Am J Physiol Renal Physiol 284: F743–F752, 2003.
First published November 26, 2002; 10.1152/ajprenal.00376.2002.

Heme activates the heme oxygenase-1 gene in renal epithelial cells by stabilizing Nrf2

Jawed Alam,[1,2] Erin Killeen,[1] Pengfei Gong,[1] Ryan Naquin,[1] Bin Hu,[1] Daniel Stewart,[1,2] Julie R. Ingelfinger,[3] and Karl A. Nath[4]

[1]*Department of Molecular Genetics, Ochsner Clinic Foundation, New Orleans 70121;* [2]*Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, New Orleans, Louisiana 70112;* [3]*Pediatric Nephrology Unit, Massachusetts General Hospital, Boston, Massachusetts 02114; and* [4]*Division of Nephrology, Mayo Clinic Foundation, Rochester, Minnesota 55905*

Submitted 18 October 2002; accepted in final form 19 November 2002

Alam, Jawed, Erin Killeen, Pengfei Gong, Ryan Naquin, Bin Hu, Daniel Stewart, Julie R. Ingelfinger, and Karl A. Nath. Heme activates the heme oxygenase-1 gene in renal epithelial cells by stabilizing Nrf2. *Am J Physiol Renal Physiol* 284: F743–F752, 2003. First published November 26, 2002; 10.1152/ajprenal.00376.2002.—The mechanism of heme oxygenase-1 gene (*ho-1*) activation by heme in immortalized rat proximal tubular epithelial cells was examined. Analysis of the *ho-1* promoter identified the heme-responsive sequences as the stress-response element (StRE), multiple copies of which are present in two enhancer regions, E1 and E2. Electrophoretic mobility shift assays identified Nrf2, MafG, ATF3, and Jun and Fos family members as StRE-binding proteins; binding of Nrf2, MafG, and ATF3 was increased in response to heme. Dominant-negative mutants of Nrf2 and Maf, but not of c-Fos and c-Jun, inhibited basal and heme-induced expression of an E1-controlled luciferase gene. Heme did not affect the transcription activity of Nrf2, dimerization between Nrf2 and MafG, or the level of MafG, but did stimulate expression of Nrf2. Heme did not influence the level of Nrf2 mRNA but increased the half-life of Nrf2 protein from ~10 min to nearly 110 min. These results indicate that heme promotes stabilization of Nrf2, leading to accumulation of Nrf2·MafG dimers that bind to StREs to activate the *ho-1* gene.

Nrf2 transcription factor; stress-response element; protein stabilization

HEME, A TETRAPYROLE with a redox active iron center, is a lipophilic molecule of limited solubility in aqueous environments.[1] Consequently, it is typically associated with (either covalently or noncovalently), and functions as a cofactor for, various proteins such as hemoglobin, myoglobin, mitochondrial and microsomal cytochromes, catalases, nitric oxide synthase, guanylate cyclase, and cyclooxygenases (11, 36, 48). In this capacity, heme is essential for many biological activities, including oxygen transport, energy production, and xenobiotic detoxification. Tissue damage or cell injury, either in pathological states or in response to noxious stimuli, can destabilize heme proteins, resulting in altered associations and even release of the heme moiety. Due to its lipophilic nature and the reactive iron molecule, "free" heme can damage cellular components and disrupt cellular function through mechanisms that are, at least in part, prooxidant in nature (9, 10, 20, 39–41). For instance, heme is known to promote oxidative degradation of proteins (2) and DNA (1) and amplify hydrogen peroxide-mediated endothelial cell dysfunction (11).

Elimination of excess free heme is essential for maintenance of cellular integrity and is largely the responsibility of heme oxygenases (HOs), enzymes that catalyze the initial and rate-limiting step in heme catabolism, the oxidative cleavage of the porphyrin ring to generate biliverdin IXα with the release of the heme iron and carbon monoxide. Of the two functional HO isoforms thus far identified (HO-1 and HO-2) (32), HO-1 plays a particularly important role in this process as the expression of this protein, and consequently overall HO activity is potently stimulated in response to heme. In addition to the substrate, a variety of physiological and nonphysiological stimuli such as inflammatory cytokines, hyperthermia, UV-irradiation, heavy metals, and arsenite, all of which are potentially injurious to cells and can lead to heme protein instability, also induce HO-1 expression. Induction of HO-1 is regulated primarily at the level of *ho-1* gene transcription.

Our previous studies have demonstrated the importance of induction of HO-1 expression and of ensuing HO activity in the protection against heme-mediated injury. For instance, in experimental rhabdomyolysis and hemolysis, heme molecules derived from myoglobin and hemoglobin released during injury to skeletal muscle and red blood cells, respectively, readily accumulate in kidney epithelial cells and lead to renal dysfunction. Prior induction of HO-1 in this experimental model, however, protected rats from renal failure and mortality; the opposite effect was observed after

Address for reprint requests and other correspondence: J. Alam, Dept. of Molecular Genetics, Ochsner Clinic Foundation, 1514 Jefferson Highway, New Orleans, LA 70121 (E-mail: jalam@ochsner.org).

[1]Hemin (ferriprotoporphyrin IX chloride) was used in this study but is referred to as heme (ferriprotoporphyrin IX) in the text.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

pharmacological inhibition of HO activity (38, 53). Furthermore, we have directly demonstrated the indispensability of HO-1 induction in protecting against heme protein toxicity by showing that *ho-1*-targeted mice are exquisitely sensitive to such insults (41).

Although the cytoprotective function of HO-1 activity during heme-mediated cellular injury is now readily obvious and has been experimentally verified in multiple studies, the mechanism by which heme activates the *ho-1* gene, in the kidney or other organs, is less well understood. Here we examine this mechanism in renal proximal tubular epithelial cells and show that heme-dependent *ho-1* gene activation is mediated by the stress-responsive DNA elements (StREs) and transcription factor Nrf2. Additional studies indicate a novel mechanism for regulation of Nrf2 activity and subsequent *ho-1* gene activation, namely posttranscriptional stabilization of the Nrf2 polypeptide in response to heme.

MATERIALS AND METHODS

Materials. Tissue culture media were from Life Technologies, and fetal bovine serum was obtained from Mediatech. Restriction endonucleases and other DNA-modifying enzymes were purchased from either Life Technologies or New England Biolabs. Oligonucleotides were synthesized by Integrated DNA Technologies. Radiolabeled nucleotides were obtained from NEN. Reagents for luciferase assays were purchased from Sigma Chemical. MafG antisera were kindly provided by Dr. V. Blank, and anti-rat HO-1 was obtained from StressGen Biotech. All other antibodies were purchased from Santa Cruz Biotechnology. All other chemicals were reagent grade.

Plasmids. The wild-type and mutant *ho-1* promoter/luciferase fusion constructs have been described previously (7). pFRluc, containing 5 tandem copies of the Gal-4 binding site, was obtained from Stratagene. Plasmid pCMV/β-gal, encoding the *Escherichia coli* β-galactosidase gene, was kindly provided by Dr. Ping Wei. Construction of the dominant-negative mutants of Nrf2 and c-Jun has been previously reported (6). The dominant mutant of MafK (31) and the A-Zip mutants derived from c-Fos and cAMP response element binding protein (CREB) sequences (3, 44) were kindly provided by Drs. Stuart Orkin and Charles Vinson, respectively. Mammalian two-hybrid vectors pEG, containing the DNA-binding domain of Gal-4 (Gdbd), and pAD, containing the transcription activation domain of Nrf2, have been described (19). Full-length human MafG (kindly provided by Dr. Volker Blank), full-length mouse Nrf2 (amino acid residues 1–597), or truncated Nrf2 (ΔN, amino acids 329–597) were cloned in-frame with the Gdbd in pEG. Full-length rat HO-1 and MafG sequences were also cloned into the pAD vectors.

