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Hemopexin-dependent heme uptake via endocytosis regulates the Bach1 transcription repressor and heme oxygenase gene activation

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ABSTRACT

Background: Intracellular heme plays versatile roles in a variety of physiological processes including mitochondrial respiration. Heme also induces the expression of genes such as heme oxygenase-1 (HO-1) by inactivating the transcription repressor Bach1 through direct binding. However, the source of heme for the regulation of the Bach1–HO-1 axis has been unclear. Considering that extracellular heme exists as a complex with hemopexin (Hx) in serum under the physiological conditions, heme–Hx complex may deliver heme for the gene regulation.
Methods: Using a mammalian expression system, high secretory recombinant Hx (rHx) was developed. We examined the effects of rHx-bound heme on HO-1 expression and Bach1 in Hepa-1c1c7 liver cells and THP-1 macrophage cells. We investigated the uptake pathway of rHx-bound heme by treating cells with chlorpromazine (CPZ).
Results: rHx-bound heme induced the expression of HO-1 and decreased the level of Bach1 protein. CPZ inhibited the induction of the HO-1 expression by rHx-bound heme.
Conclusion: rHx-bound heme was internalized into the cells via endocytosis, resulting in HO-1 expression and inactivation of Bach1.
General significance: The Bach1-dependent repression of the HO-1 expression is under the control of the Hx-dependent uptake of extracellular heme. Heme may regulate Bach1 as an extracellular signaling molecule.

1. Introduction

The transcription repressor Bach1 plays a critical role in constraining the expression of oxidative stress response genes [1]. For example, Bach1 represses the expression of heme oxygenase-1 (HO-1), which is one of the major oxidative stress-inducible enzymes protecting cells from oxidative stressors, such as cadmium or 4-hydroxy-2-nonenal treatment [2–6]. Bach1 binds to the multiple Maf recognition elements (MAREs) within the enhancer regions of HO-1 gene under normal conditions [7]. Under oxidative stress, Bach1 protein undergoes nuclear export, allowing the rapid induction of the HO-1 expression by transcriptional activators such as Nrf2 [4,8]. The HO-1 expression is similarly induced by its own substrate, heme, in a Bach1-dependent fashion. Heme binds to Bach1 through its multiple cysteine–proline (CP) motifs, resulting in inhibition of DNA binding, induction of nuclear export, and polyubiquitination and subsequent degradation [9–12].

However, the source of heme that regulates Bach1 has been unclear. In the experiments examining the effect of heme upon Bach1, free heme was added to the culture media [7,9–11]. Such an experimental condition may mimic only a pathological state of heme which is normally present as protein-bound forms. Heme functions as a prosthetic group of hemeproteins (e.g., hemoglobin, myoglobin, catalase and peroxidase) in the aerobic activities of various cells and tissues. In various pathological states, such as vascular hemolysis, rhabdomyolysis, or necrosis, free heme is liberated from hemeproteins into the blood. Extracellular heme may penetrate cell membrane due to its hydrophobicity [13] to affect Bach1. However, blood contains abundant levels of heme-scavenging proteins such as the serum glycoprotein hemopexin (Hx). Hx binds to heme with extremely high affinity ($Ka = 10^{14}\ M^{-1}$ [14]). Heme-bound Hx is subsequently taken up by low-density lipoprotein receptor-related protein 1 (LRP1)-mediated endocytosis [15–17]. Therefore, the majority of heme released into the blood may be chaperoned by Hx to be taken up by cells for not only iron reutilization but also the regulation of Bach1. Thus far, there has been no report on the effect of heme-bound Hx upon Bach1.

