Role of Bach-1 in Regulation of Heme Oxygenase-1 in Human Liver Cells

INSIGHTS FROM STUDIES WITH SMALL INTERFERING RNAs*

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Ying Shan‡§§, Richard W. Lambrecht§§∥, Tahereh Ghaziani‡§§, Susan E. Donohue‡§§, and Herbert L. Bonkovsky‡§§

From the ‡Departments of Medicine and ∥Pharmacology and the ¶Liver-Biliary-Pancreatic Center and the General Clinical Research Center of the University of Connecticut Health Center, Farmington, Connecticut 06030

Heme oxygenase-1 is an antioxidant defense enzyme that converts heme to biliverdin, iron, and carbon monoxide. Bach-1 is a bZip protein that forms heterodimers with small Maf proteins and was reported recently to down-regulate the HO-1 gene in mice. Using small interfering RNAs targeted to human Bach-1 mRNA, we investigated whether modulation of human hepatic Bach-1 expression by small interfering (si)RNA technology influences heme oxygenase-1 gene expression. We found that Bach-1 siRNAs transfected into HuH-7 cells significantly reduced Bach-1 mRNA and protein levels ~80%, compared with non siRNA-treated cells. In contrast, transfection with the same amounts of nonspecific control duplexes or LaminB2-duplex did not reduce Bach-1 mRNA or protein levels, confirming the specificity of Bach-1 siRNA. Expression of the heme oxygenase-1 gene in Bach-1 siRNA-transfected cells was up-regulated 7-fold, compared with cells without Bach-1 siRNA. The effect of increasing concentrations of heme to up-regulate levels of heme oxygenase-1 was more pronounced when Bach-1 siRNA was present. Taken together, these results indicated that Bach-1 has a specific and selective ability to repress expression of human hepatic heme oxygenase-1. Silencing of Bach-1 by siRNAs is a useful method for up-regulating HO-1 gene expression. Exogenous heme produces additional up-regulation, beyond that produced by Bach-1 siRNAs, suggesting that heme does not act solely through its effects on Bach-1.

Heme oxygenase (HO, E.C. 1.14.99.3) is the rate-controlling enzyme of heme catabolism (1–5). It carries out the specific cleavage of the α-methylene bridge of the macrocycle with the liberation of one molecule of carbon monoxide, iron, and biliverdin. Recent studies (4–6) have highlighted important biological effects of these HO reaction products, which display antioxidant, anti-inflammatory, and anti-apoptotic functions. Three isoforms of HO, termed HO-1, -2, and -3, have been described (7–9). Among the three isoforms of HO, only HO-1 is highly inducible. Earlier work from our and other laboratories established that HO-1 could be up-regulated markedly by a variety of stressful stimuli, as well as by heme or certain other metalloporphyrins, particularly, cobalt protoporphyrin (10–14). The primary mechanism for up-regulation of the HO-1 gene is by increased transcription of the gene (15), and the induction by such stressors as sodium arsenite or other arsenicals (which produce a chemical oxidative stress), by transition metals, such as cadmium or cobalt, hydrogen peroxide, other reactive oxygen species, or heat shock are clearly different in mechanism from the up-regulation produced by metalloporphyrins (13, 14, 16–19). For example, earlier work from our laboratory showed that cMye/Max and upstream stimulatory factor elements in the 5′-untranslated region of the HO-1 gene played the key role in inductions by cadmium or cobalt (11). In contrast, inductions by sodium arsenite or phenylarsine oxide depend primarily upon activation of the mitogen-activated protein kinases leading to increased levels of AP-1 proteins, which bind to several AP1-consensus elements found in the HO-1 promoter (18, 20, 21). In contrast, the up-regulation of the HO-1 gene produced by heme or cobalt protoporphyrin is not mediated by these classic stress pathways or kinase cascades but rather depends upon several heme-responsive elements (and a metalloporphyrin-responsive element), which are distinct from other consensus promoter elements, found in the 5′-untranslated region of rodent, human, and avian HO-1 (12).

Further progress in understanding the molecular mechanisms that underlie the up-regulation of the HO-1 gene by heme has come from the characterization of Bach-1, one of the family of leucine b-Zip proteins. Bach-1 was isolated as a brown protein and was shown to contain heme bound to multiple cysteine-proline motifs (22–24). It is highly conserved, and Bach-1 proteins have now been described in avian through mammalian species. Recent work of Igarashi and others (22–26) has been interpreted to indicate that Bach-1, under baseline conditions, forms heterodimers with small proteins of the Maf family, and these heterodimers repress transcription of the HO-1 gene by binding to the heme-responsive elements (HeRE) in the 5′-untranslated region of the HO-1 promoter. Under conditions of excess heme, increased binding of heme to Bach-1 leads to a conformational change and a decrease in DNA-binding activity. This permits other Maf-Maf, Nrf2-Maf, and other activating heterodimers to occupy the HeRE sites in the HO-1 promoter and leads to increased transcription and up-regulation of expression of the gene (24–26). An additional or alternative mechanism for the heme effect, namely, a stabilization of Nrf2 proteins, which are positive transcriptional regulators of HO-1, has recently been proposed by Alam et al. (27).
bacterial based upon studies with immortalized proximal tubular epithelial cells.