Cell culture, transfection, and enzyme assays. Immortalized rat proximal tubular epithelial cells (IRPTCs), developed and characterized as previously described (51), were cultured in a humidified atmosphere (95% air, 5% CO_2) at 37°C in DMEM containing 0.1% glucose, 0.1 mM nonessential amino acids, 5% fetal bovine serum, and 50 μg/ml gentamicin. Transient transfections were carried out with Fugene6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's recommendations. Briefly, cells were seeded (7×10^4/well) in 12-well plates and, 20 h after plating, cells in each well were transfected with a FuGene6-DNA mixture consisting of 50 ng of the luciferase plasmid, 25 ng of pCMVβ-gal and 175 ng of empty vector, or the indicated effector plasmid. The transfection media were removed 24 h later, and the cells were exposed to vehicle [DMSO, final concentration of 0.5% (vol/vol)] or 10 μM heme in DMSO for 5 h in serum-free medium. Preparation of cell extract and measurement of reporter enzyme activities were carried out as described (4).

Electrophoretic mobility shift assay. IRPTCs were seeded (1×10^6 cells/10-cm plate) and cultured for 48–72 h in complete medium and then treated with vehicle or 10 μM heme in serum-free medium for 3 h. Whole cell extracts (WCEs) were prepared as described previously (16). The standard binding reaction mixture (12.5 μl) contained 18 mM HEPES (pH 7.9), 80 mM KCl, 2 mM $MgCl_2$, 10 mM DTT, 10% glycerol, 0.2 mg/ml bovine serum albumin, 160 μg/ml poly(dI-dC), 20,000 counts per minute [γ-^{32}P]ATP-labeled probe, and 3.5–5 μg of WCE. Reaction mixtures were incubated at 25°C for 20 min and analyzed by native 5% polyacrylamide gel electrophoresis and autoradiography as described previously (16, 37). A double-stranded oligonucleotide containing the sequence 5′-TTTTCTGCTGAGTCAAGGTCCG-3′ was used as a probe in EMSA reactions (core StRE sequence is underlined). In supershift assays, 1 μl of preimmune serum or anti-MafG serum and preimmune IgG or anti-transcription factor IgG (2 μg/μl) were added to the reaction mixture and incubated for 20 min at room temperature before electrophoresis.

Protein and RNA blot analyses. IRPTCs were plated in 10-cm (1×10^6 cells) or in six-well plates (2×10^5 cells/well) and cultured for 48–72 h. The culture media were removed, and cells were exposed to vehicle or 10 μM heme for 0–4 h in serum-free medium. Whole cell, cytoplasmic, and nuclear protein extracts were prepared as previously described (15). Extracts were electrophoresed on a 4–12% gradient SDS-PAGE gel (Invitrogen), and proteins were transferred to a polyvinylidene difluoride membrane. The membrane was blocked overnight in Tris-buffered saline containing 0.1% (vol/vol) Tween 20 and 5% (wt/vol) nonfat dry milk and then incubated with the primary antibody (1:1,000 dilution) for 3 h. Treatment with the secondary antibody and antigen detection were carried out using the ECL system (Amersham Pharmacia Biotech) according to the manufacturer's recommendations. Total RNA was isolated by the procedure of Chomczynski and Sacchi (12), and RNA dot blot analysis was carried out as previously described (7). Successive hybridizations were carried on the same filter using cDNA probes encoding mouse Nrf2, rat HO-1, and rat ribosomal protein S3. Hybridization signals were quantified using a phosphorimager (Packard). Relative mRNA levels were calculated after correcting for RNA adsorption by normalizing the primary hybridization signals with the S3 signal.

RESULTS

StREs within the E1 and E2 enhancers mediate heme-dependent activation of the ho-1 gene. We have previously isolated and characterized a 15-kbp region of the mouse *ho-1* gene (5) and identified two 5′-distal enhancer regions, E1 and E2, that mediate gene activation in response to nonheme HO-1 inducers such as cadmium (7) and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (17) (Fig. 1). Both E1 and E2 contain three copies each of a sequence motif termed the StRE; core StRE consensus sequence = 5′-[(T/C)GCTGAGTCA-3′] that are essential for induction by these agents. To characterize the

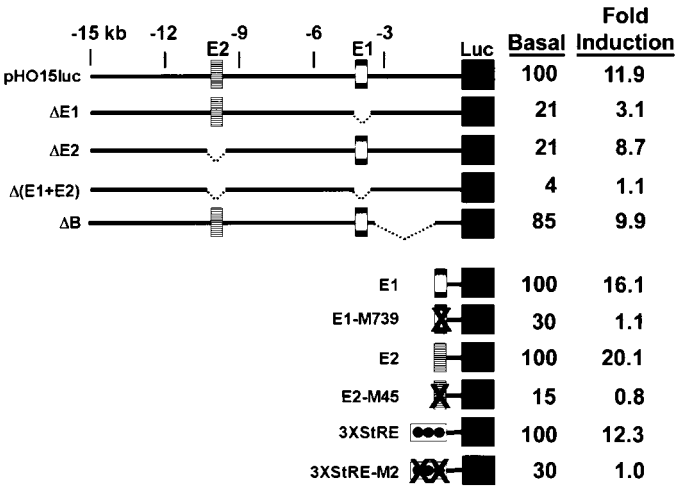

Fig. 1. Identification of the *ho-1* promoter regions responsible for heme-mediated gene induction. Plasmid pHO15luc contains ~15 kbp of the 5′-flanking region of the mouse *ho-1* gene fused to the firefly luciferase (Luc) reporter gene. The relative locations of the previously identified E1 and E2 enhancers are indicated. Derivatives of pHO15luc, generated by internal deletion of the promoter, and other fusion constructs are diagrammed. X, mutation of the stress-response elements (StREs) within the indicated regulatory region. Transfection and treatment of immortalized rat proximal tubular epithelial cells (IRPTCs) were carried out as described in MATERIALS AND METHODS. Aliquots representing 8% and 4% of the cell extracts were used for luciferase and β-galactosidase assays, respectively. Background luciferase activity (from mock-transfected cells) was subtracted from each experimental measurement, and the resulting value was corrected for variation in transfection efficiency by normalization with background-subtracted β-galactosidase activity. Normalized luciferase activity in the absence of heme (basal) was arbitrarily assigned a value of 100 for each wild-type plasmid; fold induction by heme is provided for each construct. Each data point represents the mean from at least 3 independent experiments. SE varied from 8–22%.

mechanism of *ho-1* gene activation by heme and to determine the role of StREs in this response, we examined the transcription activity of the wild-type mouse *ho-1* promoter and appropriate mutants in reporter gene transfection assays. As shown in Fig. 1, expression of pHO15luc, a chimera containing the full-length 15-kbp promoter and the firefly luciferase gene, was stimulated ~12-fold after treatment of IRPTCs with 10 μM heme, a concentration that elicited maximal induction (data not shown). Targeted deletion of the E1 enhancer (ΔE1) inhibited heme responsiveness by ~75%, whereas deletion of the E2 fragment reduced induction by only 25%, indicating a greater importance of E1 in this response. Both enhancers, however, are required for optimal induction since deletion of E1 and E2 completely abolished heme responsiveness and also drastically attenuated basal luciferase activity. The specificity of this response is demonstrated by the fact that deletion of sequences between −1.3 kb and −3.5 kb (ΔB) only minimally affected basal and heme-induced luciferase expression.

