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Original Contribution

The mitochondrial thioredoxin system regulates nitric oxide-induced HIF-1α protein

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Abstract

Hypoxia-inducible factor-1 (HIF-1), consisting of two subunits, HIF-1α and HIF-1β, is a key regulator for adaptation to low oxygen availability, i.e., hypoxia. Compared to the constitutively expressed HIF-1β, HIF-1α is regulated by hypoxia but also under normoxia (21% O_2) by several stimuli, including nitric oxide (NO). In this study, we present evidence that overexpression of mitochondrial-located thioredoxin 2 (Trx2) or thioredoxin reductase 2 (TrxR2) attenuated NO-evoked HIF-1α accumulation and transactivation of HIF-1 in HEK293 cells. In contrast, cytosolic-located thioredoxin 1 (Trx1) enhanced HIF-1α protein amount and activity under NO treatments. Taking into consideration that thioredoxins affect the synthesis of HIF-1α by altering Akt/mTOR signaling, we herein show that p42/44 mitogen-activated protein kinase and p70S6 kinase are involved. Moreover, intracellular ATP was increased in Trx1-overexpressing cells but reduced in cells overexpressing Trx2 or TrxR2, providing thus an understanding of how protein synthesis is regulated by thioredoxins.

Keywords: Mitochondria; Protein translation; MAPK; ATP; Free radicals

Hypoxia-inducible factor-1 (HIF-1) is composed of the two subunits, HIF-1α and HIF-1β, and senses low oxygen availability to coordinate a number of pathophysiological responses [1,2]. Under normoxia (21% O_2), HIF-1α protein is continuously degraded via the 26S proteasome, whereas HIF-1β is constitutively present. Low oxygen tension (hypoxia), iron chelators, or nitric oxide (NO) attenuate hydroxylation of HIF-1α at prolines 564 and/or 402. This blocks the binding of the von Hippel-Lindau protein (pVHL), which in turn abrogates polyubiquitination. As a result, proteasomal destruction is impaired and HIF-1α protein accumulates. Alternatively, increased translation provokes the synthesis of HIF-1α in response to growth factors, hormones, or cytokines [1,3]. To enhance HIF-1α protein synthesis, phosphatidylinositol 3 kinase (PI3K)/Akt and/or mitogen-activated protein kinase (MAPK) signaling pathways phosphorylate and thus activate components required for the translation of the HIF-1α gene. Phosphorylation targets comprise p70S6 kinase (p70S6K), eukaryotic initiation factor-4E (eIF-4E), and the eIF-4E binding protein-1 (4EBP-1).

The molecular mechanism of NO action revealed decreased ubiquitination of HIF-1α and an abrogated binding of pVHL to HIF-1α [4], which suggested that NO directly attenuated hydroxylation of HIF-1α. Indeed, in an in vitro HIF-1α–pVHL capture assay the NO donor GSNO (*S*-nitrosoglutathione) dose-dependently attenuated PHD (prolyl-4-hydroxylase domain-containing enzyme) activity. However, NOC-18, a chemically distinct NO donor, was reported to use a PI3K, MAPK, and cap-dependent translation control system to express HIF-1α [5]. Apparently, diverse NO donors can use distinct pathways to affect the levels of HIF-1α, either depending on concentrations being used or acting cell-type specifically.

Thioredoxins (Trx) are small multifunctional 12-kDa proteins containing two cysteine residues in the Trp-Cys-Gly-Pro-Cys motif. The cysteines can be reversibly reduced and oxidized, the reduced being the "normal active" in vivo state. Trx are reduced from the oxidized (inactive) form by the flavoenzyme thioredoxin reductase and NADPH. The thior-

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edoxin system is the major ubiquitous disulfide reductase responsible for maintaining cytosolic proteins at their reduced state [6]. There are two distinct forms of thioredoxins, i.e., thioredoxin 1 (Trx1) and thioredoxin 2 (Trx2). Whereas Trx1 is present in the cytosol, Trx2 is located in the mitochondrial matrix [7].

Overexpression of Trx1 stimulated HIF-1 activity [8–10] and provoked a HIF-1α protein increase, under normoxia as well as hypoxia [10,11]. In addition, attenuating the activity of thioredoxin reductase 1 blocked HIF-1 transactivation [12]. Moreover, Trx1 may affect HIF-1α degradation during reoxygenation [13], perhaps via the pVHL-mediated pathway [14]. Recently, we reported molecular details for the interplay between thioredoxins and HIF-1. We showed that Trx1 enhanced, but Trx2 attenuated, HIF-1α protein translation [10]. Moreover, thioredoxin 2 overexpression altered the level of reactive oxygen species, known to influence NO-induced signaling [15,16]. We further wanted to examine whether Trx1 or Trx2 affects NO-stimulated HIF-1α regulation by using HEK293 cells that overexpress Trx1, Trx2, or thioredoxin reductase 2 (TrxR2). Although mitochondrial Trx2 or TrxR2 attenuated the accumulation of HIF-1α and the activity of HIF-1 in response to NO, the response was enhanced by Trx1. The opposed actions of Trx1 vs Trx2/TrxR2 resulted from an enhanced vs attenuated translation of HIF-1α, with p42/44 MAPK and p70S6K being involved. Moreover, differentially altered ATP levels by Trx1 or Trx2/TrxR2 contributed to the expression regulation of HIF-1α.

