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Hypoxia Inducible Factor 1 as a Therapeutic Target in Ischemic Stroke

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Abstract

In stroke research, a significant focus is to develop therapeutic strategies that prevent neuronal death and improve recovery. Yet, few successful therapeutic strategies have emerged. Hypoxia-inducible factor 1 (HIF-1) is a key regulator in hypoxia. It has been suggested to be an important player in neurological outcomes following ischemic stroke due to the functions of its downstream genes. These include genes that promote glucose metabolism, angiogenesis, erythropoiesis, and cell survival. Many lines of evidence have shown that HIF-1 is induced in ischemic brains. Importantly, it seems that HIF-1 is primarily induced in the salvageable tissue of an ischemic brain, penumbra. However, the effect of HIF-1 on neuronal tissue injuries is still debatable based on evidence from *in vitro* and preclinical studies. Furthermore, it is of importance to understand the mechanism of HIF-1 degradation after its induction in ischemic brain. This review provides a present understanding of the mechanism of HIF-1 induction in ischemic neurons and the potential effect of HIF-1 on ischemic brain tissue. The author also elaborates on potential therapeutic approaches through understanding of the induction mechanism and of the potential role of HIF-1 in ischemic stroke.

Keywords

HIF-1; stroke; free radical; antioxidant; neuroprotection; cerebral ischemia

1. Introduction

HIF-1 is a master regulator of hypoxia-responsive genes. It regulates the expression of a broad range of genes that facilitate adaptation to low O₂ conditions. Its targets include genes that code for molecules that participate in vasomotor control, angiogenesis, erythropoiesis, cell proliferation, and energy metabolism. All of these genes may potentially contribute to recovery of neuronal cells following cerebral ischemia and reperfusion. Because of the potentially important roles of genes regulated by HIF-1 following ischemia, it has been suggested that regulating HIF-1 induction and accumulation is a highly promising therapeutic approach for cerebral ischemia. Thus, the mechanism of HIF-1 regulation in ischemic neurons has drawn a lot of attention and is under vigorous investigation. Nevertheless, HIF-1 may also contribute to cellular and tissue damage by stabilizing p53 and promoting angiogenesis. Indeed, both neuroprotective and detrimental effects of HIF-1 have been observed in preclinical models of ischemic stroke. Understanding the mechanism of HIF-1 expression will provide new insight into self-protection mechanisms of ischemic neurons and help in the design of new pharmacological strategies. Thus, the objective of this review is to provide insights into the accumulation mechanism of HIF-1 and its roles in ischemic stroke. This is presented from

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aspects including HIF-1 expression in hypoxic and ischemic brains, therapeutic effects of HIF-1 in ischemic stroke, mechanism of HIF-1 accumulation in ischemic stroke, and our opinions on future studies.

2. HIF-1 expression in hypoxic and ischemic brains

HIF-1 as a transcription factor in response to changes in oxygen levels has been found to be expressed in brains after hypoxic and ischemic exposures. In 1996, it was first reported that both subunit (α and β) mRNAs of HIF-1 were induced in the brains of rats or mice when they were exposed to reduced ambient O_2 concentrations for 30 to 60 min¹. Systemic hypoxia for the durations of 1, 3, or 6 hours rapidly increased the nuclear content of HIF-1 α , the subunit that primarily determines HIF-1 activation in mouse brains². Another study showed that HIF-1 α was maximally expressed after 5 hours of continuous hypoxia and declined to basal levels after 12 hours³. Hypoxic and $CoCl_2$ preconditioning could increase HIF-1 α expression in newborn rat brain⁴. HIF-1 α was significantly induced in a rat cerebral cortex after one hour of recovery from cardiac arrest, and remained elevated for over 12 hours⁵. Experimental evidence has revealed that HIF-1 α is induced in the focal ischemic brain. In 1999, Bergeron *et al.* first reported that after focal ischemia in an adult rat brain, mRNA encoding HIF-1 α was up-regulated in the brain⁶. The up-regulation was observed 7.5 hours after the onset of ischemia and increased further at 19 and 24 hours. Significantly, in the ischemic brain, HIF-1 α appeared to be mostly induced in the penumbra, the salvageable tissue⁶. In contrast to Bergeron's results⁶, Demougeot *et al.* reported that the HIF-1 α level decreased gradually from the ischemic core to more distant regions⁷. A recent study showed a biphasic activation of HIF-1 lasted for up to 10 days in wild type mice⁸. In addition, the activation of HIF-1 DNA binding was reported in primary cultured neurons in an oxygen and glucose deprivation model⁹. These results provide evidence that HIF-1 α is induced in ischemic brains and that the level of its expression is heterogeneous.