Abbreviations: Hx, hemopexin; rHx, recombinant hemopexin; HO-1, heme oxygenase-1; Igκ, immunoglobulin κ; LRP1, low-density lipoprotein receptor-related protein 1; PTM, posttranslational modification; ER, endoplasmic reticulum; CBB, Coomassie Brilliant Blue; CPZ, chlorpromazine; Chx, cycloheximide; PMA, phorbol 12-myristate 13-acetate; 2-ME, 2-mercaptoethanol; LPS, lipopolysaccharide; ROS, reactive oxygen species; Hb, hemoglobin; Hp, haptoglobin

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In most biochemical studies on Hx, native Hx proteins are prepared from the blood of rabbits using multiple steps to remove the many contaminants of major serum proteins, including albumin, transferrin, macroglobulin and immunoglobulin (reviewed in [18]). Recombinant Hx (rHx) proteins have been expressed in *Escherichia coli*, insect cells and *Pichia pastoris* [19–21]. However, it is unclear whether these rHx proteins exhibit properties and functions similar to those of native Hx due to possible differences in glycosylation and the disulfide bond status. Therefore, more suitable systems for obtaining the rHx protein are required to understand the role of Hx in extracellular heme-dependent stress responses.

In this study, we investigated the cellular uptake pathway of extracellular heme and the effects of internalized heme on Bach1 using purified rHx protein expressed in the human cell line, HEK293. To simplify the purification procedures for excluding contaminants, we improved the expression vector and purification methods. First, the signal sequence of Hx was replaced with that of immunoglobulin κ (Igκ) in order to increase the efficiency of secretion [22]. Second, we introduced His-tag into the carboxy-terminus of Hx for affinity purification and immunodetection. Lastly, we generated the HEK293 cell line stably expressing rHx using the FLP–FRT site-specific recombination system [23] in order to constantly obtain the recombinant protein. Using the rHx protein, we compared the effects of rHx-bound heme with those of free heme in terms of the Bach1-regulated HO-1 mRNA expression and found that the former was dependent on endocytosis, while the latter was not. Therefore, our results show that extracellular Hx-bound heme enters cells via Hx-mediated endocytosis, leading to the inactivation of the Bach1 repressor activity and hence the activation of HO-1 gene transcription and oxidative stress defense.

2. Materials and methods

2.1. Plasmids

The Igκ secretion signal sequence was amplified from pDisplay (Invitrogen) using PCR with the following synthetic primers: 5′-AAGCTTACCATGGAGACAGACACACTCCTG-3′ (underline; HindIII site), and 5′-GGATCC CTCGAG GTCACCAGTGGAACCTGG-3′ (underline; BamHI site, double underline; XhoI site). The amplified fragment was subcloned into a pCR-Blunt II-TOPO vector (Invitrogen) and sequenced. The fragment digested with HindIII and BamHI was further ligated into a pcDNA5–FRT vector to generate pcDNA5-Igκ. The 5xHis-tag sequence was introduced into the BamHI site to generate pcDNA5-Igκ-5xHis. Human hemopexin cDNA lacking its signal sequence was amplified using PCR of the cDNA clone of the human hemopexin gene (Open Biosystems). The primer set was 5′-CTCGAGACCCCTCTTCCTCCGACTAG TGCC-3′ (underline; XhoI site) and 5′-GGATCCGTGAGTGCAGCCCAGG AGACTGGT-3′ (underline; BamHI site). The amplified fragment was digested with XhoI and BamHI and subcloned into a XhoI/BamHI-digested pcDNA5-Igκ-5xHis vector to obtain pcDNA5-Igκ-Hx-5xHis. A plasmid expressing a FLAG-tagged mouse Bach1 used in this study has been described previously [24].

2.2. Cell culture

Flp-In 293 cells (Invitrogen) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 10 U/ml of streptomycin and 100 μg/ml of penicillin. THP-1 cells [25] were maintained in RPMI1640 supplemented with 10% FBS, 10 U/ml of streptomycin and 100 μg/ml of penicillin. The THP-1 cells were differentiated into macrophages by adding 10 nM phorbol 12-myristate 13-acetate (PMA) for 24 h. Hepa-1c1c7 cells were maintained in DMEM supplemented with 10% FBS, 10 U/ml of streptomycin and 100 μg/ml of penicillin.

