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Review Article

Need (more than) two to *Tango*: Multiple tools to adapt to changes in oxygen availability

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Abstract

Oxygen is a fundamental element for the life of a large number of living organisms allowing an efficient energetic utilization of substrates. Organisms relying on oxygen evolved complex structures for oxygen delivery and biochemical machineries dealing with its safe utilization and the ability to overcome the potentially harmful consequences of changes in oxygen availability. On fact, cells composing complex Eukaryotic organisms are set to live within an optimum narrow range of oxygen, quite specific for each cell type. Minute modifications of oxygen availability, either positive or negative, induce the expression of specific genes, the major actors of this responses being the transcription factors HIF and Nrf2 that control the attempt to cope with low oxygen (hypoxia) or to either high oxygen or to an oxygen "overflow," respectively. This review describes the interaction between these two transcription factors and their interaction with the transcription factor NF-κB acting as a pivotal determinant of final cell response. © 2018 BioFactors, 44(3):207–218, 2018

Keywords: *hyperoxia; hypoxia; Nrf2; HIF; NF-κB*

Abbreviations: *$O_2^{-\bullet}$, superoxide anion; H_2O_2, hydrogen peroxide; HO•, hydroxyl radicals; NOS, nitric oxide synthase; NO, nitric oxide; $ONOO^-$, peroxynitrite; PHD2, prolyl hydroxylase domain-containing protein 2; HIF, hypoxia-inducible factor; HRE, cis-acting hypoxia response element; VHL, von Hippel-Lindau protein; FIH, factor inhibiting HIF; NFEL2L2/Nrf2, nuclear factor (erythroid-derived 2)-like 2; EpRE/ARE, electrophile/antioxidant responsive element; Keap1, Kelch ; ECH, associating protein 1; NF-κB, nuclear factor k-light-chain-enhancer of activated B cells; IκB; NF-κB, inhibitory protein; IKK, IκB kinase; AP-1, activator protein-1*

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1. The raise of oxygen raiders

Roughly, 2.5 billion years ago, for the first time, oxygen reached a significant part as component of Earth's atmosphere [1]. The most credited hypothesis for this event is in the progressive outbreak of *Cyanobacteria* (or blue–green algae), the first microbes producing oxygen at the end of photosynthesis [2].

The progressive rise of atmospheric O_2 levels severely challenged living organisms that had to face the presence of a novel massive abundance of a "peculiar" chemical element. In fact, O_2 is a relatively stable but highly reactive free radical, capable of combining with most other elements. The presence of two unpaired electrons with parallel spin partially quenches the reactivity of molecular oxygen, as only one electron at a time can be accepted from a donor molecule. In spite of this spin restriction, O_2 is still a very reactive element and in face of this novel challenge, existing living organisms had few options: either die, or shelter in anoxic environment [3] or evolve to adapt [2].

As a result of evolution, living organisms not only learnt how to cope with oxygen high reactivity, but also to utilize this

specific feature. O_2 is now needed by all higher eukaryotes as the terminal electron recipient within the process of ATP synthesis. The "invention" of an electron transport chain and the rise of an authentic endosymbiosis leading to its permanent localization of cellular "breathing" into separate intracellular organelles, the mitochondria, tremendously boosted the energetic capability of living organisms, creating in turn novel evolutionary opportunities. In fact, at this point of the evolutionary history of life, primitive metazoan had dimensions small enough to allow the diffusion of O_2 to the few thousand cells composing the ancestral organism, but cells equipped with mitochondria had the bioenergetics features to realize eukaryotic cell complexity [3,4]. The evolutionary discovery of systems able to supply air and deliver O_2 to all the cells within the body opened the avenue to the development of bigger, more complex organisms. The evolution of a complex respiratory, circulatory, and nervous systems able to modulate efficiently the take of O_2 and its distribution throughout large bodies, composed of several billions of cells eventually completed the path to the utilization of O_2 in vertebrates [5].

In order to get to this goal, cells had evolved to equip themselves not only with complex devices aimed to the delivery oxygen throughout the body, but also with machineries able to overcame the consequences of a severe "oxidative challenge," potentially able to threaten life by scavenging reactive species, or quench their reactivity or repair oxidative damage as, some of the negative consequences due to oxygen reactivity, mentioned above, have not been completely subdued.

Mitochondria are one of the most important source of reactive oxygen. Even though mitochondrial electron transport is very efficient, electrons can escape from their path and form partially reduced reactive O_2 species such as superoxide ($O_2^{-\bullet}$) eventually leading to hydrogen peroxide (H_2O_2) generation [6]. In the presence of a heavy bout of exercise, usually associated with high mitochondrial activity, this leakage is likely to increase and potentially induce specific responses aimed to cope with oxygen-induced challenge [7]. It is generally accepted that real culprit for most of the oxidative damage to cellular macromolecules is due to a further reaction of these molecules with transitional metals (Fe and Cu) generating hydroxyl radicals (HO•), a three electron reduction of O_2 [8]. Similarly, a distinct electron transport system, nitric oxide synthase (NOS) uses NADPH to synthesize nitric oxide (NO), a mildly reactive free radical playing specific roles in several tissues, including the regulation of vascular tone, the immune response and neuronal cells communication and, like many of these types of oxido-reductases, can also produce $O_2^{-\bullet}$ [9]. A further reaction between NO and $O_2^{-\bullet}$ generates peroxynitrite ($ONOO^-$) [10], another highly reactive oxidizing and nitrating molecule, potentially able capable of damaging all cellular macromolecules [11].

Oxidative damage exerted by reactive oxygen and nitrogen species (frequently referred as RONS) to biological structures is well known and has been the target of a very rich and fortunate vein of research: in fact, not only the normal physiology, but also the majority of pathologies (if not all) share an oxidative component within their biochemistry. A detailed description of both the oxidative damage and antioxidant defenses is out of the scope of this review. Hereby, the major cellular oxygen sensing mechanisms and their interplay in composing the response (and possibly the repair of damages) associated to positive or negative fluctuations of oxygen availability will be discussed.

