Heme: a determinant of life and death in renal tubular epithelial cells

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Gonzalez-Michaca, Luis, Gianrico Farrugia, Anthony J. Croatt, Jawed Alam, and Karl A. Nath. Heme: a determinant of life and death in renal tubular epithelial cells. Am J Physiol Renal Physiol 286: F370–F377, 2004; 10.1152/ajprenal.00300.2003.—Heme oxygenase-1 (HO-1) and p21 influence cell fate, and genetic HO-1 overexpression upregulates p21 and confers resistance to apoptosis. The present study examined the effects of heme, a metabolite inactivated in renal injury, on sensitivity to apoptosis and cell growth in conjunction with cellular expression of HO-1 and p21. Immortalized rat proximal tubular epithelial cells (IRPTCs) were exposed to heme (10 μM) in serum-deplete media (0.1% FBS) and in standard cell culture media (5.0% FBS). In the presence of 0.1% FBS media, heme induced p21 through an HO-dependent, p53-independent mechanism; certain products of HO activity (iron and carbon monoxide), but not others (ferritin, apoferritin, bilirubin), recapitulated these inductive effects on p21 expression. Along with this inductive effect on HO-1 and p21, heme worsened apoptosis, the latter exacerbated by the inhibition of HO activity and loss of p21 expression. In IRPTCs maintained in 5% FBS, heme induced HO-dependent p21 expression, provoked cell cycle arrest, and inhibited cell growth without inducing apoptosis; this inhibitory effect of heme on cell growth was blocked by the concomitant inhibition of HO activity and loss of p21 expression. We conclude that heme is a potent HO-dependent inducer of p21 and that heme increases the sensitivity to apoptosis in serum-deplete conditions and decreases cell growth in serum-replete conditions; inhibiting HO activity and concomitantly ablating p21 expression exacerbate apoptosis and reverse the growth-inhibitory actions of heme. We suggest that these effects of heme may influence the nature of, and recovery from, ischemic and nephrotic insults to the kidney.

Heme-mediated toxicity is particularly relevant to the kidney (2, 15, 19, 42). This organ may be injured by increased amounts of heme from heme proteins present within the kidney, as occurs after ischemic (24) and nephrotoxic (2, 15, 19, 42) insults; the kidney can also be damaged by large amounts of heme derived from heme proteins that originate elsewhere (such as myoglobin and hemoglobin) and are avidly taken up by the kidney, as occurs in such conditions as rhabdomyolysis and hemolysis (26, 27, 29, 42–44). The toxicity of heme is facilitated by its lipophilicity, which allows heme to permeate the plasma and organellar membranes, and by the intracellular canalicul system, which readily channels heme throughout the cell (13, 25). The damaging effects of heme arise, at least in part, from its prooxidant actions and its oxidative denaturation of lipid, DNA, and cytoskeletal and other proteins (13, 25).

Despite the recognition that heme accumulates in the injured kidney and may be damaging, the influence of heme on the basic processes that govern cell fate in the injured kidney, apoptosis on the one hand and proliferative cell growth on the other, remains unknown. Our present study addresses the effects of heme, in pathophysiologically relevant concentrations, on the sensitivity to apoptosis and growth responses in renal tubular epithelial cells. As an accepted model of apoptosis, cells were exposed to serum-deplete (0.1% FBS) cell culture conditions, whereas to examine growth responses, we studied the growth of these cells in serum-replete (5% FBS) media.

