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*Chapter 11*

# HYPOXIA AND OXIDATIVE STRESS: CELL SIGNALING MECHANISMS AND PROTECTIVE ROLE OF VITAMIN C AND CILNIDIPINE

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## ABSTRACT

Hypoxia is a pathological condition that can directly impair the metabolic pathways in the living cells. Interestingly, physiological hypoxia is an important micro-environmental signal, in a range of processes including new blood vessel formation (angiogenesis) during development, wound healing, regulation of vascular tone and response to exercise. Its effects are usually mediated via the activation of hypoxia inducible factor 1 (HIF-1α), besides nitric oxide (NO) - an important key factor of hypoxia-induced responses. The low oxygen sensing at the cellular level exerts its defense through HIF-1α by increasing hypoxia adaptability but it cannot prevent the generation of free radicals through endothelial cellular oxidative stress which may lead to lysosomal, mitochondrial and microsomal damage, resulting in organelle dysfunction. Beside these actions, hypoxia induced oxidative stress greatly impairs cell signal transduction by altering gene expression in hypoxia sensitive tissues. It has also been found that cellular adaptation to low oxygen is compromised in the presence of hyperglycemia, culminating in increased cell death and tissue dysfunction. An excessive accumulation of reactive oxygen species can elevate antioxidant enzymes and then impair

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beta cell functions. Recent observation reveals that chronic intermittent hypoxia (CIH) activates NADPH oxidase which is very important for HIF-1α expression and ROS production. NADPH oxidase hyperactivity also changes intracellular calcium homeostasis and stimulate further HIF-1α production, subsequently resulting in more ROS generation. High concentration of ROS excites carotid bodies that influences sympathetic adrenergic activities via chemoreflex, alters catecholamine and insulin secretary mechanisms.

A link between hypoxia and glucose homeostasis has already been established. Present authors further ascertained that glucose homeostasis due to hypoxia can be modulated by supplementation of either vitamin C or L/N type calcium channel blocker, cilnidipine.

This chapter provides understanding of the relationship between hypoxia induced increased sympathetic activation and consequent impaired glucose homeostasis. The chapter also highlights how hyperglycemia augments oxidative stress and induces the overproduction of ROS which modulates HIF-1α regulation and possible protective actions of antioxidant (vitamin c) and L/N type of $Ca^{++}$ channel blocker (cilnidipine) against hypoxia induced altered pathophysiology in mammalian systems.

## INTRODUCTION

The relationship between hypoxia and oxidative stress is really interesting in the domain of hypoxia research or free radical biology research. Various evidences suggest that hypoxia induced stress results in alterations in glucose homeostasis (Pi and Collins, 2010). Free radicals are reactive species which have unpaired solitary electron in its outer atomic orbital; these include the hydrogen atom ($H^{\bullet}$); the diatomic oxygen molecule $O_2$, which possesses two unpaired electrons with the same spin in two separate orbitals; Nitric oxide ($NO^{\bullet}$), superoxide ($O^{\bullet -}_2$); hydroxyl radical ($^{\bullet}OH$); and transition metals, such as copper and iron. While $O_2$ qualifies as a radical by having two unpaired electrons, its reactivity with nonradical compounds is limited because the unpaired electrons in molecular oxygen have the same spin state. For molecular O2 to react with a non radical, one of the two electrons involved in its covalent bond need to undergo "spin inversion'; so that both the electrons exert an anti spin action on molecular oxygen in a slow reactive process.

$O_2$ does react readily with radicals, accepting one electron at a time to form the very reactive superoxide radical $O^{\bullet -}_2$, which has one unpaired electron (Poyton, 2009). It has been noticed that most reactive oxygen species (ROS) are generated in cells by the mitochondrial respiratory chain. Mitochondrial ROS production is regulated largely by the rate of electron flow through respiratory chain complexes. Recently, it has become clear that under hypoxic conditions, the mitochondrial respiratory chain also produces nitric oxide (NO), which can generate other reactive nitrogen species (RNS). Although excess ROS and RNS can lead to oxidative and nitrosative stress, moderate to low levels of both can modulate cellular signaling pathways. Especially important are the roles of these mitochondrially generated free radicals in hypoxic signaling pathways, which have important implications in cancer, inflammation and a variety of other diseases (Dugan and Choi, 1999). Hypoxia and free radicals like reactive oxygen and nitrogen species, can alter the functions of the transcription factor- hypoxia-inducible factor 1 (HIF1). It has also been found that free radicals generated by hypoxia, hypoxia–reoxygenation cycling and immune cell infiltration after cytotoxic therapy strongly influence HIF1 activity (Mark et al., 2008). Physiological hypoxia is found