Experimental procedures

Materials

Cell culture complete medium and supplements were purchased from PAA (Linz, Austria). Methionine-free and glucose-free media were bought from Promocell (Heidelberg, Germany). Fetal calf serum (FCS) was from Biochrom (Berlin, Germany). GSNO was synthesized as described previously [21]. Anti-actin antibody was ordered from Sigma (Schnelldorf, Germany). Nitrocellulose membrane and horseradish peroxidase-labeled anti-mouse or anti-rabbit secondary antibodies were delivered by GE Healthcare (Munich, Germany). Antibodies to phospho-p42/44 MAPK, p42/44 MAPK, phospho-p70S6K (Thr421/Ser424), and p70S6K were from Cell Signaling Technology (Beverly, MA, USA). The anti-HIF-1α antibody was purchased from BD Biosciences (Heidelberg, Germany). The plasmid pHRE-Luc harboring three erythropoietin hypoxia-responsive elements (HRE) was provided by Dr. T. Kietzmann (Technical University of Kaiserslautern, Kaiserslautern, Germany). Reporter plasmid pCap-Luc and luciferase activity assay kit were supplied by Promega (Mannheim, Germany). Plasmids expressing active ERK2–MEK1 fusion protein (pERK2-MEK1-LA) and the inactive kinase mutant ERK–MEK1–K52R (pERK2-MEK1-KR) were kindly provided by Dr. S. Weg-Remers (Institute of Toxicology and Genetics, Research Center Karlsruhe of the Helmohotz-Society, Karlsruhe, Germany) [17,18]. All other chemicals were of the highest grade of purity and commercially available.

Cell culture

Human embryonic kidney (HEK293) cells were transfected with a control plasmid (HEKi) or with plasmids encoding Trx1, Trx2, or TrxR2 as previously described [10,19,20]. Cells were cultured in Dulbecco's modified Eagle medium with 4.5 g/L D-glucose, 10% FCS, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 500 μg/ml G418. Mouse macrophage RAW267.4 cells were cultured in RPMI 1640 medium containing 10% FCS, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were kept in a humidified atmosphere of 5% CO_2 in air at 37°C and HEK cells were maintained in medium without G418 for 24 h before experiments.

Western blot

HEK293 cells (1×10^6) were seeded in 6-cm dishes 1 day before experiments. After treatments, cells were scraped off, lysed in 150 μl WB-lysis buffer (50 mM Tris/HCl, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet-40, protease inhibitor cocktail, pH 7.5), and sonicated. After centrifugation (15,000*g,* 15 min, 4°C) the protein content of the supernatants was determined by a protein assay kit (Bio-Rad, Munich, Germany). One hundred micrograms of protein was added to the same volume of 2× SDS–PAGE sample buffer (125 mM Tris/HCl, 2% SDS, 10% glycerin, 1 mM DTT, 0.002% bromophenol blue, pH 6.9) and boiled for 5 min. Proteins were resolved on 10% SDS–polyacrylamide gels and blotted onto nitrocellulose membranes. Nonspecific binding sites were blocked with 5% (w/v) defatted milk powder in TTBS (50 mM Tris/HCl, 140 mM NaCl, 0.05% Tween 20, pH 7.2) for 1 h at room temperature. Primary antibodies (1:1000 in 1% milk/TTBS or 5% bovine serum albumin/TTBS) were added and incubated overnight at 4°C. Afterward, nitrocellulose membranes were washed three times for 5 min each with TTBS. Blots were then incubated with goat anti-mouse or goat anti-rabbit secondary antibodies conjugated with peroxidase (1:2000 in 1% milk/TTBS) for 1 h at room temperature, washed three times for 5 min each with TTBS, followed by ECL detection (GE Healthcare, Munich, Germany).

Coculture of HEK and RAW264.7 cells

Cells were cocultured by using transwell inserts (BD Biosciences, Heidelberg, Germany) in order to separate cells by a 1-μm porous membrane. For these experiments 1×10^6 RAW264.7 cells were plated in the bottom of six-well plates and stimulated with LPS (1 μg/ml) and IFF-γ (100 U/ml) for 16 h. RAW267.4 cells were then washed with PBS and reconstituted with fresh medium in the presence or absence of 1 mM L-nitroarginine methyl ester (L-NAME). At the same time, 1×10^6 HEK cells seeded on coculture inserts were added. Cells were cocultured for 8 h.