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Hypoxia, Lead Toxicities and Oxidative Stress: Cell Signaling, Molecular Interactions and Antioxidant (Vitamin C) Defense

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Abstract: Hypoxia inducible factor-1α is an important transcription factor which is necessary for hypoxic gene expression that responds to changes in oxygen level in cell. HIF-1α remained stable and active in the occurance of Fe^{2+} and oxygen but it is depleted through the Von Hippel Lindau protein (pVHL) or Ubiquitin or Proteosome pathway. Restriction of oxygen induces inhibition of prolyl hydroxylase and accumulation of HIF-1α which in turn translocates to the nucleus to form a heterodimer with HIF-1β. Chronic hypoxia stimulates both KLF6 and NF-κB gene expressions and it reduces KLF4 which further enhances8 iNOS expression. Over expression of iNOS leads to rise in NO production and increase formation of peroxynitrite ($ONOO^-$). This event will lead to swelling of mitochondria and release cytochrome. Among the various heavy metals lead (Pb) is found to be a potent inducer of oxidative free radicals along with suppressor of cellular antioxidant defense system. The mechanism involves destruction of glutathione or inhibition of sulfhydryl dependent enzymes or altering intracellular oxidant and antioxidant balance which greatly affect cellular integrities. Heavy metals like lead (Pb), nickel (Ni) and cobalt (Co) can activate the hypoxia signaling pathways through Akt/ERK1/2 and induce HIF-1α accumulation.

This review analyzed the significant impact of sustained hypoxia in physiological system alone or along with simultaneous exposure of lead and possible protective role of vitamin C as antioxidant in rats.

Keywords: HIF-1α, hypoxia, NOS, oxidative stress, Pb, VEGF, vitamin C.

1. INTRODUCTION

Hypoxia is one of 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. Physiological hypoxia induces cell signaling process for the formation of new blood vessels (angiogenesis) to regulate vascular tone during developmental stage [1].

Physiological oxygen levels (PO_2) in healthy body varies from -100 Torr in the alveoli to < 10 Torr in medulla of kidney and retina [2]. Tissue exposure to low oxygen tension is observed in several physiological and pathological conditions like ischemia for shorter duration or in case of the high altitude inhabitants or any other chronic diseases for longer duration of hypoxic exposure. In both the case, hypoxic cells are programmed to rapid adjustment to maintain O_2 supply to most vital organs like heart and brain. It is understood that atherosclerosis, stroke or vascular occlusion leads to tissue ischemia followed by hypoxia. Tissue hypoxia also develops through immune cell infiltration in vascular dysfunction during chronic inflammation process [2, 3]. It has been observed that hypoxia absurdly stimulates free radicals release from the mitochondria that control the transcriptional and posttranslational response to low-oxygen conditions [4]. Hypoxia induced generation of reactive oxygen species [ROS] has been a subject of theoretical and practical dispute as experimental designs able to quantitatively evaluate ROS formation. Under normoxic conditions, ROS (constantly generated in erythrocytes) are mostly counteracted by their endogenous (superoxide dismutase, glutathione peroxidase, catalase or reduced glutathione) or exogenous (vitamin C, vitamin E etc.) antioxidant defense systems. However, under the conditions of hypoxia, hemoglobin is auto oxidized and facilitated an increased release of superoxide radicals [5, 6].

Lead is reported to be one of the oldest occupational toxic heavy metals, and lead poisoning has been evidenced since Roman era. Since 5000 years, when industrial lead manufacturing began lead poisoning also started from that period. These incidences of poisoning were not only restricted to lead exposed workers but also in the general inhabitants who could also be significantly rendered to lead due to poorly polished ceramic ware, the use of lead solder in the food canning industry, lead tainted drinking water, lead resources in spray paint and cosmetics, deposition on crops and motor vehicle sources. During mining, smelting, processing, recycling or disposal, lead and its various compounds directly enter in the occupational environment. Air borne lead can be deposited on soil and water and may enter human body *via* food and beverages. Because of lead in air, food, water, soil and dust greatly varies throughout the world. The extent of lead contamination depends upon the extent of industrialization, urbanization and lifestyle features [7, 8]. A large amount of lead used by industry 'primarily' comes from excavated and 'secondarily' from scrap metal or