2. Which oxygen concentration is "good"? and how much is either "not enough" or "too much"?

Considering that mammalian skin cells are protected by the epidermis, a barrier composed an outermost layer of differentiated, not vascularized keratinocytes enveloped by the protein keratin [12], the only cells "normally" exposed to atmospheric O_2 pressure and concentrations are those composing the lens of the eyes, and cells of the upper airways, including mouth and nose and upper regions of the lungs.

Complex organisms like mammals developed a first defense against oxygen toxicity by maintaining a gradient of oxygen tension, from the environmental level of 20% to a tissue concentration of about 3–4% (or less). Starting from atmospheric air to individual cells, pO_2 decreases from approximatively 150 mm Hg in the upper airway to about 30 mm Hg in most tissues and finally to as low as 5 mm Hg in peripheral tissues, and these values are quite well maintained [13].

Atmospheric oxygen passively diffuses across the alveolar-capillary membrane in the blood, according to concentration gradients. In normal conditions, arterial pO_2 is 75–100 mm Hg with an oxygen percentage of about 13% while venous pO_2 is obviously lower, being between 30 and 40 mm Hg and about 5% [14].

Data obtained in rats indicate that oxygen tension is highest in the bladder (~60 mm Hg), followed by muscle (~40 mm Hg), liver (~20 mm Hg), and renal cortex (~15 mm Hg) [15]. Furthermore, oxygen gradients exist within each tissue depending on the distance of cells from the closest oxygen-supplying blood vessel and the cellular metabolic demand [16].

Independently of the total amount of oxygen that reaches specific tissues and the amount actually utilized to implement different cell functions, the optimal oxygen concentration seems to be restricted to a narrow range quite specific for each cell type. Accordingly, cells efficiently respond to minute modifications of oxygen availability by specifically expressing genes in the attempt to cope with oxygen deficiency (hypoxia), a circumstance occurring when oxygen demand exceeds its availability in (para-)physiological conditions or, more dramatically, in the presence of a severe impairment of blood delivery, eventually resulting in ischemia. Human tissues undergo hypoxia during sepsis, sleep apnea, chronic obstructive

pulmonary disease (COPD), diabetic kidney disease, wound healing and in the context of the solid tumor microenvironment [17].

Conversely, in the presence of high blood flow, such as that occurring in the skeletal muscle that during heavy exercise [7,18], while breathing high partial pressures of oxygen or normoxic high pressure breathing associated to hyperbaric therapy but also to recreational scuba diving [19–21], cells and organs are exposed to oxygen "overflow." These events are likely to induce a condition frequently referred as "oxidative stress" [22]. This term, probably one of the vaguest and often abused terms in science, is intended to describe "an imbalance between oxidants production and antioxidant and repair defenses, resulting in the increased steady-state levels of oxidized cellular macromolecules" [23].

3. Sensing variations: A delicate balance between harsh extremes

The survival of an organism integrated in a relatively variable environment is strictly bound to the ability to cope with variable and potentially stressful conditions, and overcome them by the ability to trigger cellular adaptive compensatory changes and complex "system" responses [24].

As mentioned above, environmental conditions, and specific events, including high workout bouts, can affect oxygen availability, and lead to variations that drive its concentration far from the tight "optimum." Therefore, cells and consequently tissues, organs and whole organisms must temporarily set themselves to respond to these emerging situations. According to specific events, cells react in opposite directions either to a reduced availability of oxygen (hypoxia) or to an excess of oxygen availability. Definite, relatively rapid and efficient cellular mechanisms exist to adapt to emerging environmental conditions involving oxygen partial pressure and in general oxygen availability.

3.1. HIF: Not enough oxygen...

Humans display specific adaptations to chronic hypobaric hypoxia. Adaptable characters have been found in "Highlanders" (Quechuas and Sherpas) and "lowlanders," independently on their common ancestry. These characters are expressed both at the whole body levels of organization and at the biochemical level (*e.g.*, differential reliance on glycolysis or ATP yield per mole of oxygen consumed) [25]. Similarly, Tibetans show a stable adaption to the chronic hypoxia associated to high altitude as a genetic signature in prolyl hydroxylase domain-containing protein 2 (PHD2) gene encoding for a prolyl hydroxylase controlling the most important cellular response to hypoxia [26].

The major factor controlling the response to lower levels of oxygen is the hypoxia-inducible factor (HIF). This name identifies the members of a family of DNA-binding transcription factors that associates with specific nuclear cofactors under hypoxia to transactivate a large spectrum of genes, triggering different adaptive and developmental responses associated to lower oxygen availability [27].

Hypoxia-inducible factor family is composed of three members, HIF-1 and HIF-2 being the best characterized. To date, hundreds target genes are known to be transactivated by binding of HIFs to a cis-acting hypoxia response element (HRE) located either within the target gene or at its flanking sequences [28]. These genes are involved in a wide spectrum of responses associated with the control cellular process including angiogenesis, erythropoiesis, glycolysis, iron transport, cell proliferation and survival, and vascular remodeling. HIF-3 is the more distant, still scarcely known related member of the family. Its protein structure and role in regulating gene expression differ from those of HIF-1 and HIF-2. HIF-3α is considered a negative modulator of the expression of HIF-regulated genes by competing with HIF-1α and HIF-2α in binding to transcriptional elements in target genes during hypoxia, probably playing a role in fine-tuning the cellular response [29]. This review will be mainly focused on HIF-1 activity and functions. However, it is important to mention that HIF-1 and HIF-2 share similar domain structure, heterodimerize with HIF-1β, and bind to DNA at the same responsive element [30] but their effect on gene expression, in particular related to the regulation of angiogenic response, is significantly different. In fact, although both isoforms upregulate the expression of pro-angiogenic vascular endothelial growth factor (VEGF), HIF-1 activity associates with a decrease of the expression of interleukin-8 (IL-8) by inhibiting the activity of Nrf2 transcription factor (see Chapter 3.3 of this review). Conversely, the upregulation of IL-8 expression by HIF-2 is associated to the activity of SP-1 transcription factor and independent of Nrf2 activation [31].

