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Cellular Protection Mechanisms against Extracellular Heme

HEME-HEMOPEXIN, BUT NOT FREE HEME, ACTIVATES THE N-TERMINAL c-Jun KINASE*

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Hemopexin protects cells lacking hemopexin receptors by tightly binding heme abrogating its deleterious effects and preventing nonspecific heme uptake, whereas cells with hemopexin receptors undergo a series of cellular events upon encountering heme-hemopexin. The biochemical responses to heme-hemopexin depend on its extracellular concentration and range from stimulation of cell growth at low levels to cell survival at otherwise toxic levels of heme. High (2–10 μM) but not low (0.01–1 μM) concentrations of heme-hemopexin increase, albeit transiently, the protein carbonyl content of mouse hepatoma (Hepa) cells. This is due to events associated with heme transport since cobalt-protoporphyrin IX-hemopexin, which binds to the receptor and activates signaling pathways without tetrapyrrole transport, does not increase carbonyl content. The N-terminal c-Jun kinase (JNK) is rapidly activated by 2–10 μM heme-hemopexin, yet the increased intracellular heme levels are neither toxic nor apoptotic. After 24 h exposure to 10 μM heme-hemopexin, Hepa cells become refractory to the growth stimulation seen with 0.1–0.75 μM heme-hemopexin but HO-1 remains responsive to induction by heme-hemopexin. Since free heme does not induce JNK, the signaling events, like phosphorylation of c-Jun via activation of JNK as well as the nuclear translocation of NF κ B, G₂/M arrest, and increased expression of p53 and of the cell cycle inhibitor p21^{WAF1/CIP1/SD1} generated by heme-hemopexin appear to be of paramount importance in cellular protection by heme-hemopexin.

Generation of reactive oxygen species as well as radical reactions occur upon tissue and red blood cell damage and release of heme, for example, hydroxyl radical production by hemoglobin and myoglobin from hydrogen peroxide (1). Heme is considered to be one causative agent in organ failure after ischemia reperfusion since heme oxygenase-1 (HO-1)¹ is induced in

heart and kidney (2). The forms of heme in the circulation which initially predominate after an injury are hemoglobin, heme-hemopexin, hemoglobin-haptoglobin, and heme-albumin (3). Haptoglobin does not recycle and is readily depleted, and heme rapidly transfers to hemopexin from heme-albumin (4) and from deoxyhemoglobin in the presence of hydrogen peroxide (5). Hemopexin, because of its avidity for heme ($K_d < 1 \text{ pM}$; Ref. 6), has a protective role in hemolysis, trauma, and ischemia reperfusion and is in the first line of defense against heme-mediated oxidative damage (7–9). Hemopexin binds heme in a low spin complex, thereby decreasing heme's peroxidative activity, and transports it via endocytosis into cells expressing hemopexin receptors (10, 11). Moreover, hemopexin is resistant to depletion since it recycles after heme delivery (12–14) and remains in circulation to protect against heme-stimulated peroxidation.

On the other hand, while hemopexin (about 10–20 μM in plasma) is protective extracellularly, the increase in intracellular heme and iron consequent to hemopexin-mediated transport might increase the intracellular oxidation state and possibly lead to biochemical responses to prevent oxidative damage to cell constituents. However, the induction of HO-1 by heme-hemopexin (15) as well as by hypoxia ischemia (16), cytokines, and hydrogen peroxide (17) is considered to be protective for cells, since the heme catabolites biliverdin and bilirubin are anti-oxidants *in vitro* (18). In addition, iron sequestration by ferritin protects cells against the toxic effects of iron released by heme oxygenase, and hemopexin induces ferritin (9). Furthermore, the gene expression of metallothionein-1 (MT-1) is also induced by heme-hemopexin (15); in part by activation of signaling pathways (19, 20). We proposed (20) that MT induction helps the cells to maintain homeostasis as intracellular heme and iron levels increase since the sulfhydryl-rich MT protects against radicals (21) and also sequesters zinc, which might otherwise occupy iron sulfur protein active sites as well as copper, which is redox active. MT functions normally in copper (22) and zinc homeostasis (23), but recent studies with MT-null mice support the role in oxidative stress conditions (24). Heme-hemopexin targets heme to a discrete set of tissues and cells via selective expression of the hemopexin receptor and protects receptor null cells, including endothelial cells lining blood vessels, by preventing diffusion of free heme. Receptors for heme-hemopexin are expressed by liver parenchymal cells (25), lymphocytes (26), and cells of barrier tissues, *e.g.* retinal pigment epithelia (9) and probably also neurons of the peripheral (27) and central nervous systems (28). Heme-hemopexin has also recently been shown to act as a growth factor by providing a source of nutrient iron and activating PKC in human T-lymphocytes (26). As with other growth factors, the growth response curve is bell-shaped, raising the possibility that high levels of transported heme are deleterious to the cells or even toxic, although heme-hemopexin induces

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¹ The abbreviations used are: HO-1, heme oxygenase-1; heme, iron-protoporphyrin IX; NF κ B, nuclear factor κ B; CoPP, cobalt-protoporphyrin IX; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MT-1, metallothionein-1; PKC, protein kinase C; GST, glutathione S-transferase; LSD-MEM, Dulbecco's modified Eagle's medium containing 0.5% FBS; PARP, poly(ADP-ribose)polymerase; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; FBS, fetal bovine serum.

HO-1 and ferritin (9, 15), and the heme-iron is rapidly incorporated into ferritin (29). If the heme handling system is overloaded, heme can participate in intracellular oxygen radical reactions that lead to the degradation of proteins, lipids, carbohydrates, and DNA (30).

To address the cellular and biochemical consequences of increasing intracellular heme via the specific hemopexin receptor, we investigated the effects of nanomolar to micromolar levels of heme-hemopexin on the cellular oxidation state as well as on cell growth and activation of signaling cascades known to respond to growth factors and stress that ultimately activate mitogen-activated protein kinase (MAPK) family members. Here, the focus is on the N-terminal c-Jun kinase (JNK), also known as stress-activated protein kinase (SAPK), one member of the MAPK family activated by exposure of cells to UV radiation, heat shock, or inflammatory cytokines. Other members include extracellular signal regulated kinase, which responds to binding of growth factors to receptor tyrosine kinases via Ras, and p38 kinase (reactivating kinase or MAP2), which is activated in response to inflammatory cytokines, endotoxins, and osmotic stress (reviewed in Ref. 31). These kinases play key roles in determining cellular responses to many extracellular stimuli which activate small G-proteins such as Rho, Rac, and Ras which in turn stimulate MAPK kinase cascades and lead to the phosphorylation of transcription factors to produce a unique, appropriate transcriptional activity for a particular external stress. We report here additional evidence for the pleiotropic protective effects of heme-hemopexin, particularly in increasing cell survival under heme stress and generating high levels of a DNA-binding form of two transcription factors, c-Jun and NF κ B.

MATERIALS AND METHODS

Reagents—Metalloporphyrin (Porphyrin Products, Logan, UT) concentrations were determined spectrophotometrically in dimethyl sulfoxide using published procedures (19). Camptothecin and hydroxyurea were obtained from Sigma; doxorubicin from Calbiochem (San Diego, CA); and dinitrophenyl hydrazine and guanidine from Aldrich (Milwaukee, WI). Commercial antibodies to bcl-2 were obtained from Oncogene Research Products and Santa Cruz Biotechnology Inc., Santa Cruz, CA.

Hemopexin Isolation and Preparation of Heme-Hemopexin Complexes—Intact rabbit hemopexin was purified and stoichiometric 1:1 heme-hemopexin complexes (>90–95% saturated) characterized and quantitated as described previously (19, 32) keeping the Me₂SO concentration less than 5% (v/v), and using extinction coefficients (A_m⁻¹·cm⁻¹) of 1.1 × 10⁵ at 280 nm for apo-hemopexin; 1.2 × 10⁵ at 280 nm and 1.3 × 10⁵ at 405 nm for rabbit mesoheme-hemopexin. Cobalt-protoporphyrin IX-hemopexin complexes were similarly made using published extinction coefficients (19) and all hemopexin complexes were dialyzed against PBS at 4 °C before use.

