Hypoxia: a Review

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ABSTRACT

Tissue hypoxia occurs where there is an imbalance between oxygen supply and consumption. Growing evidence from experimental and clinical studies points to the fundamental and patho-physiologic role of hypoxia in cancer, ischemic tolerance, and stroke. Hypoxia-induced changes in ion homeostasis, erythropoiesis, angiogenesis, proliferation and differentiation. This review outlines hypoxia effect at molecular level and describes briefly hypoxia role in the physiological and pathological conditions.

Keywords: Chronic Hypoxia; HIF; Ischemic Tolerance; Stroke; Cancer

1. INTRODUCTION

In the life of aerobic organisms, oxygen is an essential element. The central role of oxygen is due to the fact that it is the final acceptor of electrons in the mitochondrial respiratory chain. This allows the ultimate process of oxidative phosphorylation and the generation of cellular energy, in the form of adenosine triphosphate (ATP). ATP is used in most reactions that are necessary to maintain cellular viability. Under normoxia a cell continuously maintains a high and constant ratio of cellular ATP/ADP ratio in order to survive. The dependence of cells on a high constant ATP/ADP ratio means a dependence on oxygen. Therefore, a reduction of the normal oxygen supply (hypoxia) will have consequences on the cell viability [1,2].

Hypoxia is encountered not only in different conditions including the patho-physiological conditions, such as atherosclerosis, obstructive sleep apnea, mountain sickness, ischemic diseases (stroke) and cancer, but also in physiological processes, such as embryonic development [3-6]. Different terms are given in the literature about the reduction of oxygen supply. The term hypoxemia is defined as a reduced oxygenation of the blood. Hypoxia is defined as a decrease in the oxygen supply to a level insufficient to maintain cellular function. Hypoxia-ischemia stands for the processes of hypoxia combined with ischemia. Ischemia differs from hypoxia in that it is not only a decrease in the oxygen supply; it also involves a reduction of the blood flow which leads to a decrease of nutrient supply and an accumulation of metabolic products, including CO_2, lactic acid and ammonia [7-9].

Additionally, hypoxia response can be divided in different time scales, including an acute, an intermediate and a chronic response, and in different levels of oxygen concentration, including a moderate (5-8% O2) and an anoxic level (<1 O2) (normoxia is 21% O2) [3,10,11]. The brain is regarded as the most hypoxia-sensitive organ because of its need for a high oxygen supply, whereas the skeletal muscle is amongst the most hypoxia-tolerant [12].

The first part of this review will provide a broad description of responses to chronic moderate hypoxia. In the second part of the review, the role of hypoxia in the physiological and pathological conditions will be described.

2. Sensing hypoxia

Hypoxia orchestrates a multitude of processes of molecular pathway responses. However, in the higher organisms, the cellular oxygen sensor itself is unknown [1,13]. Several mechanisms have been proposed as to how a cell senses the lack of oxygen.

The traditional mechanism of hypoxia sensing involves a heme protein (Figure 1). This protein has been suggested because most proteins capable of binding O_2 contain iron, which usually is in the center of a heme moiety [13].

Hypoxia could be detected by a reversible binding of O_2 at the heme site, which causes an allosteric shift in the hemoprotein, inactive (oxyform) to active (deoxy) form [15]. There are many kinds of heme containing oxygen binding proteins, but no real candidate has been found yet [13].

Another mechanism, better known as the "membrane hypothesis" or "membrane model", involves ion channels. It is reported that the ionic currents/conductance are inhibited during hypoxia in the O_2-sensitive channels, K+- selective, Ca_2+ and Na+ channels.

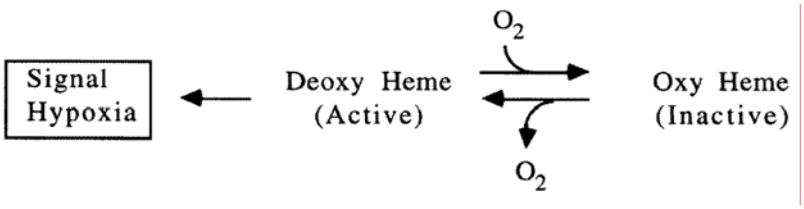

Figure 1. The activity of a hemoprotein is determined by the presence or absence of bound oxygen [14].

In addition, these channels are considered to be O_2-sensitive because their modulation by O_2 occurs without known modifications in cytosolic variables such as pH, $[Ca^{2+}]$ or ATP [1,16]. However, how O_2 interacts with the channels is not known. It is suggested that the channels' gates are changed either by direct allosteric] interactions (where the sensor switches conformation between deoxy and oxy form) or by means of a mediator [1,15,17,18]. Another problem that occurs in this model is the fact that the O_2-sensitive ionic currents/conductance are either inhibited or increased in the same channel type in different cells [1,18].

The mitochondrion itself has been suggested to be the site of hypoxia sensing. This is based on the fact that the mitochondria binds O_2 and represents the primary site of oxygen consumption in the cell [19]. Furthermore, experiments of agents mimicking hypoxia have shown an inhibition of mitochondrial function (oxidative phoshorylation) [20]. The main problem in "the mitochondrial hypothesis" lies in how the mitochondria can detect differences in the O_2 supply [15]. But the mitochondrial involvement is an attractive proposal since it provides a link between O_2 ensing and metabolism.

However, the search for an oxygen sensor is wide open and none of the different suggested mechanisms can be excluded.

3. Responses to chronic moderate hypoxia

Under chronic moderate hypoxia multicellular organisms trigger a multitude of cellular responses in order to survive and maintain the oxygen homeostasis in function of time (Figure 2). Here the most important responses will be described.