HIF-1 and HIF-2 have also an opposite effect on the oncoprotein c-Myc activity. HIF-1α induces an increase of c-Myc activity by stabilizing its interaction with the myc-associated factor X (Max) promoting cell cycle progression. On the other hand, HIF-1 inhibits c-Myc, probably by competing to the binding to Max and induces the arrest of cell cycle at G1/S phase [30].

The effects exerted by both HIF isoforms and their complex interaction with other transcription factors are presently undergoing to intensive research addressing in particular their role in angiogenesis [32].

At the molecular level, HIF-1 is composed of two subunits: HIF-1α and HIF-1β, each containing basic helix-loop-helix-PAS (bHLH-PAS) domains that mediate heterodimerization and DNA binding. HIF-1β is constitutively expressed regardless of oxygen availability [33] whereas HIF-1α protein has a short half-life ($t^1/_2 \sim 5$ min) and is highly regulated by oxygen [34]. Even though transcription and synthesis of HIF-1α are constitutive and seem not to be affected by oxygen availability [33,35], in normoxic conditions it is rapidly degraded, following a mechanism described below and no HIF-1α protein is essentially detectable. The β subunit, present in excess, heterodimerizes with other bHLH-PAS proteins making HIF-1α

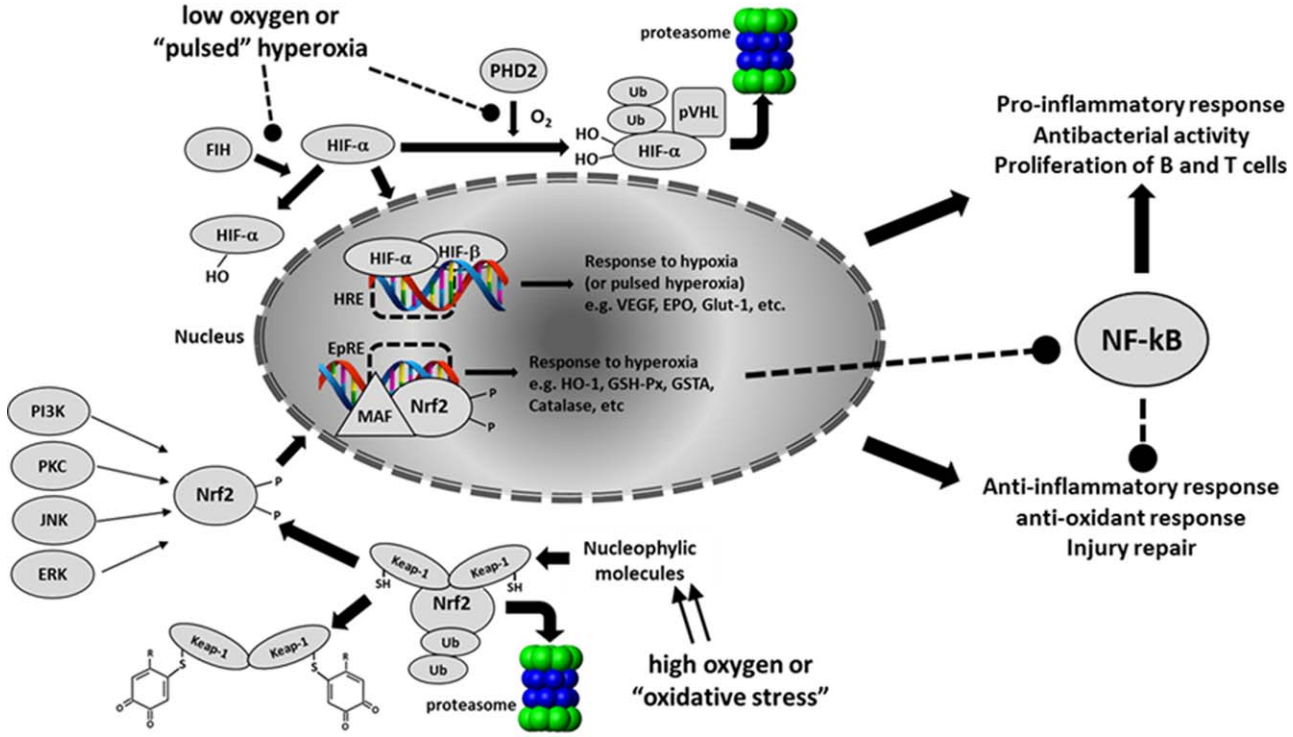

FIG 1

Prolylhydroxylase–hypoxia-inducible factor (PHD–HIF) pathway under hypoxia is described in the upper part of the figure: HIF-α is constitutively transcribed and translated. Its level is primarily regulated by its rate of degradation controlled by oxygen availability through its enzymatic hydroxylation by PHDs. Hydroxylated HIF-α is recognized by Hippel–Lindau protein (pVHL) and rapidly degraded. Non-hydroxylated HIF-α does not undergo proteolysis and binds to its heterodimeric partner HIF-β and transactivates genes involved in the adaptation to hypoxic–ischemic stress. PHD activity is inhibited under hypoxia or by nitric oxide, oxidative stress, transition metal chelators, 2-oxoglutarate analogs, and other intermediates of metabolism. Keap1–Nrf2 pathway is described in the lower part of the figure: Nrf2 is constitutively expressed. The major proportion of Nrf2 bounds to Keap1 and is subjected to proteasome degradation. Under conditions of chemical/oxidative stress, specific sensing thiol groups of keap1 react with nucleophilic molecules, inducing its dissociation from Nrf2 allowing the escape from proteolysis and its accumulation within the nucleus where it heterodimerizes with a small Maf protein and binds to the antioxidant/electrophile-responsive element (ARE/EpRE). Other regulatory mechanisms involve the phosphorylation of Nrf2 mediated by protein kinase C/mitogen-activated protein kinase (MAPK), leading to an enhanced stability and/or release of Nrf2 from Keap1 and histone acetyl transferase (HAT)/histone deacetylase (HDAC)-mediated acetylation. NF-κB is the third actor playing downstream as a fine modulator of HIF and Nrf2 mediated responses, driving cells toward a pro-inflammatory-proliferative response eventually leading to apoptosis or toward an antioxidant response accompanied by injury repair, respectively.

protein levels the decisive factor in activating the transcriptional activity [36].

