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Zinc mesoporphyrin induces rapid and marked degradation of the transcription factor bach1 and up-regulates HO-1

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Abstract

Heme oxygenase 1 (HO-1) is the first and rate-controlling enzyme in heme degradation. Bach1 is a mammalian transcriptional repressor of HO-1. To understand how zinc mesoporphyrin (ZnMP) induces the expression of HO-1, we investigated the effects of ZnMP on Bach1 mRNA and protein levels in human hepatoma Huh-7 cells by quantitative RT-PCR and Western blots. We found that ZnMP markedly up-regulated HO-1 mRNA and protein levels, and rapidly and significantly decreased Bach1 protein levels by increasing degradation of Bach1 protein [half life ($t_{1/2}$) from 19 h to 45 min], whereas ZnMP did not influence Bach1 mRNA levels. The proteasome inhibitors, epoxomicin and MG132, significantly inhibited degradation of Bach1 by ZnMP in a dose-dependent fashion, indicating that the degradation of Bach1 by ZnMP is proteasome-dependent. Purified Bach1 C-terminal fragment bound heme, but there was no evidence for binding of ZnMP to the heme-binding region of Bach1. In conclusion, ZnMP produces profound post-transcriptional down-regulation of Bach1 protein levels and transcriptional up-regulation of HO-1. Our results indicate that ZnMP up-regulates HO-1 gene expression by markedly increasing Bach1 protein degradation in a proteasome-dependent manner.

Keywords: Zinc mesoporphyrin; Post-transcriptional regulation; Proteasome; Bach1; HO-1; Heme

1. Introduction

Heme oxygenase (HO) is the rate-controlling enzyme in heme degradation, generating ferrous iron, carbon monoxide and biliverdin, which have anti-oxidant and anti-inflammatory activities in vivo [1–4]. HO has two isoforms: HO-1 and HO-2. The HO-1 gene is highly inducible by chemical and physical stress [e.g., reactive oxygen species (ROS), arsenicals, transition metals, heat shock]; indeed, another name for HO-1 is heat shock protein 32 (Mr=32 kDa) [4]. HO-1 is also highly inducible by heme, its physiologic substrate, and by other selected metalloporphyrins (MePns), including cobalt protoporphyrin (CoPP) [5,6].

Bach1, a basic leucine zipper (bZip) mammalian transcriptional repressor, is a sensor and effector of heme [7,8]. Bach1 forms antagonizing heterodimers with the Maf-related oncogene family. These heterodimers bind to Maf recognition elements (MAREs) and suppress expression of genes (e.g., HO-1 and NQO1) that respond to Maf-containing heterodimers and other positive transcriptional factors [8–10]. After binding heme, Bach1 loses its DNA binding activity, after which it is exported out of nuclei [11]. Recent work has established that heme and CoPP lead to the up-regulation of the HO-1 gene by influencing intranuclear levels and binding of Bach1 and small Maf proteins to key regulatory sites of the HO-1 promoter [5,12]. These studies show that heme enters the nuclei, binds to

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CP-containing motifs of the C-terminal region of Bach1 (amino acids 417–739), and leads to the dissociation of Bach1 from a heterodimeric repressor complex with small Maf proteins [8]. A separate synergistic effect of MePns is to stabilize Nrf2, which then can bind to the small Maf proteins and enhance gene transcription [5,13].

It is well documented that zinc mesoporphyrin (ZnMP), an analogue of heme, is a competitive inhibitor of HO activity [14,15]. In previous studies, we have characterized the regulation of the HO-1 promoter activities and mRNA levels by MePns. Recently we found that ZnMP up-regulates the HO-1 promoter activity in human hepatoma Huh-7 cells, but does not significantly influence HO-1 promoter activity or mRNA levels in chick hepatoma LMH and primary cultures of chick embryo liver cells [6]. Other recent studies from our laboratory have shown that ZnMP up-regulates HO-1 mRNA and protein levels in NIH 3T3 cells (unpublished). However, little is known about the mechanism by which this occurs. To understand how ZnMP induces the expression of HO-1, in the work reported here we investigated the effects of ZnMP on Bach1 mRNA and protein levels. We found that ZnMP markedly decreases Bach1 protein levels but does not influence its mRNA levels. In addition, we expressed and purified the recombinant C-terminal heme-binding region of Bach1, containing amino acids 417–739, named Bach1C, to investigate whether ZnMP is able to bind to Bach1. Binding assays showed that ZnMP does not bind to the heme-binding region of Bach1. Proteasome inhibitors, epoxomicin and MG132, significantly block the degradation of Bach1 by ZnMP. Our results indicate that ZnMP up-regulates human HO-1 gene expression by inducing Bach1 protein degradation in a proteasome-dependent manner.

