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Chapter 1

Introductory Chapter: Primary Concept of Hypoxia and Anoxia

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1. Introduction

Hypoxia is a pathological condition in which the body as a whole (generalized hypoxia) or a region of the body (tissue hypoxia) is deprived of adequate oxygen supply. Variations in arterial oxygen concentrations can be part of the normal physiology, for example, during strenuous physical exercise. A mismatch between oxygen supply and its demand at the cellular level may result in a hypoxic condition. Hypoxia in which there is complete deprivation of oxygen supply is referred to as anoxia. Hypoxia belongs to the most serious factors that can directly impair the function of metabolic pathways in the animal cell. The exposure of experimental animals to hypoxia has been widely used in many morphological and physiological studies. These studies dealt mostly with changes in the structure of pulmonary vessels. In healthy humans, there is a range of physiological oxygen levels within the tissues of the body, ranging from PO2 values of -100 Torr in the alveoli of the lungs to less than 10 Torr in tissues such as the medulla of the kidney and the retina [1] [Taylor 2008]. Within all of these tissues, the chemical reduction of molecular oxygen in the mitochondria of individual cells during the process of oxidative phosphorylation is central to oxidative metabolism and bioenergetic homeostasis. Because of this, any insufficiency in the availability of molecular oxygen represents a severe threat to continued physiological function and indeed survival. Hypoxia, which occurs when oxygen levels in the microenvironment of a cell, tissue, or organism are reduced relative to the normal physiological state, is associated with a range of physiological and pathophysiological processes [2]. [Semenza 2007].

Hypoxia may limit the energy budget or scope for growth and activity of an organism, it may cause an organism to alter its behavior, and or it may limit the tolerance of an organism to other environmental challenges. In most tissues of the body, the response to hypoxia is vasodilatation by widening the blood vessels, the tissue allows greater perfusion. The contrast is in the lungs where the response to hypoxia is vasoconstriction. This is known as "Hypoxic pulmonary vasoconstriction", Physiological hypoxia is an important micro environmental signal in a range of processes including new blood vessel formation (angiogenesis) during development and wound healing, the regulation of vascular tone, and the response to exercise [3] [Ratcliffe 1998]

2. Hypoxia Pathophysiology

Tissue hypoxia is also associated with a diverse and wide range of pathophysiological processes including (but not limited to) vascular disease, chronic inflammation, and cancer [3] [Ratcliffe,1998]. In vascular diseases such as atherosclerosis and stroke, vascular occlusion leads to acute or chronic tissue ischemia with resultant hypoxia. In chronic inflammatory disease, the greatly increased metabolism of inflamed tissue due to immune cell infiltration matched with vascular dysfunction leads to tissue hypoxia [4] [Das et al 2016]. In cancer, the growth of a tumor away from the local blood supply eventually leads to tumor hypoxia. In all of these cases, the induction of a genetic response to hypoxia leads to the expression of genes that are essentially adaptive (or maladaptive in the case of cancer). Seminal discoveries in the last 20 years have greatly enhanced our understanding of the molecular mechanisms underpinning this critical response [2] [Semenza 2007]. Hypoxia results from conditions such as ischemia, hemorrhage, stroke, premature birth, and other cardiovascular difficulties, among which hemorrhagic shock is the leading cause of death and complications in combat casualties and civilian settings. It has been shown to cause systemic inflammation response syndrome (SIRS), multiple organ dysfunctions (MOD), and multiple organ failure [5] [Kiang 2006].

It is an often seen problem and to find useful remedies that are capable of ameliorating its casualty is an essential effort in combat medicine, trauma injury, and cardiovascular related scenarios. Hypoxia has been shown to lead to increases in intracellular free calcium concentration (Ca^{2+}), 5-lipoxygenase, lipid peroxidation, cycloxygenase (COX), constitutive nitric oxide synthase (cNOS), leukotriene B4 (LTB4), prostaglandin E2 (PGE2), interlukins, tumor necrosis factor-α (TNF- α), caspases, complement activation, kruppel-like factor 6 (KLF6), inducible nitric oxide synthase (iNOS), heat shock protein 70 kDa (HSP-70), and hypoxia-inducible factor-1α (HIF-1α). The sequence of their occurrence provides the useful information for studying the mechanisms underlying the hypoxia-induced injury as well as therapeutic

targets to prevent or ameliorate the injury [6] [Kiang 2004.]. It is also to be mentioned that spontaneous hypoxic injury causes "accidental cell death" or necrosis by cells swelling, plasma and nuclear membrane disruption, cellular lysis in association with acute inflammation that may exacerbate the initial hypoxic injury response. However the alternative mode of cell death, apoptosis, is also possible. During apoptosis, the cells use their molecular machinery to shrink or expand into membrane-bound apoptotic bodies, with or without nuclear fragments that are easily phagocytosed by adjacent tissue cells or macrophages and minimize any acute inflammatory response. [7] [Das & Saha 2014].

