Available online at www.sciencedirect.com

SCIENCE DIRECT

Free Radical Biology & Medicine 39 (2005) 558 – 564

www.elsevier.com/locate/freeradbiomed

Original Contribution

Catalytically inactive heme oxygenase-2 mutant is cytoprotective

Yun-Sook Kim, Sylvain Doré*

Department of Anesthesiology/Critical Care Medicine, Johns Hopkins University School of Medicine, ACCM Dept., Neuro. Res. Div., 720 Rutland Ave., Ross Research Bldg. 365, Baltimore, MD 21205, USA

Received 19 November 2004; revised 10 March 2005; accepted 10 April 2005
Available online 28 April 2005

Abstract

Heme oxygenase (HO) catalyzes the rate-limiting step in heme degradation, producing iron, carbon monoxide, and bilirubin/biliverdin. HO consists of two isozymes: HO-1, which is an oxidative stress-response protein, and HO-2, which is constitutively expressed. HO-2 accounts for most HO activity within the nervous system. Its posttranslational modifications and/or interactions with other proteins make HO-2 a unique regulator of cellular homeostasis. Our previous results revealed that brain infarct volume was enlarged in HO-2 knockout mice. A similar neuroprotective role of HO-2 was shown using primary cortical neurons. To better understand the neuroprotective mechanism of HO-2, we used a catalytically inactive mutant, HO-2_{H45A}, and investigated its cellular effects in response to hemin and hydrogen peroxide-induced cytotoxicity. We observed that HO-2_{WT} overexpression in the HEK293 cell lines became less sensitive to hemin, whereas the inactive mutant HO-2_{H45A} was more sensitive to hemin as compared to control. Interestingly, HO-2_{WT}- and HO-2_{H45A}-overexpressing cells were both protected against H_2O_2-induced oxidative stress and had less oxidatively modified proteins as compared to control cells. These data indicate that when HO-2 cannot metabolize the prooxidant heme, more cytotoxicity is found, whereas, interestingly, the catalytically inactive HO-2_{H45A} was also able to protect cells against oxidative stress injury. These results suggest the multiplicity of action of the HO-2 protein itself.

Keywords: Hemin; Bilirubin; Carbon monoxide; Hydrogen peroxide; Iron

Introduction

Heme oxygenase (HO) catalyzes heme (iron-protoporphyrin IX) degradation into iron, carbon monoxide, and biliverdin/bilirubin. Heme is essential for the function of all aerobic cells. It is at the core of numerous hemoproteins (such as myoglobin, catalase, glutathione peroxidase, cytochrome, soluble guanylate cyclase, superoxide dismutase, and nitric oxide synthases) and plays a key role in controlling numerous cell functions. Cellular heme levels are tightly controlled, as heme can generate reactive oxygen species in the redox reaction of heme with oxygen, which is achieved by a fine balance between heme biosynthesis and catabolism by HO. Free heme is considerably more toxic than heme present within proteins. These reactions that cause free radical production lead to increased membranar peroxidation and damage to proteins and DNA [1–3]. Two catalytically active heme oxygenase isozymes have been reported: HO-1, an inducible form, and HO-2, which is constitutively expressed. HO, unlike nitric oxide synthase, does not have the reductase activity within its structure; consequently, it needs NADPH-cytochrome P450 reductase (CPR), as an electron donor, for its oxidative cleavage. The first step of HO reaction is to oxidize heme into α-*meso*-hydroxyheme, which subsequently reacts with oxygen to generate verdoheme and carbon monoxide. Finally, the verdoheme, in a reaction with CPR and oxygen, is converted to biliverdin and ferrous iron (Fe^{2+}) [4].

Abbreviations: CPR, cytochrome P450 reductase; DNPH, 2,4-dinitrophenylhydrazine; HEK, human embryonic kidney; HO, heme oxygenase; MTT, 3-(4,5-dimethylthiazol-2yl)2,5-diphenyl tetrazolium bromide; NADPH, nicotinamide adenine dinucleotide phosphate; PBS, phosphate-buffered saline; WT, wildtype.

* Corresponding author. Fax: +1 410 955 7271.
E-mail address: sdore@jhmi.edu (S. Doré).
URL: www.hopkinsmedicine.edu/dorelab (S. Doré).

0891-5849/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2005.04.009

The homology of the amino acid sequences of HO-1 and HO-2 is approximately 40% [5]. Histidine 25 in human HO-1 and histidine 45 in human HO-2 are important amino acids in the active site structure [6,7]. However, HO-1 and HO-2 may perform distinct protective functions against tissue injury. Their synthesis and activities are differentially regulated in mammalian cells [8]. HO-1, a heat shock protein, is induced by most factors, while HO-2, under normal conditions, accounts for almost all HO activity in the brain. It is now generally accepted that HO plays a crucial role in controlling cellular homeostasis and acting as a physiologic regulator.