Cultured Cells—Mouse hepatoma cells (Hepa) were cultured in Dulbecco's minimal essential medium (DMEM) containing 5% FBS as described (33). For growth studies, cells seeded at an initial density of 2000 cells per well in 96-well plates were synchronized by incubation in DMEM containing 0.5% serum (LSDMEM) for 16 h before addition of increasing concentrations of sterilized (0.2- μ m nylon) heme-hemopexin. Cell number was assessed using the Promega Cell Titer proliferation assay as described previously for MOLT-3 cells (26). Initial experiments using direct cell counting confirmed the accuracy of this method under the experimental conditions investigated. Although liver cells are differentiated and not actively dividing, mouse Hepa cells were used because they have well characterized responses to heme-hemopexin (15, 25) and are models for replicating cells with liver-specific functions like interleukin-6 responsiveness² and plasma protein synthesis (34).

Cell Cycle Control—Hepa cells were synchronized by incubation in low serum medium for 16 h after seeding (2 × 10⁶ cells per T75 flask) in normal culture medium and attachment during 5 h incubation. Then the medium was changed to LSDMEM containing heme-hemopexin or the DNA intercalator, doxorubicin (0.2 μ g/ml), or the ribonucleotide reductase inhibitor, hydroxyurea (10 mM), which were used to induce

G₂/M and G₁/G₀ arrest, respectively. After 3, 8, 24, and 48 h cells were fixed and the DNA stained with propidium iodide (50 μ g/ml) containing RNase (60 μ g/ml). The amount of DNA was measured using Immuno-count II with an Orthocytrofluorescence flow cytometer (Orthodiagnosics, Raritan, NJ) and the data analyzed and correlated with changes in the cell cycle using Modfit (Verity House Software, Topsham, ME).

Detection of the Protein Carbonyl Content of Cells—After the usual subculture and synchronization, cells (6 × 10⁶ cells per T-75) were incubated for up to 2 h in the presence or absence of 2–10 μ M heme-hemopexin, 2–10 μ M CoPP-hemopexin, 0.5–10 μ M free heme, or 400 μ M hydrogen peroxide (stock concentration was determined spectrophotometrically using a millimolar extinction coefficient of 43.6 M⁻¹ cm⁻¹ at 240 nm). The cellular carbonyl content of oxidatively modified proteins in cell extracts was determined using a published protocol (35) and contaminating nucleic acids were removed from the cell extracts (1 mg of protein) by precipitation upon addition of streptomycin sulfate (1% w/v). Proteins were recovered by precipitation and the protein carbonyl content of the final washed pellet, dissolved in 6 M guanidine-HCl, pH 2.3 (600 μ l), was calculated from the maximum absorbance (360–390 nm) using a molar absorbance coefficient of 22 M⁻¹ cm⁻¹. To minimize variation, the amount of protein recovered in the final pellet after solubilization in 6 M guanidine was quantitated using the bicinchoninic acid assay (Pierce, Rockford, IL) and the carbonyl content of cell samples expressed per mg of protein.

Detection of NF κ B in Nuclear Extracts—Forty eight hours after seeding (4 × 10⁶ cells/T150 flask) Hepa cells were rinsed and incubated for 1 h in serum-free HEPES-buffered DMEM, pH 7.4, supplemented with heme-hemopexin (50 nM to 10 μ M). Nuclear extracts were then prepared from these cells essentially as described previously for HeLa cells (36). Electrophoretic mobility shift assays were carried out using a 4% (80:1 acrylamide:bis-acrylamide) polyacrylamide gel after incubation of nuclear extracts (3 μ g) for 20 min at room temperature with 0.035 pmol of an radiolabeled oligonucleotide probe encoding a consensus sequence for the transcription factor NF κ B (Promega, WI). The oligonucleotides were radiolabeled using T₄ polynucleotide kinase (Promega, Madison, WI) and [γ -³²P]ATP (NEN Life Science Products Inc., specific activity 10 μ Ci/ μ l). The identity of the specific NF κ B-DNA complex was confirmed by separate competition studies with 50 M excess of the unlabeled NF κ B consensus oligonucleotide (AGTTGAGGGGACTTCCCAGGC from Promega, Madison, WI) as well as by supershift assays using a rabbit polyclonal anti-p65 NF κ B antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Determination of JNK/SAPK Activity—Synchronized Hepa cells (seeded at 1.3 × 10⁶ cells per T-75 flask) were incubated in LSDMEM for up to 6 h in the presence of 1–10 μ M heme-hemopexin, 2–10 μ M CoPP-hemopexin, or 0.4–10 μ M free heme and cell extracts prepared using Tris-Triton X-100 buffer, pH 7.4, containing 1 mM sodium vanadate (New England BioLabs, Beverly, MA) as phosphatase inhibitor. Using c-Jun-glutathione S-transferase bound to Sepharose (2 μ g) as substrate, the amount of activated JNK/SAPK in cell extracts (1 mg of protein) was estimated using an *in vitro* kinase assay (New England BioLabs, Beverly, MA) followed by quantitation of phospho-c-Jun-glutathione S-transferase produced by Western analysis using a polyclonal anti-phospho-c-Jun (Ser-63) antibody followed by detection using ECL (Amersham) and quantitation using the NIH Image program.

Detection of Wild-type p53 in Hepa Cells by Immunoprecipitation—Due to the low abundance of p53, Hepa cells were first treated with doxorubicin (0.2 μ g/ml) for 48 h to induce p53 expression. Cell lysates were prepared in PBS, pH 7.4, containing 1% (v/v) Triton X-100, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS with 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA, 5 μ g/ml leupeptin, and 1.0 μ g/ml pepstatin (PBS-TDS buffer) and after preclearing by incubation with 1 μ g of mouse IgG and "protein G-plus-agarose," p53 was recovered by incubating the lysates with 1 μ g of antibodies to wild type and mutant p53 (Ab-4 and Ab-3, respectively; Oncogene Research Products, Cambridge, MA). The antibody-bound p53 was recovered by addition of protein G-plus-agarose and detected by Western analysis after SDS-PAGE using as primary antibody Pantropic Ab-7 to p53 (diluted 1:2500) followed by detection using biotinylated rabbit anti-sheep IgG and streptavidin horseradish peroxidase with the ECL system and quantitated as described above.

Detection of Poly(ADP-ribose)polymerase (PARP) Expression Using Western Immunoblotting—After the usual subculture and synchronization, Hepa cells (seeded at 1.3 × 10⁶ per T75) were then incubated in LSDMEM in the presence or absence of 10 μ M heme-hemopexin for 21 h. Cell extracts were prepared from washed cells by solubilization in Laemmli buffer and sonication, and PARP detected after SDS-PAGE electrophoresis of cell extracts (equivalent to 1 × 10⁵ cells) and transfer

² A. Smith, unpublished observations.

to polyvinylidene difluoride membranes (Bio-Rad) using monoclonal antibodies (diluted 1:10,000) which recognize both the intact protein and the 86-kDa proteolytic fragment (C2-10 from Dr. Guy Poirier, Research Center of the Central Hospital of Laval University, Quebec, Canada) were detected with donkey anti-mouse IgG horseradish peroxidase and carried out as usual with the ECL system. Cleaved PARP present in extracts of ectoposide (VP16)-treated HL-60 cells (also from Dr. Guy Poirier) was used as a marker.

Detection of p21^{WAF1/CIP1/SD11} Using Western Immunoblotting—After the usual subculture and synchronization, Hepa cells (seeded at 1.3×10^6 per T75) were then incubated in LSDMEM in the presence or absence of heme-hemopexin or doxorubicin at various times and concentrations given in the figure legends. Cell extracts were prepared from washed cells by solubilization in PBS-TDS buffer and p21^{WAF1/CIP1/SD11} detected after SDS-PAGE electrophoresis of cell extracts (25 μ g of protein) and transfer to polyvinylidene difluoride membranes (Bio-Rad). Anti-murine p21 antibodies (Ab-5 from Oncogene Research Products) were detected using donkey anti-rabbit IgG horseradish peroxidase and the ECL system.

RESULTS

Heme-Hemopexin Increases the Protein Carbonyl Content of Cells—To establish whether events associated with receptor binding and hemopexin-mediated heme transport increase the intracellular oxidation state, the production of protein carbonyls was measured after incubation of Hepa cells with heme-hemopexin. Concentrations in the range 2–10 μ M represent a “heme load” at sites of localized trauma injury since circulating plasma hemopexin levels are approximately 20 μ M. Within 15 min, 10 μ M heme-hemopexin doubled the cell carbonyl content to about 7 nmol/mg of protein, equivalent to the level produced in response to 400 μ M H₂O₂ (Fig. 1A). This effect of heme-hemopexin is dose-dependent from 2 to 10 μ M (Fig. 1B) and transient since carbonyl levels declined after 30 min. As expected, free heme also rapidly increased carbonyl content (Fig. 1C).