3. Discovery of HIF-1

Before HIF-1 was found, HRE had been identified in the 3′-enhancer region of the erythropoietin gene, whose transcription is up-regulated by more than 100-fold by severe hypoxia. Semenza and Wang defined a binding site critical for the hypoxia-inducible function, which involves a transcription factor induced by hypoxia. Subsequently, they purified a DNA-binding complex bound to the HRE by affinity-purification using oligonucleotide with the HRE sequence and thus identified the encoding cDNAs). HIF-1 was found to be a heterodimer composed of two basichelix- loop-helix (bHLH) proteins of the PAS family, HIF-1 and HIF-1. Of these, HIF-1 had previously been identified as the aryl hydrocarbon nuclear receptor translocator (ARNT), which is dimerized with the aryl hydrocarbon receptor. However, HIF-1 was a newly defined protein and uniquely associated with the transcription of the hypoxia-inducible genes [8] [Semenza 1991]. Later, homology searches in the gene bank and cloning experiments found other members of this family, such as HIF-2 (also known as endothelial PAS protein-1) and HIF- 3. HIF-2 is also tightly regulated by oxygen tension and its complex with HIF-1 appears to be directly involved in hypoxic gene regulation, as is HIF-1. However, although HIF-3 is homologous to HIF-1 , it might be a negative regulator of hypoxia-inducible gene expression [9]. [Wang, 1995].

Hypoxia, or inadequate oxygenation, causes various responses within the body. Its effects are usually mediated via the activation of hypoxia inducible factor 1 (HIF-1). HIF-1 activation can lead to up-regulation of various genes such as erythropoietin and growth factors that help tissues adjust to the decreasing oxygen availability. Another key molecule within this hypoxia-induced response is the presence of nitric oxide [NO]. NO is an ubiquitous gaseous molecule within our

body. It is synthesized by nitric oxide synthases (NOS) and its release can be stimulated as a result of inflammatory responses, sympathetic activation and drop in oxygen levels [5].[Kiang 2006].

3.1. HIF -1α as transcriptional regulator

When the protein levels of HIF-1 increase, *e.g.* in response to hypoxia, it translocates to the nucleus, dimerizes with the alpha subunit and activates the transcription of a number of target genes displaying an HRE motif. Nuclear localization signal (NLS) domains in the alpha and beta subunit confer autonomous translocation into the nucleus 248 [10] [Das et al 2016].

