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Original Contribution

Catalytic inactive heme oxygenase-1 protein regulates its own expression in oxidative stress

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Abstract

Heme oxygenase-1 (HO-1) catalyzes the degradation of heme and forms antioxidant bile pigments as well as the signaling molecule carbon monoxide. HO-1 is inducible in response to a variety of chemical and physical stress conditions to function as a cytoprotective molecule. Therefore, it is important to maintain the basal level of HO-1 expression even when substrate availability is limited. We hypothesized that the HO-1 protein itself could regulate its own expression in a positive feedback manner, and that this positive feedback was important in the HO-1 gene induction in response to oxidative stress. In cultured NIH 3T3 cells, transfection of HO-1 cDNA or intracellular delivery of pure HO-1 protein resulted in activation of a 15-kb HO-1 promoter upstream of luciferase as visualized by bioluminescent technology and increased HO-1 mRNA and protein levels. These effects were independent of HO activity because an enzymatically inactive mutant form of HO-1 similarly activated the HO-1 promoter and incubation with HO inhibitor metalloporphyrin SnPP did not affect the promoter activation. In addition, HO-1-specific siRNA significantly reduced hemin and cadmium chloride-mediated HO-1 induction. Furthermore, deletion analyses demonstrated that the E1 and E2 distal enhancers of the HO-1 promoter are required for this HO-1 autoregulation. These experiments document feed-forward autoregulation of HO-1 in oxidative stress and suggest that HO-1 protein has a role in the induction process. We speculate that this mechanism may be useful for maintaining HO-1 expression when substrate is limited and may also serve to up-regulate other genes to promote cytoprotection and to modulate cell proliferation.

Keywords: Heme oxygenase-1; Transcription regulation; Protein delivery; Promoter; siRNA; Cell proliferation

Introduction

Heme oxygenase (HO) is the rate-limiting enzyme in heme degradation to biliverdin. This reaction generates equimolar concentrations of ferrous iron, biliverdin, and carbon monoxide (CO). The inducible form, HO-1, is regulated by various stimuli such as heavy metals, inflammation, UV radiation, and oxidative stress [1–4]. Many have suggested that HO-1 mediates its cytoprotective effects via the degradation of heme and the formation of the reaction by-products; however, when substrate availability is limited, the cytoprotective functions of HO are difficult to explain and the induction of HO-1 should not occur yet it does. Enzymatically inactive HO-1 mutant protein is cytoprotective against hydrogen peroxide-induced oxidative stress [5]. Furthermore, transfection of a cDNA encoding for an inactive HO-1 mutant protein increases catalase gene expression, suggesting a regulatory role for the HO-1 protein itself [5]. Recent evidence documents nuclear localization of a truncated form of the HO-1 protein after cleavage of the membrane-bound C-terminus [6]. Nuclear extracts enriched with this form of HO-1 protein were devoid of normal HO activity. This further suggests

Abbreviations: CO, Carbon monoxide; DE, Distal enhancer; DMEM, Dulbecco's minimal essential medium; FBS, Fetal bovine serum; HO, Heme oxygenase; PCR, Polymerase chain reaction; SnPP, Tin protoporphyrin.

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that there is a role for the HO-1 protein itself in nuclear functions. Because HO-1 is readily inducible even when limited substrate is present, we hypothesized that HO-1 protein could serve to regulate its own expression. Recent studies showed that non-heme-induced HO-1 does not alter cellular iron metabolism, further suggesting nonenzymatic roles of HO-1 in cytoprotection [7]. Autoregulation has been demonstrated in various examples [8,9] where this leads to amplification or maintenance of gene function. Kravets et al. have also reported that biliverdin reductase (BVR), which catalyzes reduction of the HO activity product, biliverdin, to bilirubin, can induce the expression of HO-1 protein, suggesting that various components of the bilirubin system could regulate the expression of genes in the pathway [10].

Previous studies in rodents have identified key regulatory regions within the genomic sequence upstream of the HO-1 gene. Distal enhancer (DE) 1 and DE2 located at −4 and −10 kb upstream of the transcription start site, respectively, are critical for HO-1 induction by most stimuli, including heme, heavy metals, and hydrogen peroxide [11,12]. Both DE1 and DE2 regions contain multiple stress-responsive elements (StRE) that represent binding sites of regulatory proteins such as AP-1, Jun, CREB, Maf, and the Cap'n'collar/basic-leucine zipper (CNC-bZIP) transcription factors. Among the CNC-bZIP proteins, the NF-E2-related factor-2 (Nrf2) functions as a transcriptional activator of HO-1 promoter whereas Bach-1 serves as negative regulator of HO-1 transcription [13–15]. Several binding partners have been reported to interact with Nrf2 and Bach-1 in response to different cellular stimulations. It is conceivable that transcription regulatory protein complexes composed of multiple factors are crucial to the regulation of HO-1 expression. MAPK signaling pathways have been shown to be activated during HO-1 inductions, including after ischemia-reperfusion lung injury [16]. It is important to test whether the MAPK pathways, including p38 and ERK signaling, are involved in the HO-1 self-regulation.