The heme-dependent transcription activities of E1 and E2 were tested directly using constructs in which each of the enhancers was fused to the minimal *ho-1* promoter (−44 to +73). In this context, E1 and E2 mediated an ~16- and 20-fold stimulation, respectively, of luciferase expression. Mutation of all three StREs in either enhancer (E1-M739 or E2-M45) reduced basal activity and abolished heme inducibility. Furthermore, a synthetic regulatory sequence composed of three tandem copies of an individual StRE (3× StRE) was as responsive as the full-length *ho-1* promoter. Mutation of the first three residues within the core StRE (3× StRE-M2) completely abrogated heme-dependent gene activation. From these results, we conclude that the StRE is sufficient and necessary for heme-mediated *ho-1* gene induction in IRPTCs.

StRE-binding activities in IRPTCs. EMSA reactions using WCEs from IRPTCs were carried out to identify DNA-binding proteins potentially responsible for heme-mediated *ho-1* gene induction. With the use of extracts from vehicle-treated cells, six SRE-protein complexes of relatively similar intensities were typically observed (Fig. 2, *lane b*). Heme treatment of IRPTCs significantly altered the subsequent EMSA profile: some of the "control" complexes (complexes 1, 3, and 4) decayed while an apparently novel complex (Fig. 2, arrow) was formed in a time-dependent manner. Of course, it is possible that this band represents multiple distinct, but comigrating, complexes. The abundance of other complexes (e.g., 5 and 6) varied among experiments, and their apparent increase in response to heme was not observed consistently (for instance, see

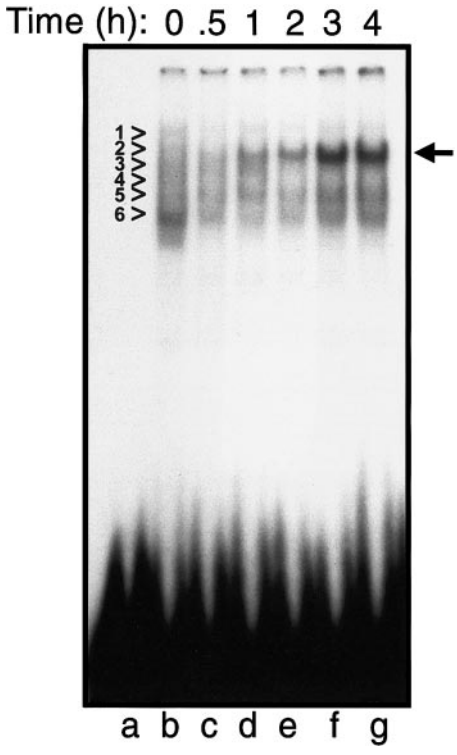

Fig. 2. StRE-binding activities in IRPTCs: IRPTCs were treated with 10 μM heme for the indicated time period (*lanes b–g*). Preparation of cell extracts and EMSA reactions were carried out as described in MATERIALS AND METHODS. The DNA-protein complexes typically observed in untreated cells (1–6) and the heme-induced complex (arrow) are indicated. *Lane a*, no protein extract.

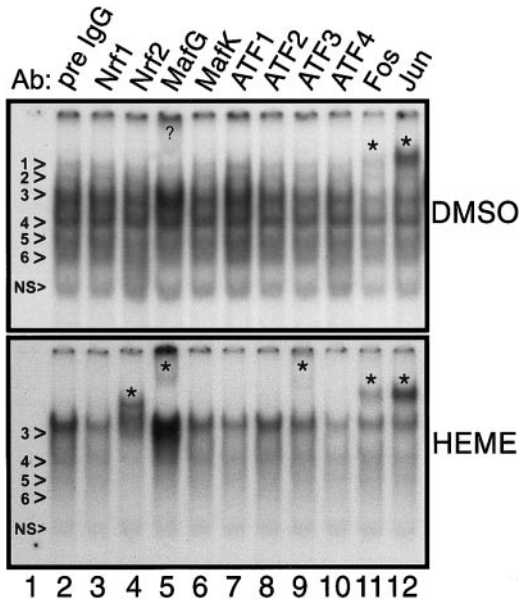

Fig. 3. Identification of SRE-binding proteins using antibody supershift EMSA. Extracts prepared from IRPTCs treated with vehicle (DMSO) or with 10 μM heme for 3 h were used in EMSA reactions. Supershift analysis using preimmune IgG (pre IgG) or antibodies (Ab) directed against the indicated transcription factors was carried out as described in MATERIALS AND METHODS. *Supershifted complexes. The control complexes (1–6, *top*) are identified, and the corresponding migration positions of some of these complexes are indicated in the heme-treated panel. NS, nonspecific complex; *lane 1*, no protein extract. ATF, activating transcription factor.

Fig. 3). The specificity of the complexes generated was confirmed using wild-type and mutant StRE oligonucleotides in competition experiments (data not shown).

Multiple basic region/leucine zipper proteins bind to StRE. The consensus StRE resembles the consensus binding sites for the Fos and Jun [TGA(G/C)TCA] (27), activating transcription factor (ATF)/CREB (TGACGTCA) (18), Maf [TGCTGA(G/C)TCAGCA or TGCTGACGTCAGCA] (28), and Cap 'N' Collar (CNC)-basic region/leucine zipper (bZIP) [(T/C)GCTGA(G/C)TCA(C/T)] (8) subfamilies of bZIP transcription factors that function as homo- or heterodimers. For instance, Fos family members dimerize most commonly with Jun proteins, and CNC-bZIP factors such as Nrf1 and Nrf2 dimerize most efficiently with small Maf proteins, including MafG and MafK. "Supershift" EMSA reactions using antibodies directed against bZIP proteins were carried out to identify specific StRE-binding proteins (StRE-BPs) and potential heme-responsive transcription factor(s). Of several factors tested, only Fos and Jun proteins were consistently detected in control complexes (Fig. 3, *top*, *lanes 11* and *12*). These proteins were also detected in the "heme" complexes, and the intensity of the supershifted bands did not change appreciably in response to heme (*bottom*). Because pan-Fos and pan-Jun antibodies were used, individual family members were not identified in this analysis. Heme-induced StRE-BPs included Nrf2, MafG, and ATF3 (the latter exhibited a weak signal but was consistently observed in multiple experiments, whereas MafG was observed inconsistently in the control complexes but was routinely detected at higher intensity in the heme complexes). Other transcription factors tested, including Nrf1, MafK, ATF1, ATF2, and ATF4, were not detected in the absence or presence of heme.

Inhibition of gene activity by dominant-negative mutants of Nrf2 and Maf. To explore the functional role of the StRE-BPs identified in *Multiple basic region/leucine zipper proteins bind to StRE* in heme-regulated *ho-1* gene expression, we examined the effect of appropriate dominant-negative mutants (DNMs) on E1 transcription activity. Because Fos family members do not homodimerize and heterodimerize most efficiently with Jun proteins, the Fos DNM would be expected to directly inhibit endogenous Jun factors. Conversely, the Jun DNM would be expected to inhibit Fos family members. As shown in Fig. 4, overexpression of the Fos or Jun DNM did not appreciably alter basal or heme-dependent E1 activity, suggesting that such proteins are not involved in heme-mediated gene activation. The CREB DNM, which would most efficiently inhibit CREB/ATF-type factors, increased basal luciferase activity but did not influence heme-induced luciferase expression. On the other hand, the Nrf2 and MafK DNMs, which would most effectively inhibit small Maf and CNC-bZIP proteins, respectively, significantly attenuated both basal and heme-dependent luciferase activity. The MafK DNM was more effective in inhibiting heme-dependent luciferase activity than basal activity (13 and 42% of controls, respectively), whereas the Nrf2 DNM exhibited similar inhibitory activities toward both heme-induced and basal luciferase expression (8 and 13% of controls, respectively). Although a DNM of MafK was used in this experiment, an analogous mutant of MafG would be expected to behave in a similar manner because of the structural and func-

