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Peroxynitrite as an alternative donor of oxygen in HIF-1α proline hydroxylation under low oxygen availability

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Abstract
In the last years, nitric oxide (NO) mediated signaling became an integral component in understanding physiological and pathophysiological processes of cell proliferation, death or cellular adaptation. Among other activities, NO affects multiple targets that allow regulation of gene expression. Recently, NO was found to attenuate accumulation of hypoxia inducible factor-1alpha (HIF-1α) under hypoxic conditions because of several mechanisms: redistribution of oxygen toward non-respiratory oxygen-dependent targets (like HIF-1α proline hydroxylases—PHDs, which perform hydroxylation of Pro402/564 of HIF-1α leading to its proteasomal degradation); in addition, peroxynitrite formed during interactions between NO and mitochondria derived superoxide leads to an increase in cytosolic iron/2-oxoglutarate (2-OG), which required for PHD activation. Here, we propose a hypothesis that peroxynitrite, formed in the cells upon exposure to NO under low oxygen availability, serves as an alternative donor of oxygen for activated PHDs so they can perform HIF-1α proline hydroxylation to de-accumulate the protein.

Keywords: *Hypoxia, nitric oxide peroxynitrite, proline hydroxylases, hypoxia inducible factor 1*

Introduction

Hypoxia-inducible factor-1 (HIF-1), a general hypoxia-inducible heterodimeric transcription complex, which plays pivotal role in mediating cellular responses to hypoxia [1,2]. HIF-1α is the oxygen-regulated subunit, rapidly accumulating in cells exposed to hypoxia [3]. The activity of HIF-1 is primarily determined by stability regulation of its alpha subunit, which is stabilized under hypoxia but degraded during normoxia. Under well-oxygenated conditions, HIF-1α protein undergoes rapid ubiquitination and degradation by the proteasome system [4,5]. HIF-1α is ubiquitinated by the von Hippel–Lindau protein (pVHL) [6], which binds directly to the oxygen-dependent degradation domain of HIF-1α and targets it for proteasome degradation [6,7]. VHL is associated with elongins B and C, cullin-2 and likely other factors that constitute part of a multiprotein complex [7]. Interaction between pVHL and a specific domain of the HIF-1α subunit is regulated through hydroxylation of proline 402/564 residues by enzymes—prolyl hydroxylases 1, 2 and 3 (PHDs) [8–10]. In recent years, there is an increasing interest to the phenomenon of nitric oxide (NO) dependent regulation of HIF-1α protein stability [11]. On one hand, it was reported that the protein is accumulated upon NO impact under normal oxygen conditions through inhibition of PHDs and S-nitrosation [12–15]. On the other hand, there is clear evidence that under hypoxic conditions, the activity of PHDs is restored by NO and therefore HIF-1α protein stabilization is attenuated [16–18], however, this

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phenomenon is not fully understood. Here, we propose a hypothesis explaining the mechanism employed to down-regulate HIF-1α protein stability based on recruitment of alternative donor of oxygen by HIF-1α PHDs.

Hypothesis of alternative donor of oxygen used by cellular HIF-1α PHDs upon exposure to nitric oxide under low oxygen availability

HIF-1α PHDs have a requirement for dioxygen, iron (prostetic group) and 2-OG [8,9]. Under hypoxic conditions, prolyl hydroxylation of the HIF-1α subunit is suppressed, causing stabilization of the protein. Lack of oxygen availability, which takes place under hypoxia leads to decrease in PHD activity. However, inhibition of mitochondrial respiration under hypoxic but not anoxic conditions is followed by PHD-dependent degradation of HIF-1α protein [19,20] more likely because of redistribution of molecular oxygen toward non-respiratory oxygen-dependent targets such as PHDs so that they do not react strongly to hypoxia [19]. It was also reported that, HIF-1α PHD-2 is over expressed upon lack of oxygen. Therefore, despite the deficiency of oxygen cannot be covered, the probability of interactions between redistributed oxygen and PHD is increased [19].

Recently, there was obtained clear evidence that NO inhibits HIF-1α protein accumulation by activating PHDs [21]. We have currently observed that, NO inhibits HIF-1α accumulation under low oxygen (1%) conditions. The effect is supported by an increase in tyrosine nitration and caused by the formation of peroxynitrite in the cells, which leads to damage of mitochondria and their respiratory chain followed by the increase in 2-OG and iron (the components needed for activation of HIF-1α proline hydroxylases) concentrations in cell cytosol. The inhibiting effect of NO on HIF-1α accumulation was not observed in the cells lacking mitochondria. On the other hand, the depletion of intracellular glutathione (GSH) was observed upon cell treatment with NO donors under hypoxic conditions. Treatment of those cells with *N*-acetyl-cysteine increased the amount of intracellular GSH, attenuated the NO effect and abolished the damage of mitochondria as well as the release of 2-OG/iron [22].

