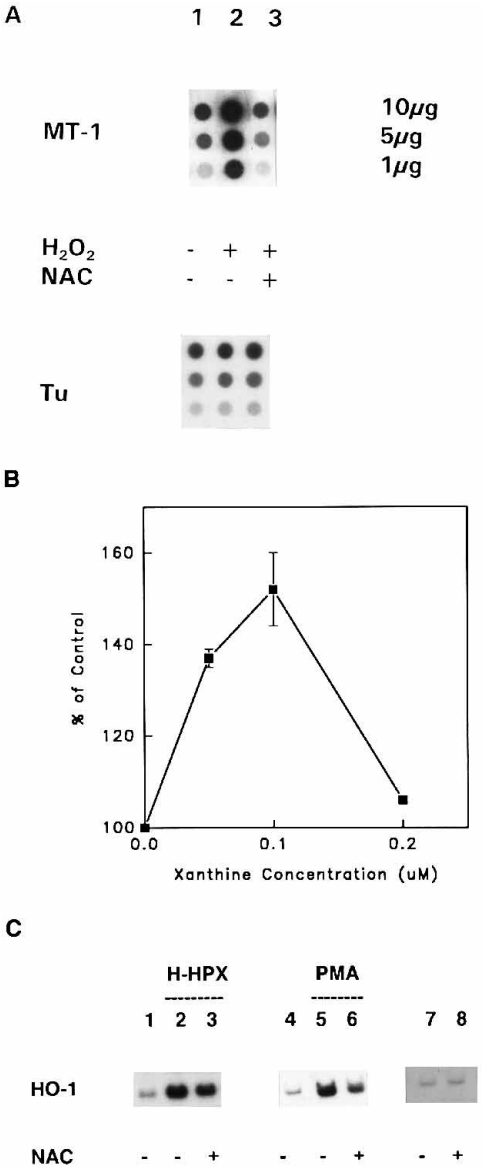


FIG. 2. **Effects of hydrogen peroxide and a superoxide generating system, xanthine oxidase, on MT-1 expression.** Hepa cells were incubated in serum-free, Hepes-buffered Dulbecco's modified Eagle's medium containing either 100 μM hydrogen peroxide, in the presence or absence of 30 mM NAC as indicated, for 3 h. Total cellular RNA was isolated as described under "Materials and Methods." *Panel A* shows a dot blot analysis of MT-1 mRNA in 5 and 10 μg of total cellular RNA. Tubulin mRNA levels are also shown. *Panel B* shows the results of transient transfection with −150MTβGeo and induction of reporter gene activity after incubation of Hepa cells with increasing concentrations of xanthine and 70 units of xanthine oxidase to generate superoxide. In control experiments when cells were incubated with either xanthine or xanthine oxidase alone, there was no detectable change in reporter gene activity (data not shown). *Panel C* shows the effects of NAC on basal and heme-hemopexin (*H-HPX*)-induced HO-1 mRNA levels in response to heme-hemopexin and PMA.

−750MTβGeo and −150MTβGeo, the former as effectively as heme-hemopexin and the latter somewhat less effectively than heme-hemopexin but similar to the levels seen with PMA (see below). This shows that the effects of receptor-occupancy on regulation are maintained in these constructs. H7 blocks the increase in expression of −750MTβGeo by both heme- and CoPP-hemopexin and of −150MTβGeo by heme-hemopexin (Table I).

The 110 bp of the shortened promoter in −150MTβGeo contain the putative ARE, four metal responsive elements (MRE a–d, which share the heptad core TGCPuCNC), a major late transcription factor consensus sequence, and an Sp1 site which overlaps with MREc (43–45). The five copies of MREd′, the element which confers the highest response to Zn(II) (43), in MREβGeo did not restore induction by heme-hemopexin suggesting that other or multiple elements (see below) are major factors in regulation.

PMA, which activates the AP-1 family of transcription factors, also induced expression of the fusion genes in −750MTβGeo and −150MTβGeo, although less effectively than heme-hemopexin (Fig. 1). Thus, the putative AP-1 site at −545 to −538 bp is not required for transcriptional regulation by heme-hemopexin or PMA in transiently transfected Hepa cells. PMA can activate via antioxidant response elements as discussed below.