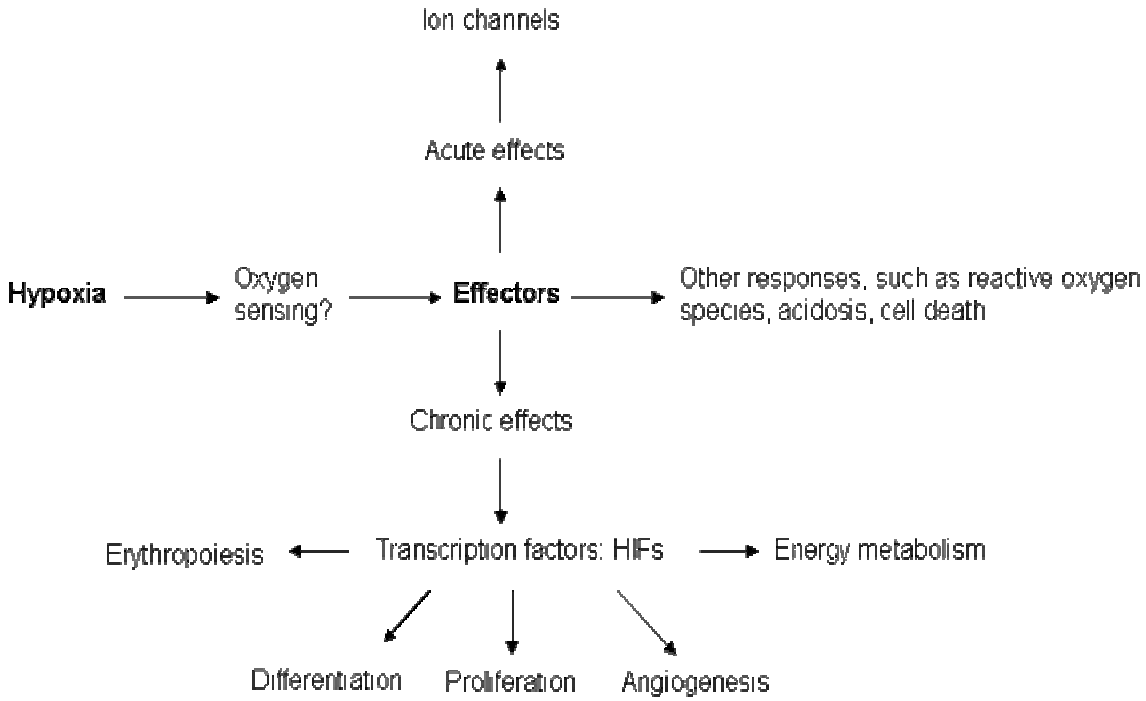

Figure 2. Main responses to hypoxia

3.1. Ion homeostasis

The acute response to hypoxia is believed to be significant disturbances of the ionic homeostasis. It is suggested that the ion channels are some of the first proteins to sense the low oxygen level, as mentioned earlier (hypotheses of oxygen sensing). The obvious choice of the ion channels comes from the fact that cells spend much of their ATP production to maintain the ionic gradient. Under normoxic condition, it is estimated that up to 60% of the ATP production is used by the ion-motive ATPases, such as the Na^+/K^+ ATPase and Ca^{2+}-ATPase [21].

During hypoxia a decrease in the intracellular ATP/ADP ratios causes an enhanced cellular K^+ efflux and Na^+ and Ca^{2+} influx [22]. With the decreased activity of the K^+ channels, the membrane depolarizes and activates the voltage-gated Ca^{2+} channels, causing an increase in the concentration of intracellular Ca^{2+}. The overload of intracellular Ca^{2+} causes among other things (1) changes in the mitochondrial metabolisms, (2) an activation of lipases and proteases which leads to membrane damage and a release of free fatty acids and proteins, (3) an activation of endonuclease and (4) a generation of reactive oxygen species (ROS) [22-25]. The bridge between the acute (depolarization) and chronic (changes in gene and protein expression) responses to hypoxia is believed to be this increase in the intracellular Ca^{2+}. Under chronic hypoxia, changes in the gene expression are regulated by the oxygen-regulated transcription factor HIF-1 (see 3.2). The key protein, which links the acute and chronic responses, is believed to be the CaM kinase II. The increase in the intracellular Ca^{2+} levels, and its subsequence binding to Calmodulin, leads to an activation of CaM kinase II, which phosphorylates the co-activator (p300) of the HIF-1 complex. This phosphorylation induces the HIF-1 transcriptional activity [26].

Because of the limited information about the effect of hypoxia on Na^+, the exact contribution of Na^+ under hypoxia is not known [27]. It is proposed that the increased intracellular Na^+ concentration during hypoxia contributes to the increase in the intracellular Ca^{2+} concentration by reversing the Na^+/Ca^{2+} exchanger [28,29]. However, under chronic hypoxia, a decreased and an increased expression of the Na^+ channels have been reported [30].

3.2. Hypoxia-inducible factors (HIFs)

While triggering ion channels is an acute response, at the molecular level, the hronic response to hypoxia involves changes in the gene expression [14]. The essential step in this process is an activation and stabilization of the hypoxia-inducible factors (HIFs) [31]. HIFs are universally used as transcriptional regulators, which are "turned on" by chronic hypoxia. The HIF family comprises three members, HIF-1, HIF-2 and HIF-3, with the function of HIF-3 poorly understood. HIF-1 is ubiquitously expressed, whereas HIF-2 is only expressed in endothelial cells and in the kidney, heart, lungs and small intestine [32-34]. HIF-1 transcriptional complex was the first transcription factor to be discovered. It binds to DNA at hypoxia response elements (HRE) in the enhancer or promoter region of target genes [35]. HIF-1 complex is a heterodimer consisting of an inducible O_2-regulated HIF-1α subunit and a constitutively expressed HIF-1β [31].

The HIF-1 activity is tightly regulated, (for reviews see: [4,13,36-39]). During normoxia, HIF-1α is subjected to multiple modes of post-translational modifications (Figure 3). HIF-1α is hydroxylated on two proline residues in the oxygen-dependent degradation domain by the oxygen-sensitive prolyl hydroxylase domain (PHD) proteins. This modification leads to an interaction with the ubiquitin E3-containing ligase von Hippel Lindau complex (pVHL), which targets HIF-1α by attaching polyubiquitin chains for proteosomal degradation. Furthermore, HIF-1α is hydroxylated by another oxygen-sensitive enzyme, Factor Inhibiting HIF-1 (FIH-1), on the asparagine residue in the C-terminal transcriptional activation domain to prevent interaction with the transcriptional co-activator CBP/p300, and thereby represses the transactivational activity of HIF-1. These post-translational modifications keep HIF-1 in an unstable and inactive state. The half-life time of HIF-1α is only a few minutes (<5 minutes) under normoxia [1]. During hypoxia the PHDs and the FIH-1 become inactive. The lack of hydroxylation results in stable HIF-1α, which will dimerize with HIF-1β. The heterodimer is then nuclear translocated and binds to the HRE on the target genes. Since FIH-1 is inactive, the co-activator CBP/p300 is recruited for activation of the transcription.