It has been shown that, the destabilization of HIF1α upon inhibition of mitochondrial respiration in hypoxia is dependent on PHD activity. Because, inactivation of PHDs in hypoxia is a result of limited O_2 availability, it is reasoned that respiratory inhibitors might increase the availability of nonrespiratory O_2 and consequently reactivate the enzymes. The experiments with Renilla luciferase indicate that, inhibition of mitochondrial respiration can indeed increase cellular O_2 availability. This increase would be significant in hypoxia when the cellular O_2 concentration becomes limiting for enzymes such as PHDs, which have a higher K_m for O_2 than does cytochrome *c* oxidase. It was suggested that, NO acts as an endogenous regulator of intracellular O_2 availability in mammalian cells [19]. In addition, it was demonstrated that inhibition of mitochondrial O_2 consumption creates the paradox of increased O_2 availability for PHDs of HIF1α, leading to a situation in which the cell may fail to register hypoxia. It is possible that, NO-dependent diversion of O_2 may reactivate other enzymes whose activities are reduced in hypoxia.

On the other hand, the amount of redistributed oxygen is probably not enough to restore PHD activity. Another reason for that is the increase in oxidation processes upon inhibition of respiratory chain. When NO inhibits complex IV of the mitochondrial respiratory chain, other complexes (in particular, complexes I and III) start producing reactive oxygen species (ROS), that mediate oxidation processes [23], upon which Fe^{2+} prostetic groups become oxidized to Fe^{3+} and cannot use molecular oxygen as the donor for generation of OH-group due to iron-dependent activation of molecular oxygen becomes impossible. In the case of complex I, inhibition by ROS (more likely by peroxynitrite formed) under hypoxic conditions, the decrease in intracellular GSH is significant, that confirms the oxidation [20]. Therefore, upon increased production of ROS, oxidation and low oxygen availability (1% or less), it is doubtful that there will be enough fully active PHDs as well as molecular oxygen to attenuate stabilization of HIF-1α protein by proline hydroxylation. However, it is known that HIF-1α protein accumulation is blocked in the cells upon exposure to hypoxia/NO exactly by PHD-dependent hydroxylation [22,23]. Therefore, there is evidence that under such conditions, PHDs use some other oxygen donor, which is different from the molecular oxygen.

We have recently observed that, NO is converted to peroxynitrite in the cells exposed to low (1%) oxygen conditions, which was confirmed by an increase in nitrotyrosine formation upon described conditions [22]. Ebselen, the peroxynitrite scavenger restored HIF-1α protein accumulation, however, there were no changes in oxygen availability. On the other hand, ebselen is also a hydrogen peroxide scavenger. The possibility of H_2O_2 participation was ruled out in the control experiment, where it was found that, the amount of hydrogen peroxide was decreased in the cells exposed to low (1%) oxygen conditions and NO compared to the cells treated only with hypoxia (1% oxygen) as well as were not significantly different from the non-treated control cells as measured by widely described method [22,24]. On one hand, the inhibition of the mitochondrial respiratory chain by peroxynitrite was attenuated but there could be another reason—loss of second donor of oxygen. Given that, iron in the PHDs could be in its oxidized form (Fe^{3+}) under such conditions, PHDs might need reactive, negatively charged oxygen-containing molecule, which could be converted in the way similar to those taking part in the

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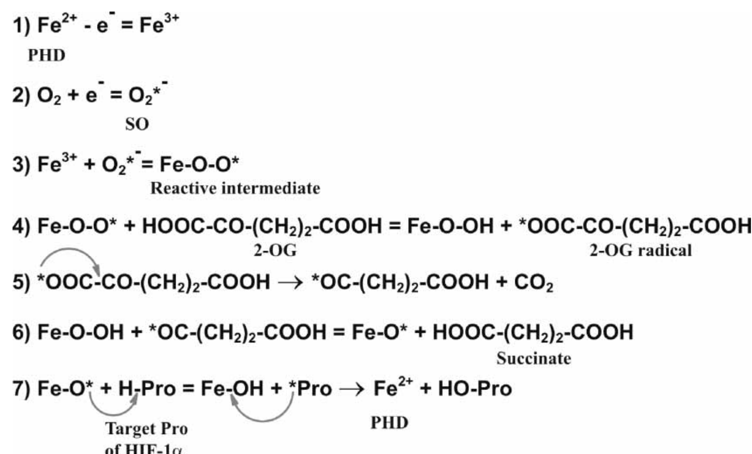

Figure 1. Consequence of electron and radical transfers during HIF-1α PHD reaction. Molecular oxygen activated by enzymatic iron is used as a supplier of oxygen atom used in proline hydroxylation process. 2-OG is employed as acceptor of electrons, which are not used in proline hydroxylation. Abbreviations: SO, superoxide; Pro, proline; HO-Pro, 4-hydroxyproline.

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case of molecular oxygen recruitment. In this respect, peroxynitrite ideally fits as a donor. The normal PHD reactions are outlined in the Figure 1. From here, it is clear that iron needs to activate oxygen for further interactions and donations. Active oxygen in the form of negatively charged superoxide radical is able to interact with Fe^{3+} forming reactive intermediate used for the substrate hydroxylation. Peroxynitrite has the negative charge similar to the one of superoxide radical, therefore, it might be able to interact oxidized iron of PHDs. Then the conversions of peroxynitrite and substrate hydroxylation could be performed in the way similar to the one, where molecular oxygen is employed (see Figure 2 for details).