The catabolism and cellular effect of heme are determined by the prevailing levels of heme oxygenase (HO: 1, 2, 8, 15, 19, 20, 32), the heme-degrading enzyme. HO catalyzes the conversion of heme to biliverdin, a reaction yielding iron and carbon monoxide; biliverdin is subsequently converted to bilirubin, whereas iron is subsequently stored in ferritin, the latter synthesized in increased amounts as HO activity is increased; these products, i.e., iron, ferritin, bilirubin, and carbon monoxide, are considered the proximate effectors of many of the main cellular actions of HO. Three isozymes possess heme-degradative capacity: HO-1 and HO-2 are the inducible and constitutive isoforms, respectively, whereas HO-3 is an isoform with trivial heme-degrading capacity. HO-1 and HO-2 are the inducible and constitutive isoforms, respectively, whereas HO-3 is an isoform with trivial heme-degrading capacity. Considerable interest is focused on HO-1 because the induction of HO-1 confers protection against tissue injury caused by diverse stressors including hypoxia, hyperoxia, ischemia, inflammation, toxic insults, and oxidative stress. The protective effects of HO-1 against tissue injury arise from such actions as vasorelaxation, anti-inflammatory properties, and cytoprotective and antiapoptotic effects (1, 2, 8, 15, 19, 20, 32).

We have recently demonstrated that HO-1-overexpressing renal epithelial cells exhibit a markedly altered phenotype (16):...
these cells proliferate at a slower rate compared with wild-type cells and exhibit resistance to apoptosis. In exploring potential mechanisms, we directed attention to the cyclin-dependent kinase inhibitor p21 (p21Cip1/WAF1/SDI1); p21 not only inhibits cell proliferation by inducing cell cycle arrest, but as recently recognized, p21 is also antiapoptotic in certain settings (11, 40). In these HO-1-overexpressing cells, expression of p21 was increased and appeared linked to resistance to apoptosis (16); analogous findings have been obtained in vascular smooth muscle cells (9).

The present study examined whether heme, a pathophysiologically relevant inducer of HO-1, as distinct from a genetic approach of upregulating HO-1, induces p21 and the mechanisms accounting for such upregulation of p21; additionally, the phenotypic changes in cells experiencing heme-driven upregulation of p21 were explored, focusing, in particular, on the sensitivity to apoptosis and the effect on cell growth.

METHODS

Cell culture studies. All reagents employed were obtained from Sigma (St. Louis, MO) unless otherwise stated. Immortalized rat proximal tubule cells (IRPTCs; 93-p-2–1, developed and characterized as previously described, 38) were cultured at 37°C in 95% air and 5% carbon dioxide in DMEM (Invitrogen, Grand Island, NY) containing low glucose (1 g/l); the medium was supplemented with 5% FBS, 20 mM HEPES, 0.1 mM nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin (18, 38). Some protocols were performed using cell culture medium that had the same components as the full culture medium except that it was supplemented with 0.1% FBS instead of 5% FBS. In some studies, IRPTCs were exposed to heme (10 μM, iron protoporphyrin IX) or zinc protoporphyrin (ZnP; 10 μM, Porphyrin Products, Logan, UT); concentrated stocks of these solutions were freshly made in 0.05 N sodium hydroxide and used to achieve the final concentration by appropriate dilution with incubation media. In addition, we exposed these cells to bilirubin (0.1, 1, 5, and 10 μM); iron as ferrous ammonium sulfate (10 M); hydroxyquinoline (10 M); iron as ferrous ammonium sulfate (10 M); and the carbon monoxide-releasing compound trimercarboxylichlororhelenium II at a concentration of 100 μM.

Determination of HO-1, p21, p53, and caspase-3 protein expression in IRPTCs. Western blot analyses were performed as described in detail in our prior publications (16, 27). Briefly, protein was extracted from these cultures using the TRIzol method (Invitrogen, Carlsbad, CA), and samples containing 100 μg of protein were separated on 15% Tris-HCl gels and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). For analysis of expression of HO-1 protein, a rabbit anti-rat polyclonal HO-1 antibody was employed (SPA8955, StressGen Biotechnologies, Victoria, BC) followed by a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (SAB 300, StressGen Biotechnologies) for a secondary antibody. Rat HO-1 (rat HSP 32, SPP 730, StressGen Biotechnologies) was used as a positive control. Expression of p21 protein was analyzed using a mouse anti-p21 monoclonal antibody (65961A, Pharmingen, San Diego, CA) as the primary antibody and a polyclonal goat anti-mouse IgG antibody (SAB 300, StressGen Biotechnologies) for a secondary antibody. Protein from a cellular extract of MCF-7 cells was used as a p21 positive control. In addition, we used a purified mouse p53 antibody (14461A, Pharmingen) and a rabbit polyclonal active caspase-3 antibody (55115, Pharmingen); a rabbit polyclonal β-actin antibody (A-2066, Sigma) was used to assess equivalency of loading for Western blot analyses. Detection was achieved by using the chemiluminescence method (Amersham Pharmacia Biotech, Piscataway, NJ).