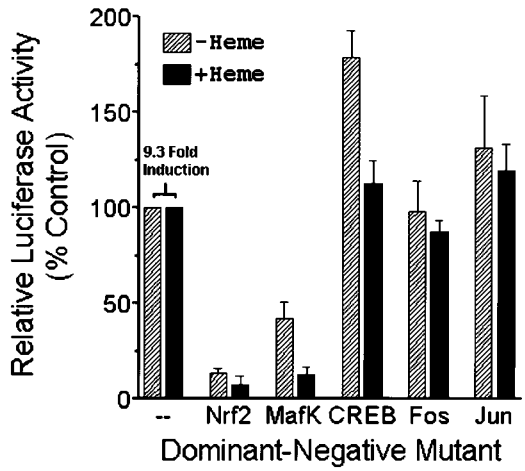

Fig. 4. Dominant-negative mutants of Nrf2 and MafK inhibit E1 activity. IRPTCs were transfected, treated with vehicle or heme, and processed as described in MATERIALS AND METHODS and in the legend to Fig. 1. Data are presented as percent of normalized luciferase activity in the absence of exogenous dominant-negative mutants, and each data point represents the average ± SE from 3–5 independent experiments. CREB, cAMP response element binding protein.

tional similarities of small Maf proteins (35). Because MafG, but not MafK, was detected in the EMSA analysis, we conclude that Nrf2·MafG heterodimers are at least partly responsible for *ho-1* gene activation in response to heme.

Heme stimulates Nrf2 expression. To explore the mechanism(s) by which heme regulates Nrf2·MafG function, we first examined the effect of this agent on the steady-state level of each of these proteins. As shown in Fig. 5*A*, the abundance of Nrf2, but not of MafG, in WCEs increased in a time-dependent manner after treatment of IRPTCs with 10 μM heme. At the last time point tested, Nrf2 levels were >10-fold higher than in untreated cells. The temporal pattern of Nrf2 induction was qualitatively similar to that observed for HO-1. Previous studies (13, 22, 25, 46, 55) have suggested that Nrf2 is sequestered in the cytoplasm in an inactive form, and oxidants and xenobiotics activate Nrf2 in part by permitting transport into the nucleus. To determine whether heme regulates Nrf2 activity in this manner, we monitored Nrf2 levels in cytoplasmic and nuclear extracts of cells treated with vehicle (DMSO) or heme for different time periods. The underlying assumption of this experiment is that regulated transport would reveal a time-dependent decay in the level of cytoplasmic Nrf2 with a concomitant increase in nuclear Nrf2. Low levels of Nrf2 were observed in the nuclear fraction, and this level increased substantially after 3 h of treatment with heme (Fig. 5*B*, *lanes 6-10*). However, no Nrf2 was detected in the cytoplasmic fraction (*lanes 1-5*) at any time point tested using this assay. The integrity of the cytoplasmic and nuclear fractions was confirmed by analysis of the compartment-specific α-tubulin and histone H1 proteins.

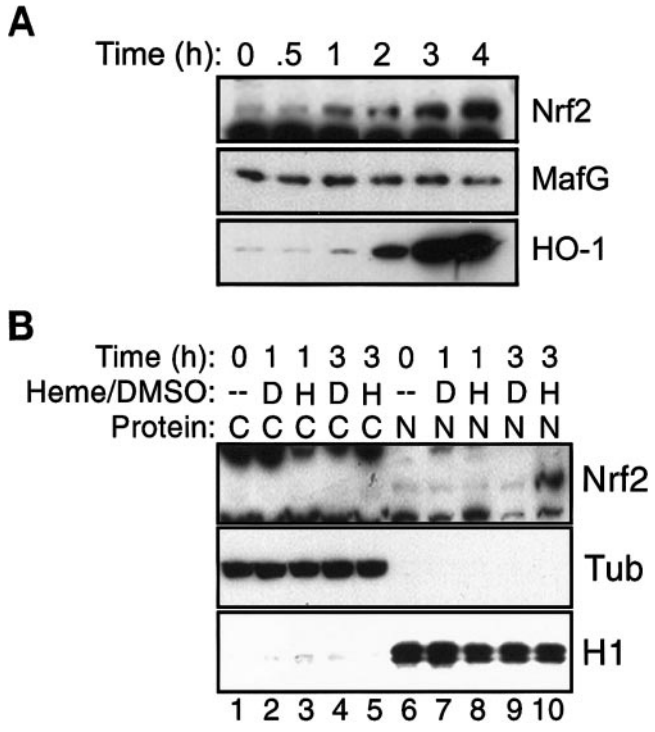

Fig. 5. Heme induces expression of Nrf2. IRPTCs were treated with DMSO (D) or heme (H) for the indicated time period. Whole cell extracts (*A*) or cytoplasmic (C; *B*) and nuclear (N) fractions were prepared and subjected to Western blot analysis using the indicated antibodies as described in MATERIALS AND METHODS. Tub, α-tubulin; H1, histone H1; HO-1, heme oxygenase-1.

Heme decreases the rate of Nrf2 degradation. To delineate the mechanism of Nrf2 induction, we first examined the effect of heme on the level Nrf2 mRNA. As shown in Fig. 6*A*, treatment of IRPTCs with heme for up to 4 h did not alter the steady-state level of Nrf2 transcripts. As expected, HO-1 mRNA abundance increased by >40-fold during this period. This result suggests that heme regulates Nrf2 expression by a posttranscriptional mechanism(s). Pulse labeling experiments indicated that heme does not influence the rate of Nrf2 synthesis (data not shown), suggesting it may regulate Nrf2 stability. Nrf2 stability was examined by monitoring the decay of basal Nrf2 in the absence of heme and of heme-induced Nrf2 in the presence of heme after inhibition of protein synthesis by cycloheximide. On the basis of these experiments, the half-life ($t_{1/2}$) of Nrf2 in unstimulated cells was calculated to be ~9.7 min. Heme stimulation increased the $t_{1/2}$ to 107 min (Fig. 6*B*). Similar values were obtained in pulse-chase experiments (data not shown). As control, we monitored the induction and decay of JunD, both of which were not appreciably altered in response to heme (Fig. 6*C*).

Heme does not stimulates the transcription activity of Nrf2 nor does it affect Nrf2·MafG dimerization. Because heme could potentially regulate Nrf2 and MafG function at additional levels, we also examined the effects of this agent on the transcription activity and heterodimerization potential of Nrf2 and MafG by mammalian one-hybrid and two-hybrid assays. For these experiments, transcription factor sequences were fused in-frame to the Gdbd, and the fusions were tested for their ability to *trans*-activate a luciferase reporter gene under the control of five copies of the Gal-4 recognition sequence. Additionally, for two-hybrid assays, appropriate sequences were cloned into the pAD "activation domain" vector and tested for their ability to interact with, and potentiate the activity of, Gdbd fusions. The parent Gdbd vector does not encode a transcription activation domain and thus did not promote luciferase expression (Fig. 7). Similarly, MafG also does not contain such a domain, and the Gdbd-MafG was transcriptionally inactive. Nrf2, on the other hand, encodes a potent activation domain and sponsored a high level of luciferase activity. Nrf2-mediated *trans*-activation, however, was not affected by heme. Deletion of the NH_2-terminal half of Nrf2 eliminates the activation domain, resulting in a protein (Nrf2ΔN) with no transcription activity. Nrf2ΔN does retain the leucine zipper dimerization domain, and association with the AD-MafG fusion elicited a high level of luciferase activity. AD-MafG also readily dimerized with full-length Nrf2; the rate and/or the extent of Nrf2·MafG dimerization, however, was not affected by heme. HO-1 is not expected to associate with Nrf2 and was used as a negative control for these studies.