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batteries recycling. However, most lead today is "secondary" lead, acquired from lead-acid batteries and 97% of these batteries are reported to be recycled [8]. In India 10% of total lead metal utilized is employed in the paints manufacturing which will definitely causes the increase possible risk for human lead exposure. Spray painters and small children are at more risk of lead exposure because there is likely to be a reservoir of painted articles in residential complex in India [9]. Gurer H, Ercal N (2000) reported that unlike hypoxia exposure, lead (Pb) intoxication also causes oxidative stress by inducing the generation of ROS and changing intracellular oxidant/antioxidant balance which leads to alteration of cell membrane integrity and fatty acid composition [10]. 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 through oxidative stress. Studies also show that wild-type Human hepatoma cells (Hep3B) increase ROS generation during metal activated some cell signaling pathways during hypoxia. Valko M *et al.* (2005) stated that hypoxia activates gene transcription *via* a mitochondria dependent signaling process induces increased ROS [11]. 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. 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 [12]. Heavy metals induced alteration of the hypoxia signaling system influenced by metal-induced oxidative stresses are responsible for the progression of metastasis [13].

This review elaborates a brief understanding of current state of knowledge of chronic hypoxia and its influence on the generation of ROS by inducing oxidative stress in the physiological system. The review will also provide recent update of heavy metal lead toxicities on oxidant and antioxidant balance and molecular interaction of chronic hypoxia and heavy metal lead (Pb) in the physiological system *in vivo*.

2. HYPOXIA

Cellular hypoxia causes an initiation of hypoxia-response genes responsible for angiogenesis, oxygen transport, and metabolism [14]. Hypoxia leads to alter intracellular chemical microenvironment by increasing calcium concentration ([Ca2+]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 heat shock protein 70 kDa (HSP-70), and hypoxia-inducible factor-1α (HIF-1α) [15-16]. Chronic hypoxia stimulates both KLF6 and NF-κB gene expressions and it reduces KLF4 which further leads to an enhanced iNOS expression (Fig. **1**). Moderate hypoxia is an important regulator to maintain pulmonary vascular tone [17].