Heme-Hemopexin Stimulates Nuclear Translocation and DNA Binding of Nuclear Factor κ B (NF κ B)—The nuclear concentration of NF κ B is induced by extracellular hydrogen peroxide, cell damage, and growth arrest (37). Within 1 h, heme-hemopexin (2–10 μ M) induces NF κ B DNA binding approximately 5–7-fold as shown by electrophoretic mobility shift assays (Fig. 1D). Identification of NF κ B was confirmed by the supershift of the radiolabeled DNA-protein complexes in the presence of an antibody to the p65 subunit of NF κ B (Fig. 1D, lane 6) and by specific competition with a non-radiolabeled oligonucleotide encoding an NF κ B consensus binding site (Fig. 1D, lane 8). Low levels of heme-hemopexin (*i.e.* 0.01–1 μ M), which stimulate cell growth in MOLT-3 cells (26) and in Hepa cells (see below), do not detectably increase the DNA binding of NF κ B.

Heme-Hemopexin Induces the MAPK Family Member JNK/SAPK—As a third parameter for the increased oxidation state we investigated whether heme-hemopexin activated JNK/SAPK, known to respond to a variety of stresses including DNA damage, heat shock, or tumor necrosis factor β . Exposure of cells to 2–10 μ M heme-hemopexin caused extensive activation of JNK/SAPK within 30 min, which continues to increase for 1 h (Fig. 2). The extent of JNK/SAPK activation is proportional to the extracellular concentration of heme-hemopexin (Fig. 2B). JNK/SAPK activity exhibits a biphasic response: declining by 3 h but increasing again by 6 h. Phospho-c-Jun levels are maintained for at least 6 h, with a small decline at 3 h (Fig. 2A) and there is no apparent change in the amount of total c-Jun, as determined by Western analysis, over this time (Fig. 2A, lower panel).

Heme-Hemopexin Supports Cell Growth at Low but Not High Concentrations—Hepa cells express relatively abundant, high affinity receptors for hemopexin (25) and proliferate normally using 0.05 to 1 μ M heme-hemopexin as the sole iron source (Fig. 3A), as previously reported for human lymphocytes (MOLT-3

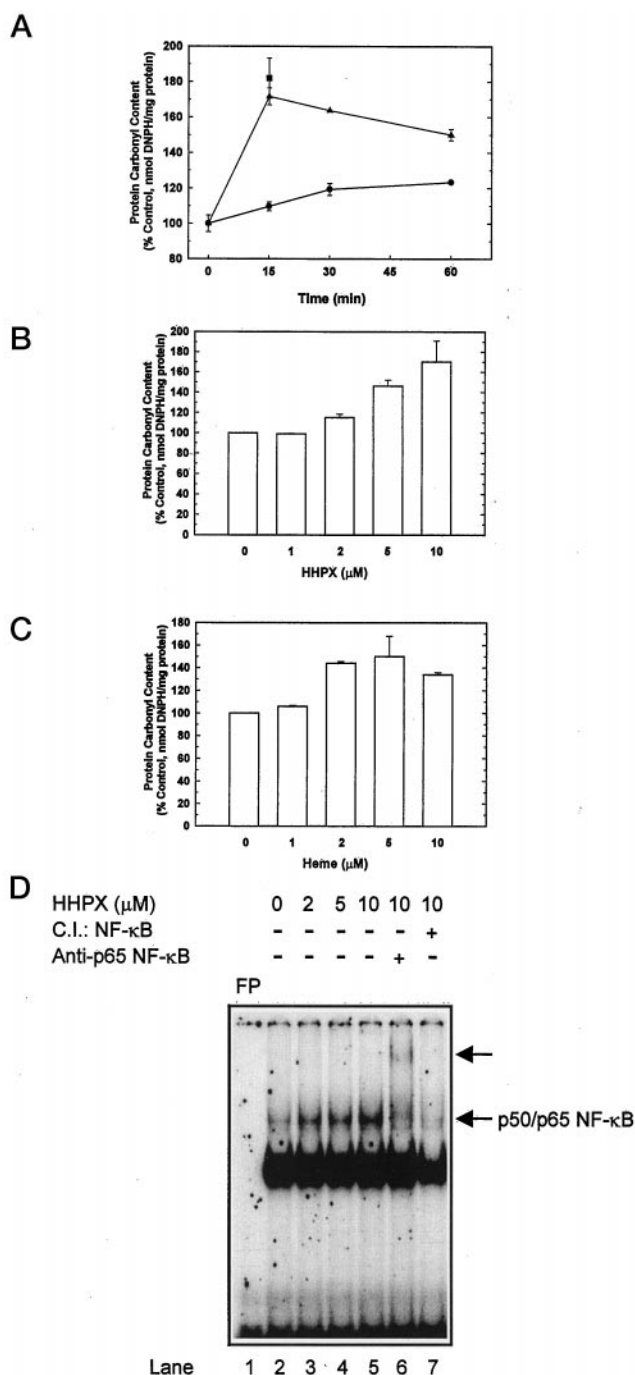


FIG. 1. Increased oxidation by high concentrations of heme-hemopexin (HHPX): a rapid increase in protein carbonyl levels and nuclear translocation of NF- κ B. The data in panel A show the level of protein carbonyls in Hepa cells incubated in DMEM containing 0.5% serum for up to 1 h as indicated with: PBS (●), 10 μ M heme-hemopexin (▲), or 400 μ M hydrogen peroxide (■). Panels B and C show the data from Hepa cells incubated for 15 min with increasing concentrations of heme-hemopexin and free heme, respectively. The data in panel D show the results from an electrophoretic mobility shift assay using nuclear extracts (3 μ g of protein), from Hepa cells incubated for 1 h with 0, 2, 5, or 10 μ M heme-hemopexin (lanes 2–5), with a radiolabeled oligonucleotide probe containing the consensus binding site for NF κ B (lane 1, free probe). In lane 6, addition of antibody to the p65 subunit retarded migration of the DNA-protein complex (top arrow), and there was a decrease in the amount of radioactive complex in the presence of a 50-fold excess of unlabeled NF κ B oligonucleotide (lane 7).

cells) and polymorphonuclear lymphocytes (26). However, in the presence of higher concentrations of heme-hemopexin (2–10 μ M), cell growth was arrested. Stimulation of cell growth

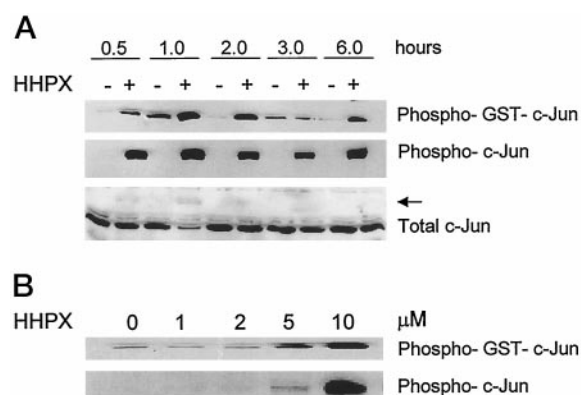


FIG. 2. Activation of JNK/SAPK by heme-hemepexin (HHPX). Hepa cells were incubated for up to 6 h in LSDMEM in the presence or absence of 0–10 μM heme-hemepexin. The activity of JNK/SAPK is determined in whole cell extracts as described under “Experimental Procedures” using the phosphorylation of c-Jun as substrate, the amount of which was then quantitated by Western immunoblotting as shown. *Panel A* shows the time course in response to 10 μM heme-hemepexin of, from top to bottom, JNK/SAPK activation, levels of phospho-c-Jun, and the levels of total c-Jun (the arrow indicates the phosphorylated form due to a small degree of cross-reactivity of the anti-c-Jun antibody with phosphorylated c-Jun). The dose-response of c-Jun levels after a 1-h incubation with 1–10 μM heme-hemepexin is shown in *panel B*.

was equivalent for cells of either low (passage 39; data not shown) or high (passage 99; Fig. 3A) passage number, and there was no loss of viability as judged by trypan blue staining in cells exposed to 10 μM heme-hemepexin (data not shown). Heme-hemepexin also overcame the growth inhibition by the permeable iron chelator, desferrioxamine (Fig. 3B), as previously reported for MOLT-3 cells (26), further substantiating that the iron released by heme catabolism was used for cell growth.