The purpose of the work described in this paper was to investigate whether down-regulation of Bach-1 expression in human hepatocytes would lead to up-regulation of the HO-1 gene. We chose small interfering RNAs (siRNA) directed at Bach-1 as a convenient means to down-regulate the Bach-1 gene. A portion of this work has been presented in abstract form (28).

EXPERIMENTAL PROCEDURES

Materials—The human hepatoma cell line, Huh-7, was purchased from the Japan Health Research Resources Bank (Osaka, Japan). Ferric (Fe^{3+})–protoporphyrin IX·Cl· (heme) was from Porphyrin Products (Logan, UT). Dimethyl sulfoxide (Me2SO) was purchased from Fisher-Biotech (Fair Lawn, NJ). RNAzol was from Biotex (Houston, TX). Goat anti-HO-1, anti-β-actin polyclonal antibodies, and rabbit anti-goat IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-HO-1 monoclonal antibody (OSA-111) was purchased from Stressgen (Victoria, Canada). Rabbit anti-mouse IgG and ECL-Plus were purchased from Amersham Biosciences. Nonspecific control duplexes-XIII and LaminB2 duplex were purchased from Dharmacon (Lafayette, CO).

Cell Cultures and Preparation of Chemicals—Huh-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% (v/v) fetal bovine serum (Invitrogen) and were routinely passaged twice a week. Heme was dissolved in Me2SO and stored at −20 °C; it was further diluted with 1% human serum albumin in 40 mM Tris-HCl (pH 7.4) just before use.

Bach-1 siRNAs Preparation and Transfection—We used Bach-1 siRNAs, targeting the following positions of the human Bach-1 mRNA (Accession Number NM_001186), 331–349, 573–591, 1673–1691, and 2296–2314. The forward sequences of Bach-1-duplex siRNAs were

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\text{Bach-1 antisense primer, 5'-TCGATCGTGCTTCGGAATGCGAAGTGA}
\]

\[
\text{HO-2 sense primer, 5'-AGTGGTAGGACCCATCGGGAAGA; glyceraldehyde-3-phosphate dehydrogenase sense primer, 5'-TTGTTGCCATCAATGACCC; glyceraldehyde-3-phosphate dehydrogenase antisense primer, 5'-TGAATGTCAGCGGAAGTGGA}.
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**Fig. 1.** Effect of Bach-1 siRNA on Bach-1 mRNA and protein levels in Huh-7 cells. Huh-7 cells were transfected with 0–200 nM Bach-1 siRNA as shown and cultured for 24 or 72 h, after which cells were harvested, and mRNA and protein were quantified, as described under “Experimental Procedures.” A, results for Bach-1 siRNA levels at 24 and 72 h. B, results for Bach-1 protein isolated from the same cells as in A. Data are presented as means ± S.E. from three independent experiments. *, differs from no Bach-1 siRNA, p < 0.05; **, differs from no Bach-1 siRNA, p < 0.01.
protein, after 4 h treatment, was 2-fold. A concentration of heme that led to detectable up-regulation of HO-1 gene expression in primary cultures of chick embryo liver cells (29, 30). Recently, we identified four HeREs and one metalloporphyrin response element within the HO-1 promoter region that mediated heme-dependent activation of the HO-1 gene in chick LMH cells (12, 13). To characterize the mechanism whereby heme up-regulates HO-1 gene expression in human liver cells, we first examined HO-1 mRNA and protein levels after treatment with heme in Huh-7 cells. As expected, heme induced HO-1 mRNA and protein levels in a time-related and dose-dependent fashion (data not shown). HO-1 mRNA levels started to increase after 10 μM heme treatment for 2 h, reached a peak (25-fold increase) at 5–10 μM heme treatment for 4 h, and the maximum was reached at 5–10 μM heme treatment for 4 h. A concentration of heme as low as 1 μM significantly up-regulated HO-1 mRNA by 18-fold, and the maximum was reached at 5–10 μM heme treatment for 4 h. The lowest concentration of heme that led to detectable up-regulation of HO-1 protein, after 4 h of treatment, was 2 μM. Thus, low concentrations of heme produce marked up-regulation of the HO-1 gene in human Huh-7 cells, as in numerous other cell types (13, 27, 31–34). As previously demonstrated in chick embryo liver cells (15, 30, 35), the marked up-regulation is relatively short-lived, although a significant (~5-fold) up-regulation persists for at least 24 h.