Antisense oligonucleotides and transient transfection. In addition to inhibiting HO activity with ZnP, we used HO-1 antisense oligonucleotides to decrease the expression of HO-1 protein (6, 39). We synthesized antisense phosphorothioates oligonucleotides complementary to the HO-1 sequence (5'-CCC TTC TGA AAG TTC-3') in the rat mRNA between 81 and 95 base pairs; as a control, we used the HO-1 sense sequence (5'-GAA CTT TCA GAA GGG-3'). IRPTCs were plated into six-well plates at a density of 1 × 10⁵ cells/well and grown in 5% FBS medium without antibiotics to 70–80% confluence, at which time they were transfected with either antisense or sense oligonucleotides using the Lipofectamine 2000 transfection reagent (Invitrogen) in 5% FBS media without antibiotics. After incubation for 16 h at 37°C, hemin or vehicle was added; 4 h later, the cells were harvested, protein was extracted, and expression of HO-1 and p21 was determined by Western blot analysis.

Terminal transferase-mediated dUTP nick end-labeling method. Apoptosis was assessed by the terminal transferase-mediated dUTP nick end-labeling (TUNEL) method using the Apoptag Plus Peroxidase In Situ Apotosis Detection Kit (Intergen, Purchase, NY) as previously described (16). IRPTCs were seeded into six-well plates and grown to confluence in standard cell culture medium containing 5% FBS. The monolayers were then incubated in the 0.1% FBS-containing medium for 24 h and exposed to hemin (10 μM) or vehicle in the presence or absence of ZnP (10 μM). IRPTCs and monolayers of cells that had detached from the monolayers were collected by gentle centrifugation of the incubation medium for each well and combined with cells subsequently harvested by trypsinization from the bottom of the wells. The resulting cell pellets were washed once with PBS and fixed by resuspension in 0.5 ml methanol-free formaldehyde (1% in PBS, Polysciences, Warrington, PA) for 15 min at room temperature. Fifty microliters of these cell suspensions were then droiited onto glass microscope slides that had previously been coated with a 0.1% poly-l-lysine solution (Polysciences). Apoptosis was assessed using the Apoptag Plus Peroxidase In Situ Apoptosis Detection Kit, which detects DNA fragmentation associated with apoptosis by labeling of 3'-OH DNA termini with digoxigenin-nucleotides, a process facilitated by terminal deoxynucleotidyl transferase (TdT); these labeled fragments were then allowed to bind to an anti-digoxigenin antibody conjugated with peroxidase, and 3,3’-diaminobenzidine was used as the substrate for the peroxidase. A sham incubation (without TdT) was performed for each cell preparation as a negative control.

DNA laddering assay. After incubation with hemin (10 μM) or vehicle for 24 h, cells were harvested and DNA laddering was assessed (22). The cell pellets were suspended in 50 μl TE (10 mM Tris, pH 8.0, 10 mM EDTA) to which 0.9 μl of lysis buffer (0.5% Triton X-100 in TE buffer) was added and incubated on ice. The lysates were then centrifuged at 12,000 g for 15 min at 4°C, and RNase A was added to the supernatants, which were incubated at 37°C for 1 h. An additional 2-h incubation at 50°C was performed after the addition of proteinase K and SDS (final concentrations of 100 μg/ml and 0.5%, respectively). The samples were extracted with phenol/chloroform, and DNA was precipitated with sodium acetate/ethanol and overnight incubation at −20°C. The pelleted DNA was washed with 70% ethanol, dried, and dissolved in TE buffer. Aliquots of the samples were separated by electrophoresis on a 2% agarose gel containing ethidium bromide to visualize laddering.