to be an important cellular signal that modulates new blood vessel formation (angiogenesis) during wound healing, regulation of vascular tone and response to exercise (Ratcliffe, 1998). However, tissue hypoxia is also associated with a diverse and wide range of pathophysiological processes including (but not limited to) vascular diseases, chronic inflammation and cancer. 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 (Taylor, 2008; Fraisl et al., 2009). A relation between increased sympathetic activation and hypoxia with consequent impaired glucose homeostasis provides possible counteraction with N-type calcium channel blockers, which are normally used to control hypertension or heart diseases (Peltonen, 2012). Studies further revealed that sympathetic over activities may be regulated by either direct influences of hypoxia via chemoreceptor or through increase in HIF-1α or through ROS (Das et al., 2016).

## HYPOXIA: AN OVERVIEW

Low oxygen sensing limits prolyl hydroxylase activity and HIF-1 α ubiquitination process and activates HIF-1 transcription factors. However, the level of HIF-1 α also increases via an $O^{2-}$independent mechanism (Jiang et al., 2001). Expression of HIF-1 α induces several growth factors, such as epidermal growth factor, heregulin, insulin-like growth factors (IGFs) I and -II, and insulin induced expression of these protein under nonhypoxic conditions (Isaacs et al., 2002; Semenza, 2003). These factors bind to cognate receptor tyrosine kinases and activate the PI3K or mitogen-activated protein kinase (MAPK) pathway which in turn increases the rate of HIF-1 α protein synthesis itself. PI3K-Akt and MAPK have also been implicated in the stabilization of HIF-1 α induced by oncogenes, hypoxia and growth factors (Semenza, 2003). HIF-1 α also associates with the molecular chaperone heat shock protein 90 (Hsp90); pharmacologic disruption of this association promotes the ubiquitination and proteasome-mediated degradation of HIF-1 α, independent of oxygen and VHL (Isaacs et al., 2002), suggesting that inhibitors of HIF-1 α and Hsp90 could be used to regulate the expression of hypoxia- or IGF-I induced HIF-1 α protein. It has been found that hypoxia leads to increase in intracellular free calcium concentration ($[Ca^{2+}]i$), 5-lipoxygenase, lipid peroxidation, cycloxygenase (COX), constitutive nitric oxide synthase (cNOS), leukotriene B4 (LTB4), prostaglandin E2 ($PGE_2$), 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) (Moore et al., 1994; Kiang, 2004).