It should be mentioned that the mechanism responsible for the post-translational modifications of HIF-1α is not known. But it is suggested that PHD might be involved in

this regulation [40].

It is believed that approximately 1-5% of the genome is transcriptionally regulated by hypoxia and many of these genes are known to be regulated by HIFs [41]. So far, more than 200 target genes have been reported to be induced by the HIF complex. These genes are involved in many different biological processes, including erythropoiesis, angiogenesis, proliferation, energy metabolism or apoptosis [38,39,42]. However, the gene expression pattern in response to the HIF activation is cell-specific. Hence, the protective response by the HIF activation in one cell lineage may not be evident in other cell types [42].

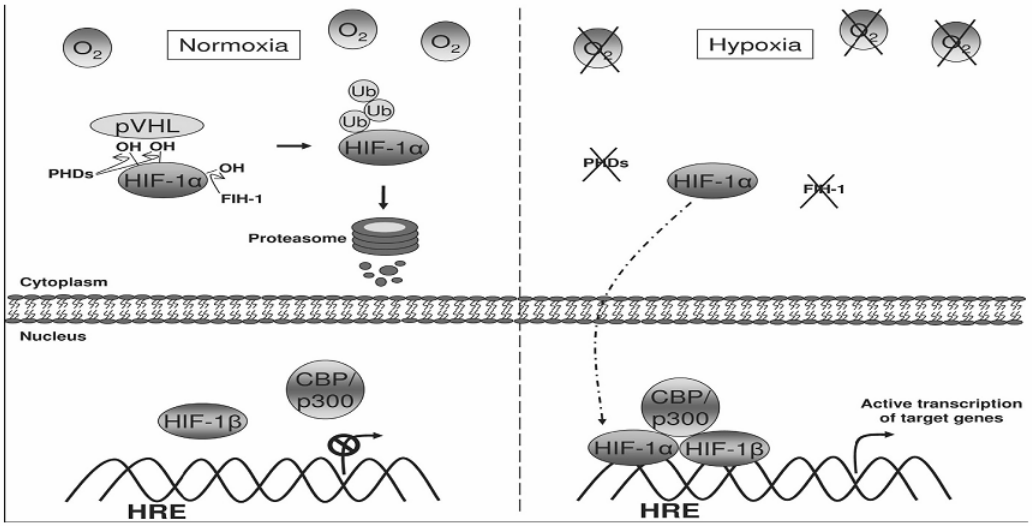

Figure 3. Regulation of HIF-1α during normoxia and hypoxia. [37].

3. 3. Erythropoiesis

In response to chronic hypoxia, the capacity of red blood cells to transport oxygen is up-regulated by the expression of genes involved in erythropoiesis. Most notably, the erythropoietin (EPO) gene is increased by hypoxia, which is required for the formation of red blood cells. An increase of the number of the red blood cells enhances the delivery of oxygen to tissues [43]. Furthermore, iron is required for heme formation and is the most limiting factor in erythropoiesis. Hypoxia increases the expression of the iron-metabolizing genes, including transferrin, transferrin receptor and ferroxidase. The increase of these genes supports the iron supply to the erythroid tissues [44]. It is well established that the expression of genes involved in the erythropoiesis is regulated by HIF-1. Indeed, oxygen-regulated EPO, transferring receptor and ferroxidase expression have been reported to be controlled by HIF-1 [13, 45].

3.4. Angiogenesis

Chronic hypoxia induces angiogenesis. Angiogenesis is the process by which new blood vessels develop from existing vasculature. Angiogenesis can be defined as a multistep process, which involves endothelial cell activation, increased blood vessel permeability, and local rearrangement of the basal membrane and extracellular matrix. Angiogenesis provides a principle mechanism for the maintenance of an adequate blood flow to areas of insufficient oxygen supply [46]. However, angiogenesis requires production and secretion of the so-called angiogenesis factors, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PFGF), fibroblast growth factor (FGF), and interleukin-8 [47]. In keeping with the central role of HIFs in responses to chronic hypoxia, it is shown that HIF-1 directly activates the expression genes involved in angiogenesis, including VEGF, VEGF receptors FLT-1, transforming growth factor-β3 (TGF- β3), angiopoietins and genes involved in matrix metabolism [48,49]. The most notable and characteristic angiogenesis factor induced by HIF-1 is VEGF. It is essential for the proliferation and migration of vascular endothelial cells, thereby enabling the formation of new blood vessels [50]. Several studies using both HIF mutant cell lines and murine model systems have shown that HIF signaling is required for the regulation of VEGF. However, the relative contribution of individual HIF family members in the induction of VEGF expression and

angiogenesis process is controversial. The individual contributions appear to be cell-type dependent [51, 52]. However, blood flow under pathophysiological conditions is controlled by modulations of the vascular tone through a production of NO (inducible nitric oxide synthase), CO (heme oxygenase 1), endothelin 1, adrenomedullin, or an activation of the α_{1B}-adrenergic receptor, all of which involve HIF-1 target genes, too. Therefore, HIF mediates angiogenesis by mechanisms, far more complex than the simple VEFG induction [45].