HIF-1 is expressed by all multicellular eukaryotic organisms and, in normoxic conditions specific for each tissue, the α subunit is bound to the von Hippel-Lindau (VHL) protein that recruits an ubiquitin ligase, tagging HIF-1α for degradation by the 26S proteasome [37] (see Fig. 1). VHL binding follows the hydroxylation of a specific proline residue in HIF-1α by PHD2 that utilizes molecular oxygen to oxidize a prolyl residue at 564 position of HIF-1α. The absolute requirement of oxygen as a co-substrate suggests PHDs as the oxygen sensor in cells [38,39]. Under hypoxia, less oxygen is available and PHDs catalytic activity is hindered and prolyl hydroxylation of HIF-1α abrogated. This event allows its escape from recognition by the pVHL ubiquitin–ligase complex and the transfer to the nucleus, where eventually HIF can exert its transactivating activity [27]. Other cellular factors, including miRNA-155 [40] and mRNA-destabilizing proteins [41] contribute to the stabilization of HIFα and to its activation.

The level of several cellular cofactors available in the cellular environment such as Ferric Iron, 2-oxoglutarate, and ascorbate positively contributes to modulate the catalytic activity of PHDs [27,42]. Conversely, metabolic intermediates of the tricarboxylic acid cycle (*e.g.*, succinate and fumarate), $O_2^{-\bullet}$, H_2O_2, and HO• have been reported to inhibit HIF activity [42,43]. Beside the modulation by metabolic intermediates, intracellular reduction of oxygen give rise to superoxide anions and eventually to H_2O_2 and hydroxyl radicals levels and affect HIF pathway. H_2O_2 has been proposed to directly inhibit PHD enzymes by oxidizing the essential non-heme-bound iron [44]. At cellular level, it has been proposed that chronic hypoxia is associated with high intracellular H_2O_2, generated by mitochondria at the Q0 site of complex III of electron transport chain [45], by NADPH oxidase, xanthine oxidase and by the

endothelial form of NOS [46]. Increased levels of H_2O_2 inhibits PHD and stabilizes HIF-1α and thereby activates its downstream pathways [28,47]. This evidences contribute to explain the HIF-activating effect of "pulsed hyperoxia" that has been reported in different experimental models [48–50].

However, the role of reactive oxygen species generated under hypoxia in inhibiting PHD activity is controversial. The dramatic inhibition of HIF-1α prolyl hydroxylation in hypoxia does not correlate with the modest effects of H_2O_2 on prolyl hydroxylation. Moreover, in general, there is no consensus as to whether hypoxia actually causes an increase or a decrease in mitochondrial release of $O_2^{-\bullet}$ [51].

Accordingly, Finley and coworkers [52] reported that the mitochondrial deacetylase sirtuin-3 (SIRT3) destabilizes HIF- thereby promoting maximal prolyl-hydroxylase activity of PHDs 1α by inhibiting reactive oxygen species production, as detected by a low specificity probe, the cell-permeant 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA). In fact, SIRT3-knockout mouse embryonic fibroblasts display a gene expression profile describing a hypoxic phenotype, associated to the increase of glucose uptake, lactate production, and glucose-dependent proliferation rates relative to wild-type cells. These observations link SIRT3 function to $O_2^{-\bullet}$ and H_2O_2 intracellular production in modulating HIF activity. In fact, stable knockdown or knockout of SIRT3 resulted in an intracellular increase in H_2DCFDA signal suggesting an increased generation of reactive oxygen species, which as mentioned, have been reported to stabilize HIF-1α protein levels under normoxia and during intermittent hyperoxia.

Another hydroxylase-domain protein, the factor inhibiting HIF (FIH), also operates a negative regulation of HIF-1α. In the presence of oxygen, FIH catalyzes the hydroxylation of the asparagine-803 residue within the C-terminal transactivation domain (CTAD) sterically inhibiting the interactions between HIFα and the transcriptional coactivators, CREB binding protein (CBP) and p300 coactivator protein, both needed to DNA binding [53].

Different NO donors have been reported to inhibit in vitro 2-oxoglutarate-dependent oxygenases, including PHD, and HIF transcriptional activity. This inhibition has been explained by the competition of NO with O_2 for the iron binding at the 2-oxoglutarate-dependent oxygenases active site [54].

Hypoxia-inducible factor HIF-1 has been shown to be mandatory for the cellular adaptation to low oxygen levels. Interestingly, as mentioned above, "pulsed hyperoxia" has been observed to induce HIF activation and the expression of genes involved in the response to low oxygen [48–50]. These observations clearly indicate that relative changes of oxygen availability, rather than steady-state hypoxic (or hyperoxic) conditions, coordinate HIF transcriptional effects. According to this hypothesis describing the "normobaric oxygen paradox," normoxia following a hyperoxic event is sensed by tissues as an oxygen shortage, upregulating HIF-1 activity. However, the HIF system therefore seems to be set differently in different cells, in order to allow a tissue-specific control of oxygen homeostasis [55].

Following HIF activation, genes involved in adaptation to low glucose levels like the glucose transporter Glut-1, carboanhydrase IX (CAIX) that regulates pH, and VEGF and erythropoietin (EPO), insulin-like factor-2 (IGF2) and transforming growth factor-α (TGFα) which are key factors in angiogenesis are expressed. The binding of these growth factors to their cognate receptors activates signal transduction pathways that lead to cell proliferation/survival and stimulates expression of HIF-1α itself.