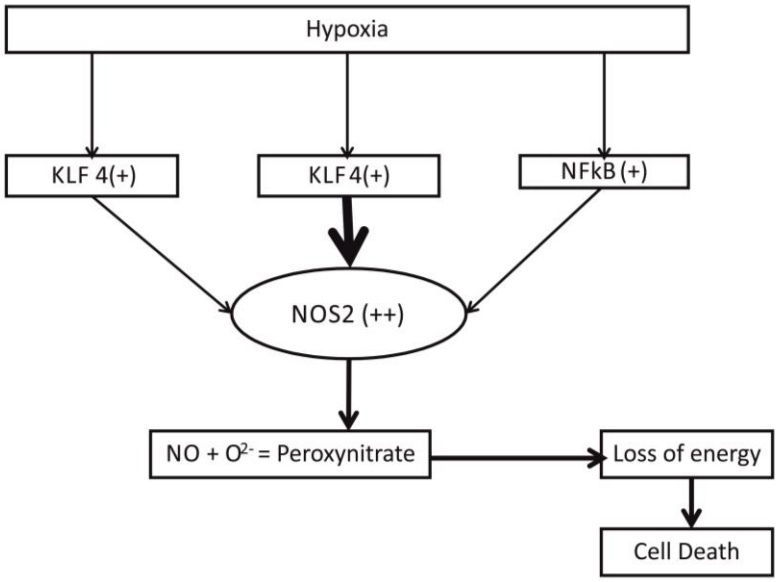


Figure 1. Hypoxia-induced cellular malfunctions. KLF4: Kruppel-like factor 4

**HIF-1 α:**Hypoxia-inducible factor-1 (HIF-1) is a heterodimer consisting of regulatory subunit HIF-1α and constitutive subunit HIF-1β (Wang et al., 1995). In oxygen deficiency, HIF-1 α expression is regulated by a post-translational protein stabilitymechanism mediated by a family of prolyl hydroxylases (PHDs). HIF-1 is also activated in normoxic condition by several physiological stimuli like growth factors, hormones, cytokines, transition metals and infectious agents (Richard and Berra, 2000; Salnikow et al., 2004). An interesting link between insulin regulatory several genes which are important for energy and iron homeostasis mediated by HIF-1 in hepatic and skeletal muscle cells was found (Treins et al., 2002). It was also noticed that HIF-1 α target genes are involved in the adaptive response facilitating oxygen delivery to oxygen-deprived tissues. This includes genes coding for erythropoietin, VEGF-A and inducible NOS (NOS2). The *erythropoietin* (EPO)gene, encoding a kidney hormone, was discovered as the first true hypoxia-inducible gene in 1992. EPO stimulates red blood cell production (erythropoiesis), thereby increasing oxygen delivery (Das and Saha, 2014).

**HIF-1 α and ROS:**ROS mediated NF-kB activation has so far been reported to regulate HIF-1a transcription (Bonello et al., 2007). ROS is either added as $H_2O_2$, generated by NOX activator thrombin or by over-expression of subunit NOX4 which in turn induces NF-kB to promote HIF-1a transcription (Gorlach et al., 2001). Interestingly, basal expression of HIF-1a is controlled by NF-kB and MAPK in vivo. The mechanisms of ROS generation in hypoxia through HIF-1α by different agonists play the role of critical determinants of adopting specific cellular signaling pathway that lead to specific set of gene regulations (Singh et al., 2005; Tapryal et al., 2010). Whether a similar pathway is involved in different mechanisms of HIF-1 activation by different cellular sources of ROS need to be investigated further.

Although ROS found to have some regulatory functional control on HIF-1, but it is still debatable whether hypoxia elevated or reduced ROS levels. Contradictory results may occur due to differences in cell type, mode of generating hypoxia, oxygen levels and assays used to

measure ROS (Das and Saha, 2014). Recent findings suggest that the primary sensor of hypoxia for the development of pulmonary vasoconstriction is the PASMC (pulmonary artery smooth muscle cell) mitochondria, which increases the production of ROS at low pressure of O2, probably in the complex III of the electron transport chain. It is possible that there are secondary sensing mechanisms that contribute to this effect, which increase the production of ROS during hypoxia such as sarcolemmal NADPH oxidase from pulmonary vasculature. Researchers have demonstrated an increase in the mitochondrial ROS generation in various tissues in response to hypoxia, including PASMC (Das and Saha, 2014; Wang et al., 2007). Besides ROS, excessive nitrite as nitric oxide, is also produced due to hyper activities of NOS2 gene through HIF-1α. It causes cytotoxicity due to pathological manifestation of altered normal cell metabolism.

Usually high concentration of intracellular NO also induces a negative feedback mechanism to control HIF-1α transcriptional factor gene (Das, 2009).


Figure 2. Hypoxia signaling through ROS.

## HYPOXIA AND OXIDATIVE STRESS – MOLECULAR INTERACTIONS

Oxygen free radicals produced during stress are unstable and potentially interact with other cellular components or molecules. ROS signals generated during hypoxia; activate protective responses, including HIF activation (Mungai, 2011). It is well established that hypoxia, mainly mediated through the hypoxia-inducible factors (HIFs), enhances the "Warburg effect" by up-regulating glycolytic genes such as hexokinases, LDH-A, and GLUT (Dang, 2007). These findings reveal close and complex interaction between cell metabolism and its microenvironments. ROS is also believed to play a role in the HIF-1 signaling pathway during hypoxia. Cells with non-functional mitochondria, therefore, reduced ROS levels, were unable to stabilize HIF-1α in response to hypoxia (Chandel et al., 2000; Mansfield, 2005). Oxidative stress which increased HIF-1α levels, enhanced HIF-1 DNA binding and increased activation of HIF-1 regulated gene promoters. This results in increased levels of hypoxia regulated proteins such as VEGF and cyclooxygenase-2 (COX-2) (Jones et al., Csiki et al., 2006).