High Concentrations of Heme-Hemepexin Induce Cell Arrest Not Apoptosis—When, after an initial 24-h period, the culture medium containing 10 μM heme-hemepexin is removed and replaced with fresh medium lacking heme-hemepexin but containing FBS, cell growth resumes and 24 h later cell number is increased in proportion to the FBS concentration (Fig. 4A). After 24 h incubation in the presence of 10 μM extracellular levels of heme-hemepexin, the cells are refractory to a previously stimulatory concentration of heme hemepexin (0.1 μM , see Fig. 4B; 0.5 and 0.75 μM (data not shown)) but are neither dead nor undergoing apoptosis. This was confirmed by examining whether cleavage of PARP by activated caspases was occurring (38). There was no detectable PARP cleavage after 21 (Fig. 4C) or 48 h (data not shown) exposure of the cells to 10 μM heme-hemepexin. As a positive control, the DNA topoisomerase I inhibitor, camptothecin, was shown to induce apoptosis in Hepa cells, producing typical morphological changes within 3 h (data not shown). The expected reduction in volume of cytoplasm and release of vesicles without cell lysis as well as cleavage of PARP were apparent at 21 h. Thus, cell arrest, not apoptosis nor necrosis, occurs at heme-hemepexin concentrations greater than 1 μM .

Although cells treated with 10 μM heme-hemepexin are arrested for a time, they do retain hemepexin receptors and remain responsive to other biological consequences of heme-hemepexin. In particular, HO-1 protein is induced within 3 h by 10 μM heme-hemepexin, and increases for at least the next 4 h before declining to basal levels at 20 h (data not shown). A second exposure to heme-hemepexin 20 h after the first induces HO-1 again and to a similar extent over time. Taken together, these observations show that cell cycle regulation has taken

place with no obvious loss of hemepexin receptors at the cell surface or ability to induce HO-1 and catabolize heme.

Hepa Cells Express Wild-type p53—Since the tumor suppressor gene product, p53, increases in response to DNA damage (39) and since many transformed cell lines express mutant forms of p53, we investigated whether Hepa cells express wild type p53, and whether changes in p53 expression occur in response to high extracellular levels of heme-hemepexin. Immunoprecipitation studies reveal that Hepa cells express wild type p53, which is induced by the DNA intercalator, doxorubicin (data not shown). When cells are grown for 48 h in medium containing low levels of serum, p53 expression is also induced by high levels of heme-hemepexin, but a growth stimulating concentration (0.5 μM) of heme-hemepexin consistently causes a 50–60% decrease in immunodetectable p53 (data not shown).

High Concentrations of Heme-Hemepexin Cause Partial Cell Arrest and Increase the Expression of p21^{WAF1/CIP1/SDI1} but Not Protein Kinase B/Akt1—Heme-hemepexin increases the expression of the cell cycle inhibitor p21^{WAF1/CIP1/SDI1}, as assessed with two different protocols, and prevents apoptosis due to withdrawal of serum factors (Fig. 5). Somewhat unexpectedly, using a protocol for induction of apoptosis by staurosporine in a p53-null lung cancer cell line (40), two immunopositive p21^{WAF1/CIP1/SDI1} species are induced by heme-hemepexin (Fig. 5A). The faster migrating form of p21^{WAF1/CIP1/SDI1} was ascribed to be the PKC-dependent cleaved form reported to be associated with G₂/M arrest (40). Cells treated for 48 h with 0.2 $\mu\text{g/ml}$ doxorubicin, which causes DNA damage and G₂/M arrest before apoptosis (41), express only the faster migrating immunoreactive species of p21 as do the low serum control cells.

Using a second protocol (Fig. 5B), p21^{WAF1/CIP1/SDI1} levels are twice as high as the control levels in cells incubated with heme-hemepexin for 6 h (Fig. 5B) and remain elevated for 48 h. As in the previous protocol, at 48 h the cell morphology indicated that the doxorubicin-treated cells were arrested. In contrast to the cells in LSDMEM which cease to grow, become small, round, and birefringent and die (Fig. 5C, *i*), the morphology of cells incubated with heme-hemepexin is that expected for arrested cells, *i.e.* they are enlarged compared with normal (Fig. 5C, *ii*) like cells exposed to doxorubicin (Fig. 5C, *iii*). Although p21^{WAF1/CIP1/SDI1} is initially increased in the control cells by the reduced serum concentration during the overnight incubation for synchronization, cells do not survive if heme-hemepexin is not added, demonstrating that heme-hemepexin acts as a survival factor. Furthermore, 10 μM heme-hemepexin increases the level of p21^{WAF1/CIP1/SDI1} in cells growing in as little as 0.5% serum to the level normally found in cells growing in growth medium containing 2% serum (Fig. 5D).

The flow cytometry data summarized in Table I demonstrate that after synchronization a significant proportion of the Hepa cells growing in medium supplemented with 10 μM heme-hemepexin remain in the G₂/M phase at 8 and 24 h compared with the low serum control cells. In control experiments, doxorubicin and nocodazole cause G₂/M arrest, and the ribonucleotide reductase inhibitor, hydroxyurea, causes G₁/G₀ arrest; and the effect of these chemicals is apparent within 8 h and is complete at 24 h. Thus, in response to heme-hemepexin cells do not progress through G₂/M normally as do cells growing in DMEM containing 5% FBS. At 48 h the percentage of cells in S phase incubated with high concentrations of heme-hemepexin is twice that of cells in LSDMEM, indicating that the cells are progressing through the cycle and those in LSDMEM become arrested in G₀/G₁. Cells growing in LSDMEM supplemented with 0.5 μM heme-hemepexin also have more cells in S-phase than those in LSDMEM alone indicating continued growth. Since the Hepa cells are not clonally derived and are not com-