3. 5. Proliferation

Chronic hypoxia induces expressions of the various growth factors that are known to promote cell proliferation. This proliferation is normally involved in initiating cell migration and regeneration after acute or chronic hypoxia damage [53]. Several growth factors, most notably insulin-like growth factor 2 (IGF-2) and transforming growth factor-α, are HIF-1 target genes. Binding of these growth factors to their cognate receptors, the insulin-like growth factor 1 receptor (IGFIR) and epidermal growth factor receptor (EGFR) respectively, activates signal transduction pathways that lead to both HIF-1α expression and to cell proliferation/survival [48]. Furthermore, it is shown that the p42/p44 mitogen activated protein kinase (MAPK), which regulates cell proliferation in response to extracellular growth factors, phosphorylate HIF-1α and activate transcription of HIF-1 target genes [53].

In addition, phosphatidylinositol 3-OH kinase (PI3K) activity is also increased in some cell types under hypoxic conditions. PI3K is one of the key downstream mediators of many tyrosine kinase signaling pathways involved in regulating cell proliferation and suppression of apoptosis [54].

3.6. Differentiation

Accumulating evidence suggests that hypoxia promotes cell differentiation in a variety of cell types. A clear link has been demonstrated between hypoxia, HIFs and molecules that are critical for the regulation of the differentiation of cells, including Notch, Oct-4 and MYC [55]. Recently, it was observed that hypoxia directly influences the Notch signalling pathway activity in the cell differentiation process [56]. Notch mediates cell-cell signalling between adjacent cells that express Notch receptors (Notch 1-4) and Notch ligands (Delta, Serrate and Lag-2). In response to ligand presentation from neighbouring cells, Notch receptors undergo proteolytic activations to liberate the Notch intracellular domain (ICD). Subsequently, ICD forms a complex with DNA binding protein CSL and co-activators, such as p300/CBP, to activate Notch target genes. These in turn negatively regulate the expression or activity of the differentiation factors [57]. HIF-1α has been shown to associate physically with ICD, promoting its stability. A model is proposed in which HIF-1α interacts with the Notch-CSL transcriptional complexes at Notch-responsive promoters in hypoxic cells to control the differentiation status [56]. However, the protein "bridging" direct interaction between HIF-1α and ICD is unknown [58]. Other molecular pathways underpinning the hypoxic control of the cell differentiation process involves OCT4 and MYC transcription factors which are directly regulated by HIF-2α [55, 59]. However, how HIF-2α leads to the activation of these transcription factors are poorly understood. It is shown that when the Oct-4 locus is an open configuration, HIF-2 complex binds and induces its expression [60]. In the case of MYC, it is postulated that HIF-2α directly interacts with MYC-MYC-associated protein X (MAX) complex which leads to a stabilization of this complex and a transcriptional activation [59].

A search in the literature shows that a significant amount of work is performed to show that hypoxia plays an important role in the cell differentiation process. However, the molecular pathways involved in this process are poorly understood [61].

3.7. Energy metabolism

A key adaptive response to chronic hypoxia is a switch from oxidative phosphorylation to anaerobic glycolysis. Under normoxic conditions, cell energy in the form of ATP is mainly generated through the oxidative metabolism of carbohydrates, fats and amino acids. During hypoxia, ATP generation by oxidative phosphorylation is arrested. This will stimulate glycolysis with an increase in glucose consumption and lactate production [62,63]. The switch of the respiratory pathway to anaerobic glycolysis leads to a significant reduction of the ATP/ADP ratios. Because of the reduced energy supply, the hypoxic cells will further response by shutting down the non-essential energy consuming mechanisms, such as protein synthesis, and relocate the energy to more critical functions, such as the maintenance of the ion homeostasis and membrane potential [12,21]. Additionally, the glucose metabolism is regulated by the HIF

pathway. HIF-1 complex induces virtually all genes encoding glucose transports and glycolytic enzymes [64,65]. It is also suggested that HIF-1 represses mitochondrial respiration. This occurs by an induction of pyruvate dehydrogenase kinase 1 that inhibits pyruvate dehydrogenase, the enzyme that converts pyruvate to acetyl-CoA for entry to the Krebs cycle [66]. In addition, it is postulated that HIF-1 induces expressions of some proteins in the mitochondria for a more efficient transfer of electrons to oxygen [3]. A switch in the metabolic pathway to anaerobic glycolysis leads to an increase in the glucose consumption. The largest store of glucose equivalents is glycogen.
Indeed, it is observed that during hypoxia, glycogenolysis is induced [67].

Recently another pathway has been suggested to play an important role during hypoxia by regulating the energy metabolism: the AMP-activated protein kinase (AMPK) pathway [20,68]. AMPK is regarded as an energy-sensing enzyme. It is a heterotrimeric complex composed of a catalytic α-subunit and regulatory β- and γ-subunits [69,70]. As mentioned earlier, hypoxia results in a fall in the cellular energy status. As a consequence, the enzyme adenylate kinase will catalyze the reaction: 2 ADP ↔ AMP + ATP in order to maintain cellular ATP levels. This will results in a large increase in the AMP levels. AMP activates the AMPK complex in three independent ways: 1) allosteric regulation via the γ-subunit; 2) promotion of phoshorylation of AMPK by one or more upstream kinases; and 3) inhibition of dephosphorylation of AMPK [69].

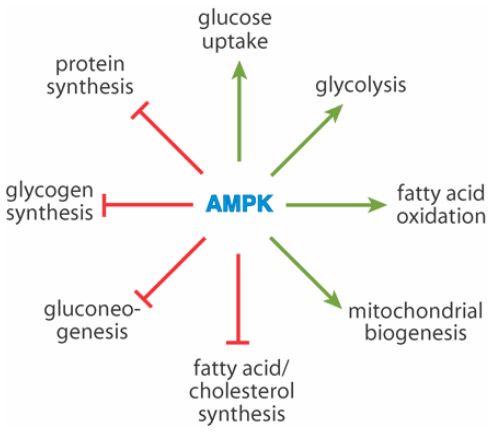

Figure 4. Key processes of the energy metabolisms that are regulated by AMPK. The multiple effects of AMPK regulate some of these processes [69]

Furthermore, the AMPK complex can be activated by a rise in intracellular Ca^{2+}, which is seen in hypoxia, through phosphorylation by a Ca^{2+}-dependent kinase activity [70,71]. The activated AMPK complex has a lot of downstream targets in cells or tissues [2]. In general, stimulation of the AMPK pathway promotes catabolic pathways that generate ATP, in order to maintain the ATP supply, while switching off non-essential ATP consuming (anabolic) pathways. This is done by direct phosphorylation of the Regulatory proteins involved in the process, and by an indirect effect on gene expressions [72]. Figure 4 shows some of the key effects of AMPK on the energy metabolism.