Roles for ROIs in Hemopexin-mediated MT-1 Gene Regulation—Incubation of Hepa cells with heme-hemopexin in the presence or absence of Cu-Zn superoxide dismutase or catalase decreased by 30 and 44%, respectively, the induction of steady state MT-1 mRNA levels (Table II, left column). Furthermore, superoxide dismutase together with catalase abrogated the effect of heme-hemopexin while neither superoxide dismutase nor catalase alone had any effect on basal MT-1 mRNA levels (Table II). The same effects of superoxide dismutase and catalase were also apparent in transient transfection assays with −150MTβGeo, which contains the 110-bp promoter region responsive to hemopexin (Table II, right column). Since superoxide dismutase converts superoxide anion radicals to H_2O_2 and oxygen, and catalase converts H_2O_2 to water and oxygen, the interference of these enzymes with MT-1 mRNA induction by heme-hemopexin suggests that MT gene regulation is due, in part, to the generation of superoxide and H_2O_2. Both of these ROIs cross membranes and both generate additional ROIs in the presence of iron. Superoxide reduces ferric irons and oxidizes ferrous irons, and hydrogen peroxide interacts with iron to create hydroxyl radicals (1).

ROIs themselves induce MT-1 gene expression in Hepa cells. Hydrogen peroxide caused a 3–4-fold increase of MT mRNA levels within 3 h (Fig. 2*A*) and an increase in transcription of −150MTβGeo that was dose-dependent. Superoxide, generated extracellularly by xanthine oxidase, increased transcription of −150MTβGeo (Fig. 2*B*) in transient transfection assays.

N-acetyl-L-cysteine (NAC), a precursor of GSH that scavenges ROIs (46–48), prevented the increase in MT-1 mRNA levels in response to H_2O_2 (Fig. 2*A*, *lane 3*), to heme-hemopexin or CoPP-hemopexin, or to PMA (Table III). In some cases treatment with NAC produces MT-1 mRNA levels lower than the controls, but this was not seen when GSH was added extracellularly in transient transfection experiments with −750MTβGeo (Table III, discussed below). As additional controls, the effects of NAC on tubulin and HO-1 mRNA levels were investigated (Fig. 2*C*). The HO-1 gene contains two AP-1 binding sites in an enhancer element required for increased transcription by heme (49) and is also induced by PMA (10). Heme-hemopexin and PMA raised HO-1 mRNA levels ~5- and 6-fold by 3 h, respectively (Fig. 2*C*, *lanes 2* and *5*), while NAC lowered the induced HO mRNA levels by about 40% (Fig. 2*C*, *lanes 3* and *6*), but NAC did not affect basal levels of HO-1 (Fig. 2*A*, *lane 8*) or tubulin mRNA (data not shown). Thus, basal and heme-hemopexin- or PMA-induced HO-1 expression is significantly less sensitive to NAC than the MT-1 gene. A 12-h exposure of Hepa cells to 30 mM NAC produces no discernable toxic effects or abnormal morphology.

The extent of changes in reporter gene activity in the transient transfection assays are essentially equivalent to those in the endogenous MT-1 gene measured by Northern blot analy-

TABLE III

Effects of NAC and GSH on heme-hemopexin-, CoPP-hemopexin-, and PMA-induced mouse MT-1 gene transcription

The left columns summarize the Northern analyses results from two independent experiments while the right columns summarize the data from six transient transfections with plasmid −750MTβGeo or −150MTβGeo as described under "Materials and Methods." Hepa cells were incubated with either 10 μM heme-hemopexin (H-HPX), 10 μM CoPP-hemopexin, or 50 ng/ml PMA in the presence or absence of 5, 15, or 30 mM NAC, or of 5, 10, or 15 mM GSH (−150MTβGeo), and 30 mM NAC or 10 mM GSH for −750MTβGeo. Since the lowest concentration of NAC and GSH was as effective as the highest concentration, the results are expressed as a mean ± S.D. for all three concentrations of NAC and GSH for −150MTβGeo. The -fold changes in fusion gene expression were calculated from the β-galactosidase reporter gene activity as described in Tables I and II. The inhibitory effects of NAC on MT-1 mRNA transcription were dose-dependent over the range 5–30 mM (data not shown).