3. Effect of HIF-1 on outcomes of ischemic stroke in *in vitro* and preclinical models

3.1. Neuroprotective effect of HIF-1 in ischemic stroke

HIF-1 regulates the expression of a broad range of genes that facilitate cellular adaptation to low O_2 conditions. Its targets include genes that code for molecules that participate in vasomotor control, angiogenesis, erythropoiesis, cell proliferation, and energy metabolism^{10–12}. Each of these functions potentially contributes to the survival of neuronal cells. The neuroprotection of HIF-1 has been extensively reported in various cerebral ischemic models during the last ten years. For example, preconditioning has been proven to be neuroprotective in experimental ischemic models. HIF-1 has been implicated in the neuroprotection induced by hypoxic/chemical preconditioning in brains^{2, 4, 6, 13–16}. Increased HIF-1 α expression by inhibiting prolyl 4-hydroxylase has been shown to provide neuroprotection in permanent focal ischemia *in vivo*¹⁷. Distinct iron chelators, deferoxamine mesylate and mimosine, protected neurons from apoptosis through HIF-1 activation *in vitro*^{18,19}. The neuroprotective effects of deferoxamine were also attributed to the activation of HIF-1 in focal ischemia *in vivo*^{15, 20}. These results reveal that pharmacological induction of HIF-1, as well as HIF-1 induction by ischemia itself, can produce protection against cerebral ischemia^{8, 17, 19, 21–23}. A number of mechanisms have been proposed to account for the neuroprotective effect of HIF-1²⁴:

- a. Expression of its downstream gene product erythropoietin (EPO) has been found to protect cells from hypoxic/ischemic injury^{18, 25–28}.
- b. Vascular endothelial growth factor expression, another downstream gene of HIF-1, counteracts detrimental ischemic injuries^{29, 30}.

- c. HIF-1 may prevent apoptotic cell death through inhibition of cytochrome c release, caspase activation, and PARP cleavage^{31, 32}.
- d. HIF-1 may have the ability to suppress p53 activation and thereby maintain cell survival³³. However, Piret *et al.* suggests differently³⁴. This may be dependent on ischemic severity.

In addition to the four mechanisms, increased glucose transport and glycolytic flow as a result of HIF-1 activation by hypoxia has been linked to tissue viability and cell survival^{15, 18–20, 22, 35}. One important function of glucose metabolism is to sustain a cellular-reducing environment by generating reducing equivalent through oxidative phosphorylation, glycolysis, and the pentose phosphate pathway. Guo *et al.* recently demonstrated that inhibiting HIF-1 α expression by HIF-1 α -specific small interfering RNA (siRNA) transfection increased reactive oxygen species (ROS) generation and cell death²⁴. In contrast, increasing HIF-1 α expression by desferrioxamine induced a more reducing environment and reduced cell death. These results suggest that maintenance of cellular redox status by HIF-1 protects cells from hypoxia and ischemia mediated injuries. In addition, it has been suggested myc is an integral part of a novel HIF-1 α pathway, which regulates a distinct group of Myc target genes in response to hypoxia³⁶. c-Myc plays a critical role in hypoxia-induced proliferation and survival of SOD₂^{−/−} astrocytes by overcoming injury caused by oxidative stress³⁷.