In this study, we evaluated whether transfection with HO-1 cDNA or intracytoplasmic delivery of HO-1 protein could modulate the activation of a 15-kb mouse HO-1 promoter upstream of luciferase. By comparing the effects of a wild-type (active) and inactive mutant HO-1 protein, we determined whether HO enzymatic activity was required to mediate these effects. We also assessed the role of HO-1 protein on enhancing HO-1 gene transcription resulting from common HO-1 inducers such as hemin and cadmium chloride. Furthermore, we have identified the genomic regions in the HO-1 promoter that respond to HO-1 protein and the essential domains within the HO-1 protein required for the self-activation. Lastly, we examined the activation of MAPK signaling pathway in the process of this HO-1 self-regulation.

Experimental procedures

Cell culture

NIH 3T3 cells were obtained from American Type Culture Collection (Manassas, VA). NIH3T3 cells stably transfected with a 15-kb HO-1 promoter-luciferase construct (3T3-HO-1/*luc* cells) were grown in Dulbecco's minimal essential medium (DMEM; Life Technologies, Rockville, MD) supplemented with 10% FBS and 1% antibiotic-antimycotic.

HO-1 cDNA constructs

Full-length rat HO-1 cDNA was amplified by PCR with primers 5′-AGGATCCAGATGGAGCGCCCACAG and 3′-GACTCGAGCCAGATCCTCTTCTGAGATG. The PCR product was cloned into the BamHI/XhoI site of pcDNA3.1/His (Invitrogen) to generate HisHO1myc construct. The HisHO1myc fragment was then subcloned into p3XFLAGCMV (Sigma) to generate HisHO1FLAG. A rat HO-1 cDNA (HisHO1mutFLAG) expressing an enzymatically inactive HO-1 with a substitution of histidine 25 to alanine was derived from the HisHO1FLAG by PCR using a site-directed mutagenesis kit from Stratagene (QuickChange II, Cat. No. 200523-4).

Transient transfection with HO-1 cDNA

Transient transfection was performed in NIH3T3 cells using Lipofectamine 2000 (Invitrogen). Briefly, 1 day before transfection, 10^5 cells were seeded in 24-well plates with antibiotic-free growth medium. DNA (0.8 μg) and 2 μl of lipofectamine 2000 reagent were diluted into 50 μl of DMEM separately. After 5 min incubation, the DNA and lipofectamine solution were mixed and incubated for additional 20 min at room temperature. The DNA/Lipofectamine complex was then added to the cells. Cells were grown for 48 h before being subjected to assays.

Intracytoplasmic delivery of HO-1 protein

A GST HO-1 fusion construct was expressed in the *Escherichia coli* strain BL21 (Invitrogen.). Bacteria were grown to an OD of 0.6–0.8. Thereafter, the fusion protein was induced with 100 μM IPTG at 30 °C. After 5 h, bacteria were harvested and sonicated for 5 min. The fusion protein was purified using a GST-purification module (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instruction. Ten micrograms of GST HO-1 protein was delivered in the 3T3-HO-1/*luc* cells using the Pro-Ject system (Pierce, Rockford, IL) as per the manufacturer. Briefly, the Pro-Ject reagent was solubized in 250 μl methanol. The methanol was evaporated overnight under a sterile hood and the dried Pro-Ject was stored at -20 °C until use. Purified HO-1 protein was diluted in PBS and added to the dried Pro-Ject reagent. After 5 min incubation, the mixture was added to the cell culture medium with 5% serum. After 3 h, cells were washed with PBS, and maintained in serum-free medium for the first 4 h of the incubation. Thereafter, 5% FBS was reincorporated. In other experiments, purified mutant HO-1 protein (His25Ala) devoid of catalytic activity (gift of Paul Ortiz de Montellano, UCSF, San Francisco, CA) was delivered to the cells as described above.

siRNA mediated gene knockdown of HO-1

Control siRNA and HO-1-specific siRNA oligos were purchased from Dharmacon, Inc. The sequences of the HO-1