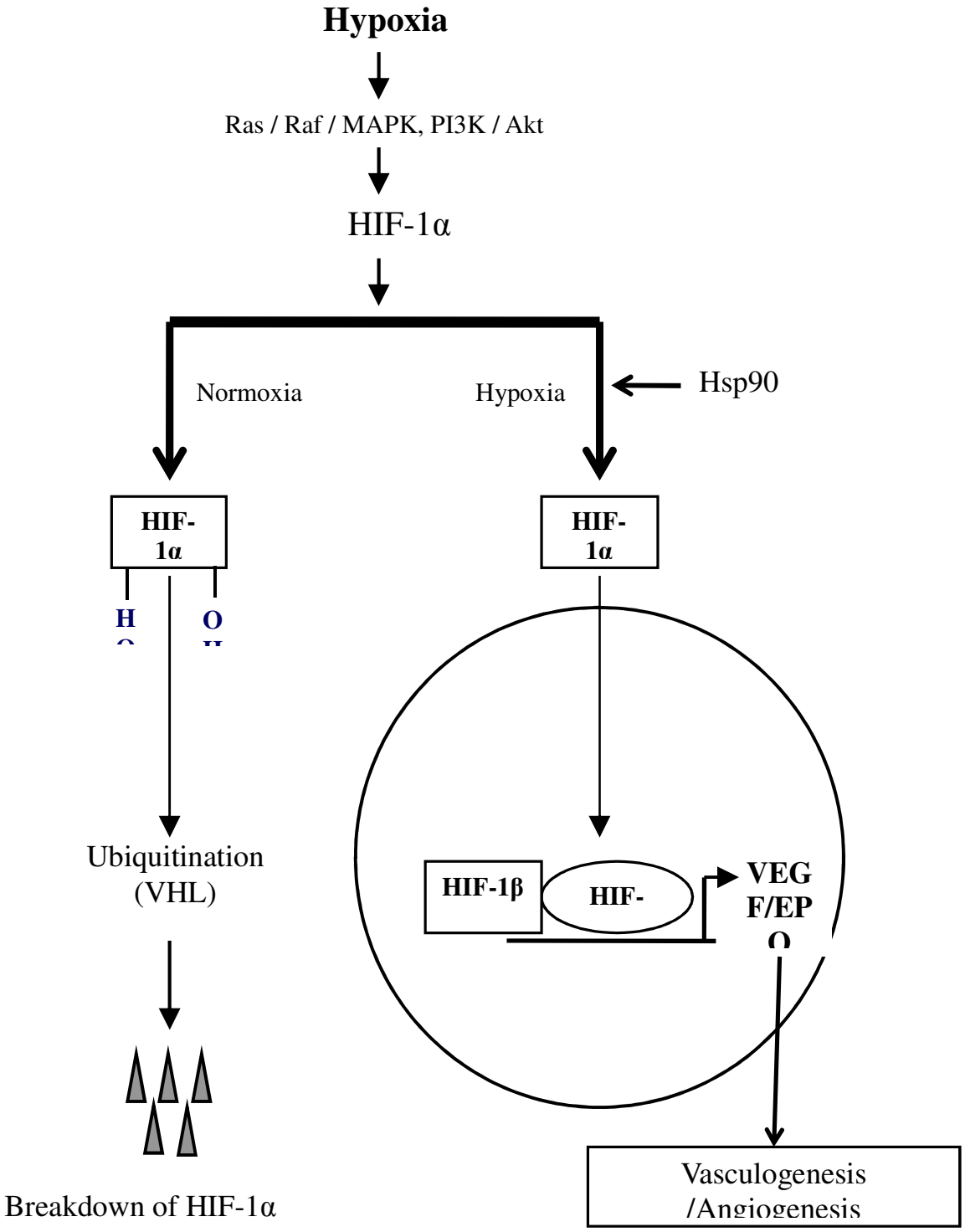


Figure 1.Schematic representation of the cell signaling events leading to ubiquitin mediated degradation of hypoxia-inducible factor 1α (HIF-1 α) and hypoxia mediated expression of VEGF and EPO protein. VEGF, vascular endothelial growth factor; EPO, erythropoietin

One group of HIF-1 target genes is involved in the adaptive response facilitating oxygen delivery to oxygen-deprived tissues. It includes *e.g.* genes coding for erythropoietin, VEGF-A and inducible NOS (iNOS) [11-12] [Jośko 2004; Tjong 2008].(Figure 1). The *erythropoietin (Epo)* gene, encoding a kidney hormone, was discovered as the first true hypoxia-inducible gene in 1992 226. EPO stimulates red blood cell production (erythropoiesis), thereby increasing oxygen delivery. Hypoxia also promotes iron uptake and transport by increasing the expression of transferrin and the transferrin receptor 249,250. Another well-known hypoxia-regulated gene is *Vegf-a*, which plays a crucial role in development and growth of blood vessels [13] [Shweiki 1992]. Under normoxic conditions, HIF-1-prolyl hydroxylases (PHD) hydroxylate the prolyl residues at amino acid 402 and 564. These enzymes require di-oxygen, Fe2+, ascorbate, and 2 oxoglutarate for activity. The hydroxylated peptides interact with an E3 ubiquitin-protein ligase complex composed of pVHL(von Hippel-Lindau tumor suppressor protein), elongin B & C, and Cullin 2 (CUL2), and then poly-ubiquitinized, resulting in HIF-1α degradation by the 26S proteasome. Under hypoxic conditions, HIF-1α is not hydroxylated because the major substrate, dioxygen, is not available. The unmodified protein escapes the VHL-binding, ubiquitination, and degradation, and then dimerizes HIF-1α and stimulates the transcription of its target genes [14]. [Chun 2002] (Figure 1).