Assessment of proliferative cell growth. To determine the effect of hemin on proliferative cell growth, IRPTCs were plated in six-well culture dishes at an initial density of 5 × 10⁵ cells/well (16). One day later, hemin, in the absence or presence of ZnP, was added. Each day for 2 successive days, and after trypsinization of the cell monolayer, cell counts were performed using a Coulter Counter. Counting of the cells retrieved from the incubation medium was also performed each day for 2 successive days. Fluorescence-activated cell sorter analysis. Cell cycle analysis was performed by fluorescence-activated cell sorter analysis (FACS) as previously described (16). Approximately 5 × 10⁴ IRPTCs were
plated into six-well culture dishes, and after 24 h hemin (10 μM) or vehicle was added. After an additional 24 h, cells were collected from the cell culture media and combined with cells harvested from the corresponding wells by trypsinization and centrifugation at 200 g for 5 min. Cells were washed in cold PBS and resuspended in 0.5 ml of PBS, to which 1.5 ml of 70% ethanol was added dropwise over 1 min. After incubation at 4°C for at least 16 h and washing, cells were incubated with 1,000 U of RNase A (Roche, Basel, Switzerland) and propidium iodide (20 mg/ml) in the dark for 30 min at room temperature. Flow cytometry was performed using a Becton Dickinson FACScan (San Jose, CA) with an excitation wavelength of 488 nm and emission wavelength of 585 nm; ModFit software (Verity Software House, Topsham, ME) was employed for the analysis of data.

Statistics. Data are expressed as means ± SE and considered statistically significant for *P* < 0.05. For multiple comparisons, analyses of variance and the Student-Newman-Keuls test were employed.

RESULTS

Effects of hemin and other reagents in serum-deplete media (0.1% FBS). Hemin induced p21 in IRPTCs, with such expression occurring at 2 h and persisting thereafter; this upregulation of p21 was accompanied by upregulation of HO-1 (Fig. 1A). In contrast to these inductive effects of hemin on p21 and HO-1, hemin suppressed p53 in a dose-dependent fashion (Fig. 1B).

To determine whether HO activity contributed to such upregulation of p21, we examined the effect of ZnPP, the competitive inhibitor of HO activity, on hemin-induced upregulation of p21. As shown in Fig. 2A, whereas ZnPP per se exerted no effect on the expression of p21 and HO-1 protein in IRPTCs, ZnPP prevented the upregulation of p21 by hemin. Thus inhibition of HO activity by ZnPP blocked hemin-induced upregulation of p21.

Because HO activity reflects activity arising from constitutive HO-2 as well as induced HO-1, we sought to determine the extent to which induction of HO-1 contributed to hemin-induced expression of p21 using an antisense approach to inhibit HO-1. As shown in Fig. 2B, HO-1 antisense oligonucleotides significantly reduced expression of HO-1 (66% by standardized densitometry), an effect not evinced by HO-1 sense oligonucleotides. HO-1 antisense oligonucleotides, but not HO-1 sense oligonucleotides, significantly reduced hemin-driven upregulation of p21 (56% by standardized densitometry).

To determine the mechanisms whereby increased HO activity facilitated upregulation of p21, we examined whether products of HO activity (for example, iron, carbon monoxide, bile pigments), proteins induced with HO-1 (for example, ferritin), or oxidant stress resulting from exposure to hemin (a prooxidant) may be involved. As shown in Fig. 3A, the iron chelator DFO failed to significantly reduce the expression of p21 in response to hemin. However, increased iron levels can induce p21 because IRPTCs exposed to iron-hydroxyquinoline (a cell-permeant form of iron) evinced marked induction of p21, an effect that was blocked by DFO. We also examined whether the iron-binding protein ferritin (which is induced in conjunction with HO-1 in response to hemin) upregulates p21; we examined ferritin as well as apoferritin, the latter representing the moiety not containing iron. As shown in Fig. 3B, neither apoferritin nor ferritin induced expression of p21.