3.8. Acidosis

A switch from aerobic to anaerobic glycolysis decreases the consumption of H^+ due to the lowered production of ATP (by oxidative phosphorylation) on the one hand, and a generation of more H^+ by other metabolic reactions, such as the ATPases reaction ($ATP + H_2O \leftrightarrow ADP + P_i + H^+$) on the other hand [62]. During hypoxia, the cellular pH homeostasis is disturbed [9]. A gradual decrease in both extracellular and intracellular pH is observed [73]. Studies have shown an approximately drop of 0.8-1.2 pH units [74]. It is postulated that acidosis might be protective to hypoxic cells. Acidosis can slow down some of the enzymatic processes, reduce energy consumption and ROS production [9]. Additionally, acidosis inhibits ion fluxes through cellular membrane channels, thereby reducing the energy required for maintaining ion gradients across the plasma membrane [75]. Hypoxia-induced acidosis plays an important role in the stabilization of HIF-1α. It is shown that a decrease in pH triggers re-localization of the von Hippel-Lindau protein (pVHL) from diffuse nuclear-cytoplasmic pattern to nucleoli. This nucleolar sequestration of pVHL stabilizes HIF-1α by not ubiquitinylating the HIF-1α for protesomal degradation [76]. HIF-1 complex contributes to the cells' surviving the metabolic acidosis by enhancing several genes of membrane located transporters (e.g. the H^+/lactate monocarboxylate), exchangers (e.g. the Na^+/H^+ exchanger), pumps and ecto-enzymes (e.g. carbonic anhydrase IX) [3].

3.9. Reactive oxygen species (ROS)

The term reactive oxygen species (ROS) encompasses wide range of molecules. Free radicals are chemical species containing one or more unpaired electrons. The unpaired electrons of oxygen react to form partially reduced highly reactive species that are

classified as ROS, including superoxide (O_2), hydrogen peroxide (H_2O_2), hydroxyl radical, and peroxynitrite. Under normoxia, ROS can be generated at several cell sites and organelles, though the major site of ROS production is the mitochondrial electron transfer chain (ETC) [77]. It is estimated that under normoxia up to 2% of the electron flow on the ETC leads to ROS production [37]. ROS has also been suggested to be an essential participant in cell signaling, acting as a second messenger [78-80]. Under hypoxia, it is unclear how ROS is formed. Therefore, it has become the most debated subject of cells or tissues exposed to hypoxia. Controversial results of the generation of ROS during hypoxia have been published. Investigators have observed both decreases and increases of ROS levels in the cells or tissues exposed to hypoxia [37,81,82]. Inconsistencies in studies on the direction and degree of ROS level production come from the differences in cell types and sub-cellular compartments examined. Additionally, there are no direct methods for measuring intracellular ROS levels [83]. However, it seems that it is accepted that exposure of cells to a chronic moderate hypoxia leads to a relative increase in ROS generation [84]. Increases in cellular ROS levels are regarded as toxic. Cells possess several antioxidant systems, such as the enzyme superoxide dismutase and non-enzymatic antioxidant NADPH-coupled reactions, to protect themselves from these toxic species [85,86]. If one accepts that the increase of the ROS levels during hypoxia comes from the mitochondria site, it is postulated that it plays an important role in HIF-regulation [19,37,83]. It is shown that HIF-1 activation directly correlates with changes in ROS [87]. But it is not known how mitochondrial ROS regulate HIF-1 stability. It is proposed that the PHDs might be the key element. It is suggested that ROS triggers an unknown signal transduction cascade, which results in post-translational modifications of PHDs. These unknown post-translational modifications will make these proteins inactive. Inactivation of the PHDs leads to a stabilization of HIF-1α [19,68,83].

3.10. Cell death

Paradoxically, cell adaptation to hypoxia leads not only to cell proliferation/survival but also to cell death in some circumstances. When the protective adaptive mechanism, initiated by HIF-1 is not sufficient, cell death occurs [33]. Hypoxia induces cell death by a number of HIF-1-mediated and –independent pathways [53]. It is demonstrated that chronic moderate hypoxia alone is not sufficient to cause cell death [88,89]. A cell death requires a combination of factors/events, such as an increased level of Ca^{2+}, a generation of ROS, a change in the cellular energy levels and acidosis etc. [9,88,90-92]. There are different forms of cell death, including necrosis, apoptosis and autophagy. There are extensive reviews about these cell death mechanisms, which will not be discussed here [90,91,93].

Necrosis is defined as a passive form of cell death, since it can occur in absence of ATP [94]. It is believed to be caused by a disruption of cellular ionic gradients in association with a reduced ATP/ADP ratio [95]. In contrast, apoptosis is an energy dependent and delayed form of cell death that occurs as the result of an activation of a genetic program [89,96]. The autophagy has been described as an alternative form of programmed cell death, a non-apoptotic form. During this process, the cell "eats itself" [93].

When and which form of cell death takes place during the hypoxic condition is debated. It is suggested that an increase of ionic Ca^{2+} leads to a rapid or a slow consumption of ATP. It is postulated that a rapid use of ATP leads to necrosis, whereas a slow use of ATP leads to apoptosis [8,97]. Furthermore, it has recently been suggested that the autophagy form of cell death can be involved in hypoxia. This form of cell death has been postulated to represent an early survival mechanism. In this strategy, cells switch to a catabolic metabolic program in which cellular constituents are degraded for energy production [3].