3.2. Detrimental effect of HIF-1 in ischemic stroke

Nevertheless, HIF-1 may contribute to cellular and tissue damage. It has been reported that HIF-1 may mediate apoptosis during hypoxia/ischemia. HIF-1-induced apoptosis has been observed in embryonic stem (ES) cells under hypoxic conditions³⁸. The study indicates that in response to hypoxia, HIF-1 α accumulates, directly associates with, and stabilizes active wild-type p53. It is possible that this increase in p53 protein is responsible for the apoptosis reported in the hypoxia ES cells. Similarly, it has been observed that HIF-1 α signaling elicits delayed death involving the participation of p53 in ischemic primary cortical neurons *in vivo*³⁹ and *in vitro*⁴⁰. Recently, Chen *et al.* showed inhibition of HIF-1 by tricyclodecan-9-yl-xanthogenate (D609) that was modulated thorough PC-PLC_DAG_phosphatidic pathway decreased the VEGF expression and BNIP3 and offered protection⁴¹. It has been suggested that HIF-1 may induce BNIP3, which can cause mitochondrial dysfunction, membrane depolarization, MPTP opening, and a form of cell death having features of both necrosis and apoptosis⁴². It is noteworthy that HIF-1 regulation and effect of HIF-1 on ischemic outcomes may be affected by ischemic duration such as the results of Baranova's 30-min tMCAO⁸ vs. Helton's 75-min bilateral common carotid artery occlusion⁴³ (see detailed discussion in 3.3). Whether short ischemic period may offer limited transcriptional activity in the absence of p300 vs. full transcriptional function in the presence of p300 needs to be considered⁴⁴. In addition, increased nitric oxide from inducible nitric oxide synthase⁴⁵, dopamine from tyrosine hydroxylase⁴⁶, and lactate from lactate dehydrogenase⁴⁷ might also cause potentially detrimental effects on ischemic brain tissue.

3.3. Link between the detrimental and beneficial effects of HIF-1 in ischemic stroke

Using the same neuron-specific HIF-1 α knock-out mice, Baranova *et al.*⁸ and Helton *et al.*⁴³ have reported distinct effects of HIF-1 on neuronal injuries following ischemia. Baranova *et al.* found that the neuron-specific knockdown of HIF-1 α increased tissue damage and reduced the survival rate of middle cerebral artery occlusion (MCAO) mice, indicating that HIF-1 is neuroprotective in their ischemic model. In contrast, Helton *et al.* observed that the knock-out of HIF-1 α reduced ischemic damage, suggesting that decreasing the level of HIF-1 can be neuroprotective. In other words, Helton's results indicate that HIF-1 may induce tissue damage in brain ischemia. Interestingly, the ischemic model that Baranova *et al.* used was 30 min ischemia with unilateral common carotid artery occlusion (mild ischemia) while Helton's

model was 75 min ischemia with bilateral occlusion (severe ischemia). The two observations support a notion that HIF-1 α may induce cell death in a severe and prolonged ischemia, and promote cell survival following mild ischemic insults.

In summary, HIF-1 plays an important role in the fate of ischemic neurons. HIF-1 appears to be involved in the cell's response to ischemic insults with a double-edged sword effect. Its effects possibly depend on the degree of severity of the insults. Understanding the mechanism of HIF-1 accumulation will undoubtedly shed new light on its role in cerebral ischemia and provide potential approaches to regulate its expression.

4. Mechanism of HIF-1 accumulation in ischemic stroke

4.1. General mechanism of HIF-1 degradation

HIF-1 is a heterodimeric transcription factor induced by hypoxia that is composed of HIF-1 α and HIF-1 β protein subunits. HIF-1 α protein is continuously synthesized, but is rapidly degraded and is almost absent in normoxic cells. HIF-1 β is constitutively expressed in cells and does not respond to changes in O₂ tension, though it is essential for hypoxia-induced transcriptional changes mediated by HIF-1^{48, 49}. Thus, HIF-1 activation is primarily dependent on the HIF-1 α protein level^{50, 51}. Exposure to low O₂ results in HIF-1 α accumulation, and exponentially increases in its cellular levels^{52–55}.

Extensive studies have revealed that the HIF-1 α degradation pathway in normoxia includes HIF-1 α hydroxylation by prolyl hydroxylases, binding with von Hippel-Lindau tumor suppressor (pVHL), and degradation by 26S proteasomal system (scheme 1). The mechanism of HIF-1 induction/accumulation under hypoxic conditions has been vigorously investigated, primarily in cancer cells. Heme proteins and ion channels have been suggested to involve in the induction of HIF-1. More recent evidence suggests that HIF-1 can be induced by free radicals, especially reactive oxygen species (ROS). Analysis of events upstream of HIF-1 α suggests a crucial role for ROS generation upon hypoxia via mitochondria as a sensor mechanism of the oxygen level, and that ROS may be involved in stabilizing HIF-1 α ⁵⁶. ROS originating from the mitochondria has been attributed to triggering the increase of the transcription factor in the human hepatocellular carcinoma cell line HepG2⁵⁷, Hep3B cells⁵⁸, cardiomyocytes⁵⁹, endothelial cells^{60, 61}, and renal cells⁶². In addition, the addition of antioxidants pyrrolidine dithiocarbamate or ebselen abolished transcriptional activation of many genes during hypoxia or CoCl₂ exposure in embryonic stem cells, and abolished the hypoxic induction of HIF-1 α , EPO, glycolytic enzymes, and vascular endothelial growth factor⁵⁸.

