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Research Paper

Mitochondria-targeted heme oxygenase-1 induces oxidative stress and mitochondrial dysfunction in macrophages, kidney fibroblasts and in chronic alcohol hepatotoxicity ☆

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ABSTRACT

The inducible form of Heme Oxygenase-1 (HO-1), a major endoplasmic reticulum (ER) associated heme protein, is known to play important roles in protection against oxidative and chemical stress by degrading free heme released from degradation of heme proteins. In this study we show that induced expression of HO-1 by subjecting macrophage RAW-264.7 cells to chemical or physiological hypoxia resulted in significant translocation of HO-1 protein to mitochondria. Transient transfection of COS-7 cells with cloned cDNA also resulted in mitochondrial translocation of HO-1. Deletion of N-terminal ER targeting domain increased mitochondrial translocation under the transient transfection conditions. Mitochondrial localization of both intact HO-1 and N-terminal truncated HO-1 caused loss of heme aa-3 and cytochrome c oxidase (CcO) activity in COS-7 cells. The truncated protein, which localizes to mitochondria at higher levels, induced substantially steeper loss of CcO activity and reduced heme aa3 content. Furthermore, cells expressing mitochondria targeted HO-1 also induced higher ROS production. Consistent with dysfunctional state of mitochondria induced by HO-1, the mitochondrial recruitment of autophagy markers LC-3 and Drp-1 was also increased in these cells. Chronic ethanol feeding in rats also caused an increase in mitochondrial HO-1 and decrease in CcO activity. These results show that as opposed to the protective effect of the ER associated HO-1, mitochondria targeted HO-1 under normoxic conditions induces mitochondrial dysfunction.

Introduction

Heme oxygenases (HO) represent a family of evolutionarily conserved endoplasmic reticulum (ER) enzymes that have been described as fonts of multiple messengers [1]. HO's are widely considered as the central components of mammalian stress response and defense against oxidative stress [2–5]. Three different isoforms of HO have been described in mammalian systems including the inducible HO-1; constitutive HO-2; and a newly identified HO-3, which is not catalytically active [6,7]. Although its function remains obscure, HO-3 may be involved in heme binding or heme sensing [8]. Out of the three isoforms, the inducible HO-1 is highly concentrated in tissues that are heavily involved in the catabolism of heme proteins [9]. The HO's catalyze the oxidative cleavage of protoheme to biliverdin, liberating CO and free iron. The enzyme requires NADPH–cytochrome–P450-reductase (NPR) as the donor of electrons for substrate metabolism by HO-1[10–12].

The human HO-1 is comprised of a protein fold that primarily contains α-helices. The heme is held between two of these helices. The HO-1 acts as the cytoprotective stress protein, and provides defense against oxidative stress by accelerating the degradation of pro-oxidant heme and hemoproteins to the radical scavenging bile pigments, biliverdin and bilirubin [13–16]. This protein is also induced in response to variety of stimuli such as free iron, inflammation, heavy metals, UV radiation and various oxidative stress conditions including hypoxia or conditions that produce ROS [4,5,17–21]. Under oxidative injury in some tissues heme-derived Fe and CO may exacerbate intracellular oxidative stress and cellular injury by promoting free radical generation in mitochondria and other cellular compartments [22,23]. HO-1 overexpression is also known to promote mitochondrial sequestration of non-transferrin iron and induce macroautophagy contributing

Abbreviations: HO-1, Heme Oxygenase-1; ROS, Reactive Oxygen Species; NPR, NADPH cytochrome P 450 reductase; CcO, cytochrome c oxidase; ER, Endoplasmic reticulum; DCFH-DA, Dichlorofluorescein diacetate

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to the pathological iron deposition and bioenergetic failure in age related neurodegenerative disorders [24–32].

Studies also suggest the contribution of oxidative stress, chemical stress and Reactive Oxygen Species (ROS) in inducing the expression of HO-1. A study by Han et al. [33] suggested that mitochondria-derived H_2O_2 plays an important role in the intracellular signaling pathways, leading to up-regulation of HO-1 transcription in cultured endothelial cells. Some studies also suggested that increased intramitochondrial heme and subsequent ROS generation may be the driving force for mobilizing HO-1 in mitochondria [34].

In this study we examined the fate of induced HO-1 in macrophages exposed to physiological or chemical hypoxia. We have found that HO-1 is not only significantly induced but also a substantial portion of the induced protein is localized inside mitochondria. We further analyzed the N-terminal sequence motifs of the protein and found that a higher percentage of expressed N-terminal 16 amino acid lacking (ΔN16) protein is localized to mitochondria. An important consequence of mitochondria targeted HO-1 is the formation of shortened mitochondrial fragments as seen by immunocytochemistry, indicative of cellular toxicity and mitochondrial fission. Increased mitochondrial localization of HO-1 also induced inhibition of cytochrome c oxidase (CcO) activity and caused higher production of ROS. The mitochondria-targeting of HO-1 also promotes autophagy as evident by increased mitochondrial localization of LC3 and Drp-1. These results show that HO-1 induces mitochondrial dysfunction, and cellular pathology under certain growth conditions.