4. Hypoxia and oxidative Stress

During normoxia, about 2-3% of oxygen consumed by the mitochondria is incompletely reduced yielding reactive oxygen species (ROS) [15] Hamanaka RB and Chandel NS, 2009. The mitochondrial ROS route to the cytosol and at low to moderate concentrations act as signaling molecules for a number of biological functions like cell growth, differentiation and metabolism and immune functions. Although at high concentrations they can adversely modify the cell components like lipids, proteins and DNA. However the cells are well equipped with antioxidants that are capable of mounting an adequate antioxidant defense against ROS. Oxidative stress results when there is a shift in the balance between the oxidants and antioxidants in favor of oxidants disrupting redox signaling and control and /or inducing molecular damage (as defined by H Sies and D P Jones) [16] Jones DP 2006 In addition to a host of factors both low and high oxygen levels are capable of inducing increased ROS formation and ultimately oxidative stress. Since oxygen is essential for formation of all ROS several controversies exist and it appears paradoxical for

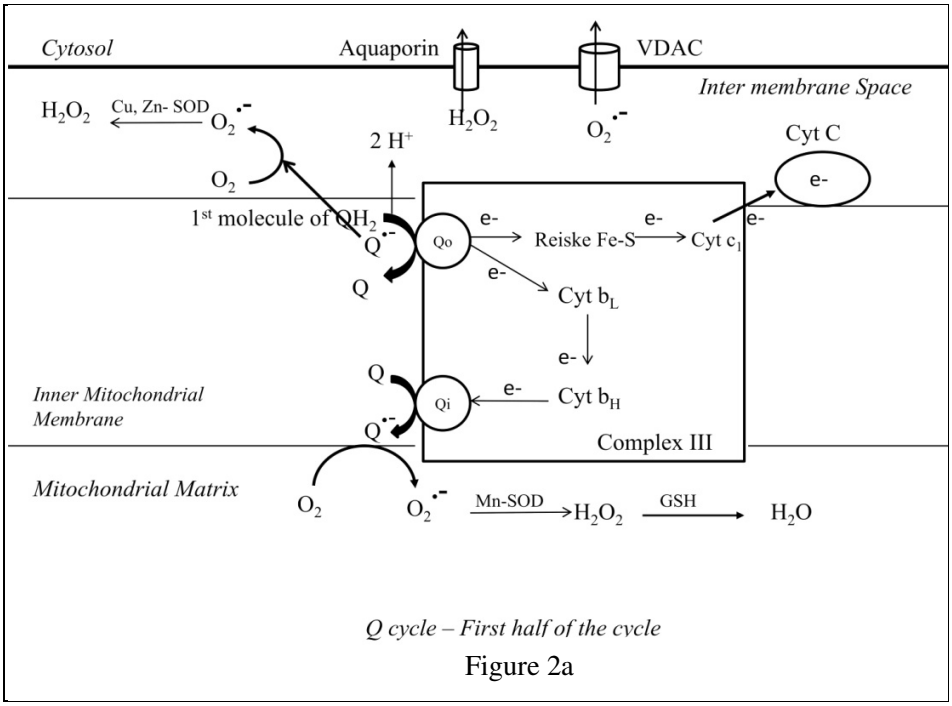
increased ROS formation in low oxygen microenvironment like hypoxia. However there is enough evidence in support of increased ROS formation and oxidative stress in hypoxia.

Hypoxia and Mitochondrial ROS generation:

Mitochondria have been considered the main source of ROS generation (particularly H_2O_2) during hypoxia. Bell ET et al have demonstrated a dose dependent relationship between ROS and available oxygen levels with an increase in intracellular ROS with increasing severity of hypoxia [17] Bell EL et al 2007. During mitochondrial respiration, electrons from NADH and $FADH_2$ are transferred successively through several electron carrier molecules of the electron transport chain (respiratory chain). The electron transport chain consists of series of proteins and organic molecules located in the inner mitochondrial membrane (IMM) and organized as four membrane bound complexes (Complex I to IV) which generate a proton gradient across the inner mitochondrial membrane and two mobile carriers - Cytochrome c and Ubiquinone (coenzyme Q) and a ATP synthase (complex V, F1F0 - ATPase) that uses the proton gradient for ATP synthesis. Complex I (NADH dehydrogenase) and Complex II (succinate dehydrogenase) transfer electrons from NADH and FADH2 respectively to coenzyme Q (ubiquinone). Ubiquinone can accept one electron to form Ubisemiquinone or two electrons to form ubiquinol. Because of its lipid solubility and small size it is freely mobile within the lipid bilayer of the inner mitochondrial membrane (IMM). Reduced coenzyme Q further feeds the electrons to complex III of the ETC. Complex III pushes the electrons to complex IV (cytochrome oxidase) via cytochrome c, second mobile carrier of the ETC. At this stage, complex III plays a crucial role of transferring single electrons sequentially to cytochrome c and complex IV since they can accept single electrons at a time unlike complex I and complex II [18]. Guzy and Schumacker 2006. Complex IV transfers the electrons from cytochrome c to final electron acceptor Oxygen which is reduced to water in the process. As the electrons travel through ETC they move downhill from a higher to lower energy level. The energy released in this downhill movement of electrons is used to pump protons (H^+) (by complexes I, III and IV) from the mitochondrial matrix to the intermembrane space creating a proton gradient. The generated proton gradient drives formation of energy in the form of ATP from ADP and Pi by ATP synthase. During the ETC, ROS are produced at complex I, II and III.