HIF-1 contribution to cell death is also debated. It is discussed that HIF-1 can either be anti-apoptotic or pro-apoptotic, but it depends on the cell type and experimental conditions [33, 94]. Under chronic hypoxia, HIF-1 induces apoptosis. It is shown that HIF-1 increases the expression of various pro-apoptotic members of the BCL-2 (B-cell lymphoma-2) family [11,94,98]. The BCL-2 family is separated in three classes: the first class inhibits apoptosis, the second class promotes apoptosis, and the third class can bind and regulate the anti-apoptotic BCL-2 proteins to promote apoptosis [99]. HIF-1 complex has been shown to specifically induce the second class members of the BCL-2 family [98,100]. These classes lead to permeabilization of the outer mitochondrial and the subsequent release of apoptogenic molecules, such as cytochrome c, which leads to an activation of the caspase proteins. Caspases, which are cysteinyl aspartate

proteases, in turn cleave a series of substrates, activate DNases and orchestrate the demolition of the cell [99].

Furthermore, chronic hypoxia induces the stabilization of tumor suppressor p53 protein [101,102]. Traditionally, p53 protein controls cellular homeostasis by affecting cell cycle progression and apoptosis. Under normoxia, p53 has a very short half-life and the protein is often at an undetectable level. Cell exposure to stress such as DNA damage or chronic hypoxia, leads to a stabilization of p53. As a consequence, p53 becomes activated as a transcription factor and promotes transcription of genes involved in cell cycle regulation, apoptotic event etc. [103]. It is argued that a direct interaction between p53 and HIF-1α leads to p53 stabilization, which results in an inhibition of HIF-1 dependent transcription [33,104,105]. Furthermore, it is proposed that p53 leads to a targeting of HIF-1α for ubiquitination and subsequent proteasomal degradation [106]. However, a search in the literature shows that a unifying picture concerning hypoxia and cell death is lacking and how cells balance between adaptation and cell death is still an unanswered question.

4. Physological and Pathological responses to hypoxia

Hypoxia has been implicated in a range of pathological and physiological conditions, and it can be harmful or beneficial, depending on the circumstances. In the following text the most well known hypoxic conditions will be described.

4.1. Detection of hypoxia

Chronic hypoxia is a strong prognostic factor for the outcome of various diseases. Currently, the use of 2-nitromidazole drugs that specifically bind to hypoxic cells has been largely advocated, pimonidazole and EF5 being the most well known [107]. Reductive enzymes metabolize these drugs in the presence of oxygen, while when oxygen is absent, the extra electrons are not removed, and the drugs are converted to highly reactive free radical molecules that covalently bind to protein and DNA. The drug-protein adduct can then be detected by specific antibodies [5,108]. However, the disadvantage of these drugs is that they have to be administered before sampling the tissue. Therefore, the search for possible surrogate markers for hypoxia is still growing.

4.2. Hypoxia preconditioning/ Ischaemic tolerance

Although hypoxia is correlated with pathological conditions, in the recent years it has been used for preconditioning stimuli. Preconditioning is defined as a stressful but non-damaging stimulus to cells, tissues or organisms to promote a transient adaptive response, so that injury resulting from subsequent exposure to a harmful stimulus is reduced [109].

Hypoxic preconditioning, or hypoxia-induced tolerance, refers to a brief period of hypoxia that protects against an otherwise lethal insult (for example, stroke) minutes, hours or days later. Hypoxic preconditioning protects the brain, heart and retina against several types of injury, including ischemia [110]. It is proposed that the protective response induced by hypoxic preconditioning is due, at least in part, to hypoxic induction of HIF isoforms and HIF isoforms target genes. Indeed, several studies have shown systemic hypoxia, which produced hypoxic preconditioning and protected the brain against ischemia, increased the levels of HIF-1α in neonatal and adult rodent brains [111,112]. Additionally, hypoxic preconditioning can also be achieved by known chemical inducers of HIF-1α such as $CoCl_2$ and desferrioxamine, suggesting that the preconditioning phenomenon is mainly mediated by HIF-activity [113]. Although, hypoxia leads to an increase level of HIF, it is probably the HIF target genes that provide the protection against subsequent ischemia and other types of injury. Indeed, induction of some of these genes products, such as EPO and VEGF, has been shown to protect the brain against ischemia on their own. In particular, it is shown that EPO both protects the brain against ischemia and produce an EPO-mediated preconditioning [114, 115]. Furthermore, it is reported that an overexpression of glucose transporter 1, which is important for the glucose metabolism, protects cells *in vitro* and *in vivo* against ischemia and other types of injury [116]. However, hypoxic preconditioning is more complex than the involvement of the transcription factor HIF. Indeed, studies have shown that other transcription factors and their target gene products might participate in this response, including activating protein 1 (AP1), the cyclic AMP-response-element-binding(CREB), nuclear factor-κB (NF-κB), early growth response 1 and the redox-regulated transcriptional activator SP1 [117-120]. Moreover, preconditioning can also be induced by hyperoxia, oxidative stress,

inflammatory cytokines, anaesthetics and metabolic inhibitors [109].

Nonetheless, the best current research strategy in order to obtain preconditioning aims to mimic the hypoxic response by increasing HIF-1 activity [121]. Induction of HIF-1 could occur either specifically, with targeted inducers, through gene therapy or through the action of hypoxia mimetics [122].

4.3. Stroke/Cerebral Ischemia and the potential role of neuroglobin

Stroke (hypoxia-ischemia) occurs when cerebral blood flow to the brain is interrupted. It restricts the delivery of substrates, particularly oxygen and glucose, and impairs the energetics required to maintain ionic gradients. With energy failure, membrane potential is lost and neurons and glia depolarize. Subsequently, the voltage-dependent Ca^{2+} channels become activated and cause accumulation of intra-neuronal free Ca^{2+} [123]. A prolonged elevation of intracellular Ca^{2+} leads to the catabolic process of vital molecules and irreversible death of neuronal cells through multiple mechanisms that involve the activation of Ca^{2+}-dependent effector proteins, such as calpains, endonucleases and caspases [124]. Furthermore, ROS are produced by the Ca^{2+}-dependent enzymes, such as nitric-oxide synthase, phospholipase A_2 and cyclooxygenase [125,126]. The important role of ROS in cell damage associated with stroke is understood by the fact that even delayed treatment with free-radical scavengers can be effective in experimental ischemia [127]. In the initial phase of a stroke, an induction of HIF-1 is reported. However, this induction seems to be by a hypoxia-independent pathway, e.g. via cytokines [110,121]. Furthermore, HIF-1 is a marker for chronic hypoxia, where failure of ion homeostasis and an increase in intracellular Ca^{2+} concentration is an acute hypoxia effect [53]. Therefore, the induction of HIF-1 during stroke remains unclear. Nevertheless, the disturbance of ion homeostasis plays an important role in the pathogenic of the stroke; other mechanisms, such as inflammation and peri-infarct depolarizations, are involved in the complex sequence of the pathological events that evolve over time and space [126].