2.3. Antibodies

For the Western blotting analysis, we used the following antibodies: anti-HO-1 (1:1000, Enzo Life Sciences), anti-His-tag (1:1000, Marine Biological Laboratory), anti-FLAG-tag (1:500, Sigma-Aldrich) and rabbit anti-actin antibodies (1:1000, Santa Cruz Biotechnology). The anti-Bach1 antibody has been previously described [9]. The secondary antibodies were horseradish peroxidase-linked anti-rabbit IgG antibodies (1:2000, GE healthcare) or anti-goat IgG antibodies (1:2000, Zymax).

2.4. Establishment of a stable cell line expressing rHx

Flp-In 293 cells were co-transfected with the plasmid pcDNA5-Igκ-Hx-5xHis and a FLP-recombinase vector (pOG44, Invitrogen) in order to generate stable integration of the chimeric hemopexin gene at the FRT-site in the genome. The cells were transfected with 3.6 μg of pOG44 and 0.4 μg of pcDNA5-Igκ-Hx-5xHis using the Gene Juice reagent (Novagen) and further cultured with the complete medium containing the antibiotic hygromycin (300 μg/ml) at 37 °C for one week to select stably rHx-expressing cells.

2.5. Western blotting analysis

Whole cell extracts were prepared as previously described [26]. Lysates or rHx proteins were resolved on SDS-PAGE using 5%–20% polyacrylamide gradient gels (ORIENTAL INSTRUMENTS LTD) and transferred to PVDF membranes (Millipore). The membranes were blocked for 1 h in a blocking buffer [5% skimmed milk in T-TBS buffer (0.05% Tween 20 in TBS)] and subsequently incubated with the primary antibodies in the blocking buffer overnight at 4 °C. After washing with the T-TBS buffer, the membranes were subsequently reacted with the secondary antibodies in the T-TBS buffer for 30 min at room temperature. ECL blotting reagents (Amersham) were used to detect immune complexes.

2.6. Purification of rHx

The stable cells expressing rHx were seeded at 2.0×10^6 cells per 10-cm diameter dishes and cultured for seven days in FreeStyle 293 medium (Invitrogen) with penicillin (100 U/ml) and streptomycin (100 μg/ml) and without FBS. The supernatant was collected and centrifuged at 300 ×*g* for 5 min. The supernatant was diluted 2.5-fold with 20 mM Tris buffer, pH 8.0. Chromatographic purification of rHx was performed using a column with anion exchangers (1 ml HiTrap Q HP, GE Healthcare) and Ni-NTA (1 ml HisTrap, GE Healthcare) on an AKTA purifier system at 4 °C. The anion exchange column absorbed proteins were eluted with a gradually increasing gradient of 20–500 mM NaCl (20 min; flow rate, 1 ml/min) or slowly increasing gradient of 20–300 mM NaCl (25 min; flow rate, 1 ml/min) in 20 mM Tris buffer, pH 8.0. The Ni-NTA column absorbed proteins were eluted with a gradient of 20–500 mM imidazole in 20 mM HEPES buffer, pH 8.0. The fractions containing the rHx protein were confirmed using SDS-PAGE and a Western blotting analysis. The rHx-containing fractions were pooled and concentrated using an Amicon Ultra-30 membrane with a molecular weight cutoff of 30,000 (Millipore). The purity of the rHx protein was estimated using SDS-PAGE.

2.7. Deglycosylation of rHx

The glycosylation status of rHx was evaluated using treatment with the deglycosylation enzymes PNGase F, Endo H_f or o-glycosidase (NEB). After the purified rHx protein was denatured in denaturing buffer (5% SDS, 0.4 M dithiothreitol) with a heating reaction at 100 °C for 10 min, the rHx protein was treated with each deglycosylation enzyme at 37 °C for 1 h according to the manufacturer's protocol (NEB). To deglycosylate the rHx protein under a native state, it was treated