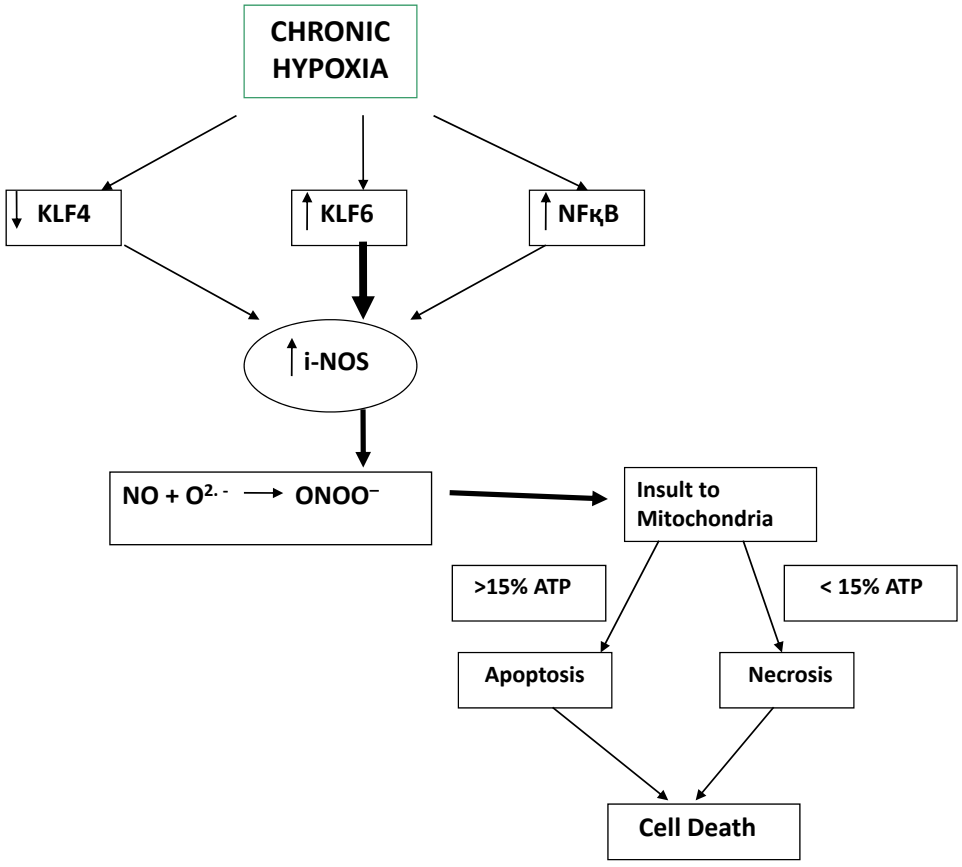

Fig. (1). Graphic representation showing hypoxia-induced cellular abnormalities. NF- kB: nuclear factor-kappa B; NO: nitric oxide; $ONOO^-$: peroxynitrate KLF4: Kruppel-like factor 4; KLF6: Kruppel-like factor 6.

Nevertheless, tissue hypoxia is also associated with a varied and extensive range of pathophysiological processes including vascular and degenerative diseases, chronic inflammation, and cancer [1].

2.1. History of HIF-1

Previous to HIF-1, HRE (hypoxia regulatory element) was found in the 3'-augmented region of the erythropoietin gene, which is found to be responsible to up-regulate more than hundred folds during extreme hypoxia. Semenza *et al.* defined a binding site that is critical for the hypoxia-inducible function involves expressing a transcription factor induced by hypoxia. Consequently, 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 [18]. HIF-1, the transcription factor is having two heterodimer subunits i.e. HIF-1α and HIF-1β. Because of hydroxylation, HIF-1α is unsteady at high oxygen concentrations. Hydroxylated HIF-1α is rapidly ubiquitinated and degraded by the proteasome. Hence oxygen inactivates HIF-1 and prevents hypoxia-inducible gene transcription [19]. At low oxygen tension, HIF-1α fails to be hydroxylated and degraded, leading to over expression of hypoxia-induced genes.

HIF-1 was previously known as the aryl hydrocarbon nuclear receptor translocator (ARNT), which is dimerized with the aryl hydrocarbon receptor. However, HIF-1 is a newly defined protein which is uniquely linked with the transcription of the hypoxia-inducible genes [18]. Further studies by molecular genetics and cloning reveals other two family members of HIF. These are HIF-2 (also known as endothelial PAS protein-1) and HIF- 3.

HIF-2 is also firmly regulated by oxygen saturation and its structural binding with HIF-1 emerges to be the factor which is directly responsible in hypoxic gene regulation, like HIF-1. Although HIF-3 is homologous to HIF-1 but possibly it might play as an inhibitory regulator of hypoxia-inducible gene expression [19-20]. All the HIF families (HIF-1,2 and 3) are having alpha- and beta-subunits belonging to the basic helix-loop-helix-PAS (bHLH-PAS) superfamily [21].

Effects of hypoxia are usually mediated *via* the activation of HIF-1, activation of which can lead to up-regulation of various genes like erythropoietin (EPO) and vascular endothelial growth factors (VEGF) that help tissues to adapt with 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 [22]. Reports on NO to cause either stabilizing or destabilizing HIF-1α at low oxygen concentrations are very much contradictory. The reasons for this variability may be due to the complicated chemistry and/or concentration of NO. It has been reported that the result of NO concentration on HIF-1α is biphasic in nature. NO concentrations up to 400 nM decrease HIF-1α stability, but concentrations above 1 μM increase HIF-1α stability [23].