The brain also activates neuroprotective mechanisms in an attempt to counteract the damaging effects of excitotoxicity [128]. By administrating EF5, a hypoxia marker (see 4.1), in an experimental animal model, it is shown that the brain is hypoxic during the first few hours of recovery, but the tissue is no longer hypoxic after 2 days [129]. This hypoxic period is believed to play an important role in protecting the brain cells from further damage. It is postulated that hypoxia induced HIF-1 levels lead to proportional increases in the HIF-1 target genes product during recovery [130]. It is well documented that during recovery, HIF-1 target genes, such as EPO, VEGF and IGF 2, involved in the erythropoiesis, angiogenesis and proliferation processes respectively, are over-expressed [110]. Additionally, it is suggested that HIF might be important in affecting cell survival and recovery through a regulation of the cellular antioxidant capacity. It is believed that the most damaging effect during recovery (reperfusion) is caused by an increased generation of ROS [42,131].

Moreover, neuroglobin (Ngb), a recently discovered protein, is suggested to be the key mediator of hypoxic-ischemic injury-repair coupling in the brain [132]. Ngb is a monomeric heme-protein, which binds oxygen reversibly, and is preferentially expressed in the neurons of the central and peripheral nervous systems (CNS, PNS) [133]. The intracellular globins play an important role in oxygen homeostasis of the animal cells, e.g. myoglobin facilitates oxygen diffusion to the mitochondria and stores oxygen for short or long term periods of hypoxia [134]. We have recently shown that Ngb is upregulated in the human neuroblastoam cell line under hypoxic condition [135]. Additionally, another group has shown that Ngb is upregulated in mice brain exposed to hypoxia-ischemia condition [136]. However, we and the other group were not able to show statistically significant upreglulation of Ngb in mice brain under hypoxic condition [137, 138]. Therefore, upregulation of Ngb in mice brain under hypoxic condition remains an open question. The mechanism for hypoxic induction of Ngb is unknown. However, both HIF-dependent and independent mechanisms of induction has been suggested [132]. It is postulated that Ngb protects neurons from hypoxic and ischemic cell death [139]. However, since Ngb is a relatively newcomer, it remains unknown how Ngb protects neurons [140]. Several neuroprotective mechanisms have been proposed for Ngb. It is suggested that Ngb might have an Mb-like function in the oxygen supply to the respiratory chain, either by facilitating oxygen diffusion or by providing a short term oxygen store [141]. We suggest that

Ngb can function as a ROS scavenger during oxidative injury. Our conclusion is based on firstly, an increase survival of cell over-expression Ngb under H_2O_2 stress and secondly, the observation that in eyes a negative correlation of Ngb and H_2O_2 levels in hypoxia/reoxygenation studies is present [137, 142]. Additionally, other studies have shown Ngb is an efficient scavenger of $NO^{\cdot}$ and peroxynitrite [143,144]. Furthermore, it has been suggested that Ngb can interact with Rho GTPases as a GDP-dissociation inhibitor (GDI). Rho GTPases are GDP-bound and maintained inactive in the cytosol, complexed with a GDI. Activation of Rho GTPases requires GDI dissociation, replacement of GDP with GTP, and intracellular translocation of GTPases from the cytoplasma to the plasma membrane. Binding of Ngb to Rho GTpases keep this protein in the inactive form [145]. Recently, it has been demonstrated that this interaction leads to inhibition of death signaling [139]. Additionally, it is suggested that Ngb inhibits apoptosis by inactivating Cytochrome c (Cyt c). It is shown that Ngb ferrous (Fe^{2+}) can reduce Cyt c ferric (Fe^{3+}), thereby inactivating Cyt c in the apoptotic pathway [146].

Based on the current observation that Ngb expression is induced by neuronal hypoxia, cerebral ischemia, ischemia/reperfusion, and probably other neurodegenerative diseases, it is suggested that Ngb has the potential to be a neuroprotective molecule [132].

4.4. Hypoxia and cancer

It was more than 50 years ago that it was first reported that human tumors grew under a condition which was termed "chronic hypoxia" [147]. It is described that hypoxia contributes to selecting cancer cells resistant to apoptosis and mediates resistance to chemotherapy and radiotherapy [148]. Therefore, a correction of hypoxia before radiation therapy is routine, by using blood transfusion to increase the haemoglobin concentration in patients, which results in a better response to the therapy [149]. Nowadays, hypoxia in human cancers, such as the head and neck, cervical or breast cancer, is associated with increased metastasis and poor survival in patients [150, 151]. As earlier mentioned, cells undergo a variety of responses to the chronic hypoxic condition, which involves different pathways. The earliest pathway was noted 70 years ago: cancer cells shift from oxidative phosphorylation to anaerobic glycolysis. This process is known as the Warburg effect and involves a decreased mitochondrial respiration and an increased lactate production, even in the presence of oxygen [152]. Furthermore, it has been known for a long time that tumorigenesis involves multiple mechanisms, including angiogenesis, proliferation, metastasis, differentiation [153, 154]. However, a better understanding of the regulation of the multiple steps in tumorigenesis was first recognized in the early 1990s when HIF-1 was identified. Today, it is well established that HIFs play an important role in tumour progression. Studies have reported that HIF-1α, HIF-2α and HIF-3 α are overexpressed in human cancers, and that the level of expression is correlated with tumorigenesis and patient mortality [155-157]. Furthermore, in support of HIF-1's role in tumour progression, it is reported that genetic or pharmacological inhibition of HIF-1 in animal model systems manifests a decrease in tumorigenesis and an increase in survival [158, 159]. Additionally, recent studies have provided evidence indicating that HIF-1 mediates resistance to chemotherapy and radiation [160,161]. Therefore, with a growing understanding of the HIF-1 pathway, the regulation of its transcriptional activity has become an attractive goal for therapeutic targeting in cancer. Currently, different approaches have been used to inhibit HIF-1α gene transcription: through inhibition of the ability of HIF-1α to interact with proteins that modulate its activity, or through inhibition of signal transduction pathways [162]. There are several approved therapeutic agents reported which are capable of inhibit HIF-1 activity, including Trastuzumab (Herceptin), Imatinib (Glivec), Camptothecin. Although, the anticancer effect of these agents might be due, in part, to their inhibition of HIF-1, none of these drugs specifically target HIF-1. The lack of selectivity increases the difficulty in correlating molecular and clinical responses in patients, but it does not disqualify these drugs as potential anticancer agents [155].