We have demonstrated the neuroprotective role of HO-2 [9]. By comparing wildtype (WT) mice with HO-2 knockout ($HO\text{-}2^{-/-}$) mice, we showed that, after subjection to a middle cerebral artery occlusion (MCAO) stroke model, infarct size was approximately twice greater in knockout mice [10,11]. Similar results were reproduced using WT mice with HO inhibitor. We also provided evidence for the antiapoptotic role of HO-2 by showing an increase in the number of in situ, end-labeled, apoptotic-like cells in $HO\text{-}2^{-/-}$ brain after stroke, by showing increased apoptotic-like cell death in cerebellar granular cell cultures in $HO\text{-}2^{-/-}$ under serum-deprivation conditions, and by showing reduced DNA ladder profiles in cells overexpressing HO-2 [11,12].

HO-2 is highly expressed in brain, suggesting a diversity of function far beyond the heme degradation, especially considering the ongoing debate regarding whether a significant intracellular pool of free heme exists within cells that is capable of generating sufficient amounts of the bioactive metabolites, which should account for all HO-2 cytoprotective action. Here, in order to acquire a better understanding of the cellular role/function of HO-2 protein, we addressed whether the catalytic activity of HO-2 is crucial against induced toxicity. We tested the cytoprotective role of wildtype HO-2 ($HO\text{-}2_{WT}$) versus a catalytically inactive mutant ($HO\text{-}2_{H45A}$), using a point mutation of histidine into alanine ($HO\text{-}2_{H45A}$), against both heme- and hydrogen peroxide (H_2O_2)-induced toxicity. As expected, cells transfected with the inactive $HO\text{-}2_{H45A}$ mutant were not protected against hemin-induced toxicity. Surprisingly, we observed that after transfection with the inactive $HO\text{-}2_{H45A}$ mutant, cells were protected against H_2O_2-induced toxicity. Together, the data show that overexpression of an inactive HO-2 mutant renders cells more resistant to H_2O_2 and prevents cell death. These results also support the concept of the array of actions involved in the HO-2 cytoprotective role.

Materials and methods

Materials

All chemicals, unless stated otherwise, were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell cultures

Human embryonic kidney (HEK293) cells were maintained, as described before, in Dulbecco's modified Eagle medium (GIBCO, Carlsbad, CA) with 10% fetal bovine serum, penicillin (100 U/ml; GIBCO), and streptomycin (100 μg/ml; GIBCO) in a humidified atmosphere of 95% air/5% CO_2 at 37°C [13].

Plasmid constructions of $HO\text{-}2_{WT}$ or mutant $HO\text{-}2_{H45A}$ and transfection

To generate the myc-tagged HO-2 expression construct, the human HO-2 cDNA was polymerase chain reaction (PCR)-amplified, and the PCR fragments were subcloned into *Eco*RI/*Not*I sites of pcDNA3.1 (Invitrogen, Carlsbad, CA). To generate catalytically inactive HO-2, histidine 45 was replaced with alanine using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The sequences of all constructs were confirmed by nucleotide sequencing with the deoxy-chain-termination method. To express $HO\text{-}2_{WT}$ or $HO\text{-}2_{H45A}$ proteins transiently, stably transfected HEK293 cells overexpressing human cytochrome P450 reductase (CPR) were transfected with pcDNA3.1-$HO\text{-}2_{WT}$ or pcDNA3.1-$HO\text{-}2_{H45A}$ construct using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After the transfection, cells were grown for another 45 h before the indicated treatment.

Hemin and hydrogen peroxide (H_2O_2) treatment

Hemin was freshly dissolved in 100 mM NaOH at a concentration of 100 mM stock solution and then diluted to the final concentrations (300 and 400 μM). H_2O_2 was freshly diluted in the culture medium at a concentration of 100 mM stock solution and directly added to the transiently transfected cells at the desired final concentrations (200 and 300 μM). All experiments were performed under dim light to prevent heme pigment photogradation.

HO assay

The HO activity was measured as previously described, with some modifications [10]. Transfected cells were harvested by a cell scraper in ice-cold phosphate-buffered saline (PBS) with protease inhibitor cocktail (Roche Biosciences, Palo Alto, CA), suspended in the lysis buffer (PBS, pH 7.4, protease inhibitor cocktail, 0.1 mM phenylmethyl sulfonyl fluoride), and sonicated by an Ultrasonic Cell Disruptor (MedSonic, Inc., Farmingdale, NY). Membrane fractions from cells (5 μg) were incubated with NADPH (1 mM) and ^{55}Fe-heme (Perkin Elmer Life Sciences, Boston, MA) for 6 min at 30°C. The assay was performed with or without the HO-specific inhibitor protoporphyrin IX (SnPPIX) at a final concentration of 5 μM. Reactions were terminated by the addition of phenol:chloroform:isoamyl