Inducer	Steady state MT-1 mRNA levels		Transient transfection, β-galactosidase activity					
			−750MTβGeo			−150MTβGeo		
		+NAC	+NAC	+GSH		+NAC	+GSH	
	-fold increase		*-fold change*			*-fold change*		
H-HPX	3.6 ± 0.1	0.3 ± 0.02	5.6 ± 0.3	1.7 ± 0.4	3.7 ± 1.5	4.1 ± 1.0	0.45 ± 0.15	0.5 ± 0.05
CoPP-HPX	5.0 ± 0.3	0.85 ± 0.04	4.75 ± 0.06	1.7 ± 0.14	1.9 ± 0.45			
PMA	2.5 ± 0.01	0.25 ± 0.02	3.1 ± 0.45	1.1 ± 0.2	1.7 ± 0.02			

sis. The increases in β-galactosidase activity of −750MTβGeo in response to heme-hemopexin, CoPP-hemopexin, and PMA were also abolished by both NAC and GSH (Table III). Taken together, the results of both Northern analyses and transient transfections are consistent with NAC and GSH acting as ROI scavengers and indicate that the maintenance of thiol levels by NAC or GSH prevents induction of MT-1 gene transcription in response to heme-hemopexin, CoPP-hemopexin, or PMA. Thus, the increase in ROIs which occurs in response to heme-hemopexin causes oxidation of a critical thiol residue and/or depletion of intracellular thiols leading to altered MT-1 gene transcription. However, NAC and GSH also chelate zinc, and thus zinc availability as well as the links between redox-mediated release of zinc from MT itself (50) and MT-1 gene expression are addressed below.

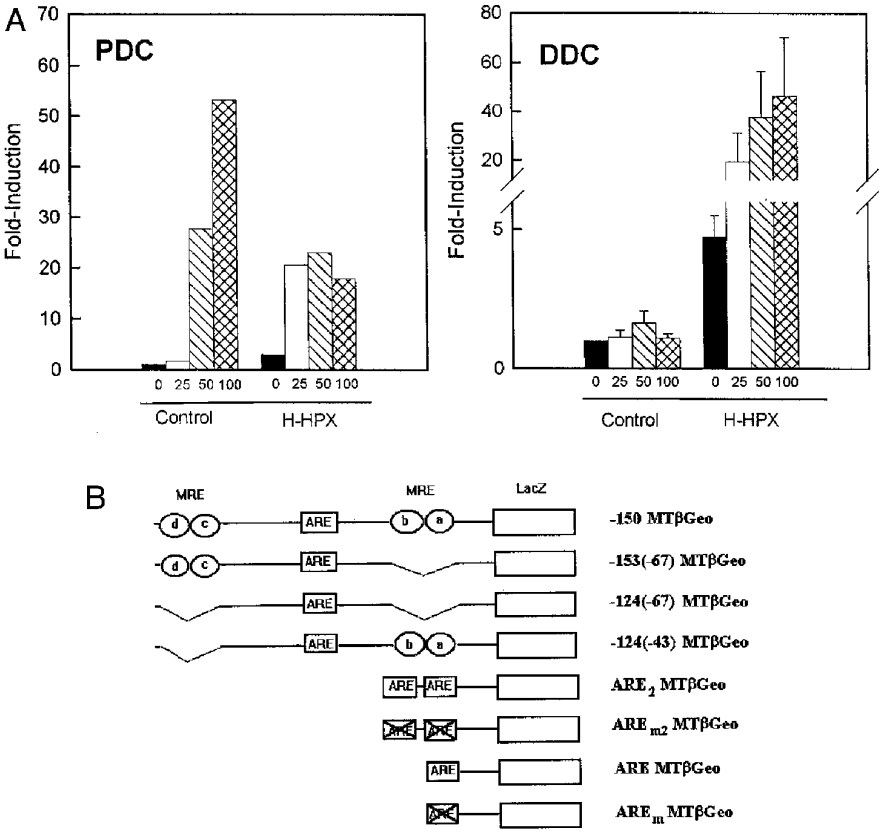
Role for ARE in Hemopexin-mediated MT-1 Gene Regulation—ROIs have been proposed to act as second messengers for a variety of agents including PMA, tumor necrosis factor, and interleukin-1 (51) which have all been reported to induce MT and HO expression. We next sought to identify and define the specific sequences in the proximal 110 bp (−153 to −42), including the putative ARE, which are responsible for the increase in transcription in response to heme-hemopexin. This response was compared with that of H_2O_2. As shown in Fig. 3, *Panel C*, β-galactosidase activity of a fusion gene containing two copies of the putative native MT-ARE, ARE_2MTβGeo, was increased ~2-fold by heme-hemopexin. When this element was mutated so that it no longer responds to chemicals such as *tert*-butylhydroquinone or β-napthoflavone (33, 52), there was no increase in activity of the fusion gene constructs in response to heme-hemopexin (Fig. 3, *Panel C*), indicating that transcriptional activation by heme-hemopexin was lost. Hepa cells transiently transfected with ARE_2MTβGeo were exposed to 100 μM hydrogen peroxide exhibited a 2-fold increase in reporter gene activity, but when transfected with the mutated element were unresponsive. Thus, the DNA sequence (5′-GTGACTATGC-3′) located at position −98 to −89 in the mouse MT-1 promoter is required for hemopexin-mediated regulation of the MT-1 gene, and since it is also activated when cells are incubated with H_2O_2, it can be designated as an ARE. However, while the response of ARE_2MTβGeo to heme-hemopexin was consistent, that of H_2O_2 was variable with respect to both the dose causing maximal response (50–500 μM in 2% serum) and the extent of induction for a specified H_2O_2 concentration. In general a 2-fold increase was seen at either 100 or 200 μM and mean maximal increases of no more than 3-fold occurred at the highest concentration. Induction by 300–500 μM of H_2O_2 was paralleled by a loss of cellular protein albeit with a proportional decrease in CAT activity (but not in galactosidase activity). This suggests that, in contrast to the induction by heme-hemopexin where no such decrease in cellular protein occurred, some toxicity was occurring at these higher concentrations of H_2O_2.