Conclusions

Summarizing the total picture of inhibition of HIF-1α protein accumulation by NO upon low oxygen availability, we would like to underline the main points of this difficult process. First of all, NO inhibits complex IV of electron respiratory chain leading to ROS generation [23]. ROS, in particular, superoxide, interact with NO forming peroxynitrite. Peroxynitrite inhibits complex I of the mitochondrial respiratory chain, inducing dysfunction of mitochondria, which results in 2-OG/iron release [22]. Both compounds are recruited by HIF-1α PHDs. On the other hand, the expression of HIF-1α PHD-2 is increased under hypoxic conditions [19,25,26]. However, ROS and peroxynitrite induce oxidation in general and more likely that the iron responsible for PHD catalytic reaction is oxidized in part of PHD molecules. Peroxynitrite probably serves as a compensatory donor of oxygen and used by PHDs to perform hydroxylation of HIF-1α proline residues. Points stated above are summarized in the scheme shown in the Figure 3.

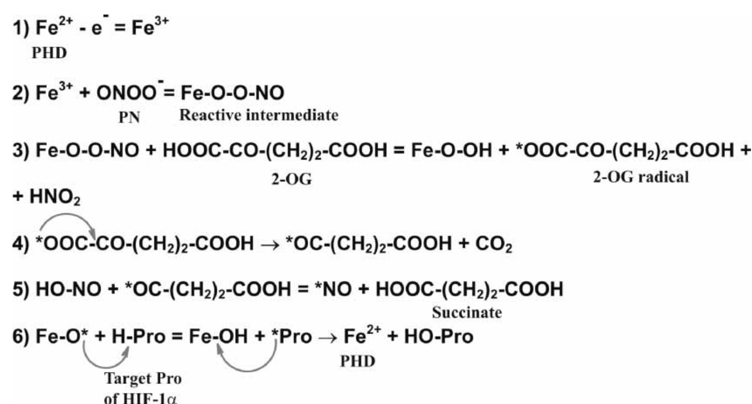

Figure 2. Hypothetic consequence of electron and radical transfers during HIF-1α PHD reaction with peroxynitrite as the donor of oxygen. Peroxynitrite interacts with oxidized PHD iron and used as a supplier of oxygen atom for proline hydroxylation process. 2-OG is also employed as acceptor of electrons, which are not used in proline hydroxylation. Abbreviations: PN, peroxynitrite; Pro, proline; HO-Pro, 4-hydroxyproline.

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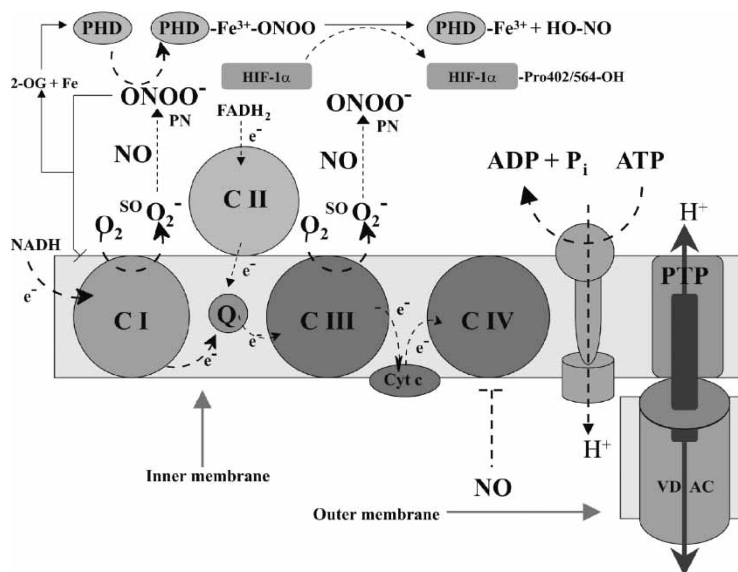

Figure 3. Summary of processes leading to inhibition of HIF-1α protein accumulation in the cells upon exposure to NO under hypoxic conditions. NO inhibits complex IV (C IV) of the electron respiratory chain. This leads to generation of superoxide radicals by complexes I (C I) and III (C III). Generated superoxide interacts with NO forming peroxynitrite, which inhibits C I and II (C II) of the respiratory chain [23]. All the events described result in the dysfunction of mitochondria followed by the release of HIF-1α PHD activating 2-OG/iron. PHD iron, could easily be oxidized to Fe^{3+} upon peroxynitrite formation leading to glutathione depletion [15,16]. According to our prediction, PHDs use peroxynitrite as oxygen donor for HIF-1α Pro 402/564 hydroxylation. Other abbreviations used: Q, co-enzyme Q; PTP, permeability transition pore; VDAC, voltage-dependent anion channel.

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