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A reducing environment stabilizes HIF-2 α in SH-SY5Y cells under hypoxic conditions

Hu Chen and Honglian Shi*

College of Pharmacy, University of New Mexico Health Sciences Center, Albuquerque, NM 87131, USA

Abstract

Accumulating evidence suggests that hypoxia-inducible factor-2 (HIF-2) is important for the cellular response to hypoxia. However, it is not clear how HIF-2 is regulated under hypoxic conditions. We investigated kinetic changes in redox status and HIF-2 α accumulation in hypoxic SH-SY5Y cells. Our results demonstrated that hypoxia causes a reducing environment and increases HIF-2 α protein levels. Experiments with redox modulations (N-acetylcysteine and L-buthionine sulfoximine) confirmed that a reducing environment induced HIF-2 α accumulation while an oxidizing environment decreased it. In addition, experiments with SOD mimic, catalase, and exogenous H₂O₂ provided evidence that the presence of H₂O₂ down-regulated the amount of HIF-2 α protein. This study offers novel evidence supporting redox status regulation of HIF-2 α accumulation under hypoxic conditions.

Keywords

HIF-2 α ; Hypoxia; Redox status; SH-SY5Y; Stroke; Brain tumor

1. Introduction

Oxygen deprivation is a central feature of cancer and ischemic diseases such as cerebral ischemia. It has been found that hypoxia-inducible factors (HIFs) act as key regulators in cells exposed to low oxygen. Each of the three members of the HIF family is composed of an oxygen-regulated functional α -subunit and a constitutively expressed β -subunit. In the last decade, HIF-1 has been a predominant target of many studies in tumor as well as ischemic brain [1]. Recently, there is accumulating evidence demonstrating a similar importance of HIF-2 in protecting cells from hypoxic injuries. For example, HIF-2 α over-expression can activate several genes with particular importance in cell survival pathways and of potential therapeutic value in stroke [2]. Other studies have proven that HIF-2 plays a more important role in regulating erythropoietin (EPO), a potential neuroprotectant, than HIF-1 in the brain [3,4]. Moreover, it has been observed that HIF-2 α accumulation is more sensitive and stable than HIF-1 α in hypoxia [5]. However, the molecular mechanism of HIF-2 α regulation in hypoxic cells is still not fully understood.

Many studies have indicated that an imbalance of redox status is involved in hypoxic exposure [6]. Cellular redox status is mainly determined by redox-regulated gene expression, levels of antioxidants, and free radical generations. It has been demonstrated that redox status can regulate HIF-1 α protein level under hypoxic conditions [7]. Structurally, HIF-2 α and HIF-1 α

*Corresponding author. Fax: +1 505 272 8306. E-mail address: hshi@salud.unm.edu (H. Shi).
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are closely related, sharing 48% overall amino acid identity and exhibiting a high degree of homology in their oxygen-dependent-degradation domains [8]. Does redox status also affect HIF-2 α accumulation in brain cells exposed to hypoxia? In fact, a few studies have shown that HIF-2 α protein level can be altered by reactive oxygen species (ROS) in nonneuronal cells [9-11]. To clarify the relationship between cellular redox status and HIF-2 α protein level in brain cells under hypoxic conditions will help understand molecular mechanisms of HIF-2 α regulation and further elucidate cellular self-protection mechanisms in hypoxic-related neuropathological conditions such as brain tumor and cerebral ischemia. We hypothesized that cellular redox status might regulate HIF-2 α protein level in brain cells under hypoxic conditions. Experiments were carried out to test this hypothesis in SH-SY5Y cells.

2. Materials and methods

2.1. Materials

The following chemicals were purchased from Sigma (St. Louis, MO): N-acetyl cysteine (NAC), L-buthionine sulfoximine (BSO), catalase, H₂O₂, and mouse anti- β -actin antibody. MnTMPyP was from A.G. Scientific (San Diego, CA). Glutathione assay kit was ordered from Cayman Chemical (Ann Arbor, Michigan).

2.2. Cell culture and hypoxia treatments

SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and antibiotics (penicillin–streptomycin 1:100) at 37°C in a humidified incubator gassed with 95% air and 5% CO₂. Medium was changed to DMEM without FBS and without antibiotics at 80% confluence. Cells were then incubated at 37 °C in a humidified hypoxia chamber (Coy laboratory products) with 1% O₂, 5% CO₂ and balance N₂ for 0, 1, 4, or 12 hours (h).