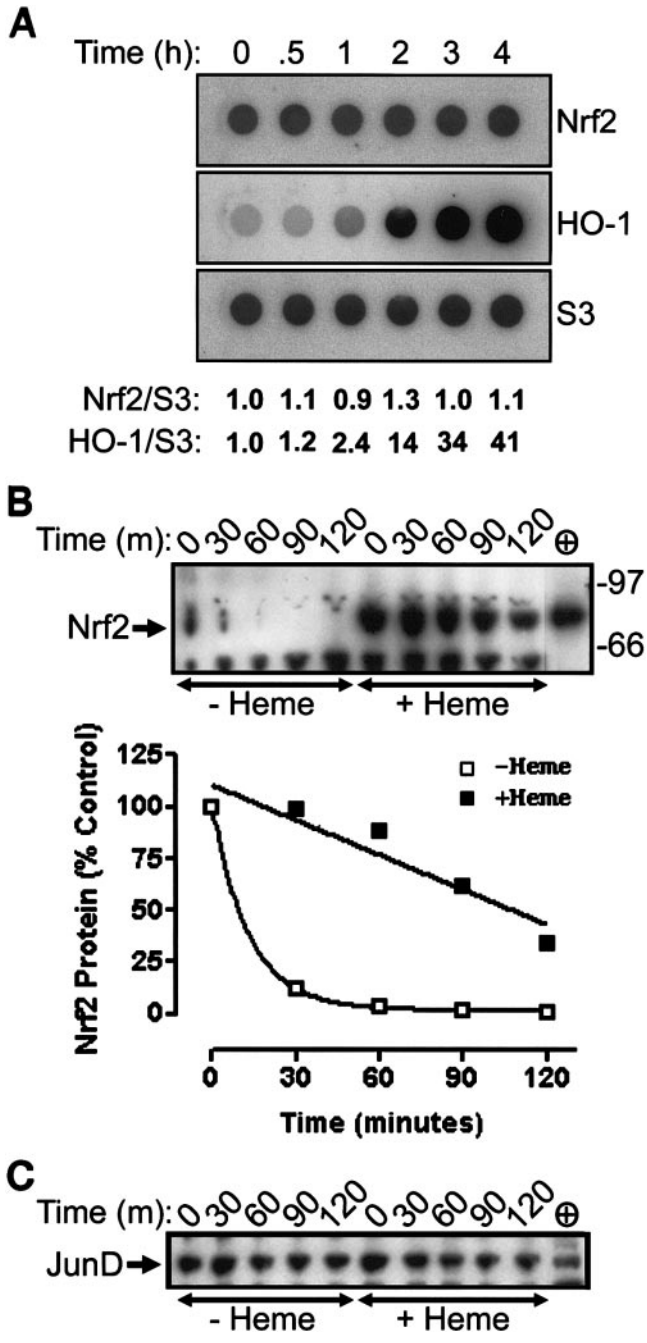

Fig. 6. Posttranscriptional regulation of Nrf2 expression by heme. *A*: heme does not affect the steady-state level of Nrf2 mRNA. IRPTCs were treated with 10 μM heme for the indicated time period. Total RNA (5-μg portions) was dot blotted onto a nylon membrane and successively hybridized to the indicated cDNA probes. Hybridization signals were detected and quantified with a phosphorimager. S3-normalized, relative mRNA levels for Nrf2 and HO-1 are presented. *B* and *C*: heme promotes stabilization of Nrf2 (*B*) but not of JunD (*C*). IRPTCs were treated with DMSO (−Heme) or 10 μM heme in serum-free medium for 2 h. The treatment media were replaced with the same media containing 100 μg/ml of cycloheximide, and cells were incubated at 37°C for the indicated time period. Twenty-microgram portions of nuclear extracts were analyzed by Western blotting as described in MATERIALS AND METHODS. Protein extract (2.5 μg) from HEK-293 cells transfected with expression plasmids encoding mouse Nrf2 or mouse JunD were used as positive controls (+). The migration of the molecular mass (kDa) markers are indicated. Relative Nrf2 levels were quantified by densitometry, and each data point represents the average value from 2 independent experiments.

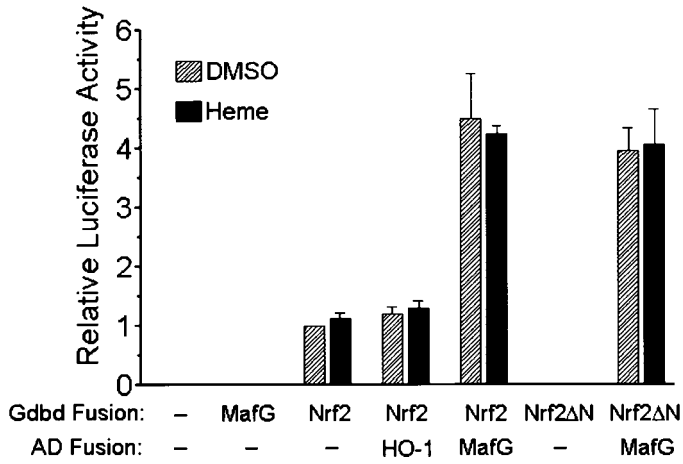

Fig. 7. Heme does not stimulate the transcription activity of Nrf2 or Nrf2·MafG dimerization. Transfection and treatment of IRPTCs, preparation of cell extract, reporter enzyme assays, and data analysis were carried out as described in MATERIALS AND METHODS and in the legend to Fig. 1. Plasmid pFRluc was used as the experimental reporter gene. Equal mass amounts of the parent DNA-binding domain of Gal-4 (Gdbd) and activation domain (AD) plasmids or the fusion plasmids were used in the indicated mixtures. Luciferase activities were normalized to that obtained with the Gdbd-Nrf2 fusion. Data are presented as means ± SE; n = 3–4. Nrf2ΔN, NH_2-terminal deleted Nrf2.

DISCUSSION

On the basis of the data obtained in this study, we propose the following model for *ho-1* gene activation by heme in renal epithelial cells (Fig. 8). In unstimulated cells, Nrf2 is expressed at constitutive levels but rapidly degraded, either in the cytoplasm or after transit into the nucleus. Other StRE-BPs (depicted by circles and diamonds) bind to the *ho-1* enhancers (squares) and effectively repress, or otherwise permit only low levels of, gene activity. One or more members of the CREB/ATF family, not all of which were tested by antibody supershift EMSA, may function in this capacity, as overexpression of the CREB DNM stimulates basal transcription activity of the E1 enhancer (Fig. 4). Other likely candidates of repressor StRE-BPs include heterodimers between Bach1 and small Maf proteins since the Bach1·MafK dimer is known to bind to the *ho-1* StREs, and targeted deletion of the *bach1* gene leads high constitutive expression of HO-1 mRNA and protein in several organs (43, 50) (studies to determine whether one or more of the 6 specific StRE/protein complexes detected by EMSA in unstimulated IRPTCs contain Bach1 protein are currently in progress). Upon cellular stimulation, heme interferes with the Nrf2 degradation pathway, permitting accumulation of the transcription factor in the nucleus, where it heterodimerizes with MafG. Nrf2·MafG heterodimers displace some of the repressor StRE-BPs bound to the *ho-1* enhancers and promote high rates of transcription. Additionally, heme may directly interfere with the binding of repressor StRE-BPs, as was recently demonstrated for Bach1·MafK heterodimers (43, 50). Overall, heme-mediated activation of the *ho-1* gene, therefore, likely reflects the net effect of relief of re-