Paradoxically, accumulated evidence shows that cellular ROS can down-regulate HIF-1 α stabilization in cancer cells. For example, exposure of Hela⁶³ and COS7⁶⁴ cells to H₂O₂ selectively blocked the accumulation of HIF-1 α protein in hypoxia. Inhibition of the Fenton reaction enhanced HIF-1 α levels, HIF-1 transactivation, and also activated expression of the HIF-1 target genes in HepG2 cells⁶⁵. In other experiments, increasing ROS also impaired the hypoxic signaling mechanism and the expression of the HIF-1 α protein in different cancer cells^{54, 66, 67}. Furthermore, over-expression of the thiol reducing protein thioredoxin potentiated hypoxia-induced gene activation in human breast carcinoma MCF-7 cells⁶⁸. The dramatic effect observed with the reducing thiol agent N-mercaptopyrrolidylglycine on HIF-1 α formation and EPO gene activation in normoxic cells provides a strong indication that ROS can, indeed, down-regulate HIF-1 α in Hep 3B and B-1 cells⁵⁴. The mechanism of destabilization of HIF-1 α by ROS may involve ROS increasing HIF-prolyl hydroxylase activity⁶⁵; ROS acting like oxygen in activating prolyl hydroxylase⁶⁷; and ROS increasing HIF-1 α protein oxidation, as a mildly oxidized protein more likely can be degraded by ubiquitin-proteasome systems^{69, 70}.

4.2. Mechanism of HIF-1 degradation in ischemic brains

HIF-1 plays an important role in cell death/survival in ischemic brain cells. To regulate HIF-1 levels would be particularly important. However, the mechanism of HIF-1 degradation/accumulation has not been studied in ischemic brain cells as extensively as in cancer cells. It is known that under normoxic conditions, HIF-1 α is rapidly degraded via the ubiquitin-dependent proteasomal (26S) pathway after hydroxylation and ubiquitination. Under hypoxic conditions, it is generally regarded that HIF-1 α is accumulated due to hydroxylation inhibition^{48, 49, 52–55, 71}. However, recent evidence has revealed that HIF-1 α can also be degraded in hypoxic conditions^{72–74}. The discussion in the previous section suggests that free radicals, especially ROS, play a very important role in the level of HIF-1 in various cancer cells under low oxygen conditions, a mechanism that may be of particular importance in the setting of ischemic brain injury. Cerebral ischemia/reperfusion is known to be associated with an increase in ROS levels and metabolism (see review articles^{75–78}). It has been demonstrated that ROS production in the ischemic core was significantly greater than in the ischemic penumbra during ischemia/reperfusion⁷⁹. Experimental evidence has suggested that ROS is involved in HIF-1 α degradation in ischemic brain cells, although contrasting results have been reported.

On one hand, it seems that ROS induces HIF-1 α accumulation in ischemic neurons and brains. Liu *et al.* reported that ROS increased HIF-1 α and its downstream gene EPO expression in neuronal cultures after hypoxia⁸⁰. The study suggested that ROS induced by preconditioning might downregulate the threshold for HIF-1 α and EPO activation. HIF-1 α accumulation was observed in both old and young rat cerebral cortex exposed to intermittent hypoxia, which increases ROS production⁸¹. Cyanide induced a rapid surge of intracellular ROS generation, followed by nuclear accumulation of HIF-1 α in an immortalized dopaminergic cell line⁸². Manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride, a potent scavenger of superoxide, not only prevented chronic intermittent hypoxia-induced increases in ROS, but also chronic intermittent hypoxia-evoked HIF-1 α up-regulation in mice⁸³. In another experiment, SOD1 Tg neuronal culture, which had low levels of ROS, resulted in a low level of HIF-1 α expression, compared to neurons from control animals⁸⁰.