2.3. Antioxidant or oxidant treatments

NAC is a thiol-containing antioxidant that increases intracellular glutathione synthesis and has commonly been used to induce a reducing environment [7]. BSO selectively inhibits GSH biosynthesis and is often utilized to create an oxidizing environment [12]. NAC (0.5 mM) was added to cells immediately before the onset of hypoxia. BSO (0.3 mM) was added to cells 12 h before hypoxic treatments according to a previous publication [7]. Catalase (500 units/ml) was used to scavenge H₂O₂. MnTMPyP, a SOD mimic to dismutate cellular superoxide, was added to culture medium at the concentration of 5 μ M [13]. Catalase and MnTMPyP were added right before the onset of hypoxic exposure.

2.4. Cell viability assay

Cell death was assessed by measuring lactate dehydrogenase (LDH) released into the culture medium using a cytotoxicity assay kit (Takara Bio, Shiga, Japan).

2.5. Redox status (GSH/GSSG ratio) assessment

Cells were washed twice with 2 ml ice-cold PBS and lysed with 1% sulfosalicylic acid. The lysates were centrifuged at 12000 rpm for 15 min at 4 °C. The supernatants were collected for analyses of GSH and GSSG according to manufacturer's instruction (Cayman Chemical).

2.6. Western blot

Western blotting was carried out according to a previous publication [7] with rabbit anti-HIF-2 α primary antibody from Novus Biologicals (Littleton, CO). Protein bands were detected by horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology, Santa Cruz, CA)

and visualized with enhanced chemiluminescence reagent (Thermo scientific, Waltham, MA). Autoradiographic signals of immunoblot were quantified using NIH ImageJ software.

2.7. Statistical analysis

Data were presented as mean $\pm$ S.E. Statistical comparisons between groups were made using one-way ANOVA. $P < 0.05$ was considered statistically significant.

3. Results and discussion

We first studied time-dependent accumulation of HIF-2 α protein in SH-SY5Y cells exposed to hypoxia to help understand the mechanism of its regulation. Cells were exposed to hypoxia (1% O₂) for 0, 1, 4, or 12 h. As shown in Fig. 1, HIF-2 α protein levels increased in a time-dependent manner during the 12 h exposure. Control cells (0 h hypoxia) had a very low level of HIF-2 α accumulation. HIF-2 α was up-regulated in the cells exposed to hypoxia for 4 h. Remarkable upregulation of HIF-2 α was observed at 12 h. This result indicates that HIF-2 α protein is accumulated continuously during the 12 h hypoxic exposure.

Next, we conducted parallel experiments to evaluate changes of cellular redox status in the cells exposed to hypoxia for 12 h. The GSH/GSSG ratio has been widely used to indicate cellular redox status. A higher ratio indicates a more reducing environment [14]. As shown in Fig. 2, exposure to hypoxia dramatically increased cellular GSH/GSSG ratio in the first 4 h. The ratio started to decrease at 4 h, but kept a higher ratio at 12 h than control cells. This observation is in agreement to previous reports that hypoxia induces more cellular reductants and a more reducing environment (higher GSH/GSSG ratio) in myocardium [15], rat brain [16], and primary neurons [7]. This result states that HIF-2 α protein level increases in a reducing environment in SH-SY5Y cells exposed to hypoxia.

The results in Figs. 1 and 2 show that the GSH/GSSG ratio does not correlate with the HIF-2 α accumulation after 4 h hypoxic exposure. The GSH/GSSG ratio entered a decreasing phase while HIF-2 α accumulation kept increasing during the period. It should be pointed out that although the GSH/GSSG ratio at 12 h was lower than at 4 h, it was still significantly higher than control cells. This indicates that the cells kept at a more reducing environment during the 12 h hypoxic exposure. As the cells were at a more reducing environment from the 4th h to the 12th h of hypoxic exposure, HIF-2 α protein kept accumulation during the period although GSH/GSSG ratio started decreasing. This result is in line with the concept that a more reducing environment increases HIF-2 α accumulation under hypoxic conditions. The mechanism of a decreasing GSH/GSSG ratio after 4 h hypoxia is of interest and needs further investigation.