HIF also plays a central role in both embryonic development and postnatal physiology [56]. According to the concept that, in mammalians, regulatory elements sensitive to oxygen availability such as HIF-1 play important roles in circulatory system development [57,58]. O_2 functions as a morphogen (through HIFs) in many developmental systems. In fact, miscarriage with cardiac malformations, vascular defects, and impaired erythropoiesis occurs in mice embryos rendered homozygous for a null allele at the locus encoding HIF-1α [57]. Overall, the activation of HIF-1 leads to the upregulation of the expression of more than 70 target genes involved in angiogenesis, cell survival and anaerobic glucose metabolism, but also in many aspects of cancer progression and invasion [59]. A detailed description of the several responses elicited by HIF and of the target genes is provided elsewhere [27].

The association between the presence of allelic variants of the gene encoding for HIF have and the risk of several diseases, including cancer, highlights the importance and the pleiotropic effect of HIF [60].

3.2. Nrf2: When too much oxygen is "too much"

Environmental conditions associated with high levels of oxygen can induce an oxidative challenge to biological systems eventually resulting in cell dysfunction/loss of function and death [61]. In the presence of these conditions, cells respond in the attempt to cope with, dispose and possibly repair damages possibly induced by the increased concentration of reactive oxygen and nitrogen molecular species inducing cytoprotective and detoxifying enzymes consisting of phase I (cytochrome P450s) and phase II (detoxifying and antioxidant proteins) enzymes. The activation of this redox-sensitive gene regulatory network mediated by the activation of nuclear factor (erythroid-derived 2)-like 2 (NFEL2L2/Nrf2) is probably one of the most important responses. Interestingly, largely before the discovery and characterization of this transcription factor, it was demonstrated the exposure of lungs to high oxygen does not immediately results in tissue injury but induces an increase of H_2O_2, eventually resulting in a subsequent damage and inducing the expression of antioxidant enzymatic defences [62].

Nrf2 belongs to a subset of basic leucine-zipper (bZip) genes sharing a conserved structural domain, termed *cap n' collar* (CNC) domain [63]. Nrf2 encoded protein is composed of 605 amino acids owing seven functional domains named Neh1–7 (Nrf2–ECH homology), responsible for different specific

molecular features, including DNA binding, heterodimerization with regulatory proteins and co-activators [64].

The key signaling protein of this adaptive mechanism is Nrf2. This transcription factor has been shown to heterodimerize with c-Jun and small Maf proteins (MafG and MafK) to bind to the *electrophile/antioxidant responsive element* (EpRE/ARE) in the regulatory regions of target genes, and Kelch ECH associating protein 1 (Keap1) [65].

This latter acts as a repressor protein by binding to Nrf2 and promoting its degradation by the ubiquitin proteasome pathway (Fig. 1). Also known as inhibitor of Nrf2 (INrf2) [66], Keap1 is normally associated with Nrf2 in the cytoplasm, and interacts with Cul3 [67], thus stimulating its ubiquitination and consequent proteasomal degradation [68]. Keap1 is a cysteine-rich protein, most of which has been demonstrated to be modified by different oxidants and electrophiles [69]. Under oxidative stress conditions, or through the activity of Nrf2 inducers, these modifications can result in a conformational change resulting in the release of Nrf2 from the low affinity binding site, finally disturbing its ubiquitination. Keap1 molecules are then saturated with Nrf2, allowing de novo synthesized Nrf2 to translocate and accumulate into the nucleus [70] where it binds to the ARE. The coordinated activity of these proteins is pivotal to the cellular response to oxidative challenges and attempts to restore the cell to an adaptive homeostatic state by conferring a resistance to stress (hormesis) [71].

Nrf2 is regarded as a ubiquitously expressed transcription factor but the levels of its mRNA vary substantially in different organs [72]. The presence of hypermethylated CpG sequences observed during prostate tumorigenesis associated with decreased Nrf2 expression supports the hypothesis that tissue specific differences are determined by epigenetic mechanism [73].

Complex regulatory mechanisms regulate Nrf2 activity at both the transcriptional and the post-translational levels. The gene encoding Nrf2, NFE2L2, contains a xenobiotic response element (XRE) and two XRE-like sequences, which can recruit the arylhydrocarbon receptor AhR, enabling NFE2L2 to be transcriptionally activated by polycyclic aromatic hydrocarbons [68].

The promoter region of NFE2L2 also contains EpRE/ARE DNA binding sites. This suggests the ability of Nrf2 to regulate its own transcription. The ARE activator D3T (3H-1,2-dithiole-3-thione) has been shown to increase Nrf2 protein and mRNA levels and that these increases were inhibited by co-treatment with the protein synthesis inhibitor cycloheximide [74]. Nrf2 expression is in part modulated by autoregulation. In fact, two electrophile/antioxidant response element-like sequences (EpRE/ARE-L1 and -L2), located at 492 and 754 bp from the transcription start site, have been reported in the promoter of the mouse orthologue of the NFE2L2 gene. Under stress conditions newly translated Nrf2 protein escapes Keap1-mediated degradation and binds to the EpRE/ARE-L1 and -L2 sequences to induce NFE2L2 gene transcription in a feed-forward manner [74,75].

The cross talk between Nrf2 and the nuclear factor k-light-chain-enhancer of activated B cells (NF-κB) will be discussed in the detail in another part of this article. However, it interesting to consider that several molecules considered as "chemopreventive agents" have been reported to activate Nrf2 while repressing NF-κB activity and that NF-κB negatively modulates the transcription of RE-dependent genes [76].

Interestingly, Keap1 has been shown to bind to IKKβ, a member of the IKK complex, promoting its ubiquitination and degradation such that Keap1 plays a role in the negative regulation of the NF-κB pathway [77].