Carbon monoxide, in contrast, vigorously induced p21, as shown in studies in which IRPTCs were exposed to the carbon monoxide donor. Figure 4 shows a marked inductive effect of the carbon monoxide-releasing compound on the expression of p21 in IRPTCs. Another product of HO activity, bilirubin, in equimolar amounts failed to induce p21 (Fig. 5A); a lack of an inductive effect on p21 was also observed at lower concentrations of bilirubin, namely, 0.1, 1, and 5 μM (data not shown). Finally, because oxidative stress is induced by hemin, and
As an additional index for apoptosis, we undertook analogous studies that examined expression of active caspase-3. As demonstrated in Fig. 8, the intensity of expression of active caspase-3 mirrored the severity of apoptosis observed in the TUNEL assay; namely, expression of caspase-3 was not observed in cells maintained in 5% FBS-containing medium; however, in 0.1% FBS-containing medium, hemin induced expression of active caspase-3, an effect markedly accentuated by ZnPP. Thus expression of active caspase-3 corroborated findings obtained with TUNEL staining.

Effects of hemin in serum-replete (5% FBS) media. We also examined the effect of hemin on the growth of IRPTCs. We reasoned that because p21 is a potent cell cycle inhibitor, then the inductive effect of hemin on HO-1 and p21 may confer a growth-inhibitory action of hemin. We thus studied the effect of hemin on proliferative cell growth; proliferative cell growth requires FBS-replete medium, and in the case of IRPTCs, 5% FBS is employed. We confirmed that hemin, under these conditions (active cell proliferation in the presence of 5% FBS), again recapitulated the inductive effect of hemin on p21 and HO-1, as observed in cells exposed to 0.1% FBS, and that this effect occurred through ZnPP-inhibitable, HO-dependent mechanisms (data not shown). The exposure of IRPTCs to hemin led to a dose-dependent reduction in cell number as determined 24 and 48 h after exposure to hemin (Fig. 9). This reduction in cell number by hemin under these conditions was not due to detachment of cells from the monolayer; cells retrieved from the cell culture medium under each condition were essentially undetectable on day 1 (a time point at which a growth-inhibitory effect of hemin was already observed), and <0.5% of attached cells on day 2. Additionally, it is unlikely that hemin-induced reduction in cell numbers was due to increased apoptosis by hemin because in cells studied in the presence of 5% FBS, hemin did not induce TUNEL staining (Figs. 6 and 7) and did not increase expression of active caspase-3 (Fig. 8). As added evidence against increased apoptosis induced by hemin as the mechanism underlying the reduced number of cells when exposed to hemin, we examined DNA laddering in IRPTCs studied under exposure to hemin.

oxidative stress may stimulate cellular expression of p21 (4, 5, 14), we examined the effect of the antioxidant NAC on cellular expression of p21. NAC, at a concentration of 1 mM, failed to decrease hemin-induced upregulation of p21 (Fig. 5B). From these studies, we conclude that such products of HO activity as carbon monoxide and iron may contribute to the inductive effect of hemin on p21, whereas neither ferritin, bilirubin, nor oxidative stress is involved.

Effects of hemin in serum-deplete (0.1% FBS) or serum-replete (5% FBS) media. Because HO activity and p21 may exert antiproliferative effects, and hemin can exert cytotoxic or cytorepressive effects, we examined the effect of hemin on cell viability, specifically, on apoptotic processes, in the absence and presence of ZnPP. In these studies, we evaluated apoptosis by TUNEL staining, undertaking parallel observations in which cells were also studied in serum-replete medium (5% FBS, the medium that sustains healthy cell growth). As demonstrated in Figs. 6 and 7, at 24 h, exposure to 5%-containing medium was attended by little if any apoptosis. In contrast, exposure to 0.1% FBS-containing medium induced apoptosis, and hemin significantly worsened the amount of apoptosis occurring in cells cultured in 0.1% FBS-containing medium. Moreover, the presence of ZnPP markedly exacerbated hemin-induced apoptosis in cells maintained in 0.1% FBS-containing medium.