The above results established an association between a reducing environment and HIF-2 α accumulation in hypoxic cells. To confirm a causal relationship between HIF-2 α and redox environment, we modulated cellular redox status in opposing directions with NAC and BSO. The result demonstrates that NAC induced a more reducing environment (higher GSH/GSSG ratio) and higher HIF-2 α protein level than control (hypoxic exposure only) after either 4 or 12 h exposure (Fig. 3). This result confirmed that a reducing environment favored HIF-1 α stabilization. Meanwhile, Fig. 3 also shows that BSO decreased GSH/GSSG ratio and HIF-2 α protein levels, compared to hypoxic treatment only. These results were evident that a more reducing environment was responsible for HIF-2 α protein stabilization in hypoxic cells but not an oxidizing one.

The primary goal of this project was to elucidate the role of redox status on HIF-2 α accumulation under hypoxic conditions, which is more relevant to pathological conditions such as brain tumor and cerebral ischemia than normoxic conditions. It is of interest to understand the relationship between redox status and HIF-2 α accumulation under normoxic conditions.

As shown in Fig. 1 (time 0 h), HIF-2 α level in the cells was very low under normoxic conditions. We also evaluated the effect of redox changes on HIF-2 α accumulation under normoxic conditions. Cells were treated with NAC and BSO in the same approaches which were used in the hypoxic experiments. HIF-2 α accumulation was estimated by Western blotting. Interestingly, we observed that HIF-2 α accumulations were very low in cells treated with either BSO (Fig. 3B, time 0 h) or NAC (data not shown), and there was no difference between control cells and treated cells. This indicates that the mechanism of HIF-2 α accumulation in normoxia may be different from that in hypoxia.

To clarify if cell death was involved in the effects of NAC and BSO on redox status and HIF-2 α accumulation under hypoxic exposure, we evaluated cytotoxicity induced by hypoxia, NAC, and BSO. We found that there were no significant differences in cell death between different groups at the same time points (Table 1). This result clarified that cell death was not involved in the effects of NAC and BSO on the changes of redox status and HIF-2 α accumulation.

If a reducing environment induced HIF-2 α accumulation, antioxidants would increase HIF-2 α accumulation as they generally reduce free radical levels and induce a reducing environment. We further investigated the effect of antioxidants on HIF-2 α accumulation in hypoxic cells. The cells were treated with MnTMPyP and catalase. MnTMPyP was used to dismutate superoxide anion radical, the primary ROS in cells. MnTMPyP readily permeates cell membranes and can effectively detoxify intracellular superoxide radical anion. Catalase was used to quench H₂O₂, which can be generated from the dismutation of superoxide anion radical [13,17]. Interestingly, we observed that MnTMPyP decreased HIF-2 α protein level (Fig. 4). In contrast, catalase significantly increased HIF-2 α accumulation. The seemingly contradictory result may be due to the function of MnTMPyP, i.e. dismutation of superoxide anion radical to H₂O₂. We further tested the effect of exogenous H₂O₂ on HIF-2 α accumulation. Similar to MnTMPyP, exogenous H₂O₂ significantly decreased HIF-2 α protein level compared to hypoxia control. This result strongly supports that H₂O₂ suppresses HIF-2 α accumulation, which is in line with the concept that a reducing environment induces HIF-2 α accumulation.

Currently there is extensive research on oxygen sensing and HIF expression in hypoxic cells [18,19]. ROS has been suggested to play a role in the regulation of HIF expression in hypoxic cells. However, there are inconsistent observations about ROS's role in HIF-2 α regulation. On one hand, ROS have been observed to up-regulate HIF-2 α in murine embryonic cells [9], Hep3B cells [10], and human renal proximal tubular cells [11]. On the other hand, Brown and Nurse have recently challenged the concept that ROS increase HIF-2 α protein expression. Their results show that ROS do not mediate HIF-2 α induction in an adrenomedullary chromaffin cell line [20]. The conflicting results may result from different cell species. However, there may be other factors that determine the HIF-2 α accumulation in hypoxic conditions. Our observation provides a novel mechanism for HIF-2 α accumulation in hypoxic conditions (i.e. a reducing environment induces HIF-2 α accumulation). The result is in consistency with previous findings that a reducing environment induces EPO [21,22], which is primarily regulated by HIF-2. The mechanism of stabilization of HIF-2 α in a reducing environment is a critical issue which needs to be addressed in future studies.