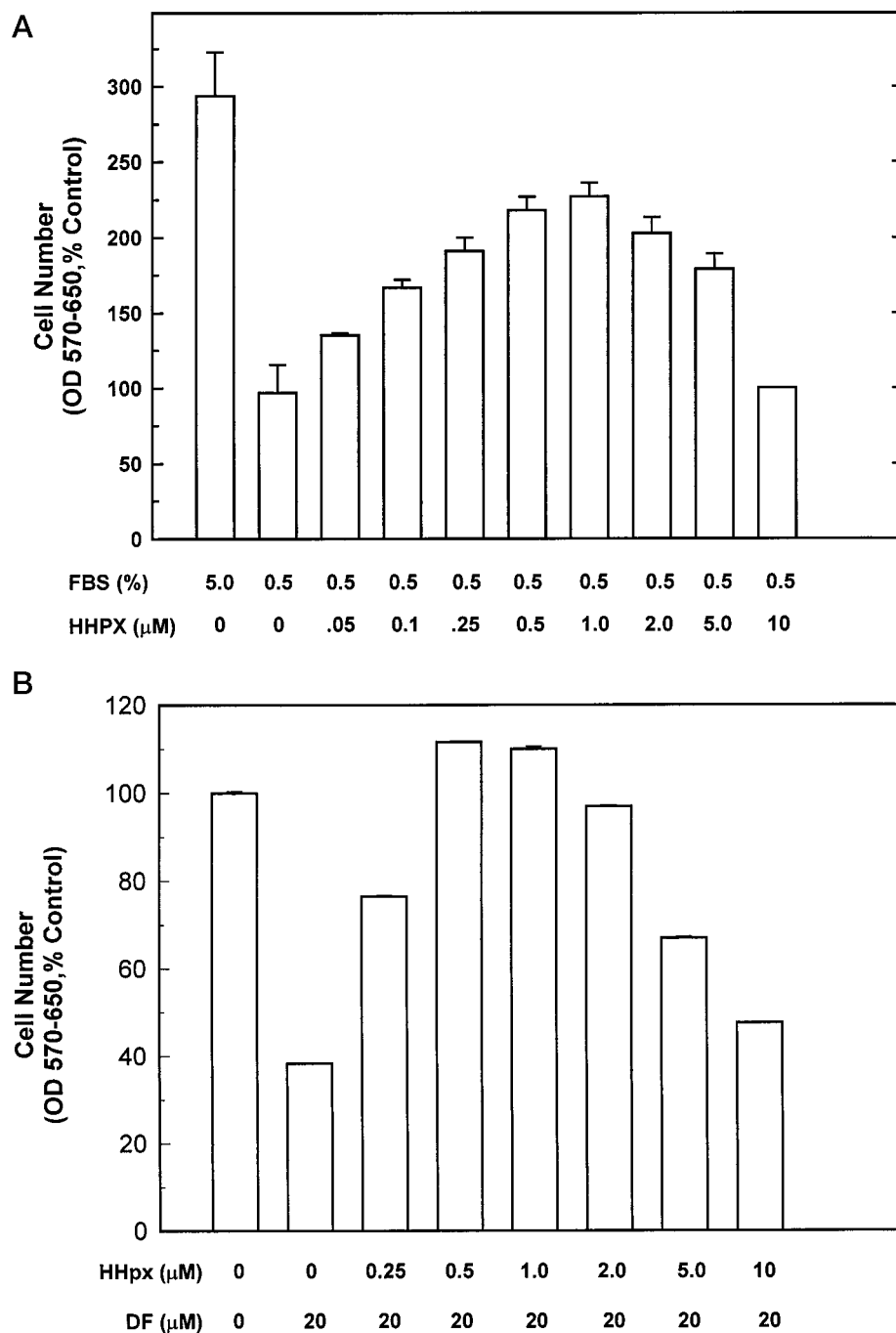


FIG. 3. Dose-response curve for the effects of heme-hemepexin (HHPX) on Hepa cell proliferation. Mouse Hepa cells (2×10^3 cells/well) were grown in microtiter plates in DMEM containing 4.5 g/ml glucose and either 5% FBS or 0.5% FBS with or without supplementation with heme-hemepexin (0.05–10 μM) as indicated. The number of viable cells was determined 48 h later using the Promega proliferation assay, as described under “Experimental Procedures.” The data in *Panel A* were obtained using cells at high (p99) passage, but essentially identical results were obtained with cells at low (p39) passage number (data not shown) routinely used. *Panel B* shows the effect of increasing amounts of heme-hemepexin on cells treated with 20 μM desferrioxamine. The means and standard deviation of triplicate samples from one representative experiment repeated at least twice are shown. The mean value for cells grown in DMEM containing 0.5% FBS was defined as 100% (O.D. 570 minus 650 nm) and was used to normalize all other values.

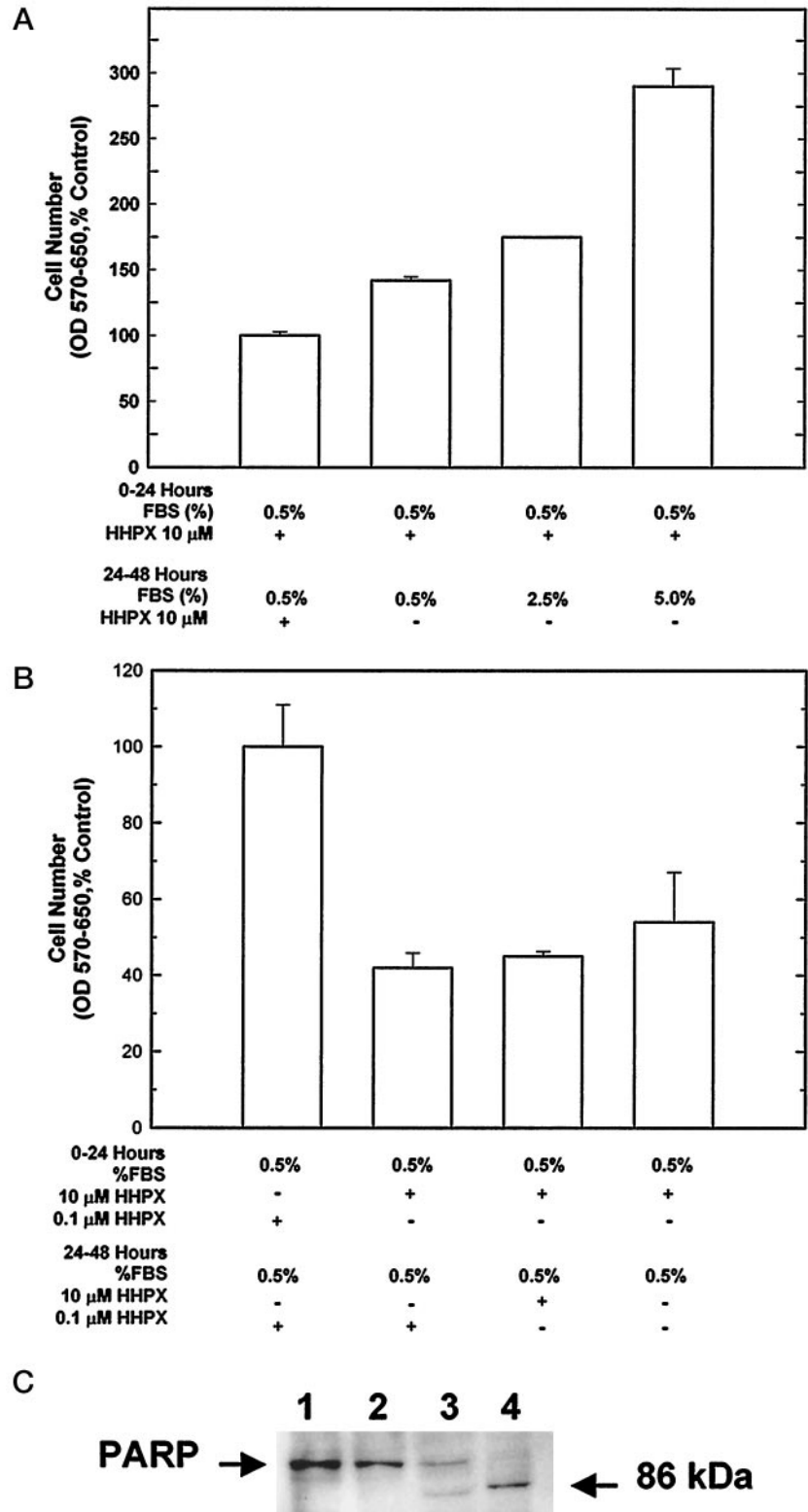
pletely synchronized by the overnight incubation in LSDEM, the data have been interpreted conservatively but are clear.

In addition to p21^{WAF1/CIP1/SDI1}, other proteins, for example, bcl-2 and the protein kinase B/Akt-1, have been identified whose expression leads to cell survival rather than apoptosis (42). Hepa cells do not express levels of bcl-2 detectable by immunoblotting, but protein kinase B/Akt1 is activated within 1 h by insulin and epidermal growth factor; however, low or high concentrations of heme-hemepexin are without effect

(data not shown). Doxorubicin induces bcl_{XL}, a partner of Bad, whereas hemepexin causes a small decrease in bcl_{XL} levels (data not shown).

Rapid Induction of p21^{WAF1/CIP1/SDI1} Requires High Concentrations of Heme-Hemepexin—Since p21^{WAF1/CIP1/SDI1} acts in cellular responses to oxidative stress and binding of p21^{WAF1/CIP1/SDI1} to JNK/SAPK inhibits the kinase and prevents apoptosis (43), we investigated the kinetics of heme-hemepexin effects on p21^{WAF1/CIP1/SDI1} levels. The data in Fig.

FIG. 4. Effects of high concentrations of heme-hemopexin (HHPX) on cell viability. The data show the growth of Hepa cells incubated for 24 h in DMEM supplemented with 0.5% FBS containing 10 μ M heme-hemopexin followed by a second 24-h period of culture in increasing concentrations of serum as indicated (*Panel A*) or in low serum medium containing either 0.75 or 10 μ M heme-hemopexin (*Panel B*). In *panel C*, PARP cleavage was used as an indicator of apoptosis. After growth for 16 h, cells were incubated for 21 h in medium containing PBS or 10 μ M heme-hemopexin (*lanes 1 and 2*, respectively) or the topoisomerase I inhibitor, camptothecin (10 μ M; *lane 3*). *Lane 4* contains the 86-kDa proteolytic fragment of cleaved PARP present in an extract of HL-60 cells (equivalent to 1×10^5 cells) after incubation with ectoposide.