As an additional index for apoptosis, we undertook analogous studies that examined expression of active caspase-3. As demonstrated in Fig. 8, the intensity of expression of active caspase-3 mirrored the severity of apoptosis observed in the TUNEL assay; namely, expression of caspase-3 was not observed in cells maintained in 5% FBS-containing medium; however, in 0.1% FBS-containing medium, hemin induced expression of active caspase-3, an effect markedly accentuated by ZnPP. Thus expression of active caspase-3 corroborated findings obtained with TUNEL staining.
As shown in Fig. 10, no evidence of DNA laddering was observed when IRPTCs were exposed to hemin in the presence of 5% FBS; in contrast, DNA laddering was observed in IRPTCs exposed to 0.1% FBS-containing medium, and this was exaggerated in the presence of hemin. Thus the exposure of IRPTCs to these concentrations of hemin inhibited cell growth without inducing cell detachment or apoptosis. Furthermore, we demonstrated that hemin led to cell cycle arrest because hemin increased the percentage of cells in the G₀/G₁ phase (51.0 ± 2.6 vs. 59.4 ± 3.4%, P < 0.05, n = 4) and decreased the percentage of cells in the S/G₂/M phase (44.7 ± 1.8 vs. 39.7 ± 3.1%, P < 0.05, n = 4).

To determine whether the growth-inhibitory action of hemin was due to HO activity under those conditions, we examined the effect of hemin on cell growth in the presence of ZnPP. Whereas ZnPP per se exerted no effect on cell growth, it prevented the inhibitory effect of hemin on the growth of IRPTCs (Fig. 11). This reduction in cell number by hemin under these conditions was not due to detachment of cells from the monolayer because cells retrieved from the cell culture medium under each condition were essentially undetectable on day 1, and <1% of attached cells on day 2. Thus ZnPP prevented the inhibitory effect of hemin on the growth of IRPTCs.

**DISCUSSION**

Our studies demonstrate that hemin induces a time-dependent and dose-dependent expression of p21 protein in IRPTCs. Such upregulation of p21 was apparently not mediated through p53 because with increasing concentrations of hemin, p53 was progressively suppressed (and eventually was no longer detected) as p21 was increasingly induced. Accompanying this upregulation of p21 was marked induction of HO-1. HO activity accounted for the upregulation of p21 in hemin-treated cells because inhibiting HO activity completely prevented upregulation of p21. Moreover, that the HO-1 isoform, specifically, contributed to the HO-dependent upregulation of p21.
was demonstrated in studies undertaken with HO-1 antisense oligonucleotides. Thus hemin-induced upregulation of p21 appears p53 independent and requires intact HO activity, the latter emanating mainly from induced HO-1.

Studies were undertaken to explore the mechanisms underlying this induction of p21. We first considered the possibility that a product of HO activity accounted for such upregulation of p21; in this regard, we examined the capacity of products of HO (iron, carbon monoxide, bile pigments) and a key protein linked to HO activity (ferritin/apoferritin) to elicit such induction.

Recent findings have demonstrated that intracellular iron is a critical facilitator of PMA-elicited upregulation of p21 (12). In our studies, we found that while low micromolar amounts of a cell-permeant form of iron induced marked upregulation of p21 via a DFO-inhibitible process, hemin-induced upregulation of p21 was not reduced by DFO. The efficacy with which DFO chelates iron released from heme is uncertain, and it is possible that iron freed from heme may not be adequately sequestered by DFO; the lack of an effect of DFO in inhibiting heme-induced upregulation of p21 thus does not preclude the possibility that iron, derived from heme, may still contribute to heme-mediated upregulation of p21. Nonetheless, our finding that low micromolar amounts of a cell-permeant form of iron induced marked upregulation of p21 uncovers and highlights the capacity of iron, a critical mediator of renal and other types of tissue injury, to stimulate p21. On the other hand, neither the iron-binding protein ferritin nor its iron-free moiety apoferritin was capable of eliciting such upregulation of p21.