In summary, our present study offers novel evidence supporting that redox status regulates HIF-2 α accumulation under hypoxic conditions. These evidences should be helpful to understand the self-protection mechanism of brain cells during pathological conditions such as brain tumor and cerebral ischemia.

Acknowledgements

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References

1. Ratan RR, et al. Harnessing hypoxic adaptation to prevent, treat, and repair stroke. *J Mol Med* 2007;85:1331–1338. [PubMed: 18043901]
2. Ralph GS, et al. Identification of potential stroke targets by lentiviral vector mediated overexpression of HIF-1 α and HIF-2 α in a primary neuronal model of hypoxia. *J Cerebr Blood Flow Met* 2004;24:245–258.
3. Chavez JC, Baranova O, Lin J, Pichiule P. The transcriptional activator hypoxia inducible factor 2 (HIF-2/EPAS-1) regulates the oxygen-dependent expression of erythropoietin in cortical astrocytes. *J Neurosci* 2006;26:9471–9481. [PubMed: 16971531]
4. Yeo EJ, Cho YS, Kim MS, Park JW. Contribution of HIF-1 α or HIF-2 α to erythropoietin expression: in vivo evidence based on chromatin immunoprecipitation. *Ann Hematol* 2008;87:11–17. [PubMed: 17712557]
5. Holmquist L, Jogi A, Pahlman S. Phenotypic persistence after reoxygenation of hypoxic neuroblastoma cells. *Int J Cancer* 2005;116:218–225. [PubMed: 15800931]
6. Clanton TL. Hypoxia-induced reactive oxygen species formation in skeletal muscle. *J Appl Physiol* 2007;102:2379–2388. [PubMed: 17289907]
7. Guo S, et al. Glucose up-regulates HIF-1 α expression in primary cortical neurons in response to hypoxia through maintaining cellular redox status. *J Neurochem* 2008;105:1849–1860. [PubMed: 18266932]
8. Hu CJ, Wang LY, Chodosh LA, Keith B, Simon MC. Differential roles of hypoxia-inducible factor 1 α and HIF-2 α in hypoxic gene regulation. *Mol Cell Biol* 2003;23:9361–9374. [PubMed: 14645546]
9. Mansfield KD, Guzy RD, Pan Y, Young RM, Cash TP, Schumacker PT, Simon MC. Mitochondrial dysfunction resulting from loss of cytochrome c impairs cellular oxygen sensing and hypoxic HIF- α activation. *Cell Met* 2005;1:393–399.
10. Guzy RD, et al. Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing. *Cell Met* 2005;1:401–408.
11. Block K, Gorin Y, Hoover P, Williams P, Chelmicki T, Clark RA, Yoneda T, Abboud HE. NAD(P) H oxidases regulate HIF-2 α protein expression. *J Biol Chem* 2007;282:8019–8026. [PubMed: 17200123]
12. Griffith OW, Meister A. Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-n-butyl homocysteine sulfoximine). *J Biol Chem* 1979;254:7558–7560. [PubMed: 38242]
13. Furuichi T, Liu W, Shi H, Miyake M, Liu KJ. Generation of hydrogen peroxide during brief oxygen-glucose deprivation induces preconditioning neuronal protection in primary cultured neurons. *J Neurosci Res* 2005;79:816–824. [PubMed: 15668910]
14. Schafer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic Biol Med* 2001;30:1191–1212. [PubMed: 11368918]
15. Zhu X, Zuo L, Cardounel AJ, Zweier JL, He G. Characterization of in vivo tissue redox status, oxygenation, and formation of reactive oxygen species in postischemic myocardium. *Antioxid Redox Signal* 2007;9:447–455. [PubMed: 17280486]
16. Maiti P, Singh SB, Sharma AK, Muthuraju S, Banerjee PK, Ilavazhagan G. Hypobaric hypoxia induces oxidative stress in rat brain. *Neurochem Int* 2006;49:709–716. [PubMed: 16911847]
17. Moldovan L, Moldovan NI, Sohn RH, Parikh SA, Goldschmidt-Clermont PJ. Redox changes of cultured endothelial cells and actin dynamics. *Circ Res* 2000;86:549–557. [PubMed: 10720417]
18. Taylor CT. Mitochondria, oxygen sensing, and the regulation of HIF-2 α . Focus on “Induction of HIF-2 α is dependent on mitochondrial O₂ consumption in an O₂-sensitive adrenomedullary chromaffin cell line”. *Am J Physiol Cell Physiol* 2008;294:C1300–C1302. [PubMed: 18463234]