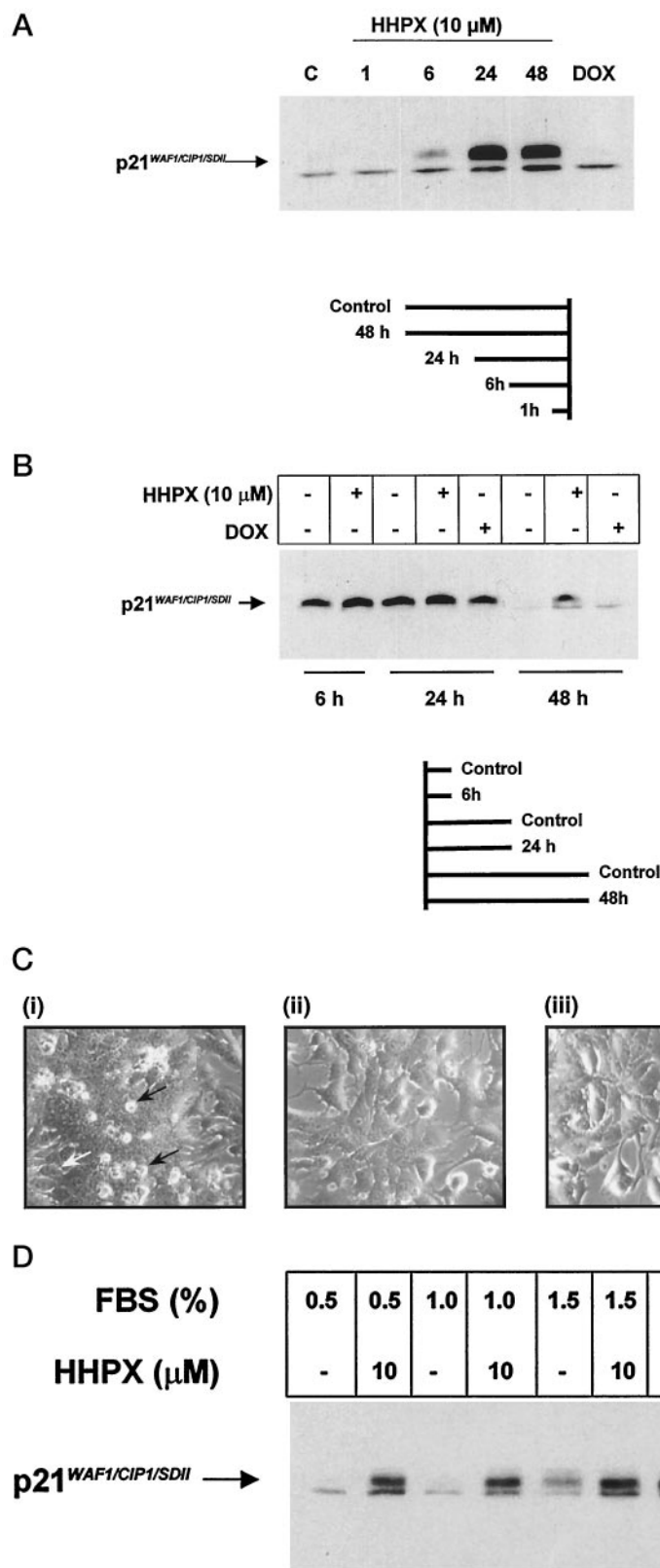
6 establish that nuclear levels of p21^{WAF1/CIP1/SDI1} are induced within 1 h when cells are incubated with 2–10 μ M heme-hemopexin, independently of any detectable changes in p53 (data not shown), but concomitantly with the rapid induction of nuclear translocation of NF κ B shown in Fig. 1. This induction of p21^{WAF1/CIP1/SDI1} takes place whether cells are incubated in LSDMEM (Fig. 6A) or in HEPES-buffered, serum-free DMEM (Fig. 6B).

Induction of p21^{WAF1/CIP1/SDI1} by heme-hemopexin is rapid

and sustained; but in contrast, JNK/SAPK activation is apparent within 15–30 min, reaches a maximum at 1 h, declines after 3 h, and increases again at 6 h, in parallel with the cellular levels of phospho-c-Jun (Fig. 2). As discussed below, the high levels of p21^{WAF1/CIP1/SDI1} and phospho-c-Jun could explain the observed absence of apoptosis despite JNK/SAPK activation.

Effect of Receptor Occupancy on JNK/SAPK Activity and on Cellular Carbonyl Production—We also investigated the role of receptor occupancy independent of tetrapyrrole transport on

FIG. 5. Effects of heme-hemepexin (HHPX) on the expression of p21^{WAF1/CIP1/SDI1}. Two protocols were employed as illustrated. The data in *panel A* show the increase in p21^{WAF1/CIP1/SDI1} when cells growing in DMEM with 0.5% FBS were incubated with 10 μ M heme-hemepexin at different times after synchronization. In *panel B*, cells were treated at time 0 and harvested subsequently at 6, 24, and 48 h. Doxorubicin (0.2 μ g/ml) was used as a positive control. In *panel C*, the data show by light microscopy the typical morphology of cells after incubation for 48 h in LSDMEM (i), 10 μ M heme-hemepexin (ii), or doxorubicin (iii). *Panel D* shows the levels of p21^{WAF1/CIP1/SDI1} detected by Western analysis in cells growing for 48 h in the presence or absence of 10 μ M heme-hemepexin in DMEM containing various amounts of FBS (0.5–2.0%) compared with cells growing exponentially in normal culture medium (DMEM supplemented with 2% FBS).



JNK/SAPK activation and cell protein carbonyl content using CoPP-hemepexin. The results reveal that JNK/SAPK is activated by this non-transportable heme-analog-hemepexin complex (Fig. 7A). However, unlike heme-hemepexin, CoPP-hemepexin does not increase Hepa cell carbonyl content (Fig. 7D). The stability of the CoPP-hemepexin complex and lack of CoPP uptake is reinforced by the ability of free CoPP to triple the

carbonyl content within 15 min³ and to extensively induce HO-1 (19). In contrast, although free heme rapidly diffuses across the plasma membrane into cells, JNK/SAPK is not activated by non-protein bound heme (Fig. 7B) despite the in-

³ A. Smith and J. D. Eskew, unpublished observations.

TABLE I
Flow cytometry analysis of the effect of heme-hemepexin on Hepa cell growth

Hepa cells, after seeding (2×10^6 cells/T75 flask) and attachment in normal culture medium for 5 h later, were synchronized by incubation in LSDMEM for 16 h and then heme-hemepexin or the DNA intercalator and nucleic acid synthesis inhibitor, doxorubicin ($0.2 \mu\text{g/ml}$), the microtubule inhibitor, nocodazole ($0.1 \mu\text{g/ml}$), or the ribonucleotide reductase inhibitor, hydroxyurea (10 mM) were added to the medium. At the times indicated afterwards, the cells were fixed and the DNA stained, and the data obtained was analyzed and correlated with changes in the cell cycle as described under "Materials and Methods."

Time in culture	Cycle	FBS (0.5%)	FBS (5.0%)	Heme-hemepexin ($10 \mu\text{M}$)	Heme-hemepexin ($0.5 \mu\text{M}$)	Doxorubicin	Nocodazole	Hydroxyurea
0	G ₀ -G ₁	46.94	39.54	ND ^a	ND	ND	ND	ND
	S	36.05	33.84					
	G ₂ -M	17.01	26.62					
3	G ₀ -G ₁	45.33	48.44	48.98	33.31	41.86	19.72	59.95
	S	41.38	38.66	40.71	42.35	45.76	37.79	30.83
	G ₂ -M	13.29	12.89	10.31	24.34	12.38	42.49	9.23
8	G ₀ -G ₁	37.89	36.64	33.55	39.71	23.56	0.00	73.69
	S	47.44	52.05	44.87	45.28	47.26	24.94	19.15
	G ₂ -M	14.67	11.31	21.58	15.01	29.13	75.06	7.16
24	G ₀ -G ₁	35.24	33.63	22.95	38.76	4.21	0	66.94
	S	51.14	49.50	44.21	42.46	6.75	0	17.40
	G ₂ -M	13.61	16.88	32.84	18.78	89.00	100.00	15.66
48	G ₀ -G ₁	56.11	43.69	35.99	50.49	3.88	4.61 ^b	55.96 ^c
	S	9.52	27.56	41.15	15.92	4.54	18.24 ^b	39.82 ^c
	G ₂ -M	34.37	28.75	22.88	33.59	70.08	77.16 ^b	4.22 ^c

^a ND, not determined.

^b Some cell debris was apparent in this sample.

^c Some toxicity was apparent in this sample.