Materials and methods

Source of antibodies

Polyclonal antibody against human HO-1 (anti-rabbit) was purchased from Life Span Biosciences Inc., Seattle, WA. Antibody to human CcO subunit 1 (anti-mouse) was from Abcam, Cambridge, MA. Antibodies against human NPR (anti-mouse) and human actin (anti-goat) were from Santa Cruz Biotech., Santa Cruz, CA. Antibody to human dynamin related protein, Drp-1 was from BD Biosciences, San Jose, CA, USA and Microtubule-associated protein 1A/1B-light chain 3, LC-3 was from MBL International, Woburn, MA. Mitotracker green was purchased from Life Technologies, Grand Island, NY

Cell culture conditions, exposure to hypoxia and $CoCl_2$ treatment

RAW 264.7 mouse monocyte macrophages were cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% heat inactivated fetal calf serum and 100 μg/ml penicillin–streptomycin. Cells were grown under normal oxygen condition of 148 Torr or 21% O_2. Cells grown up to 90% confluence under normoxia were latter exposed to hypoxia for 12 and 24 h. Simulation of realistic in vivo hypoxia requires that O_2 tension be maintained at less than 5 Torr. This hypoxic condition was achieved in a temperature controlled hypoxic chamber by a constant flow of premixed gas that was certified to contain 1 Torr of oxygen and 5% CO_2 (BOC gases, Murray Hill, NJ). For chemical hypoxia, cells grown to 70% confluence were treated with 150 μM $CoCl_2$ in fresh medium and incubated for 0–96 h.

Construction of plasmids

Full length mouse HO-1 (WT) cDNA was amplified from RNA from CoCl2 treated RAW 264.7 cells by reverse transcription followed by overlap PCR. N-terminal 16 and 33 amino acid coding region cDNA constructs (ΔN16 and ΔN33, respectively) were generated by PCR amplification of the parent cDNA using appropriate sense primers containing an ATG codon and upstream Kozak sequence. All constructs were engineered to contain 5′ Hind III and a 3′ Xba I sites and cloned in PCMV4 vector. The sequence properties of all the plasmid constructs were verified prior to use. The primers used for generating WT and mutant HO-1 are listed in Table 1.

Predictions of subcellular targeting

The Bioinformatics program, WoLF PSORT, which is an extension of the PSORT II program, converts protein amino acid sequences into numerical localization features and uses the *k* nearest neighbor classifier (kNN) to predict localization sites. This program was used to predict the putative mitochondrial targeting efficiency of the WT and N-terminal deletion HO-1 constructs.

Transient transfection of WT and mutant HO-1 in COS-7 cells

COS-7 cells were grown in high glucose, Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 0.1% gentamicin. Cells were transiently transfected with WT, ΔN16 and ΔN33 cDNA's using FUGENE HD (Roche Diagnostics, Mannheim, Germany) transfection reagent. The transfection reagent/DNA ratio was maintained at 3:2 and after 48 h, the cells were harvested, washed in 1 × phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4), and the cell pellets were used for further analyses.

Isolation of subcellular fractions from COS-7 and RAW 264.7 cells

Cells were washed twice with ice cold phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) and lysed in RIPA buffer (25 mm Tris–HCl, ph 7.4, 150 mm NaCl, 0.1 mM EDTA, 1% Nonidet P-40, 0.1% deoxycholate, 0.025% NaN_3, 1% protease inhibitor cocktail) to prepare cellular extract. Mitochondria and microsome fractions were isolated as previously described [35] with little modifications. Briefly, cells were resuspended in sucrose–mannitol buffer (20 mM Hepes, pH 7.5, containing 70 mM sucrose, 220 mM mannitol and 2 mM EDTA) and homogenized using a glass/Teflon Potter Elvehjem homogenizer (Wheaton Industries, Millville, NJ, USA) for approximately 30 strokes. The homogenate was centrifuged at 600 × *g* for 10 min followed by another spin at 650 × *g* for 10 min to remove nuclei and cell debris. The post-nuclear supernatant was then centrifuged at 8000 × *g* for 15 min to sediment the crude mitochondrial fraction. The pellet was resuspended in sucrose–mannitol buffer, layered over a 1.0 M sucrose cushion and centrifuged at 8500 × g for 20 min to purify the mitochondria. The purified mitochondria were washed with sucrose–mannitol buffer twice. The post-mitochondrial supernatant was centrifuged at 100,000 × *g* to pellet microsomes. Mitochondria and microsomes were re-suspended in 50 mM potassium phosphate buffer (pH 7.5)

Table 1
Primers used for generation of WT HO-1 and mutant constructs.

Constructs	Primer
WT HO-1	**FP:** ATCGGTACCACCGCCGTGATGGAGCGTCCACAGCCCGACAGCATG
	RP: ATCTCTAGATTACATGGCATAAATTCCCACTGCCACTGTTG
ΔN16	**FP:** ATCGGTACCACCGCCATGTTGAAGGAGGCCACCAAGGAGGTACACATC
ΔN33	**FP:** ATCGGTACCACCGCCATGAAGAACTTTCAGAAGGGTCAGGTGTCC