2. Materials and methods

2.1. Reagents and materials

Plasmid pCMV-Bach1 harboring the mouse Bach1 cDNA sequence was kindly provided by K. Igarashi (Tohoku University School of Medicine, Sendai, Japan). Prokaryotic expression vectors pQE-30 and pQE-40 were purchased from Qiagen (Valencia, CA). Ferric (Fe^{3+})-protoporphyrin IX●Cl (hemin), ZnMP and tin mesoporphyrin (SnMP) were purchased from Frontier Scientific (Logan, UT). TRIzol was from Invitrogen (Carlsbad, CA). Goat anti-human Bach1, goat anti-human GAPDH polyclonal antibodies, mouse anti-rabbit IgG, and rabbit anti-goat IgG were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Rabbit anti-human HO-1 polyclonal antibody was purchased from StressGen (Victoria, BC Canada). ECL-Plus was purchased from Amersham Biosciences Corp (Piscataway, NJ). Epoxomicin and MG132 were from Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide (DMSO) was purchased from Fisher Biotech (Fair Lawn, NJ). BCA protein assay reagent was from Pierce (Rockford, IL). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from HyClone (Logan, UT). Trypsin and isopropyl thio-β-D-galactopyranoside (IPTG) were obtained from Sigma-Aldrich. Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). Restriction endonucleases were purchased from either New England Biolabs (Ipswich, MA) or Invitrogen.

2.2. Cell cultures and treatments

Human hepatoma cell line, Huh-7 (Japan Health Research Resources Bank, Osaka, Japan) was maintained in DMEM supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% (v/v) FBS. ZnMP or SnMP was dissolved in DMSO and stored at −20 °C. The addition of DMSO to the cultures did not exceed 1 μL of DMSO/mL of media.

2.3. Transfection and luciferase activity assay

Plasmid construct, phHOGL3/11.6, containing −11.6 kb (11.6 kb from transcriptional start site) 5¢-flanking region of the human HO-1 promoter was a gift from Dr. A. Agarwal (University of Alabama at Birmingham, Birmingham, AL) [16]. Huh-7 cells were transfected with phHOGL3/11.6 using Lipofectamine™ 2000 from Invitrogen according to the manufacturer's protocol. Cells lysates were collected and luciferase reporter gene expression was assessed by quantitation of luciferase activity, normalized to β-galactosidase activity and protein content.

2.4. Quantitative RT-PCR

Total RNA from treated cells was extracted and cDNA was synthesized as described before [12]. Real time quantitative RT-PCR was performed using a MyiQ™ Single Color Real-Time PCR Detection System from Bio-Rad (Hercules, CA) and iQ™ SYBR Green Supermix Real-Time PCR kit (Bio-Rad). We included samples without template and without reverse transcriptase as negative controls, which were expected to produce negligible signals (Ct values>35). Standard curves of Bach1, HO-1 and GAPDH were constructed with results of parallel PCR reactions performed on serial dilutions of a standard DNA (from one of the controls). Fold-change values were calculated by comparative Ct analysis after normalizing for the quantity of GAPDH in the same samples.

2.5. Western blots

Protein preparations and Western blots were carried out as described before [12]. In brief, total proteins (25–50 μg) were separated on 4–15% gradient SDS-PAGE gels (Bio-Rad). After electrophoretic transfer onto ImmunBlot PVDF membrane (Bio-Rad), membranes were blocked for 1 h in PBS containing 5% nonfat dry milk and 0.1% Tween-20, and then incubated overnight with primary antibody at 4 °C. The dilutions of the primary antibodies were as follows: 1:2000 for anti-HO-1 antibody, and 1:1000 for anti-Bach1, and anti-GAPDH antibodies. The membranes were then incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (dilution 1:10,000). Finally, the bound antibodies were visualized with the ECL-Plus chemiluminescence system according to the manufacturer's protocol (Amersham). A Kodak 1DV3.6 computer-based imaging system (Rochester, NY) was used to measure the relative optical density of each specific band obtained after Western blotting. Data are expressed as percentages of the controls (corresponding to the value obtained with the vehicle-treated cells), which were assigned values of one.

2.6. Construction of prokaryotic expression vector pQE-Bach1C

pQE-30 vector was used to construct the prokaryotic expression vector pQE-Bach1C by subcloning the fragment containing the Bach1C gene [17]. The cDNA sequence encoding Bach1C was amplified by PCR from vector pCMV-Bach1 using a forward primer (P1: 5′-CGGGATCCTTATTTGAAAAGAA-AGTGTATCTCTC-3′) and reverse primer (P2: 5′-CCCGAGCTCATCAAAT-GAAGGGGCCGCACACTGAGG-3′), in which Bam HI and Hind III sites exist at the ends of Bach1C cDNA. PCR conditions consisted of 30 cycles of 94 °C for 0.5 min, 55 °C for 0.5 min, and 72 °C for 1.5 min. The PCR-amplified DNA fragment was separated on 1% agarose gel containing ethidium bromide and visualized by UV-light illumination. The amplified cDNA fragment was inserted into vector pQE-30 to construct prokaryotic expression vector pQE-Bach1C and this was verified by sequencing.

2.7. Expression and identification of recombinant Bach1C (417–739) in E. coli M15

E. coli M15 was cultured in LB medium for approximately 2 h until the cells reached an A_{600} of 0.6. Subsequently, recombinant Bach1C expression was induced by addition of IPTG to a final concentration of 1.0 mM and then incubating the cells for an additional 4 h at 37 °C [18]. Cells were finally