Involvement of cis-Acting Elements in Addition to the ARE in Hemopexin-mediated MT-1 Gene Regulation—In contrast to the inhibition of MT-1 induction by NAC and GSH, two other thiol compounds considered to be ROI quenchers, diethyl dithiocarbamate (DDC) and pyrrolidine dithiocarbamate (PDC), significantly *increased* the extent of MT induction by heme-hemopexin[3] (Fig. 3*A*). Low concentrations (25 μM) of DDC and PDC synergistically augment, up to 20–30-fold, the induction of the reporter gene of −150MTβGeo caused by heme-hemopexin (Fig. 3*A*) but do not affect basal expression. A large increase in MT-1 mRNA levels is also seen using Northern analysis (data not shown). However, higher concentrations of DDC and PDC (up to 100 μM) significantly induce basal levels of expression of −150MTβGeo.

We originally investigated the effects of DDC and PDC[3] because they are structurally related compounds and DDC inhibits superoxide dismutase by chelating zinc. Their effects on MT mRNA levels were interpreted as being due to an increase in intracellular ROIs since the regulation by heme-hemopexin appeared to be independent of the element, MREd′ (see Fig. 1). However, since NAC and GSH are inhibitory while DDC and PDC are stimulatory, they cannot act by the same mechanism. While this work was in progress, a role for MREs in the response to oxidative stress of the chick MT gene was suggested (53, 55), and a model for MT gene regulation was proposed involving increased intracellular zinc and a constitutively expressed transcription factor, MTF-1. This factor binds to the MREs upon release from a rapidly turning over inhibitor protein, MTI (54), a system analogous to NFκB and IκB.