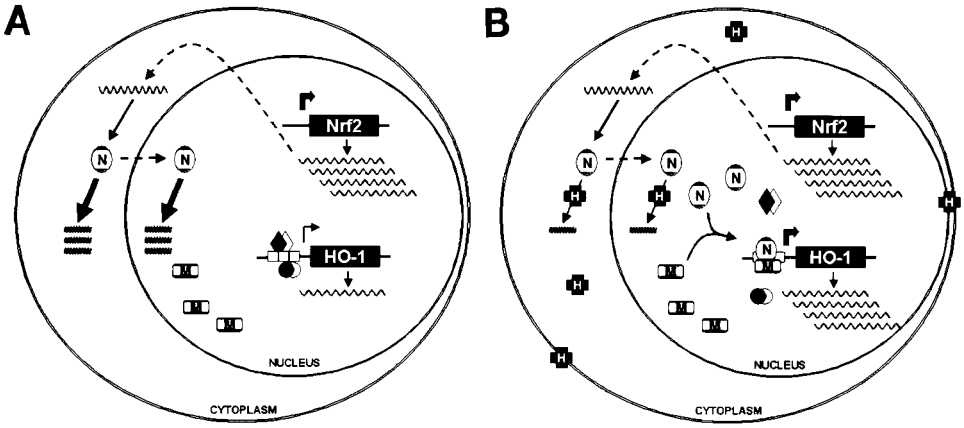

Fig. 8. Model of *ho-1* gene regulation in the absence (*A*) or presence (*B*) of heme. N, Nrf2; M, MafG; H, heme. See text for additional details.

pression (i.e., inhibition of repressor StRE-BPs) and the positive action of Nrf2. Although renal epithelial cells, a relevant target for heme released during hemolysis or rhabdomyolysis, were used in the present study, we suspect that the mechanism described herein is generally operative in other cell types since heme-mediated activation of the *ho-1* gene is an ubiquitous phenomenon.

How does heme stabilize Nrf2? We consider it unlikely that the heme molecule directly associates or interferes with a component of the Nrf2 degradation pathway as other, structurally distinct HO-1 inducers, including cadmium and arsenite, also promote Nrf2 stability (Stewart, Killeen, and Alam, unpublished observations). All of these agents have in common the ability to generate cellular oxidative stress, and it is probably this common condition, or more precisely, a signal generated during this state, that triggers the stabilization process. In a prooxidant state, increased Nrf2 levels and activity would result in activation of select genes encoding proteins, such as NAD(P)H:quinone oxidoreductase, γ-glutamylcysteine synthase, or glutathione *S*-transferase, with antioxidant and xenobiotic detoxification activities (14, 24). Induction of HO-1 would represent one component of this homeostatic response. Increased HO-1 activity will lead to elimination of the prooxidant heme molecules, those internalized from the extracellular environment and those released intracellularly on destabilization of heme proteins. In certain context, induction of HO-1 is accompanied by increased synthesis of apoferritin (38), which can sequester the released heme iron. Additionally, HO-1-catalyzed degradation of heme generates the antioxidants biliverdin and bilirubin (49), promoting further abatement of the oxidative environment. Attenuation of the oxidative stress, and return to a normal reducing environment, will lead to resumption of normal Nrf2 degradation and downmodulation of target genes such as HO-1.

In this study (and as illustrated in Fig. 8), we have identified the StREs and transcription factor Nrf2 as the key components of the heme-dependent *ho-1* gene regulatory circuit. In this and other respects, the proposed mechanism is remarkably similar to that for heme-dependent regulation of the thioredoxin gene (*trx*) in K562 human erythroleukemia cells (29). Activation of the *trx* gene is also proposed to be mediated by Nrf2 and an antioxidant response element (ARE), 5′-TGCTGAGTAAC-3′, that is very similar to the *ho-1* StREs. Furthermore, as proposed for the *ho-1* gene (see Fig. 8), activation of the *trx* gene also involves exchange of factors bound at the ARE. In unstimulated K562 cells, a dimer composed of a small Maf protein and the CNC-bZIP factor NF-E2p45 is proposed to occupy the *trx* ARE. Upon heme stimulation, an Nrf2·small Maf heterodimer replaces the p45·Maf factor and promotes higher levels of transcription. It is unlikely that the p45·Maf dimer plays a similar role in *ho-1* gene regulation in IRPTCs. Normal expression of p45 is limited to the erythroid lineage cells (8), and we did not detect this protein in antibody supershift EMSA assays (data not shown). On the other hand, activator protein-1 (Fos/Jun) proteins may function in this capacity since these proteins were identified as StRE-BPs in unstimulated IRPTCs. Interestingly, Fos/Jun factors did not bind to the *trx* ARE either in unstimulated or heme-stimulated K562 cells, pointing to mechanistic differences between *ho-1* and *trx* gene activation by heme.

The mechanism described here differs from that proposed for *trx* gene activation in an even more fundamental respect, namely the process by which heme modulates Nrf2 activity. The prevailing model for the regulation of Nrf2 function stipulates that, under normal conditions, Nrf2 exists in an inactive, cytoplasm-localized state, in part or fully as a consequence of binding to the cytoskeleton-associated protein Keap1 (25, 30). On cellular stimulation by xenobiotics, electrophiles, or oxidative stress-generating agents, the cytoplasmic-retention mechanism is inactivated, and Nrf2 is transported to the nucleus by an as yet uncharacterized mechanism(s) but one that, under certain circumstances, may involve protein kinase C-mediated phosphorylation of Nrf2 (22). In the nucleus, Nrf2 dimerizes with other bZIP factors, including Jun (52), ATF4 (19), and small Maf proteins (23, 33), and the resulting heterodimers bind to response elements to regulate target gene transcription.

In line with the above model, Kim et al. (29) have proposed that heme stimulates *trx* gene activity by promoting transport of Nrf2 from the cytoplasm to the nucleus. Ideally, for such regulated nuclear transport, and as documented for other transcription factors (26, 45, 47), one would expect a decay in the level of cytoplasmic Nrf2 concomitant with the increase in nuclear Nrf2. In our studies, however, we have been unable to detect any cytoplasmic Nrf2 in unstimulated or stimulated IRPTCs, making it difficult to implicate the Keap1-dependent pathway in regulation of Nrf2 activity by heme. Indeed, our data are more supportive of a mechanism in which Nrf2 is transported into the nucleus by a constitutive rather than a regulated transport pathway, and heme (and other stimuli) enhances Nrf2 activity by promoting Nrf2 protein stabilization.

Reconciliation of these divergent mechanisms will require additional experimentation, but one possibility is readily obvious. The functional operation of the regulated, subcellular trafficking mechanism should be critically dependent on the relative levels of Keap1 and Nrf2, which are also likely to vary in a cell type-dependent manner. In some cells, Keap1 may be expressed at extremely low levels, and the majority of the Nrf2 will be constitutively transported into the nucleus and not detected in the cytoplasmic fraction. In such cells, stimulation of Nrf2 activity will result primarily from inducer-dependent regulation of Nrf2 turnover, as described in this report. In IRPTCs, we detect very low levels of Keap1 mRNA; measurement of the amount of Keap1 protein, however, has not been possible because of the lack of anti-Keap1 antibodies (data not shown). In cells expressing higher levels of Keap1, Nrf2 will associate with Keap1 and presumably bypass the degradation apparatus, resulting in detectable levels of cytoplasmic Nrf2, as found in K562 (29) and other cells (22, 34). In such cells, it is likely that both nuclear transport and inhibition of protein degradation contribute to the overall induction of Nrf2 activity in response to stimuli.