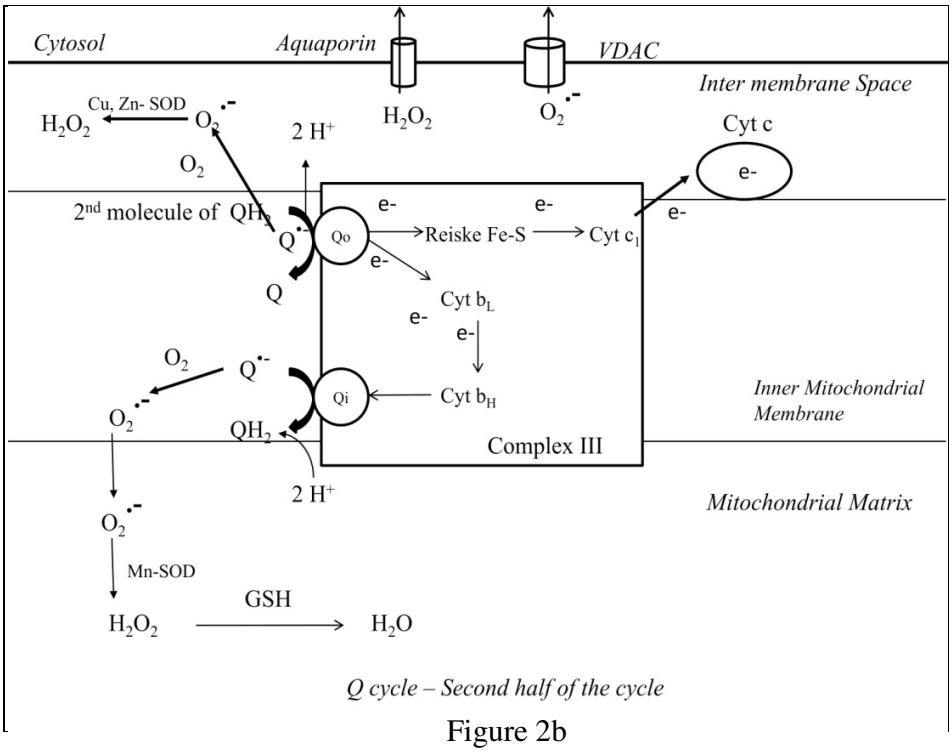
Complex I and II produce ROS only into the matrix, while complex III produce ROS on both sides of the inner mitochondrial membrane [15] Hamanaka and Chandel 2009.