The PDC-induced expression of MREβGeo in baby hamster kidney cells required very low levels (0.5 μM) of extracellular Zn(II), and it was proposed that PDC transported Zn(II) into cells and caused dissociation of the MTI·MTF-1 complex (54). However, the lack of induction of MREβGeo by heme-hemopexin (Figs. 1 and 3, *Panel C*) demonstrates that MT-1 gene regulation in response to this heme transport system is not due to either a direct or indirect effect on the MTI·MTF-1 interaction causing dissociation of MTF-1 followed by binding to the MRE. It also seems unlikely from this result that Zn(II) uptake has been stimulated. Nonetheless, the regulation of MT-1 expression by hemopexin via the ARE does not exclude or comment on regulation by changes in intracellular Zn(II) pools (see below).

As summarized in Fig. 3, heme-hemopexin induces to a similar extent the expression of three fusion genes, −124(−67)MTβGeo, −153(−67)MTβGeo and −124(−43)MT-



C

Relative Expression (fold-change)

	H-HPX			PDC	H_2O_2	H7
		+PDC	+H7			
-153(-67)	1.93 +/ 0.35	3.84 +/- 1.3	1.0 +/- 0.13	2.1+/-0.7	N.D.	1.0 +/-0.05
-124(-67)	1.5 +/- 0.15	2.35+/-0.27	0.85+/-0.27	1.1+/-0.23	1.64+/-0.33	0.93+/-0.1
-124(-43)	1.9+/-0.45	2.6+/-1.0	0.9+/-0.4	1.0+/-0.14	N.D.	0.93+/-0.2
ARE_2	1.87+/-0.40	2.76+/-0.86	1.1+/-0.35	1.3+/-0.3	1.91+/-0.13	1.02+/-0.06
ARE_{M2}	1.0+/-0.2	N.D.	N.D.	N.D.	0.92+/-0.06	N.D.
MRE	1.1+/-0.2	N.D.	N.D.	2.0+/-0.06	0.9+/-0.4	N.D.

FIG. 3. **Identification of the *cis*-acting elements in the mouse MT-1 proximal promoter required for transcriptional activation by heme-hemopexin and evidence that this is the same element involved in the response to hydrogen peroxide.** *Panel A* summarizes the data from transient transfection studies using −150MTβGeo. Hepa cells were incubated in the presence and absence of heme-hemopexin (*H-HPX*) and increasing concentrations of either PDC (*left*) or DDC (*right*), as indicated, for 24 h. *Panel B* shows a schematic representation of the MT-1 fusion genes investigated containing MRE and putative ARE elements. Constructs which contain fragments of the proximal promoter are defined by their location in the promoter. *Panel C* shows the results of the transient transfection assays carried out as described in the legend to Table I. Twenty-four hours after transfection heme-hemopexin (*H-HPX*; 10 μM) in the presence or absence of PDC or H7 was added as indicated, and the β-galactosidase activity of the fusion gene in cell extracts was measured 24 h later and normalized to the CAT activity. In additional experiments the cells were incubated with hydrogen peroxide or, as controls, H7 or PDC alone. Each data point represents the mean ± S.D. of four independent transfections (ARE_MMTβGeo and ARE_{M2}-MTβGeo) or from 6 to 10 independent transfections (−750MTβGeo, −153(−67)MTβGeo, −124(−67)MTβGeo, 124(−43)MTβGeo, AREMTβGeo, ARE_2-MTβGeo). The fusion gene containing only one copy of the MT-ARE was not induced by heme-hemopexin in six independent transfection experiments. Heme-hemopexin and hydrogen peroxide increased the expression of −150MTβGeo 4.9 ± 0.9- and 2.2 ± 0.6-fold, respectively. The stimulatory and inhibitory effects, respectively, of PDC and H7 on the induction of −150MTβGeo by heme-hemopexin are presented elsewhere in the manuscript. A 3-fold induction of MREβGeo by hydrogen peroxide was observed with 500 μM reagent.

βGeo, all of which contain the MT-ARE, either with 5′- and 3′-flanking regions, with 5′-flanking MREs c and d or with 3′-flanking MREs a and b, respectively. Their responses were, however, only ~50% that of the fusion gene −150MTβGeo containing the complete region and none were induced to higher levels than ARE_2MTβGeo. CoPP-hemopexin also induces these fusion genes but slightly less effectively than heme-hemopexin (data not shown). The combined results of deletion analyses of this 110-bp region are consistent with a mechanism whereby the increased transcription of the MT-1 gene by heme-hemopexin requires the ARE. The presence of additional elements including MREs c and d does not restore the transcriptional activity of the intact 110-bp region.