We also examined the capacity of carbon monoxide to stimulate expression of p21. We demonstrate that the carbon monoxide donor upregulated p21, thus raising the possibility that this product of HO can contribute to heme-induced, HO-mediated upregulation of p21. Other products of HO activity such as bile pigments failed to upregulate p21. Finally, because heme potently induces oxidative stress, and the latter is well recognized as a stimulus for p21 (4, 5, 14), we examined the effect of an antioxidant on hemin-induced upregulation of p21; NAC, the thiol-containing antioxidant, not only failed to mitigate hemin-induced upregulation of p21 but accentuated such expression of p21. While the cause for this apparent augmentation in the expression of p21 is uncertain, it is possible that NAC, like other thiols when in the presence of transition metals (such as iron), may exert prooxidant rather than antioxidant effects (28).

In conjunction with this upregulation of p21, hemin significantly altered the behavior of renal tubular epithelial cells in response to apoptotic stimuli on the one hand and conditions supporting cellular growth on the other. We focused on these two aspects of cellular behavior, apoptosis and proliferative cell growth, because they are fundamental to mechanisms of, and recovery from, acute renal injury and are significantly influenced by prevailing cellular expression of HO-1 and p21.

Fig. 9. Enumeration of cells grown in DMEM and 5% FBS on days 1 and 2 under control conditions and after exposure to increasing concentrations of hemin (2.5, 5, and 10 μM). Values are means ± SE; n = 4 in each group. *P < 0.05 vs. control conditions. †P < 0.05 vs. hemin (2.5 μM).

Fig. 10. A: electrophoresis of DNA from cells cultured in DMEM containing 5% FBS under control conditions and after exposure to hemin (10 μM) for 24 h. B: electrophoresis of DNA from cells cultured in DMEM containing 0.1% FBS under control conditions and after exposure to hemin (10 μM) for 24 h. Each condition was performed in duplicate.

Fig. 11. Enumeration of cells grown in DMEM and 5% FBS under control conditions and after exposure to hemin (10 μM), ZnPP (10 μM), and hemin and ZnPP (each at 10 μM) on days 1 and 2. Values are means ± SE; n = 4 in each group. *P < 0.05 vs. control conditions. †P < 0.05 vs. hemin.
IRPTCs maintained in 0.1% FBS, findings that indicate a proapoptotic effect of hemin under these conditions. This proapoptotic effect of hemin was significantly enhanced by inhibiting HO activity with ZnPP. Thus the worsening in hemin-induced apoptosis when HO activity was inhibited indicates that HO activity, under conditions of exposure to hemin, provides a countervailing response that mitigates the proapoptotic effects of hemin. We suggest that in states of exposure of cells to hemin, the concomitant induction in HO facilitates the cellular degradation of hemin, thereby limiting the extent to which cells are subjected to hemin and its attendant proapoptotic effects.

We also suggest that the exacerbation of hemin-induced apoptosis when HO activity is inhibited reflects the loss of the antiapoptotic actions of p21, the latter induced along with HO-1 in hemin-treated cells. The antiapoptotic effect of p21 may originate from cell cycle arrest and/or the inhibitory actions of p21 at certain steps in the effector pathways for apoptosis, the latter including inhibition of proapoptotic signals (for example, apoptosis signal-regulating kinase-1) (3). initiator caspases (caspase-8 and caspase-10) (41), or executioner caspases (caspase-3) (37). Relevant to the latter consideration is our finding that expression of active caspase-3 is increased when p21 expression is lost, attendant on the inhibition of HO activity. However, we emphasize that the evaluation of the functional role of p21 in the model of apoptosis examined in our study requires genetic manipulations that modulate expression of p21, as analogous pharmacological manipulations of p21 are not readily available; such studies are beyond the scope of the present study.