**RESULTS AND DISCUSSION**

**Heme-mediated Induction of HO-1 Gene Expression in Human Liver Cells**—Heme is a potent inducer of HO-1 in many cell types and systems. We have previously demonstrated heme-dependent inductions of HO-1 gene expression in primary cultures of chick embryo liver cells (29, 30). Recently, we identified four HeREs and one metalloporphyrin response element within the HO-1 promoter region that mediated heme-dependent activation of the HO-1 gene in chick LMH cells (12, 13). To characterize the mechanism whereby heme up-regulates HO-1 gene expression in human liver cells, we first examined HO-1 mRNA and protein levels after treatment with heme in Huh-7 cells. As expected, heme induced HO-1 mRNA and protein levels in a time-related and dose-dependent fashion (data not shown). HO-1 mRNA levels started to increase after 10 μM heme treatment for 2 h, reached a peak (~25-fold increase) at 4–6 h, and were lower but still significantly increased (~5-fold) after 16 h. A concentration of heme as low as 1 μM significantly up-regulated HO-1 mRNA by 18-fold, and the maximum was reached at 5–10 μM heme treatment for 4 h. A concentration of heme as low as 1 μM significantly up-regulated HO-1 mRNA by 18-fold, and the maximum was reached at 5–10 μM heme treatment for 4 h. A concentration of heme as low as 1 μM significantly up-regulated HO-1 mRNA by 18-fold, and the maximum was reached at 5–10 μM heme treatment for 4 h. A concentration of heme as low as 1 μM significantly up-regulated HO-1 mRNA by 18-fold, and the maximum was reached at 5–10 μM heme treatment for 4 h. A concentration of heme as low as 1 μM significantly up-regulated HO-1 mRNA by 18-fold, and the maximum was reached at 5–10 μM heme treatment for 4 h. A concentra-
Measuring Bach-1 mRNA concentrations in Bach-1 siRNA-transfected cells using quantitative RT-PCR, we found 100–200 nM Bach-1 siRNA at 72 h could reduce Bach-1 mRNA levels by 70% when compared with non-Bach-1 siRNA treated cells (Fig. 1 A). We next measured Bach-1 protein concentrations in Bach-1 siRNA-transfected cells using Western blots. We found that 100–200 nM of Bach-1 siRNA reduced Bach-1 protein up to 80% at 72 h when compared with non-Bach-1 siRNA-transfected cells (Fig. 1 B). To confirm the specificity of silencing the Bach-1 gene by Bach-1 siRNAs, we first tested 20–100 nM LaminB2 duplex silencing LaminB2 mRNA level by quantitative RT-PCR in Huh-7 cells. The results showed that transfected 25 nM LaminB2 siRNA for 72 h reduced by 80% LaminB2 mRNA levels. We further tested Bach-1 gene expression from the cells transfected with non-Bach-1-related siRNA, e.g. non-specific control duplexes and LaminB2 duplex. We found that there were no significant reductions of either Bach-1 mRNA or protein levels in the cells transfected with 25 and 100 nM nonspecific control duplexes and LaminB2 duplex (data shown 100 nM) when compared with cells that were not transfected (Fig. 2). Thus, we demonstrated that silencing of the human Bach-1 gene using siRNA-targeted Bach-1 mRNA was effective, specific, and selective.

Induction of HO-1 mRNA Is Associated with Reduced Expression of Bach-1 Gene Caused by Bach-1 siRNA—The transcription repressor Bach-1 forms heterodimers with one of the small Maf proteins (i.e. MafK, MafF, or MafG) that bind to the Maf recognition element and suppresses expression of genes that respond to Maf-containing heterodimers (24, 25). Recently, Sun et al. (24) showed that HO-1 is constitutively expressed at higher levels in many tissues of Bach-1-deficient mice, suggesting that Bach-1 acts as a negative regulator of transcription of the mouse HO-1 gene. We analyzed the effect of Bach-1 on expression of human HO-1 by silencing the Bach-1 gene in Huh-7 cells. Bach-1 siRNA significantly increased HO-1 mRNA levels in Huh-7 cells in a dose-dependent and time-related fashion (Fig. 3, A and B). The levels of HO-1 mRNA were significantly increased after exposure to 50 nM Bach-1 siRNA and reached a maximum level at 200 nM (7.5-fold). Induction of HO-1 mRNA expression was significant at 24 h after exposure to 100 nM Bach-1 siRNA and reached a maximum by 48 h. The expression levels of HO-1 mRNA remained increased for 72 h, and this was consistently associated with the repression of Bach-1 mRNA expression. To explore whether the increased HO-1 gene expression in the presence of Bach-1 siRNA represented a general response, we measured the levels of HO-2 and ALAS-1 mRNA in the same Bach-1 siRNA-treated cells. Bach-1 siRNA did not affect HO-2 (Fig. 3 C) or ALAS-1 (Fig. 3 D) mRNA levels in Huh-7 cells. Thus, these results demonstrated that Bach-1 is a repressor of the HO-1 gene in human hepatic cells, consistent with the results of Sun et al. (24) in mice.