Augmentation by PDC of MT-1 Gene Regulation by Heme-Hemopexin via Both MRE-dependent and MRE-independent Effects—We next tested the following model: that PDC mobilized zinc and enhanced binding of activating factors to the MRE which then interacted with the ARE to augment the effects of heme-hemopexin on MT expression. The effects of PDC alone or together with heme-hemopexin on expression of these fusion genes containing the MT-ARE in the presence or absence of flanking MREs are presented in Fig. 3, *Panel C*. PDC did not consistently increase the reporter gene activity of −124(−43)MTβGeo (which contains MREs a and b together with the ARE), but did increase the expression of −153(−67)MTβGeo (which contains the MREs c and d together with the ARE) and augmented the effect of heme-hemopexin. Furthermore, PDC stimulated the heme-hemopexin-mediated increase in expression of the fusion genes ARE_2- and −124(−67)MTβGeo (which contains the ARE). However, PDC itself was without effect on these fusion genes demonstrating an MRE-independent stimulation by PDC. The proposed release of MTF-1 from MTI by Zn(II) uptake via PDC in baby hamster kidney cells (which unlike Hepa cells do not synthesize MT) (54), provides a basis for the stimulation by PDC of the effects of hemopexin on −153(−67)MTβGeo. These data also imply an interaction between the MT-ARE and MREc and/or MREd. Since PDC and DDC increased,[4] but to a lesser extent, the effect of heme-hemopexin on the reporter gene activity of

[4] Y. Ren and A. Smith, unpublished observation.

−124(−67)MTβGeo (which lacks MREs), additional *cis*-acting elements or *trans*-acting proteins are affected by PDC in hemopexin-activated cells (which were incubated in 2% serum that would retard zinc uptake).

Effect of H7 on Activation of the MT-ARE by Heme-Hemopexin—The MT-ARE contains a core sequence to which members of the AP-1 family of transcription factors, Jun/Fos, bind and DNA-binding of these proteins is induced by PKC activated by phorbol esters. We show here that the PKC inhibitor, H7, inhibits activation by heme-hemopexin not only of −150MTβGeo but also of the −153(−67), −124(−67), −124(−43) and ARE $_2$MTβGeo fusion genes (Fig. 3, *Panel C*). This demonstrates that activation of the MT-ARE itself by heme-hemopexin is sensitive to H7.

Conclusion—The data presented indicate that an H7-sensitive process, likely PKC activation, acts in the transcriptional regulation of MT-1 by heme-hemopexin; that the increased MT-1 transcription occurs in response to signals produced by hemopexin receptor occupancy; that reactive oxygen intermediates including superoxide and hydrogen peroxide are part of the hemopexin receptor-mediated regulatory pathway to the nucleus; that H_2O_2 induces MT gene expression; that superoxide generated extracellularly also induces MT, albeit to a lesser extent than H_2O_2; and, finally, that the element at −98 to −89 bp which acts in MT-1 gene regulation by heme-hemopexin is also activated by H_2O_2, supporting its function as an ARE. Since activation of ARE$_2$MTβGeo by heme-hemopexin is inhibited by H7, the MT-ARE, which differs from the ARE core in the GSH *S*-transferase Ya gene by one nucleotide, may be a naturally occurring high affinity AP-1 binding site whose occupancy is enhanced by ROIs, as previously generated by mutation of the Ya ARE core (62). It would also appear from the deletion analysis of the 110-bp region that, while an interplay between regions including the ARE and MREc and/or MREd takes place, additional interactions are required for full expression from this region.