While the inhibition of HO activity in hemin-exposed IRPTCs markedly increased apoptosis, inhibition of HO activity in IRPTCs, maintained under control conditions of 0.1% FBS, decreased the low-grade apoptosis that occurs in this control, hemin-free condition (Figs. 6 and 7). These unexpected findings would suggest that under control conditions, HO activity in IRPTCs, maintained in 0.1% FBS, may contribute to apoptosis. This nuanced behavior of HO activity is germane to, and may offer insights regarding, the seemingly contradictory effects of HO activity described in the literature; for example, while induced HO activity in the majority of settings is generally antiapoptotic (2, 10, 15, 19, 20, 22, 33), proapoptotic and cytotoxic effects of HO activity are also clearly described (6, 10, 23, 30, 36). It is possible that proapoptotic rather than antiapoptotic actions of HO may emerge if the induction of HO is not appropriately or adequately linked to downstream products/intermediates (for example, ferritin, carbon monoxide, p21, etc.), which account for the cytoprotective actions of HO and nullify the possible toxic effects of HO (such as iron). An added consideration is that under basal conditions (that is, without exposure to hemin), HO activity reflects largely HO-2, whereas in hemin-exposed cells, the markedly increased HO activity reflects the vigorous induction of HO-1. Inhibition of HO activity in cells under basal conditions thus reflects inhibition of HO activity mainly from HO-2, whereas inhibition of HO activity in hemin-treated cells reflects inhibition of HO activity mainly from HO-1. It is quite possible that the downstream effects of HO activity may differ and differentially modulate the propensity to apoptosis, depending on the source of HO activity (HO-1 vs. HO-2). This consideration may be relevant to the reduction in apoptosis by ZnPP in IRPTCs under control conditions (ZnPP inhibiting HO activity largely from HO-2) and the marked exacerbation of apoptosis by ZnPP in hemin-treated IRPTCs (ZnPP inhibiting HO activity largely from HO-1).

The inductive effect of hemin on p21, a cyclin-dependent kinase inhibitor, also led us to examine the effect of hemin on proliferative cell growth. We thus employed cell culture conditions that, in contrast to those provided by 0.1% FBS-containing medium, would facilitate the normal proliferation of cells, the latter achieved with 5% FBS-containing medium. Under such conditions, instead of provoking apoptosis, hemin inhibited cell growth, the latter associated with cell cycle arrest. Moreover, this effect was HO dependent because ZnPP, the inhibitor of HO activity, vitiated the inhibitory effects of hemin on cell proliferation. Thus the induction of HO activity that occurs in cells exposed to hemin accounts for the inhibition of cell growth, the latter, we speculate, occurring, at least in part, through HO-mediated upregulation of p21.

That heme is inhibitory to cellular growth through HO-dependent mechanisms is relevant to at least three considerations. First, heme proteins such as myoglobin provoke growth arrest of renal epithelial cells, an effect accompanied by inhibition of protein and DNA synthesis, and prominent DNA injury (17). The extent to which these latter effects reflect the heme moiety per se is uncertain at the present time; these effects can clearly synergize with the actions of heme, described in the current study, in provoking growth arrest. Second, the pathobiological effect of heme is fundamentally determined by the conditions to which cells are exposed: in the presence of serum, the apoptotic effect of heme is forestalled and the growth-inhibitory effect of heme is disclosed. Third, these findings are relevant to the heterogeneous effects of HO on the growth of other cell types. For example, expression of HO-1 inhibits the growth of smooth muscle cells from the vasculature and the respiratory tract (10, 31, 34), whereas overexpression of HO-1 promotes endothelial cell growth and capillary formation (7, 10, 21); moreover, carbon monoxide, which we demonstrate is a potent inducer of p21, is increasingly recognized for its growth-inhibitory effects on smooth muscle cells from the vasculature and respiratory tract (10, 31, 34).

In summary, we demonstrate that heme, in pathophysiologically relevant concentrations, and a metabolite that accumulates in the kidney in ischemic and nephrotoxic states, determines cell fate in renal tubular epithelial cells. Hemin exerts proapoptotic effects in serum-deprived conditions and growth-inhibitory effects under serum-replete conditions; the concomitant downregulation of HO-1 in hemin-exposed cells restrains this apoptotic effect of hemin in serum-deprived conditions and contributes to the inhibitory effect of hemin on cell growth in serum-replete states. In either setting, such upregulation of HO-1 is accompanied by induction of p21 which, we speculate, contributes to these effects of HO activity.

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HEME INDUCES HO-1 AND p21 AND INFLUENCES CELL FATE

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