Complex III is considered as an important source of ROS during hypoxia [19] Murphy 2009. Crucial role of complex III in ROS formation in hypoxia necessitates a greater understanding of its role in ETC. Mitochondrial Complex III also referred to as cytochrome bc1 complex (cyt bc_1 or bc_1) or ubiquinol cytochrome c oxidoreductase is a dimer with each monomer made up of 11 protein subunits encoded by mitochondrial and nuclear genes [18] Guzy and Schumacker 2006. Complex III has three important subunits with known electron transport activity – binuclear Rieske Fe-S protein (2Fe-2S cluster), bis heme cytochrome b and cytochrome c_1 [20] Bell et al 2007. Cytochrome b contains two heme groups. Of the two heme groups, one is low potential heme (b_L) located near the outer surface of the inner mitochondrial membrane and the second high potential heme (b_H) at the centre of the membrane about 20 Å from b_L [21]. Matsuno-Yagi and Hatefi 1996. The complex III has two separate ubiquinol and ubiquinone binding sites - Qo and Qi. Qo is located on the P (positive) side (outer surface) of the inner mitochondrial membrane and is ubiquinol oxidation site. Qi is located on the N (negative) side (close to the matrix) of the inner mitochondrial membrane and is the ubiquinone reduction site [22]. Tian H 1998. Complex III performs an important function of transfer of single electrons sequentially to cytochrome c and then to cytochrome IV since they can accept single electrons at a time unlike complex I and complex II by a cycle called Q cycle (Figure 2a and Figure 2b). Q cycle begins with the binding of first molecule of ubiquinol to Qo site and ubiquinone at Qi site of complex III. The two electrons of ubiquinol follow two separate paths within complex III. One of the electrons from ubiquinol (yielding ubisemiquinone) is transferred to Rieske Iron-Sulfur protein to cytochrome c1 and finally to cytochrome c. The second electron from ubisemiquinone is transferred to cytochrome b and subsequently to ubiquinone bound at the Qi site of complex III converting ubiquinone to ubisemiquinone. Yet another ubiquinol binds at the Qo site with electron following the Rieske Iron-Sulfur, cytochrome c_1, cytochrome c pathway and the second electron via cytochrome b reduces the ubisemiquinone at the Qi site to regenerate ubiquinol. Thus one Q cycle involves oxidation of two ubiquinol molecules to ubiquinone with regeneration of one ubiquinol molecule and transfer of four protons from the intermembrane space from the matrix. [23] Guzy et al 2005.



Q cycle – First half of the cycle

Figure 2a



Q cycle – Second half of the cycle

Figure 2b

Figure 2a and 2b: Schematic diagram depicting the Q – cycle at complex III of the mitochondrial electron transport chain and the generation of superoxide and H_2O_2 radicals at Qo and Qi sites of complex III.

Superoxide can be generated at Qo and Qi sites at complex III by one electron reduction of oxygen to superoxide (Figure 2a and Figure 2b) [23] Guzy 2005 Ubisemiquinone that is formed repeatedly at Qo and Qi sites of complex III is the site of ROS formation in hypoxia. The molecular oxygen that is lipophilic is dissolved in the hydrophobic environment within the membrane is highly electrophilic and can potentially capture electrons from ubisemiquinone forming superoxide radical [18] Guzy and Schumacker 2006. Superoxide generated at Qo site is released into the intermembrane space and the generated at Qi site is released to the mitochondrial matrix. Superoxide is an important source of H_2O_2 during hypoxia. Superoxide can be dismutated to H_2O_2 in the matrix by Cu,Zn-SOD and in the intermembrane space by Mn-SOD [18] Guzy and Schumacker 2006. Hydrogen peroxide can travel to the cytosol via the membrane aquaporin channels [24] Tafani 2016. Superoxide may also enter the cytosol through voltage dependent anion channels (VDACs) [25] Chandel et al 2000. The mechanism by which hypoxia increases ROS generation are poorly understood, however several hypothesis have been proposed like O_2 dependent structural changes that prolong the lifetime of ubisemiquinone ('semiquinone lifetime' hypothesis), increase in the accessibility of O_2 to a site where single electrons can be captured ('oxygen access' hypothesis) or enhancement of the directional escape of superoxide to the intermembrane space versus matrix compartments ('vectoral transport' hypothesis) Guzy and Schumacker 2006 [18], [23].

The ROS produced can either participate in cell signaling or induce irreversible cellular damage and death [26] Solaini G et al 2010. There is substantial evidence to suggest the role of ROS produced at complex III of ETC in hypoxia signaling by stabilizing HIF-1α by preventing its hydroxylation by prolyl hydroxylases in low oxygen microenvironment. This allows HIF-1α translocation to the nucleus and dimerization with HIF-1β initiating transcription of target genes (Figure 3) [27] Bell EL et al 2007.

Prolyl hydroxylases (PHD) belong to a family of mixed function oxidases involved in the hydroxylation of proline residues of HIF-1α that signals it for degradation. These enzymes require 2-oxoglutarate (α-ketoglutarate) and oxygen as substrates and non-haem iron as a cofactor [18] Guzy and Schumacker 2006. The activities of PHDs have been extremely sensitive to inhibition by ROS although the mechanisms by which the ROS alter the activity of PHDs are unknown. However the following hypothesis have been put forward 1) Possible oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) forbidding the mandatory binding of ferrous iron to prolyl hydroxylases.

2) Recruiting ascorbate for free radical scavenging making it unavailable for reducing ferric iron or probably by preventing direct binding of ascorbate to prolyl hydroxylase 3) Altering the concentrations of 2-oxoglutarate and succinate that might also have an prolyl hydroxylase activity [18] [28] [29] Qutub and Popel 2008, Guzy and Schumacker 2006, Pouyssegur 2006.