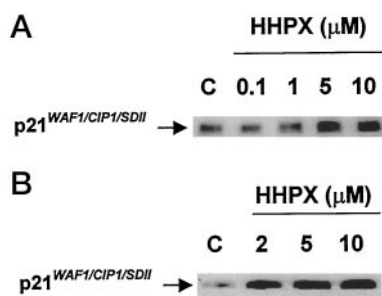


FIG. 6. Rapid effects of heme-hemepexin (HHPX) on nuclear levels of p21^{WAF1/CIP1/SDI1}. Western immunoblotting was used to measure the p21^{WAF1/CIP1/SDI1} levels in aliquots of whole cell extracts ($25 \mu\text{g}$ of protein, panel A) or nuclear extracts ($5 \mu\text{g}$ of protein, panel B) from Hepa cells incubated for 1 h with increasing concentrations of heme-hemepexin in LSDMEM or in HEPES-buffered serum-free DMEM, respectively.

creased oxidative state which heme produces and consequent increased protein carbonyl content (see Fig. 1B).

DISCUSSION

Heme-mediated oxidative stress may play a role in neurodegenerative diseases, including Alzheimer's (44), as well as contribute to aging and is considered to be a contributing factor in the pathology of ischemia reperfusion injury of kidney, heart, and brain principally due to the associated induction of heme oxygenase (2). In stroke, reperfusion injury is considered to be due to the toxic effects of heme (45) and to cascades from calcium channels and glutamate receptors (46, 47). Hemepexin has both extra- and intra-cellular protective roles and the present research begins to define the responses of cells to high concentrations ($2\text{--}10 \mu\text{M}$) of heme-hemepexin as a model for a physiological, cellular heme "load" and to determine at the molecular level some of the initial hemepexin receptor-activated and heme-related events in hemolysis, trauma, and ischemia reperfusion.

This is the first report to link cellular heme transport processes with metal-catalyzed oxidation, which is also a mixed

function oxidation since it requires molecular oxygen and reducing equivalents. Heme transport causes oxidative modification of proteins as shown by the increase in protein carbonyl content of ferritin replete Hepa cells within 15 min of exposure to heme-hemepexin. This assay is the definitive method for assessing metal-catalyzed oxidation (35), and reaction with oxygen or H_2O_2 generates an active oxygen species which oxidizes amino acids at or near the metal. Hemepexin receptor occupancy *per se*, examined using CoPP-hemepexin which binds to the receptor without tetrapyrrole transport, does not increase cellular protein carbonyl content as does free heme which is amphipathic and is nonspecifically taken up by all cells. Thus, carbonyl production is caused by the cellular uptake of heme, events associated with endocytosis of heme-hemepexin and heme catabolism. When heme is transported into cells via the hemepexin receptor the oxidation state is transiently increased, and recovery, as indicated by decreased carbonyl content, starts within 30 min presumably due to proteolysis of the oxidized proteins which are known to be unstable. The actual carbonyl levels induced in the Hepa cells by heme-hemepexin are essentially identical to those induced by $400 \mu\text{M}$ H_2O_2 or generated during ischemia reperfusion injury of brain (48).

Key transcription factors activated by stresses and producing the cellular responses to inflammation include the NF κ B Rel family and NF κ B itself which is released from the cytosolic "signalsome" complex upon phosphorylation of a component protein, I κ B (49). After incubation of Hepa cells with heme-hemepexin, NF κ B is translocated to the nucleus within 60 min. Despite increasing cellular protein carbonyl content, free heme is not as extensive an inducer of the DNA binding of NF κ B as is heme-hemepexin.⁴ Several kinases including JNK/SAPK, PKC, and an I κ B-2 kinase activated by tumor necrosis factor- α have been implicated in the phosphorylation of I κ B (see Ref. 49). Both JNK/SAPK as shown here and PKC (26) are activated

⁴ R. M. Vanacore, J. D. Eskew, P. J. Morales, L. Sung, and A. Smith, submitted for publication.

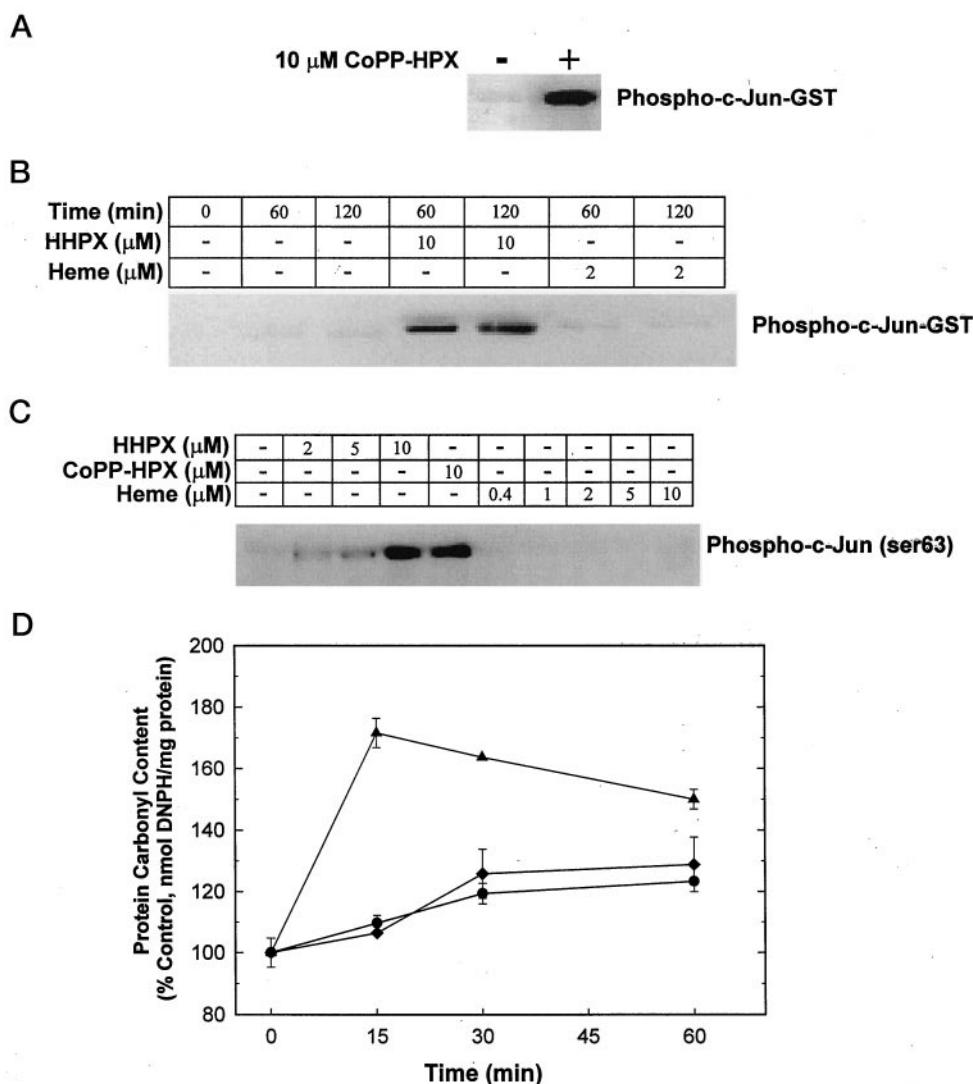


FIG. 7. Effects of CoPP-hemopexin (CoPP-HPX) on JNK/SAPK activation and cellular protein carbonyl content. The data in *panel A* show the increase in phosphorylation of *c-Jun* in response to CoPP-hemopexin (10 μM) by 3 h by Western immunoblotting as described under "Experimental Procedures." *Panel B* shows the results from the JNK/SAPK activation assay after cells were incubated with PBS, 10 μM heme-hemopexin, or 2 μM free heme (a level which generates the same intracellular concentration of heme as 10 μM heme-hemopexin, Ref. 3) for 1 or 2 h. *Panel C* shows the cellular phospho-*c-Jun* levels at 1 h after cells were incubated with PBS, heme-hemopexin, or CoPP-hemopexin or free heme at the concentrations indicated. *Panel D* shows the changes in protein carbonyl content in Hepa cells incubated for up to 60 min in PBS (●) in the presence or absence of either 10 μM heme-hemopexin (▲) or CoPP-hemopexin (◆).

by hemopexin. Clinically, NF κ B is considered to play a significant role in ischemia reperfusion injury (50) especially related to the inflammatory response (51). The promoters of many genes activated by oxidative stress contain NF κ B-binding sites including human HO-1 (52), but whether NF κ B plays a role in HO-1 induction by physiological stimuli is not yet established.

c-Jun is the major substrate of JNK/SAPK which is activated *in vivo* during reperfusion of ischemic kidney (53). Raising intracellular levels of heme via heme-hemopexin could constitute a cellular stress and, as shown here, JNK/SAPK is rapidly and extensively activated by heme-hemopexin as well as by CoPP-hemopexin implicating the hemopexin receptor itself in this process. In contrast, free heme which increases the cellular oxidation state as does heme-hemopexin does not activate JNK/SAPK, nor does 20 μM free heme in chick embryo liver cells (54). Despite sustained elevated levels of phospho-*c-Jun*, Hepa cells exposed to 2–10 μM heme-hemopexin do not undergo apoptosis: there is no abnormal morphology, no activation of caspases nor increase in bcl_{XL}. Thus, the research presented here links for the first time the stimulus of high levels of

extracellular heme, *viz.* heme-hemopexin complexes, with sequential phosphorylation events leading to the activation of the JNK/SAPK signal transduction pathway.