The findings derived from the mammalian one-hybrid and two-hybrid assays are consistent with the view that heme does not modulate the transcription potential of Nrf2 or its rate of dimerization with MafG. In this regard, we point out that the lack of activation of the Gdbd-Nrf2 fusion by heme is not necessarily at odds with a mechanism in which heme promotes stability of Nrf2, the latter mechanism possibly leading to the expectation that heme would also stimulate stabilization of the fusion protein. For example, it is quite tenable that stabilization of Nrf2 requires a protein present in sufficiently limiting amounts such that its phenotypic effects may not be observed in transient transfection assays where the exogenous protein (e.g., Gdbd-Nrf2) is expressed at artificially high concentrations per transfected cell. Alternatively, or additionally, stabilization of Nrf2 is likely to involve specific *cis*-acting structural signals or domains (21) that may be masked or become inoperative on fusion to the Gdbd. Resolution of this issue requires further characterization of the mechanism of Nrf2 stabilization, the latter residing beyond the scope of the present study.

In summary, our studies provide, to the best of our knowledge, the first mechanistic analysis of heme-mediated *ho-1* gene activation in renal epithelial cells, in the course of which we have identified the key components of this regulatory circuit, namely the StREs and transcription factor Nrf2. In addition, we have uncovered a novel system, heme-dependent stabilization of Nrf2 protein, for regulation of Nrf2 activity; notably, Nrf2 function is regulated primarily by posttranslational processes, both at the level of subcellular compartmentalization and protein turnover. Our findings add to the growing appreciation of the relevance of Nrf2 to mechanisms of renal injury, and, in this regard, it is germane that Nrf2-deficient female mice develop lupus-like autoimmune nephritis (54). In light of our prior observations demonstrating the exaggeration of renal inflammation in stressed *ho-1*$^{-/-}$ mutant mice (42) and our present observations attesting to induction of HO-1 via Nrf2-dependent pathways, we speculate that the development of lupus-like autoimmune nephritis in Nrf2-deficient mice reflects, at least in part, an inability to induce HO-1, and thereby restrain inflammatory responses in the kidney.

We thank Margaret Overstreet for assistance in preparation of the manuscript.

These studies were funded by Public Health Service National Institutes of Health Grants DK-47060, HL-55552, HL-48455, DK-58950, and DK-43135.

REFERENCES

1. **Aft RL and Mueller GC.** Hemin-mediated DNA strand scission. *J Biol Chem* 258: 12069–12072, 1983.
2. **Aft RL and Mueller GC.** Hemin-mediated oxidative degradation of proteins. *J Biol Chem* 259: 301–305, 1984.
3. **Ahn S, Olive M, Aggarwal S, Krylov D, Ginty DD, and Vinson C.** A dominant-negative inhibitor of CREB reveals that it is a general mediator of stimulus-dependent transcription of c-*fos*. *Mol Cell Biol* 18: 967–977, 1998.
4. **Alam J.** Functional analysis of the heme oxygenase-1 gene promoter. In: *Current Protocols in Toxicology*, edited by Maines MD, Costa LG, Reed DJ, Sassa S, and Sipes IG. New York: Wiley, 2000, p. 9.7.1–9.7.21.
5. **Alam J, Cai J, and Smith A.** Isolation and characterization of the mouse heme oxygenase-1 gene. Distal 5′ sequences are required for induction by heme or heavy metals. *J Biol Chem* 269: 1001–1009, 1994.
6. **Alam J, Stewart D, Touchard C, Boinapally S, Choi AM, and Cook JL.** Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. *J Biol Chem* 274: 26071–26078, 1999.
7. **Alam J, Wicks C, Stewart D, Gong P, Touchard C, Otterbein S, Choi AM, Burow ME, and Tou J.** Mechanism of heme oxygenase-1 gene activation by cadmium in MCF-7 mammary epithelial cells. Role of p38 kinase and Nrf2 transcription factor. *J Biol Chem* 275: 27694–27702, 2000.
8. **Andrews NC, Erdjument-Bromage H, Davidson MB, Tempst P, and Orkin SH.** Erythroid transcription factor NF-E2 is a haematopoietic-specific basic-leucine zipper protein. *Nature* 362: 722–728, 1993.
9. **Balla G, Jacob HS, Eaton JW, Belcher JD, and Vercellotti GM.** Hemin: a possible physiological mediator of low density lipoprotein oxidation and endothelial injury. *Arterioscler Thromb* 11: 1700–1711, 1991.
10. **Balla G, Vercellotti GM, Muller-Eberhard U, Eaton J, and Jacob HS.** Exposure of endothelial cells to free heme potenti-