**Hypoxia and cell signaling mechanisms:** Yuan and co-workers found that increase in HIF-1 through ROS may induce a $Ca^{++}$ dependent pathways. They demonstrated the involvement of calcium-calmodium dependent kinase II (CaMK II) under chronic hypoxia. CaMK II phosphorylates p300, a co-activator required for the transcriptional activity of HIF-1, thereby increasing the HIF-1 transactivation (Yuan G et al., 2005). In contrast, under acute hypoxia, HIF-1 transcriptional activity is increased as a result of a decrease in the $O_2$ dependent asparaginyl hydroxylation in the CAD region of HIF-1α, assisting in the recruitment of co-activators (Lando D et al., 2002). Another very interesting contrast observation on HIF-1 is to notice a positively targets BCL2/adenovirus E1B 19 kd-interacting

protein 3 (BNIP3) expression under hypoxic stress (Zhang, 2008). This leads to a reduction in mitochondrial activity and prevents reactive oxygen species (ROS) generation that is produced from oxidative phosphorylation. ROS production from complex III of the electron transport chain in the mitochondria has been shown to stabilize and thereby promote HIF-1 activity. This is most likely by the oxidation of $Fe^{2+}$ to $Fe^{3+}$ and inactivating PHD activity for the hydroxylation of the ODDD on HIF-1 (Guzy et al., 2005). The hypoxic cell usually takes anaerobic glycolytic pathways to generate ATP, whereas its residual low oxygen supply supports some level of oxidative production of ATP through the tricarboxylic acid cycle and electron transport chain (ETC). Electrons leaking from the mitochondrial ETC actually generate an excess of reactive oxygen species (ROS) in normal hypoxic cell. Reoxygenation or high oxygen levels following severe hypoxia further exaggerate ROS generation (Kulkarni et al., 2007). Hypoxia is found to be closely related to oxidative stress. Activation of HIF-1α reduces, whereas its inhibition increases ROS generation (Kim et al., 2006). Concurrently, oxidative stress exacerbates the status of hypoxia. In vitro studies in rat proximal tubular cells or in vivo studies in streptozotocin-induced diabetic rats show that high glucose depresses the activation of HIF, an effect fully reversed by treatment with antioxidants, such as α-tocopherol or tempol (Rosenberger et al., 2008). Beside these, NADPH oxidase activation also stimulates hypoxia (Yang et al., 2003). Hence, it may be clearly stated that hypoxia and oxidative stress are closely linked. It has been estimated that, under normoxic physiological conditions, 1–2% of electron flow through the mitochondrial respiratory chain gives rise to ROS(Turrens, 1997). It might be expected that hypoxia would decrease ROS production, due to the low level of $O_2$ and might diminish mitochondrial respiration, but ROS level is actually increased (Papandreou et al., 2006). Chandel et al., (1998) provided good evidence that mitochondrial reactive oxygen species triggers hypoxia-induced transcription and also showed that ROS generated at Complex III of the mitochondrial respiratory chain stabilize HIF-1α during hypoxia. Although others have proposed mechanisms indicating a key role of mitochondria in HIF-1α regulation during hypoxia (Rosca et al., 2008) but many scientists contradicted the role of mitochondria on HIF-1 regulation (Bell et al., 2008). During hypoxia mitochondrial electron transport system slows down and that causes augmentation of reducing equivalents resulting in production of superoxide at low oxygen concentration. It is also to be noted that hypoxia induces epigenetic repression of the PKC gene through a NADPH oxidase-independent ROS-mediated pathways (Patterson et al., 2010). Studies also demonstrated that prolonged hypoxia, in the presence of low or high glucose, significantly decreased PKC protein abundance in cultured H9c2 cells (Kim et al., 2004).