On the other hand, many investigators have observed that ROS may promote HIF-1 α degradation and decrease HIF-1 α levels in ischemic neurons and brains. Dimethyloxallyl glycine (DMOG) and ethyl 3, 4-dihydroxybenzoic acid (DHB) are prolyl hydroxylase inhibitors and well-known activators of HIF-1. Most recently, it was observed that treatments with either DMOG or DHB during nerve growth factor (NGF) deprivation suppressed the accumulation of ROS to levels that were only slightly above the basal level measured in NGF-maintained neurons⁸⁴. This result implies that inhibiting ROS may play a role in DMOG and DHB's ability to increase HIF-1 expression. Superoxide anion radical generated by xanthine/xanthine oxidase reduced HIF-1 α levels in explants of human pial arteries⁸⁵. The antioxidant pyrrolidine dithiocarbamate or coexpression of the redox factor-1 increased HIF-1 α levels in RacG12V-cells⁸⁶. Glucose is a main substrate required for production of the principal intracellular reductants NADPH and GSH by the pentose phosphate pathways^{87, 88}, and subsequently maintains a reduced cellular milieu in the brain^{89–91}. Consequently, glucose deprivation would result in a condition of metabolic oxidative stress. In fact, it has been reported that glucose deprivation increased ROS generation in neurons under hypoxic conditions^{72, 92}. A negative relationship was observed between the HIF-1 α level and the H₂O₂ level⁷². Furthermore, N-acetyl cysteine, a ROS scavenger, increased the HIF-1 α protein level while L-buthionine sulfoximine, which induces oxidative stress, inhibited HIF-1 α expression in hypoxic neurons. This result indicates that ROS does not promote the stabilization of HIF-1 α protein, but rather induces its degradation in hypoxic neurons.

Although it is not clear how ROS accelerates HIF-1 α degradation in ischemic brain, ROS may promote HIF-1 α degradation through two mechanisms (scheme 2). First, ROS may activate

the proteasomal degradation pathways. The proteasomal proteolytic pathways are among the principle mechanisms by which many intracellular proteins are degraded⁹³. These pathways are critical in regulating important cell processes including differentiation, cytokine-induced gene expression, apoptosis, and the stress response^{94–96}. The proteasome complexes exist in two forms, 26S and 20S. The two different complexes usually have different protein substrate specificities. The 26S proteasomal pathway is ubiquitin-dependent and is a major pathway for protein degradation in cells. The 20S proteasomal pathway is ubiquitin-independent and primarily degrades cellular oxidized proteins under conditions of oxidative stress^{93, 97–100}. ROS may activate the 26S pathway by increasing prolyl hydroxylase activity under hypoxia^{67, 101–103}. ROS may also elevate 20S proteasomal activity¹⁰⁴. HIF-1 α is sensitive to oxidative attacks^{63, 105} with its cysteine and methionine residues being vulnerable to oxidative damages. In fact, a recent publication has shown that the 20S degradation pathway appears to be involved in HIF-1 α 's turnover in hypoxia⁷⁴. Second, it has been reported that p53 mediates proteasomal degradation of HIF-1 α ¹⁰⁶. The protein p53 is a redox sensitive and can be activated by oxidants. ROS may activate p53 expression, which promotes the degradation of HIF-1 α . In addition, under hypoxia, HIF-1 α may transcriptionally activate its own degradation, possibly mediated by acetylation⁷³.

Due to these controversial observations, further study is needed to elucidate the exact mechanism of HIF-1 α degradation/accumulation induced by ROS generated in ischemic neurons and brains. The following specific questions may help elucidate the role of ROS in HIF-1 α degradation/accumulation in ischemic brain:

- a. Does the level of ROS have differential effect on HIF-1 α accumulation? In other words, do low levels of ROS induce HIF-1 α accumulation? For instance, Chang *et al.* observed that low levels of H₂O₂ might up-regulate HIF-1 α protein¹⁰⁷. Do high levels of ROS promote HIF-1 α degradation?
- b. Does the level of ROS have differential effect on the activities of proteasomal activities in ischemic neurons and brains? It has been suggested that the effects of ROS on the 26S proteasomes appear to depend on the severity of oxidative stress^{102, 103}.
- c. Does ROS have varied effects on the components of the 26S proteasomal degradation pathway that include protein modifications such as hydroxylation, pVHL binding, ubiquitination, and 26S proteasomes? For example, ROS do not seem to increase pVHL binding and ubiquitination activities¹⁰⁸, but might activate prolyl hydroxylase under hypoxia^{67, 101}.
- d. Which ROS is responsible for HIF-1 α degradation/accumulation because ROS include a group of oxygen centered reactive species such as superoxide anion radical, H₂O₂, and hydroxyl radical?
- e. Does ischemic severity play a role in low/high levels of ROS generation that may regulate the beneficial/detrimental effect of HIF?

5. Future directions

Stroke ranks second after ischemic heart disease as a cause of lost disability-adjusted life-years in the U.S. and worldwide¹⁰⁹. Recombinant tissue plasminogen activator (r-tPA) is the only approved therapy for selected patients with acute ischemic stroke. However, r-tPA has a very limited treatment window and may result in bleeding complications¹¹⁰. Extensive research has provided evidence that free radicals are involved in ischemic brain damage. The oxidative damage theory has led to numerous experimental and clinical studies using antioxidants as neuroprotectants^{111–113}. Many antioxidants have been tested in phase III clinical trials such as ebselen, tirilazad, edaravone, and NXY-059. There are antioxidants still undergoing phase

III clinical trials, such as uric acid¹¹⁴. However, except for edaravone, which has been approved as a stroke drug in Japan, most of these completed trials failed due to lack of efficacy. After the NXY-059 trial was recently declared a failure¹¹⁵, the research team pointed out that one major reason for the failure was incomplete understanding of the mechanisms responsible for cell death during ischemia¹¹³. Understanding the endogenous neuroprotection conferred by ischemic tolerance has been suggested a good way to unravel the molecular mechanisms, and might improve therapeutic strategies for patients with stroke^{113, 116}. As HIF-1 is a master regulator of a large number of genes that are critical in survival response to hypoxia in diverse cell types, future studies on HIF-1 regulation and its role in brain injury under ischemia will elaborate the fundamental mechanism of neuronal survival in ischemia and will assist in designing novel treatments for human stroke.

As discussed above, ROS may play a critical role in regulating HIF-1 expression in ischemic brain. This novel concept has high potential to be used to screen a broad range of antioxidant drugs. In the past clinical trials, the antioxidant drugs have been focused on their ability to scavenge ROS and to reduce lipid peroxidation. Different from these previous studies on antioxidant drugs for stroke treatments, this new screening would focus on the efficacy of potential antioxidant drugs in preventing neuronal death based on the novel pathway. This will help to find out why the failed drugs did not work, which may be due to their inability in affecting HIF-1 α stabilization. Hopefully, this will also help find antioxidants that work for human stroke treatments. It is noteworthy that one caveat for antioxidant treatments is that they are not specific to HIF-1, instead, their effects are much broader.

Furthermore, researchers in academic institutions and pharmaceutical companies currently screen small molecules that selectively inhibit HIF-specific prolyl hydroxylases (PHDs) for drug discovery in stroke treatments as increasing HIF-1 stabilization by inhibiting its hydroxylation is believed to provide therapeutic benefits for stroke patients^{17, 117–119}. However, HIF-1 α may also be degraded in hypoxia through other mechanisms as discussed above. For example, both ubiquitin-dependent and -independent proteasomal pathways could be involved in the degradation of HIF-1 α . Consideration of all these factors may provide more efficient approaches in treating the disease.

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Scheme 1.

HIF-1 α degradation through ubiquitin-dependent proteasome (26S) pathway under normoxic conditions. Note: One hydroxylated proline residue of the two present in HIF-1 is sufficient for HIF to be recognized by VHL.

Scheme 2.

Proposed HIF-1 α degradation through both ubiquitin-dependent proteasome (26S) and ubiquitin-independent proteasome (20S) pathways under hypoxic conditions. ROS may increase prolyl hydroxylation and 26S proteasomal activity, and thus promote HIF-1 α degradation. ROS may also promote HIF-1 α degradation by oxidizing HIF-1 α and elevating 20S proteasome activity.