ates damage mediated by granulocytes and toxic oxygen species. *Lab Invest* 64: 648–655, 1991.
11. **Balla J, Jacob HS, Balla G, Nath K, Eaton JW, and Vercellotti GM.** Endothelial-cell heme uptake from heme proteins: induction of sensitization and desensitization to oxidant damage. *Proc Natl Acad Sci USA* 90: 9285–9289, 1993.
12. **Chomczynski P and Sacchi N.** Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156–159, 1987.
13. **Dhakshinamoorthy S and Jaiswal AK.** Functional characterization and role of INrf2 in antioxidant response element-mediated expression and antioxidant induction of NAD(P)H: quinone oxidoreductase1 gene. *Oncogene* 20: 3906–3917, 2001.
14. **Dhakshinamoorthy S, Long DJ II, and Jaiswal AK.** Antioxidant regulation of genes encoding enzymes that detoxify xenobiotics and carcinogens. *Curr Top Cell Regul* 36: 201–216, 2000.
15. **Gong P, Hu B, Stewart D, Ellerbe M, Figueroa YG, Blank V, Beckman BS, and Alam J.** Cobalt induces heme oxygenase-1 expression by a hypoxia-inducible factor-independent mechanism in Chinese hamster ovary cells: regulation by Nrf2 and MafG transcription factors. *J Biol Chem* 276: 27018–27025, 2001.
16. **Gong P, Ogra Y, and Koizumi S.** Inhibitory effects of heavy metals on transcription factor Sp1. *Ind Health* 38: 224–227, 2000.
17. **Gong P, Stewart D, Hu B, Li N, Cook J, Nel A, and Alam J.** Activation of the mouse heme oxygenase-1 gene by 15-deoxy-delta12,14-prostaglandin j2 is mediated by the stress response elements and transcription factor nrf2. *Antioxid Redox Signal* 4: 249–257, 2002.
18. **Hai T, Wolfgang CD, Marsee DK, Allen AE, and Sivaprasad U.** ATF3 and stress responses. *Gene Expr* 7: 321–335, 1999.
19. **He CH, Gong P, Hu B, Stewart D, Choi ME, Choi AM, and Alam J.** Identification of activating transcription factor 4 (ATF4) as an Nrf2-interacting protein. Implication for heme oxygenase-1 gene regulation. *J Biol Chem* 276: 20858–20865, 2001.
20. **Hebbel RP and Eaton JW.** Pathobiology of heme interaction with the erythrocyte membrane. *Semin Hematol* 26: 136–149, 1989.
21. **Hershko A and Ciechanover A.** The ubiquitin system. *Annu Rev Biochem* 67: 425–479, 1998.
22. **Huang HC, Nguyen T, and Pickett CB.** Regulation of the antioxidant response element by protein kinase C-mediated phosphorylation of NF-E2-related factor 2. *Proc Natl Acad Sci USA* 97: 12475–12480, 2000.
23. **Itoh K, Igarashi K, Hayashi N, Nishizawa M, and Yamamoto M.** Cloning and characterization of a novel erythroid cell-derived CNC family transcription factor heterodimerizing with the small Maf family proteins. *Mol Cell Biol* 15: 4184–4193, 1995.
24. **Itoh K, Ishii T, Wakabayashi N, and Yamamoto M.** Regulatory mechanisms of cellular response to oxidative stress. *Free Radic Res* 31: 319–324, 1999.
25. **Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, and Yamamoto M.** Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev* 13: 76–86, 1999.
26. **Kallio PJ, Okamoto K, O'Brien S, Carrero P, Makino Y, Tanaka H, and Poellinger L.** Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1α. *EMBO J* 17: 6573–6586, 1998.
27. **Karin M, Liu Z, and Zandi E.** AP-1 function and regulation. *Curr Opin Cell Biol* 9: 240–246, 1997.
28. **Kataoka K, Noda M, and Nishizawa M.** Maf nuclear oncoprotein recognizes sequences related to an AP-1 site and forms heterodimers with both Fos and Jun. *Mol Cell Biol* 14: 700–712, 1994.
29. **Kim YC, Masutani H, Yamaguchi Y, Itoh K, Yamamoto M, and Yodoi J.** Hemin-induced activation of the thioredoxin gene by Nrf2. A differential regulation of the antioxidant responsive element by a switch of its binding factors. *J Biol Chem* 276: 18399–18406, 2001.
30. **Kobayashi M, Itoh K, Suzuki T, Osanai H, Nishikawa K, Katoh Y, Takagi Y, and Yamamoto M.** Identification of the interactive interface and phylogenic conservation of the Nrf2-Keap1 system. *Genes Cells* 7: 807–820, 2002.
31. **Kotkow KJ and Orkin SH.** Dependence of globin gene expression in mouse erythroleukemia cells on the NF-E2 heterodimer. *Mol Cell Biol* 15: 4640–4647, 1995.
32. **Maines MD and Panahian N.** The heme oxygenase system and cellular defense mechanisms. Do HO-1 and HO-2 have different functions? *Adv Exp Med Biol* 502: 249–272, 2001.
33. **Marini MG, Chan K, Casula L, Kan YW, Cao A, and Moi P.** hMAF, a small human transcription factor that heterodimerizes specifically with Nrf1 and Nrf2. *J Biol Chem* 272: 16490–16497, 1997.
34. **Morimitsu Y, Nakagawa Y, Hayashi K, Fujii H, Kumagai T, Nakamura Y, Osawa T, Horio F, Itoh K, Iida K, Yamamoto M, and Uchida K.** A sulforaphane analog that potently activates the Nrf2-dependent detoxification pathway. *J Biol Chem* 277: 3456–3463, 2002.
35. **Motohashi H, Shavit JA, Igarashi K, Yamamoto M, and Engel JD.** The world according to Maf. *Nucleic Acids Res* 25: 2953–2959, 1997.
36. **Muller-Eberhard U and Fraig M.** Bioactivity of heme and its containment. *Am J Hematol* 42: 59–62, 1993.
37. **Murata M, Gong P, Suzuki K, and Koizumi S.** Differential metal response and regulation of human heavy metal-inducible genes. *J Cell Physiol* 180: 105–113, 1999.
38. **Nath KA, Balla G, Vercellotti GM, Balla J, Jacob HS, Levitt MD, and Rosenberg ME.** Induction of heme oxygenase is a rapid, protective response in rhabdomyolysis in the rat. *J Clin Invest* 90: 267–270, 1992.
39. **Nath KA, Balla J, Croatt AJ, and Vercellotti GM.** Heme protein-mediated renal injury: a protective role for 21-aminosteroids in vitro and in vivo. *Kidney Int* 47: 592–602, 1995.
40. **Nath KA, Grande JP, Croatt AJ, Likely S, Hebbel RP, and Enright H.** Intracellular targets in heme protein-induced renal injury. *Kidney Int* 53: 100–111, 1998.
41. **Nath KA, Haggard JJ, Croatt AJ, Grande JP, Poss KD, and Alam J.** The indispensability of heme oxygenase-1 in protecting against acute heme protein-induced toxicity in vivo. *Am J Pathol* 156: 1527–1535, 2000.
42. **Nath KA, Vercellotti GM, Grande JP, Miyoshi H, Paya CV, Manivel JC, Haggard JJ, Croatt AJ, Payne WD, and Alam J.** Heme protein-induced chronic renal inflammation: suppressive effect of induced heme oxygenase-1. *Kidney Int* 59: 106–117, 2001.
43. **Ogawa K, Sun J, Taketani S, Nakajima O, Nishitani C, Sassa S, Hayashi N, Yamamoto M, Shibahara S, Fujita H, and Igarashi K.** Heme mediates derepression of Maf recognition element through direct binding to transcription repressor Bach1. *EMBO J* 20: 2835–2843, 2001.
44. **Olive M, Krylov D, Echlin DR, Gardner K, Taparowsky E, and Vinson C.** A dominant negative to activation protein-1 (AP1) that abolishes DNA binding and inhibits oncogenesis. *J Biol Chem* 272: 18586–18594, 1997.
45. **Roberts BJ and Whitelaw ML.** Degradation of the basic helix-loop-helix/Per-ARNT-Sim homology domain dioxin receptor via the ubiquitin/proteasome pathway. *J Biol Chem* 274: 36351–36356, 1999.
46. **Sekhar KR, Spitz DR, Harris S, Nguyen TT, Meredith MJ, Holt JT, Guis D, Marnett LJ, Summar ML, and Freeman ML.** Redox-sensitive interaction between KIAA0132 and Nrf2 mediates indomethacin-induced expression of γ-glutamylcysteine synthetase. *Free Radic Biol Med* 32: 650–662, 2002.
47. **Shuai K, Stark GR, Kerr IM, and Darnell JE Jr.** A single phosphotyrosine residue of Stat91 required for gene activation by interferon-γ. *Science* 261: 1744–1746, 1993.
48. **Stocker R.** Induction of haem oxygenase as a defence against oxidative stress. *Free Radic Res Commun* 9: 101–112, 1990.
49. **Stocker R, McDonagh AF, Glazer AN, and Ames BN.** Antioxidant activities of bile pigments: biliverdin and bilirubin. *Methods Enzymol* 186: 301–309, 1990.

50. **Sun J, Hoshino H, Takaku K, Nakajima O, Muto A, Suzuki H, Tashiro S, Takahashi S, Shibahara S, Alam J, Taketo MM, Yamamoto M, and Igarashi K.** Hemoprotein Bach1 regulates enhancer availability of heme oxygenase-1 gene. *EMBO J* 21: 5216–5224, 2002.
51. **Tang SS, Jung F, Diamant D, Brown D, Bachinsky D, Hellman P, and Ingelfinger JR.** Temperature-sensitive SV40 immortalized rat proximal tubule cell line has functional renin-angiotensin system. *Am J Physiol Renal Fluid Electrolyte Physiol* 268: F435–F446, 1995.
52. **Venugopal R and Jaiswal AK.** Nrf2 and Nrf1 in association with Jun proteins regulate antioxidant response element-mediated expression and coordinated induction of genes encoding detoxifying enzymes. *Oncogene* 17: 3145–3156, 1998.
53. **Vogt BA, Alam J, Croatt AJ, Vercellotti GM, and Nath KA.** Acquired resistance to acute oxidative stress. Possible role of heme oxygenase and ferritin. *Lab Invest* 72: 474–483, 1995.
54. **Yoh K, Itoh K, Enomoto A, Hirayama A, Yamaguchi N, Kobayashi M, Morito N, Koyama A, Yamamoto M, and Takahashi S.** Nrf2-deficient female mice develop lupus-like autoimmune nephritis. *Kidney Int* 60: 1343–1353, 2001.
55. **Zipper LM and Mulcahy RT.** The Keap1 BTB/POZ dimerization function is required to sequester Nrf2 in cytoplasm. *J Biol Chem* 277: 36544–36552, 2002.