**Hypoxia and glucose homeostasis:** The relationship between hypoxia and glucose homeostasis always generates interest. Recent observation reveals that hypoxia activates NADPH oxidase which is very important for HIF-1 expression and ROS production. NADPH oxidase hyperactivity also changes intracellular calcium homeostasis and further stimulates HIF 1 production subsequently generatemore ROS. High concentration of ROS excites carotid bodies and adrenal medulla that influences adrenergic activities via chemoreflex and alters catecholamine status (Nanduri et al., 2015). Relation between increased sympathetic activation and hypoxia with consequent impaired glucose homeostasis provides a possible protective counteraction with N-type calcium channel blockers which are normally used to control hypertension or heart diseases (Peltonen, 2012). Study revealed that fasting glycemia gets corrected after withdrawal of two weeks hypoxia exposure but insulin resistance and beta cell abnormalities remain unchanged (Polak et al., 2013). The observation on high altitude

hypoxia revealed that increase in concentrations of glucose and insulin occur due to consequence of a transient peripheral insulin resistance (Larsen et al., 1997). It is also noticed that circulatory glucagon level is also modulated by hypoxia. Some studies show that acute hypoxia or intermittent hypoxia increases plasma glucose and insulin levels in the early phase, indicating that hypoxia induces glucose intolerance in humans (Oltmanns et al., 2004) and increases insulin resistance in genetically obese mice (Polotsky et al., 2003). Based on several studies it may be postulated that exposure to hypoxia would increase whole-body insulin resistance, induce beta cell dysfunction and augment hepatic glucose output, which collectively would lead to fasting and postprandial hyperglycemia (Polak et al., 2013). The signal transduction for hypoxia and glucose homeostasis also highlight insulin sensitivity through HIF-2α, which then increases Irs2 transcription and insulin-stimulated Akt activation. HIF-2αand Irs2 are, both necessary for the improved insulin sensitivity, as knocked down of either molecule disturb the glucose tolerance and insulin-stimulated Akt phosphorylation (Cullen, 2013).

## HYPOXIA, OXIDATIVE STRESS, ANTIOXIDANTS

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 (Davies et al., 1985).The decrease in tissue oxygenation induced by hypoxia alters many physiological and psychological processes in an elevation and duration-dependent fashion. The exposure of an organism to transient hypoxic stress activates respiratory and circulatory systems and adrenal glands and affects neurotransmitter release and action in the central nervous system. Kumar et al., (1989) have found the short exposure (5 days) to an altitude of 7576 m caused increased plasma lipid peroxidation level in rats. This result was confirmed by the same experimental protocol adding vitamin E supplemented groups (Ilavazhagan et al., 2001). Elucidating the mechanisms by which mammalian cells and organisms adapt to acute and chronic perturbations in ambient oxygen tension is critical for the understanding homeostasis maintenance and consequently the development of therapeutic strategies to counteract hypoxia-induced cell damage. HIF1$\alpha$ which plays a major role in mediating hypoxia -induced toxicity in mouse embryonic fibroblasts, is constitutively expressed in all cells but is almost immediately dissociates in the presence of oxygen. However, under conditions of hypoxia, it accumulates within cells and induces transcription of its target genes. Recently, vitamin C has been found to be an essential cofactor in the HIF-1α degradation pathway. HIF-1α is a transcriptional activator that regulates the expression of a number of hypoxia-responsive genes such as erythropoietin, heme oxygenase, and vascular endothelial growth factor (Botusan et al., 2008).Under normoxic conditions, reactive oxygen species (constantly generated in erythrocytes) are mostly neutralized by their intrinsic antioxidant enzymatic and non-enzymatic defense mechanism such as superoxide dismutase, glutathione peroxidase, catalase or reduced glutathione (Kurata et al., 1993). However, under the conditions of hypoxia, autooxidation of hemoglobin is facilitated and an increased flux of superoxide radicals occurs (Rifkind et al.1991; Das, 2010).

Hypoxia inducible factor-1α (HIF-1α) transcription factor expressed in chronic sustained hypoxia provides capabilities against hypoxia injuries by increase in expression of VEGF gene or $NOS_2$ gene in all the metabolically active tissues. It was found that VEGF over expression due to chronic hypoxia exposure in rats actually causes a protective measure against hypoxia induced cellular hypoxia. Role of antioxidant vitamins supplementation found to be interesting. It counteracts excessive HIF-1α transcription factor expression, followed by VEGF gene expression. The role of antioxidant vitamins is mainly to decrease ROS production due to hypoxia exposure and enhancing intracellular heme biosynthesis and reduction of nitrite levels (Das et al., 2015).