Paddenberg R et al., demonstrated a role of complex II of the electron transport chain in hypoxia induced ROS generation particularly in the pulmonary vasculature. Complex II is the smallest of the protein complexes of the ETC located on the matrix side of the inner mitochondrial membrane. Under normoxia this complex oxidizes succinate to fumarate meanwhile transferring two electrons to ubiquinone and reducing it to ubiquinol. During hypoxia complex II switches its function from succinate dehydrogenase to fumarate reductase changing the direction of electron flow from ubiquinol to fumarate (fumarate accepting as electron acceptor) thereby generating ROS and accumulating succinate [30] Paddenberg et al 2003.

Other Sources of ROS:

NADPH oxidase (NOX) has also been considered as an important source of ROS in hypoxia. NOX is activated by mitochondrial ROS by Protein Kinase C Epsilon (PKCε) pathway furthering the ROS production and cellular damage [24] Tafani M et al 2016.

Xanthine oxidase is also an important generator ROS under hypoxic conditions. Xanthine generates superoxide radical and hydrogen peroxide by one electron and two electron reduction of oxygen respectively. Superoxide can also be generated by uncoupling of eNOS/NOS3. The uncoupled eNOS produces more superoxide radical and less of nitric oxide [31] Battelli et al 2016.

Inducible Nitric oxide synthase (iNOS), inducible cyclooxgenase (COX2), inducible 5 - lipooxygenase (5-LOX), inducible heme-oxygenase-1 (HO-1) are other potential generators of ROS [24] Tafani M et al 2016.

Effects of ROS:

The cytosolic ROS can access the nucleoplasm interacting with nucleic acids inducing DNA damage, impairing DNA repair, causing mutations and damaging other nuclear components. Additionally the cytosolic ROS gain access to the extracellular space crossing cell membrane via aquaporins and anion channels. The detrimental effects of the extracellular ROS range from damage to adjacent tissues to distant tissues and organs, signaling local damage, activating improper mechanisms of adaptation, organ remodeling and chronic damage. The extent of damage

caused by ROS is determined by the half life, reactivity, velocity of diffusion and the possibility to travel with plasma [24] Tafani M et al 2016. Polyunsaturated fatty acids are highly susceptible to the oxidative damage by ROS (Lipid Peroxidation) reflected by a rise in Malondialdehyde (MDA) levels a product of lipid peroxidation [10] Das KK et al 2016. n-3 PUFAs which are essential structural components of cell membrane phospholipids undergo oxidative damage compromising membrane lipid dynamics, structure and function of membrane associated proteins like enzymes, receptors and transporters [32] Behn C et al 2007. The extracellular Na^+ and water gain entry into the cell as a consequence of cell membrane damage leading to cell swelling. The damaged cell membrane also allows the entry of extracellular Ca^{2+} disrupting the cellular calcium homeostasis initiating a cascade of reactions of enhanced ROS production, cellular damage and death [24] Tafani M et al 2016.