Heme is a reactive form of iron able to participate in oxygen radical reactions, but hemopexin in the plasma acts as an extracellular antioxidant by coordinating and inactivating the reactive heme-iron (12). ROIs could be generated by redox cycling of heme released from hemopexin, which would require both a change in heme coordination by hemopexin, possibly induced by receptor binding because hemopexin in solution is an antioxidant, and a source of electrons. CoPP is bound to hemopexin similarly to heme (17), and cobalt can undergo redox cycling, but less readily than iron or heme under physiological conditions. However, CoPP is not extensively taken up by cells when presented as a CoPP-hemopexin complex (17). Nevertheless, since NAC abolishes CoPP-hemopexin-mediated MT gene activation, occupation of the hemopexin receptor *per se* is implicated in the pathway that generates free radicals. Binding of hemopexin complexes to the hemopexin receptor, as does binding of diferric transferrin to its receptor, may activate the transmembrane NADH oxidase which catalyzes electron transfer from NADH to molecular oxygen (57, 58). This enzyme produces superoxide and participates in ferric iron reduction as an electron source. Hemopexin binds both ferri- and ferroprotoporphyrin (59), and several parallels exist between the hemopexin and transferrin systems. An as yet to be defined "ROI-inducing effect" of PKC is thought to be needed to stimulate NF-κB DNA binding which is induced by signals involving ROIs (56). PKC activation is associated with, and may be the direct mechanism for, activation of the NAD(P)H oxidoreductase of the respiratory burst in phagocyte and leukocytes (61). The NADH oxidase in hepatic plasma membranes has several features which distinguish it from other NADH oxidoreductase or the leukocyte NADPH oxidoreductase and mitochondrial NADH oxidase (58).

Our current working hypothesis is that ROIs, including superoxide and hydrogen peroxide, are generated upon receptor occupancy as a consequence of PKC and plasma membrane NADH oxidase activation. Furthermore, production of ROIs at a low level may be a metabolic signal which helps set in motion a series of events including HO-1 and MT-1 activation to prepare the cell for survival since the presence of extracellular heme-hemopexin indicates hemolysis and/or tissue trauma. The signaling pathway results in phosphorylation or oxidation of key sulfhydryl group(s) of specific proteins of the regulatory pathway for gene regulation including transcription factors and proteins with which they associate. Possible additional sources of ROIs include redox cycling of heme and possibly of heme-hemopexin, interactions between intracellular iron and ROIs, and thiyl radicals from oxidation of a sulfhydryl group on the hemopexin receptor subunit (18).

A role for heme itself in stimulating MT-1 gene transcription is also evident from the results presented here with free heme and heme analogs. However, since −750MTβGeo, but not −150MTβGeo, responds to free heme,[5] more distal elements in the region between −600 and −150 bp appear to be involved. Heme is rapidly catabolized after uptake, and there is evidence that iron can be bound directly to MT (60) but with an affinity that makes it unlikely that iron would displace zinc.

Thus, we propose that regulation of MT-1 expression by hemopexin takes place by receptor-mediated signals from the plasma membrane which affect gene regulation following activation of signaling pathways involving PKC and ROIs. The latter act in part through the ARE, perhaps as an early defense mechanism of the cell. If additional events also occur, such as redox-sensitive release of Zn(II) from MT or increased Zn(II) uptake as in the acute phase response (62), a rapid synergistic increase in transcription would take place, probably due to released MTF-1. The inhibitory effects of NAC and GSH on hemopexin-mediated induction of MT-1 expression may be due to their ability to bind Zn(II), but it seems more likely here that they act as ROI quenchers. Quenching may prevent oxidation of a critical thiol on a transcription factor, phosphatase or other protein or prevent GSSG formation from H_2O_2 and GSH. However, since heme-hemopexin does not activate MREβGeo, redox-mediated Zn(II) release from MT is not caused by hemopexin.

It seems likely, but is not yet proven, that member(s) of the AP-1 family of transcription factors recognize the AP-1-like element within the MT-ARE. However, as elegantly shown by Nguyen *et al.* (63), an AP-1 site resembles an ARE in responding to xenobiotics if the terminal 3′-GC is present. The MT-ARE contains an internal sequence similar to an AP-1 binding site in the SV40 promoter (64). The AP-1 family are leucine zipper proteins known to act synergistically with zinc finger proteins like MTF-1. The synergistic increases by PDC of hemopexin-mediated MT induction provide an example of a process whereby a variety of stimuli at the cell surface activate MT transcription in part via the MT-ARE and MREs.

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[5] Y. Ren and A. Smith, unpublished results.

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