Regulation of HO-1 Expression by Heme and Bach-1 siRNA—We next studied the effects of combinations of heme and Bach-1 siRNA on HO-1 gene regulation in Huh-7 cells. Before testing HO-1, we first confirmed Bach-1 mRNA was repressed by more than 50% by Bach-1 siRNA. HO-1 mRNA
expression levels were markedly up-regulated by treatment with a lower concentration of heme in the presence of Bach-1 siRNA and were greater for all of the heme concentrations tested (0–10 μM). Using non-linear regression analysis, the HO-1 mRNA levels were correlated with heme concentrations in the presence or absence of Bach-1 siRNA ($r = 0.9$, $p < 0.0001$) (Fig. 4A). The two curves are significantly different from each other ($p < 0.001$) implying an additive effect of Bach-1 siRNA and heme on the up-regulation of HO-1 gene expression. As expected, HO-1 protein levels were consistent with HO-1 mRNA levels, in cells treated with heme in the presence or absence of Bach-1 siRNA (Fig. 4B).

Recently, Ogawa et al. (25) and Sun et al. (24) reported that Bach-1 is a heme-binding protein. Heme specifically binds to four dipeptide cysteine-proline motifs of Bach-1 and, when heme is thus bound, the DNA-binding and repressive activity of Bach-1/Maf hetero-oligomers is markedly inhibited. We identified four HeREs and one metalloporphyrin response element located in the 5′-untranslated region of the chick HO-1 promoter region (39). By sequence analysis, we found there are four homologous HeRE sites, but not a metalloporphyrin response element site, in the human HO-1 promoter region. Our results are consistent with the idea that the heme-dependent up-regulation of HO-1 is due, at least in part, to a Bach-1/heme interaction, which allows Maf/Maf recognition elements (HeREs) to regulate the gene.

However, the precise mode of action whereby heme up-regulates HO-1, and the identity of the binding proteins involved remains imperfectly understood. An alternative hypothesis is that heme increases the stability of the Nrf2 protein (27) leading to accumulation of heterodimers of Nrf2/MafG that bind to the HeREs, activating the HO-1 gene. Our results showed that exogenous heme produces an additional up-regulation of HO-1, beyond that produced by Bach-1 siRNAs, suggesting that heme does not act solely through its effects on Bach-1, which is consistent with this alternative hypothesis. It seems likely that the heme-mediated induction of HO-1 is a function of both Bach-1 (via heme binding with subsequent derepression of HO-1 gene expression) and of Nrf2 (via a heme-mediated increase in stability of Nrf2 with subsequent induction of HO-1 gene expression).

In summary, heme markedly induced HO-1 mRNA and protein levels in a time-related and dose-dependent fashion in human Huh-7 cells. Using siRNA technology, we specifically silenced ~80% of the human Bach-1 gene by using four siRNAs, targeted to different locations of the human Bach-1 mRNA. Bach-1 has a specific and selective effect to repress the expression of human hepatic HO-1. The silencing of Bach-1 by siRNAs is a useful method for up-regulating HO-1 gene expression in human hepatocytes. Exogenous heme produces additional up-regulation beyond that produced by Bach-1 siRNAs, suggesting that heme does not act solely through its effects on Bach-1.

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FIG. 4. Effects of heme and Bach-1 siRNAs on HO-1 mRNA and protein levels in Huh-7 cells. Huh-7 cells were cultured in 12-well plates for 1 day. Some cells were transfected with 100 nM of Bach-1 siRNA for 72 h, and some were treated with indicated concentrations of heme for 4 h. Cells were harvested, and total RNA and protein were isolated. HO-1 mRNA was quantified by quantitative RT-PCR, and HO-1 protein was detected by Western blotting, as described under “Experimental Procedures.” A, non-linear regression analyses of HO-1 mRNA levels from three independent experiments. B, Western blots of HO-1 protein isolated from the same cells as in A. Proteins (50 μg/lane) were separated by 4–15% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with anti-human HO-1- and β-actin-specific antibodies.
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