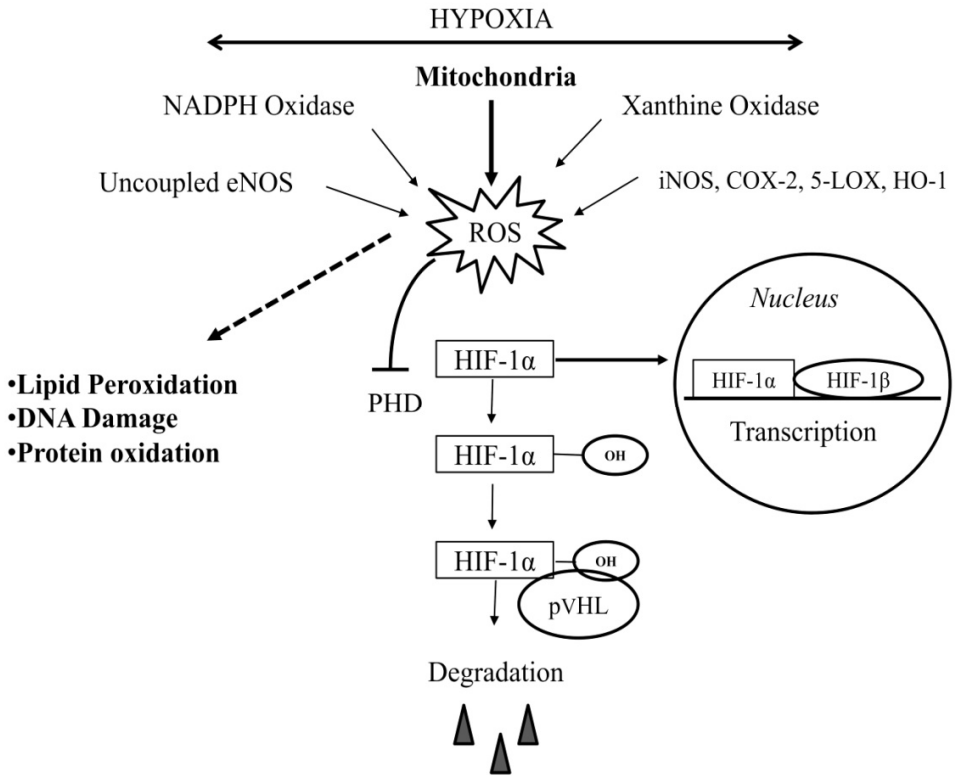


Figure 3: Schematic diagram depicting the various sources of ROD and the biological effects of ROS.

5. Hypoxia and heavy metal interactions

Heavy metals activated some of the signaling pathways observed under hypoxia and these are used to understand oxygen sensing mechanism and signaling cascades in the control of hypoxia-inducible various transcriptional gene expression. Studies also show that wild-type Human hepatoma cells (Hep3B) increase ROS generation during metal activated some cell signaling pathways during hypoxia [33] [Valko M, 2005]. Heavy metals are capable to induce expression of HIF-1 transcriptional factor and vascular endothelial growth factor (VEGF) gene through the phosphatidylinositol 3-kinase or Akt pathway or ROS [34] [Gao 2002].. Heavy metals induced alteration of the hypoxia signaling systems influenced by metal-induced oxidative stresses are responsible for progression of metastasis [35] [Galanis 2009]. Hypoxia-induced factor HIF-1 controls precise oxygen homeostasis by modulating expression of several cancer-related genes, including heme oxygenase1 and vascular endothelial growth factor. The carcinogenic metals such as lead (Pb), nickel or chromium have been known to activate HIF-1 [34] [36] [Semenza, 2000; Gao, 2002]. *In vitro* study has reported that nickel activates the HIF-1 based on the substitution of iron in the oxygen carrier by heavy metals, which leads to permanent hypoxia; thus activating HIF1 [37] [Leonard 2004]. Some heavy metals are found to cause widespread organ damage due to cellular hypoxia by inhibition of enzyme cytochrome C oxidase of mitochondria. It has been found that heavy metals intoxication under hypoxic conditions induced growth retardation in growing rats and damages on femoral and mandibular bones that predispose to fractures [38] [Arora 1995]. The mechanisms of carcinogenesis caused by heavy metals emphasizing on the involvement of the hypoxia signaling pathway by metal-induced generation of reactive oxygen species and oxidative stress generation in cancer progression [35] [Galanis 2009].

It has been further reported that divalent metal compound competitively inhibit iron (Fe^{2+}) absorption in heme synthesizing tissues. This leads to decrease production of heme biosynthesis. Beside this many divalent cation heavy metals also suppress the activities of enzyme ferrochelatase which facilitate conversion of protoporphyrin IX to Heme. Hence it is clear that lead reduces heme biosynthesis in mitochondria. Low level of heme reduces intracellular oxygen tension and simply intracellular low Fe^{2+} and low oxygen tension inhibit PHD_2 (prolyl hydroxylases) [39] [Das 2015]. Heavy metals induces ROS production, activates ERK and AKT pathways in similar fashion as hypoxia and up regulates key proangiogenic molecules HIF-1α and VEGF expression in cells. The

increase production of HIF-1α and VEGF by heavy metals actually facilitate cell to adapt from adverse micro environment in physiological system [7] [Das and Saha 2014]

Conclusion:

Both hypoxia and heavy metal exposure induce generation of ROS (reactive oxygen species) , increase expression of p53, NF-kβ, AP-1, MAPK and HIF-1α. The increase expression of all these transcription factors leads to either cellular adaptation or cell death. The mechanisms by which mammalian cells adapt to acute and chronic alteration of oxygen tension is extremely important to understand the exact homeostasis regulation to counteract hypoxia-induced cell damage as therapeutic strategy.

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