Moreover, the mouse NFE2L2 gene contains a 12-O-tetradecanoylphorbol-13-acetate-response element downstream from the transcription start site that allows it to be transcriptionally activated by oncogenic KrasG12D via c-Jun and c-Fos [78,79].

Finally, the presence of an NF-κB binding site downstream from the transcription start site of NFE2L2 gene, allows it to be induced by inflammatory stimuli [39].

Nrf2 mRNA levels have been reported to be affected by the levels of peroxisome proliferator-activated receptor α (PPARα) [80]. A dynamic reciprocal cross talk between Nrf2 and PPARα has therefore been proposed to contribute to cellular response [68].

Data obtained following forced overexpression of specific microRNA (miR) suggest that Nrf2 activity can be suppressed by targeting the 30-untranslated region of its mRNA. In fact, the ectopic expression of miR-28 in MCF-7 and the overexpression of miR-93 in MCF-10A and T47D human breast cancer cells, respectively, decreases and suppresses Nrf2 mRNA and protein levels [81,82]. Similarly, human SH-SY5Y neuroblastoma cells display reduced Nrf2 mRNA and protein levels when miR-27a, miR-142-5p, miR-144, and miR-153 are overexpressed [83].

At the post-transcriptional level, similarly to HIF, under basal conditions, in the absence of cellular stress, the nuclear content of Nrf2 is rather small and its major proportion is in the cytoplasm bound to Keap1 and subjected to proteasome degradation [84] (see Fig. 1). Under conditions of chemical/oxidative stress, specific sensing thiol groups of keap1 react with nucleophilic molecules, inducing its dissociation from Nrf2 allowing its accumulation within the nucleus. Other regulatory mechanisms involve the phosphorylation of Nrf2 mediated by protein kinase C/mitogen-activated protein kinase (MAPK), leading to an enhanced stability and/or release of Nrf2 from Keap1 [75] and histone acetyl transferase (HAT)/histone deacetylase (HDAC)-mediated acetylation. Nrf2 acetylation has been reported to enhance its transcription capacity and downstream target expression and has been shown to protect animals from diseases characterized by oxidative stress and inflammation [85,86].

As mentioned above, cells exhibit some Nrf2 activity under unstressed conditions [84]. This residual activity probably occurs because a portion of Keap1 is inhibited by endogenous electrophiles.

Once set free from Keap1, the activation of Nrf2 transcriptional activity requires its translocation to the nucleus, the formation of a transcriptionally active complex through dimerization with partner proteins (such as sMaf or c-Jun) and binding to EpRE/ARE enhancer motifs [69,87–90].

Bach-1, a protein that belongs to the CNC family of transcription factors, also forms heterodimers with sMaf proteins and binds to Maf recognition elements, such as EpRE/ARE [91,92]. Bach-1 acts as a transcriptional repressor by binding to EpRE/ARE-like enhancers and antagonizes Nrf2 binding until it becomes inactivated by oxidants/electrophiles [93].

Nrf2 and Bach1 bind to two distal EpRE/ARE sites upstream of the antioxidant gene allowing a reciprocal equilibrium inside the nucleus influences EpRE/ARE-mediated gene expression. Under basal conditions, Bach-1 binds to EpRE/ARE within the promoter of target genes. In response to stress stimuli, Bach-1 is removed from the promoter, and facilitates the dissociation of Nrf2 from Keap1, its nuclear traslocation and binding to EpRE/ARE. Thus, the interplay between Bach-1 and Nfr2 appears to be crucial for the regulation of inducible heme oxygenase 1 (HO-1) gene expression [93].

Once bound to EpRE/ARE region of target genes, Nrf2 modulates the expression of a large series of genes involved in the cellular antioxidant and detoxification response [94]. These genes include detoxifying enzyme (*e.g.*, NAD(P)H: quinone oxidoreductase 1 [NQO-1] and glutathione-S transferases), enzyme participating to the antioxidant system such as HO-1 and ferritin that contribute to maintain a redox homeostasis in the occurrence of redox perturbation, such as in inflammation, growth factor stimulation, and nutrient/energy fluxes.

Overall, Nrf2 coordinate adaptive responses to diverse forms of stress associated with the high oxygen flux, and upregulates the repair and degradation of damaged macromolecules. Beside this role, Nrf2 but also contributes to cellular adaptation through the regulation of the intermediary metabolism by inhibiting lipogenesis, enhancing β-oxidation of fatty acids, and facilitating the pentose phosphate pathway warranting NADPH regeneration and purine biosynthesis. For a more detailed listing of Nrf2 target genes see Hayes and Dinkova-Kostova review [95].

A change in expression and activation of Nrf2 has profound effects on the physiological response to oxygen levels change. A hallmark of both hyperoxia and hypoxia is an incomplete mitochondrial oxygen reduction and the generation of $O_2^{-\bullet}$ which eventually leads to increased H_2O_2 levels [96,97]. Both H_2O_2 and $O_2^{-\bullet}$ are known to affect various cellular oxidative processes, and, in addition, they can serve as signaling molecules [98]. However, although Nrf2 represents a redox sensitive sensor, many reports demonstrated that this transcription factor is not upregulated in hypoxic condition.

Nrf2 activation is regulated by both high and low oxygen in hyperoxic environment. In fact, hyperoxic conditions trigger excess production of H_2O_2 and induce expression of numerous antioxidant proteins in the lung [99]. SODs, catalase, GPx1, and GSH reductase are the most widely examined classical enzymatic antioxidants in hyperoxia models. Many observations suggest an important role for the Nrf2-driven transcriptional response in mitigating cellular stress induced by prooxidants such as higher oxygen levels.

Exposure of murine alveolar type II-like epithelial cells (C10 line) to hyperoxia (95% O_2) induces Nrf2 nuclear translocation. This effect is modulated by H_2O_2-dependent mechanism involving PI3K/Akt pathway in Nrf2 activation [100,101]. Furthermore, exposure of human lung microvascular endothelial cells to hyperoxia (95% O_2) stimulates Nrf2 translocation from the cytoplasm to the nucleus and increases Nox4 expression [102].