**Hypoxia and Vitamin C**: Antioxidants are intimately involved in the prevention of cellular damage by interacting with free radicals and by terminating the chain reaction, thereby curtailing free radical activity. L-Ascorbic acid (Vitamin C) is a dietary antioxidant that inactivates oxygen free radicals. Some studies have shown that vitamin C works in concert with vitamin E to prevent the free radical chain oxidation of lipids. Numerous reports have shown the positive effect of vitamin C as an antioxidant and scavenger of free radicals (Bulger et al., 1998). Ascorbic acid not only scavenges ROS but also reactive nitrogen species and prevent oxidative damages to macromulecules like lipids, proteins and DNA and protects individual from cardiovascular disease, stroke, cancer, neurodegenerative diseases and cataractogenesis (Halliwell and Gutteridge, 1986). Reports suggest that L-ascorbic acid can enter in to cell mitochondria in its oxidized form via GLUT-1 and protects mitochondria from oxidative injury. Mitochondria are found to generate intracellular ROS significantly. Intracellular protection on mitochondrial genome from vitamin C and surface membrane may be beneficial through vitamin C supplementation (Sagun et al., 2005). It has been noted that cellular respiratory tract lining fluid (RTLF) contains a variety of antioxidant enzymes like superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase. Vitamin C constitutes the first line defense system in RTLF against oxidants (Kasprzak et al., 2011). Various studies reveal that ascorbic acid can influence gene expression, apoptosis and protects cell death due to oxidative stress assaults (Wu et al., 2002).Rats exposed to chronic sustained hypoxia showed significant increase in serum i-NOS, serum nitrite, serum HIF-1α and serum VEGF concentration but significant beneficial changes are also noticed in serum i-NOS, serum nitrite, serum HIF-1α and serum VEGF concentration when hypoxic rats were supplemented with L-ascorbic acid (Das et al., 2016).

A possible counteracting mechanism by vitamin C through HIF-1α transcription factor expression either directly or through regulating/inhibiting ROS formation and indirectly controlling over production of reactive nitrogen species may be postulated (Das et al., 2015). Influence of vitamin C supplementation was found to be effective to counteract excessive HIF-1α transcription factor expression and subsequently VEGF gene expression. Vitamin C also improves intracellular hypoxic status by loading cells with extra vitamin C and resulted in resistance to hypoxia- and hypoxia / reoxygenation-induced cell death associated with the quenching of reactive oxygen species. It is also observed that vitamin C can down-regulate VEGF production via the modulation of COX-2 expression and that p42/44 MAPK acts as an important signaling mediator in this process (Guaiquil et al., 2004; Kim et al., 2011).

**Hypoxia, glucose homeostasis and calcium channel blocker:** Studies revealed that hypoxia causes hyperglycemia, glucose intolerance and insulin resistance in rats. These changes are related to elevated levels of HIF-1α concentration. Simultaneous treatment with antioxidant (vitamin C) and N-type calcium channel blocker (cilnidipine) were found to

ameliorate overall insulin sensitivities. The report from various studies stated that chronic and acute hypoxia can increase gluconeogenesis, increase hepatic glucose output probably through increased sympathetic drives, increased circulating steroids and enhanced HIF-1α concentration. HIF-1αconcentration in turn influence transcription of multiple enzymes required for gluconeogenesis (Polak et al., 2013). Although the link between hypoxia and altered glucose metabolism is well defined but exact mechanism of impaired glucose tolerance during hypoxia exposure is yet to be established. Studies based on calcium channel blockers, especially L- and N-type calcium channel blocker like cilnidipine, further explained the role of sympathetic nervous system during hypoxia and its regulatory actions to inhibit sympathetic overdrive and reduction of norepinephrine release from adrenergic nerve endings (Das et al.,2016). It may be postulated that increase in sympathetic activation due to hypoxia leads to increase insulin resistance by altering insulin signaling pathways or ROS generation (Peltonen et al., 2012). Treatment with L-N Type calcium channel blocker is able to control glucose homeostasis, perhaps, through either suppressing ROS productions via adrenergic system or lowering GLUT 4 expression (Tan et al., 2014).

## CONCLUSION

Hypoxia generates ROS which influences sympathetic adrenergic activities via chemoreflex, HIF-1 α and glucose homeostasis. Antioxidants like vitamin C or L- N type calcium channel blocker can reduce hypoxia or low oxygen sensing mediated cell signaling pathways especially glucose regulatory pathways.

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