19. Hagen T, Taylor CT, Lam F, Moncada S. Redistribution of intracellular oxygen in hypoxia by nitric oxide: effect on HIF-1 α . *Science* 2003;302:1975–1978. [PubMed: 14671307]
20. Brown ST, Nurse CA. Induction of HIF-2 α is dependent on mitochondrial O₂ consumption in an O₂-sensitive adrenomedullary chromaffin cell line. *Am J Physiol Cell Physiol* 2008;294:C1305–C1312. [PubMed: 18353899]
21. Neumcke I, Schneider B, Fandrey J, Pagel H. Effects of pro- and antioxidative compounds on renal production of erythropoietin. *Endocrinology* 1999;140:641–645. [PubMed: 9927288]
22. Rondon IJ, Scandurro AB, Wilson RB, Beckman BS. Changes in redox affect the activity of erythropoietin RNA binding protein. *FEBS Lett* 1995;359:267–270. [PubMed: 7867812]

Abbreviations

BSO	L-buthionine sulfoximine
h	hours
HIF-2	hypoxia inducible factor 2
NAC	N-acetyl cysteine
ROS	reactive oxygen species

Fig. 1. Time-dependent protein accumulation of HIF-2 α under hypoxic exposures. SH-SY5Y cells were exposed to hypoxia (1% O₂) for 0, 1, 4, and 12 h. HIF-2 α accumulation was detected by western blot and normalized by the level of β -actin. Data are shown as means $\pm$ S.E., $n = 3$. # $P < 0.05$, compared to control (0 h).

Fig. 2. Time-dependent redox changes induced by hypoxic exposures. SH-SY5Y cells were exposed to 1% O₂ for 0, 1, 4, and 12 h. Redox status was estimated by GSH/GSSG ratio. Data are shown as means $\pm$ S.E., $n = 4$. # $P < 0.05$, compared to control (0 h hypoxia).

Fig. 3.

Effects of redox modulations on HIF-2 α accumulation. SH-SY5Y cells were treated with 0.3 mM BSO (or 0.5 mM NAC) and 1% O₂ for 0, 1, 4, and 12 h. (A) Percentage increase of GSH/GSSG ratio over basal value (0 h hypoxia). Data are shown as means $\pm$ S.E., $n = 4$. * $P < 0.05$, compared to hypoxia group at the same time points. (B) Western blot representatives of HIF-2 α accumulation in cells treated with NAC and BSO. Western blot for treatment of hypoxia only is shown in Fig. 1. (C) Increase of HIF-2 α accumulation detected by western blot over basal value (0 h hypoxia). Data are shown as means $\pm$ S.E., $n = 3$. * $P < 0.05$, compared to hypoxia group at the same time points.

Fig. 4.

Effects of antioxidants and H₂O₂ on HIF-2α accumulation in hypoxic SH-SY5Y cells. Cells were exposed to 1% O₂ for 4 h with catalase (500 units/ml), MnTMPyP (5 μM) or H₂O₂ (100 μM). HIF-2α accumulation was detected by western blot and normalized based on β-actin levels. Data are shown as means ± S.E., *n* = 3. #*P* < 0.05, compared to control (0 h hypoxia); **P* < 0.05, compared to 4 h hypoxia (Con: control, Hypo: hypoxia, Cat: catalase, MnT: MnTMPyP).

Table 1

Effects of BSO and NAC on cell viability of SH-SY5Y cells exposed to hypoxia. Cells were treated with 0.3 mM BSO or 0.5 mM NAC in 1% O₂ for 0, 1, 4, and 12 h. Percentage of cell death was assessed by LDH release assay. Data are shown as means $\pm$ S.E., $n = 4$. No significant cell death was found between treatment groups at the same time points.

Time (h)	Hypoxia	BSO	NAC
0	0.11 $\pm$ 0.03	0.12 $\pm$ 0.05	0.14 $\pm$ 0.03
1	0.32 $\pm$ 0.09	0.34 $\pm$ 0.07	0.39 $\pm$ 0.09
4	0.54 $\pm$ 0.11	0.55 $\pm$ 0.09	0.54 $\pm$ 0.10
12	2.36 $\pm$ 0.26	2.28 $\pm$ 0.35	2.73 $\pm$ 0.52