Potteti and coworkers demonstrated the recruitment of nuclear Nrf2 to the promoters of antioxidant genes (*e.g.*, HO-1 and NQO1) in human normal small airway epithelial cells exposed to acute (up to 12 h) and chronic hyperoxia (up to 36 h) [103]. However, even if the extent of the Nrf2 binding at the HO-1 promoter was similar under acute (3 h) and chronic (more than 24 h) hyperoxic conditions. ChIP assays revealed that the enrichment of Nrf2 at the NQO1 promoter appeared to be variable and biphasic in response to hyperoxia [103].

However, although a large amount of data has been generated using 95% oxygen as hyperoxia, the effect of lesser concentrations of oxygen has not been clearly elucidated. It is critically important to determine the threshold of hyperoxic exposure that would allow cells to activate the adaptive response, driven by Nrf2, that is vital for repair of the cell damage due to high oxygen concentration. Further, the degree of hyperoxia and the duration of exposure that would allow cells to recover; and conversely, the level and duration that would inhibit recovery of cells has not been clearly established.

Cimino and coworkers investigated the alterations induced in HUVECs by exposure to mild hyperoxia (O_2 32%) [104]. Interestingly, these authors demonstrated a significant decrease in the nuclear levels of the transcription factor Nrf2, and a decrease in the expression of HO-1 and NQO-1 genes. Furthermore, pharmacological activation of Nrf2 was able to protect endothelial cells from hyperoxia-induced cytotoxicity.

Thus, it appears that Nrf2-mediated gene expression is induced to mount a cytoprotective response to preserve redox homeostasis, thereby helping to maintain cell survival or preventing lung epithelial cell death during hyperoxia. In fact, mice lacking the Nrf2 gene are more susceptible to hyperoxia-induced lung injury and death than are wild-type mice [105].

Conversely, the exposure of Rhabdomyosarcoma cells muscle cell line to hypoxia has been reported to prevent Nrf2 nuclear translocation and to result in reduced cellular antioxidant defense, further aggravated by hypoxia-induced oxidative damage [106]. Additionally, human adult cardiomyocytes exposed to hypoxic environment showed reduced nuclear translocation of Nrf2 that is actively retained in the cytoplasm by Keap1 [107]. Another study revealed reduced levels of Nrf2 in human normal renal proximal tubular epithelial cells (HK-2) and primary murine kidney epithelial (pMKE) cells exposed

to hypoxia [108]. In addition, this decrease correlates with a reduced activation or a lack of activation of expressions of several target gene [108]. In fact, a number of putative transcriptional targets of Nrf2, such as NQO1, GCLC, and GCLM, are not induced by either acute or chronic hypoxia in both human and murine kidney epithelia. Interestingly, HO-1 is induced in response to acute or chronic hypoxia in kidney epithelium, suggesting that other transcription factors are required in addition to Nrf2, in order to achieve a significant antioxidant gene expression in hypoxia. HO-1 has a functional HIF binding site so that its expression is affected by HIF signaling [36].

3.3. Cross talk between HIF-1 and Nrf2

Evidences support that Nrf2 signaling plays a role in activating and sustaining the HIF-1 response. Although HIF-1 and Nrf2 signaling are both regulated by reactive oxygen species, there are evidences that these two signaling pathways can directly and indirectly regulate one another. Evidences have shown that knockdown of Nrf2 is sufficient to decrease HIF-1 at the post-translational level, suggesting that Nrf2 or probably indirectly via its downstream targets play a role in the regulation of PHDs [109]. Oh et al. recently reported that NQO1 expression increases the half-life of HIF-1 protein. NQO1 overexpression is sufficient to stabilize HIF-1 levels in normoxia [110]. Furthermore, it has been recently suggested that Nrf2 may be indirectly responsible for the increased transcription of HIF-1 via activation of thioredoxin pathway [111]. However, these two signaling pathways do not always work in concert or reinforce each other. Loboda et al. found that HIF-1 stabilization repressed Nrf2 signaling through a Bach1-dependent mechanism, in endothelial cells [31]. In a separate study, treatment with the natural product andrographolide, an Nrf2 inducer, decreased HIF-1 expression [112]. Andrographolide pretreated cells showed increased levels of PHDs. This effect was countered by knockdown of Nrf2, suggesting that this molecule activates Nrf2 signaling to actively block HIF-1 signaling [113]. Taken together, these studies indicate that the HIF-1 and Nrf2 stress response pathways exist in a complex, interactive signaling network.

4. NF-κB: A third actor

4.1. Cross talk between HIF and NF-κB

Transcription factors are among the oxygen and targets of reactive oxygen and nitrogen species which can positively or negatively respond to nutrients by a modulation of gene expression.

Both HIF and Nrf2 have been reported to interplay with a key transcription factor in inflammation and cellular response and, in particular, with NF-κB. NF-κB is a homo or heterodimer composed of different subunits RelA (p65), RelB, c-Rel, NF-κB1 (p50/p105), and NF-κB2 (p52/p100). Its transcriptional activity can be triggered by many different stimuli, including bacterial lipopolysaccharide (LPS), viral pathogens, cytokines, or growth factors [114]. NF-κB activation follows the phosphorylation and subsequent proteasomal degradation of a family of inhibitory proteins (IκBs) by specific kinases: the IκB kinases (IKKα and/or IKK beta) and NEMO that is a regulatory subunit of IKK, which respond to diverse stimuli. Once freed from the inhibitory complex, NF-κB dimers are enabled to translocate to the nucleus and bind to a κB binding site in the enhancer/promoter of a very large number (more than 150 in humans) of NF-κB-specific target genes [115].

In addition, NF-κB activation can result from a non-canonical pathway mediated through the activation of a NEMO-independent kinase complex involving IKK1 and the NF-κB-inducing kinase [116]. In the non-canonical pathway, new synthesis of p100 and RelB allows the generation of NF-κB which is not under IκB control and thus localized to the nucleus.