4.5. Hypoxia and cancer stem cell

In recent years, it has been observed that hypoxia plays an important role in the differentiation of stem cells to cancer stem cells. Stem cells are undifferentiated cells, generally characterized by their functional capacity to both self-renew and to generate a large number of differentiated progeny cells [163]. Because of their exceptional properties, stem cells have the potential to be used for

developmental biology, drug screening, functional genomics applications, and regenerative medicine. Stem cells reside in specialized cellular contexts called stem-cell niches, which are defined as particular locations or microenvironments that provide the signals and physical support to maintain stem cells. Cancer stem cells are cancer-initiating cells that can self-renew and generate distinct cell types. These cells are capable of indefinite self-renewal and can give rise to rapidly dividing transit amplifying cells that have a limited capacity for self-renewal and whose progeny differentiate to produce the bulk of the tumor [164, 165]. The finding that many cancers are maintained by a small population of stem cells has extremely important implications for both understanding and treating cancer. A major question is how these stem cells arise. The source of cancer stem cells is not entirely clear and may differ depending on the specific disease. They can arise from normal stem cells that have sustained a mutation to make them cancerous [164]. In contrast, cancer stem cells can be derived from more differentiated cells that have undergone a mutation or epigenetic changes that give them stem cell properties [166]. It is believed that hypoxia promotes a generation of cancer stem cells. Some of the effects of hypoxia on the generation of cancer stem cells are mediated by the HIF proteins. It is striking that HIFs activate several key stem cell genes and pathways; thereby tumor hypoxia may contribute to the conversion of differentiated tumor cells into cancer stem cells [167]. It is interesting that two transcription factor markers for stem cells differentiation, Oct-4 and c-Myc, are directly activated by HIF- 2α. The activity of c-Myc, a prominent oncogene, is modulated by HIF. Through a binding to the transcription factor Sp1 under hypoxic conditions, HIF-1α antagonizes c-Myc activity and inhibits c-Myc dependent cell-cycle progression [168]. In contrast, HIF-2α potentiates c-Myc activity by enhancing its physical association with Sp1, Miz1, and Max [169]. Oct-4 transcription factor, which is not expressed in normal differentiated somatic cells, is also expressed in a variety of cancer cell lines and is induced by hypoxia in a HIF-2α expressing renal carcinoma cell line [170]. It has been demonstrated that inducible expressions of Oct-4 in transgenic mice produce reversible epithelial dysplasia, a characteristic of premalignant lesions [171]. Together, these data suggest that the Oct-4 locus, which is not expressed in normal differentiated somatic cells, may promote the proliferation of undifferentiated progenitor and/or stem cells, thereby contributing to tumor growth. However, it is not well-known to what extent Oct-4 contributes to the growth of human tumors. In addition, HIF regulation of ATP-binding cassette (ABC) glycoprotein activity may also contribute to cancer stem cells formation. Some cancer stem cells express ABC glycoprotein transporters at the cell surface, a trait shared with normal hematopoietic stem cells. These transporters remove chemotherapeutic drugs and promote the multidrug resistance (MDR), observed in a large number of cancer cell lines [172]. Finally, HIFs have been shown to activate the Notch signaling pathway that controls stem cells self renewal and multipotency and appears to have both oncogenic and tumor suppressor effects in different contexts [173, 174]. HIF-1α may also activate the expression of c-Myc by regulating Notch signaling [175]. These discoveries could help in developing novel therapeutics for cancer treatment. The fact that cancer can grow from malignant cells with stem cell properties strongly support the idea that eradicating cancer stem cells should be an important goal in curing cancer. Therefore, the HIF pathways are even more attractive as targets of therapeutic intervention [156]. Inhibiting HIF activity could promotecancer stem cells differentiation, thereby reducing their ability to repopulate tumors after chemo and radiation therapies.

5. DISCUSSION

Our understanding on the role of hypoxia in physiology and pathophysiology has increased several folds in recent years, thanks to identification of HIF which acts as a master regulator coordinating oxygen homeostasis. This review has summarized the current understanding of cell responses to chronic hypoxia and conditions in which hypoxia can be harmful or beneficial. Many questions need to be answered about how cells sense hypoxia and activate the oxygen-regulated pathways, and how the pathways are integrated. Significant studies are done to understand the hypoxia HIF-dependent responses, but limited researches available which have focused on the hypoxia HIF-independent responses. Despite the increase in knowledge of HIF activation, mechanisms that contribute to the positive and negative regulation of HIF are poorly understood. More research is required to determine the ratio of the pro-survival

pathways and cell death pathways that are activated in response to hypoxia and how these are regulated. A search in literature shows that hypoxia effects are analyzed under different conditions, such as cell types, duration of exposure to hypoxia, etc. It is well established that different cell or tissue types response differently to hypoxia. Therefore, the interpretations of the results in the literature are become more complicated. In order to obtain a unifying picture of hypoxia response a standardization of the experimental is needed. Finally, it is postulated that up to 5% of genome is transcriptionally regulated by hypoxia. Until identification of all these genes a complete picture of response to hypoxia will remain unclear.

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