Even more important is the finding potentially linking activation of a MAPK cascade by hemopexin, a receptor-mediated heme transport system, with the concomitant increase of HO-1 and MT-1 gene transcription. A role for *c-Jun*, the major substrate for JNK/SAPK, in the regulation of both HO-1 and MT-1 genes, induced by phorbol esters (20), remains to be defined. However, AP-1 sites have been identified in the promoters of these genes and implicated in both HO-1 (52, 55) and MT-1 (56) gene expression but the mode of regulation by *c-Jun* is not established and may not be a simple activation of transcription. An involvement of MAPK cascades in HO-1 and MT-1 gene transcription readily explains how so many disparate surface as well as intracellular stimuli, *e.g.* hydrogen peroxide, cytokines, metals like cadmium, chemicals like arsenite and diethylmaleate which decrease intracellular GSH, might converge or in parallel result in increased transcription of the HO-1 and MT-1 genes.

During recovery from ischemia, in addition to NF κ B (57) and immediate early genes (e.g. *c-jun*), other key regulatory proteins which can affect cell cycle distribution, rate of DNA repair and DNA replication, including p21^{WAF1/CIP1/SD11} and p53, are induced. p21^{WAF1/CIP1/SD11} inhibits all cyclin-dependent kinases and overall acts as a survival factor (58). Hepa cells express wild type p53 and hemopexin causes a rapid increase in nuclear levels of p21^{WAF1/CIP1/SD11} within 1 h, i.e. during the period of carbonyl production, in the absence of any detectable changes in p53 expression. p21^{WAF1/CIP1/SD11} is also induced independently of p53 when GSH is depleted by diethylmaleate (59). Over several days, in low levels of serum, the presence of heme-hemopexin in the medium allows the long-term survival of Hepa cells and this is associated with G₂/M arrest with induction of both p53 and p21^{WAF1/CIP1/SD11}. Furthermore, high concentrations of heme-hemopexin increased the expression of p53 whereas growth stimulatory levels consistently cause a 50% decrease. Overall, these effects of hemopexin on p21^{WAF1/CIP1/SD11} are consistent with previous observations supporting a role for p21^{WAF1/CIP1/SD11} activated independently of p53 by mitogens at entry into G₁/S but in addition to promote a transient pausing late in G₂/M (60), as discussed below. p21^{WAF1/CIP1/SD11} is a binding partner for JNK/SAPK inhibiting it and thus induction of p21^{WAF1/CIP1/SD11} after DNA damage may prevent apoptosis (43). The protective effect of hemopexin is therefore proposed to be due in part to the sustained increase in p21^{WAF1/CIP1/SD11} and p53 despite the rapid and extensive JNK/SAPK activation by heme-hemopexin. In addition, heme-hemopexin as shown here also rapidly induces NF κ B and interestingly, recent studies have shown that suppression of tumor necrosis factor- α -mediated apoptosis results from activation by NF κ B of several genes involved in cell cycle control via inhibition of caspase-8 (61).

The cellular responses to extracellular heme-hemopexin and the mechanism whereby gene regulation is linked to heme and iron metabolism and to cell cycle control is a physiologically relevant biological process that requires more definition. The time course of induction of HO-1 shown here and of ferritin⁵ is consistent with observations that overexpression of these proteins causes cell arrest (62, 63). In comparison with doxorubicin- and nocodazole-treated cells, the percentage of cells in G₂/M stage of the cycle shows that the arrest mediated by heme-hemopexin is dynamic indicative of pausing. Thus, while the maximum induction of HO-1 and ferritin occurs at about 7 h of exposure to heme-hemopexin, by 20 h HO-1 has already declined to basal levels although ferritin remains slightly elevated. Overall, the data support that the cells recover after a period of cell arrest and that processes in addition to HO-1 and ferritin induction are occurring.

The role of HO-1 and ferritin on the intracellular protection of cells against oxidative stress from heme and heme-iron derived from catabolism is supported by several lines of evidence (63–65). It is of interest to compare the effects of heme-hemopexin with those of free heme since elevated intracellular heme levels are not inconsequential to cells. In contrast to heme-hemopexin, heme does not activate JNK/SAPK nor, as shown elsewhere,⁴ extensively induce NF κ B despite increasing the cellular protein carbonyl content to levels similar to heme-hemopexin. Thus, the physiological effects of free metalloporphyrins differ from those of protein complexes likely to occur in the circulation reinforcing the need for studies using physiologically relevant heme-protein complexes.

There are four principal means whereby heme binding by

hemopexin protects cells from heme. First, coordination of the heme-iron via two histidine ligands prevents heme from acting as an extracellular oxidant; second, expression of hemopexin receptors targets heme to cells which can respond appropriately and as a corollary cells lacking hemopexin receptors are protected from exposure to heme; third, cells which express hemopexin receptors receive heme at a controlled rate and in such a manner that signals from the cell surface generated by receptor occupancy set in motion events including activation of JNK/SAPK generating phospho-c-Jun, the nuclear translocation of NF κ B for gene transcription,⁴ as well as sustained levels of both p21^{WAF1/CIP1/SD11} and p53; and fourth, hemopexin receptor-mediated uptake occurs in a way that allows the cells to respond to the intracellular heme levels in a manner where even in arrested cells HO-1 induction still takes place, which together with ferritin also induced by heme-hemopexin provide a form of intracellular protection as shown for retinal pigment epithelial cells (9). Hemopexin receptors are not ubiquitously expressed on all tissues and clearly the intracellular protection afforded by HO-1 and ferritin induction by free heme could be crucial for survival of certain cells such as endothelial cells (64, 65). Clinically, cells lacking hemopexin receptors are at risk for heme toxicity when haptoglobin and hemopexin become depleted during chronic and acute hemolysis. Intravascular hemolysis from normal “wear and tear” on red blood cells accounts for at least 10% of red cell breakdown but is pathological in the hemolytic anemias, thalassemias, in some patients with intracardiac prosthetic devices or heart valve disease, in certain viral and bacterial infections, and in crush and ischemia reperfusion injury. Although albumin can bind heme tightly (K_d 10 nM), the heme-binding site can also be occupied by hydrophobic metabolites and pharmaceuticals, which displace heme or prevent access. Binding of heme to albumin and to histidine-proline-rich glycoprotein as seen in patients (66), does not prevent heme uptake, and dissociated heme will diffuse into all cells causing oxidative stress without JNK/SAPK activation. Once heme-albumin complexes are detected in the circulation the prognosis is extremely poor for patients with life-threatening hemorrhagic shock (67), indicating that additional therapeutic measures utilizing proteins are worthy of investigation.

Thus, the hemopexin heme transport system defends mammalian cells against more than one deleterious agent or condition and as such can be considered an important member of “pleiotropic defense systems.” Taken together these observations suggest that increasing both extracellular and intracellular heme by heme-hemopexin above a certain threshold is sensed via the hemopexin receptor by cells which then undergo a series of events initiated by rapid JNK/SAPK activation with phosphorylation of c-Jun and nuclear translocation of NF κ B together with the induction and maintenance of p21^{WAF1/CIP1/SD11} and p53 to protect themselves. Consequently, dividing cells with functional p53 survive after a period of transient cell arrest. Thus, the hemopexin system provides cells with a means, including activation of separate kinases of which PKC and MAPK family member JNK/SAPK have been identified, whereby they can combat increases in cellular heme, iron, and oxidation state with the concomitant regulation of HO-1 and MT-1 gene expression among several others.

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⁵ M. Shibata-Womack, P. J. Morales, L. Sung, N. Shipulina, and A. Smith, submitted for publication.

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