Other transcription factors for which synergistic crosstalk with NF-κB has been reported are Sp1, AP-1, STAT3, and CEBP/β. In particular, Activator Protein transcription factors (AP-1), such as c-Jun and c-Fos, can stimulate p65 transactivation through κB sites even in the absence of AP-1 sites [117]. Moreover, NF-κB has been reported to directly interact with c-Jun and c-Fos and stimulate the AP-1 binding to DNA and its activation through AP-1 sites [118].

The protection conferred by NF-κB activation is most likely mediated by NF-κB-induced expression of Bcl-2 and other cytoprotective enzymes, such as MnSOD and GSH peroxidase—each of which are regulated by NF-κB [119,120]. In lung epithelial cells, NF-κB activation prevents hyperoxia induced apoptosis. In addition, the NF-κB function in reducing tissue damage in hyperoxic lung injury may also be attributed to its protective role against either $O_2^{-\bullet}$ or other reactive species [120]. However, a therapeutic approach targeting NF-κB mediated hyperoxic signaling in the lung needs to be further investigated. In fact, while NF-κB has been demonstrated to have a critical role in protecting lung cells against hyperoxia-induced cell death, many of the NF-κB modulated genes encode for pro-inflammatory cytokines such as interleukin 8 (IL-8) and TNF-α. This results in the recruitment of monocytes that, in turn, enhance inflammation [121].

According to its importance in several important functions such as inflammation, the regulation of cell differentiation, proliferation, and survival following a large spectrum of physical, physiological and oxidative stresses [122,123], NF-κB activity is strictly controlled by both positive and negative regulatory factors working at many levels [124].

In addition to its adaptive role to low oxygen availability, HIF-1 has been demonstrated to mediate a wide spectrum of cellular responses in particular those sharing a pro-inflammatory stimulus, also independently of hypoxia [28]. In fact, a complex interplay exists between HIF-1 and NF-κB. This interaction, obviously results in the immune response occurring in different diseases such as rheumatoid arthritis (RA) asthma and COPD, and cancer [125]. Interestingly, all these pathological conditions have been associated to a dysregulated redox status [126–128].

According to above, targeting the HIF pathway will affect NF-κB function. In fact, a number of studies using both genetic and chemical inhibition of the PHD proteins have demonstrated therapeutic effects in several disease models [129,130] and even in response to infection where inflammation where NF-κB play an important role [131].

4.2. Nrf2-NF-κB cross talk

Many studies reported that a variety of phytochemicals supress NF-κB and activates Nrf2 pathway clearly suggesting a cross-talk between these two transcription factors [69] although it is not clear yet if the suppression of NF-κB signaling and the activation of Nrf2 pathway are independent or regulated by an upstream controllers. In general, with few exceprions, NF-κB and Nrf2 apparently play opposite roles in the pathological processes of inflammation and cancer, and a large number of pathological stimuli, such as cigarette smoke, lipopolysaccharide (LPS) [132].

However, whether NF-κB p65 subunit suppresses the EpRE/ARE-driven gene transcription remain still in doubt. It has been hypothesized [132] that p65 subunit undirectionally antagonizes EpRE/ARE-mediated gene trascription though two distinct but interconnected mechanisms. A first one is due to the competitive interaction with the CH1-KIX domain of CBP p65 that selectively deprives CBP, a well-established coactivator of Nrf2. A second mechanism would be due to the recruitment, of histone deacetylase 3 (HDAC3), promoted by p65, leading to local histone hypoacetylation. Speciale and coworkers demonstrated the inhibitory effect of Nrf2 pathway on NF-κB transcription machinery in human endothelial cells exposed to TNF-α [133]. Interestingly, the pharmacological inhibition of Nrf2 nuclear accumulation induced by an anthocyanin was able to increase TNF-α-activated p65 nuclear translocation. Furthermore, another paper from the same group reported that knockdown of the endogenous Nrf2 by siRNA, increased IKK phosphorylation induced by palmitic acid in endothelial cells [134]. According to the evidence of reciprocal cross-talk with NF-κB, Nrf2 signaling counters NF-κB signaling with few exceptions such as in acute myeloid leukemia where Nrf2 is upregulated by NF-κB-mediated transactivation of the NFE2L2 gene by direct binding of NF-κB to NFE2L2 promoter [39].

Finally, Under physiological conditions, the interference of p65 with Nrf2 transactivation may serve as a negative regulatory mechanism for fine tuning of Nrf2-ARE signaling. If it is true, the antioxidant activity of many natural phytochemicals might be attributed not only to their direct stimulatory effect on Nrf2 signaling but also to their ability in preventing p65-mediated repression of EpRE/ARE transactivity [135].

5. Conclusions

Hypoxia-inducible factor and Nrf2 play an interactive role in cellular "adaptive homeostasis" contributing to the inducing the transient increase in protective homeostatic capacity that occurs in response to stressful conditions such as those associated to fluctuations of oxygen availability.

A third actor, NF-κB plays a central role in determining the result of the response to oxygen fluctuation and to cellular injury. The role of each transcription factor in human health and disease does not seem to be independent from the activation of the other two. This is particularly evident in the case of cancer prevention and progression where constitutive Nrf2 activation is observed in a variety of human cancers and it is highly correlated with tumor progression and aggressiveness [136]. It is possible that both NF-κB activity, and its relationship with HIF, hold the balance of the result determining the "direction" of the cellular response to stressful stimuli toward either repair or death [137].

It interesting to note that a large number of phytochemicals have been proposed to beneficially act on human health by modulating the activity of these transcription factors, therefore determining the final result of the response [138].

Within this frame, a system biology approach, based on "problem driven" design, supported by high throughput methodologies, are likely to represent an expedient strategy for a better understanding of the role of molecules of nutritional interest in the modulation of transcriptional activation and, in general, for a better characterization of the cellular response to different stimuli associated to human health and disease.

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