2.2. HIF -1α as Transcriptional Regulator

When the protein concentration of HIF-1 α increases in reaction to hypoxia, it translocates to the nucleus, dimerizes with the beta subunit and activates the transcription of a numerous target genes displaying an HRE motif. Sets of HIF-1 target genes are involved during adaptive response that favors oxygen delivery to hypoxic tissues (Fig. **2**). This includes genetic coding for erythropoietin, VEGF-A and inducible NOS (iNOS) [24, 25]. The *erythropoietin* (EPO) gene, encoding a kidney hormone, was discovered in 1992 and may be considered as the first true hypoxia-inducible gene. EPO stimulates red blood cell production (erythropoiesis), thereby increasing oxygen delivery.

At the time of growth and metastatic development, tumor cells meet several kinds of microenvironmental stresses among them hypoxia is the most important. Deprivation of oxygen regulates neovascularization, glucose metabolism

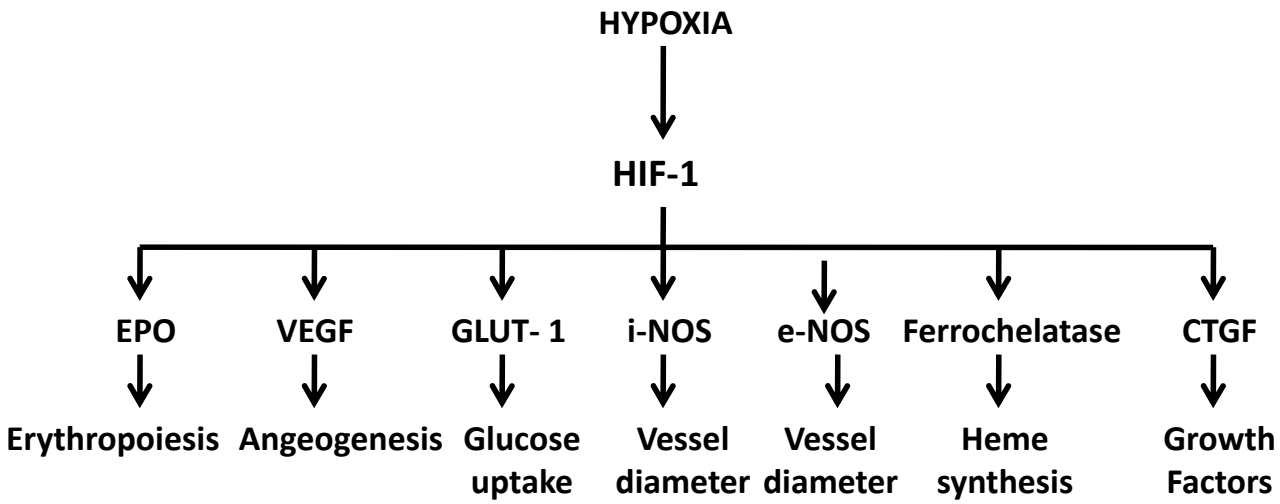

Fig. (2). Hypoxia induced HIF-1 activation pathways and transcription of target Genes. EPO, erythropoietin; VEGF, vascular endothelial growth factor; GLUT-1, glucose transporter 1; i-NOS, inducible nitric oxide synthase; e-NOS, endothelial nitric oxide synthase; CTGF, connective tissue growth factor.

and tumorogenecities. HIF-1 is responsible for this pleiotropic action and this action can be termed as angiogenic switch to overcome the limited supply of oxygen and nutrients in expanding neoplasia [26-28]. The hypoxic microenvironments can also induce alteration of cell signaling proteins including Src, STAT3, phosphoinositide 3-kinase (PI3K)/Akt, extracellular signal-regulated kinase (Erk) and glycogen synthase kinase 3β (GSK3β) [29].

3. LEAD TOXICITY

Lead is found to be absorbed by the gut *via* food, beverages and soil or dust. It has been observed that dietary factor, nutrition, chemical configuration of the metal and types of diet affect its absorption. Lead is not distributed unvaryingly throughout the body. It is rapidly up taken in blood and soft tissues (half life 28-30 days) subsequently a slower redistribution to bone (half life 27 years) [8]. Unabsorbed dietary and atmospheric lead is excreted in the feces. In human, lead can cause a wide range of biological impacts based up on the degree and duration of its exposure.

Although lead poisoning can influence most of the parts of the body, but its effects are predominant on the nervous and renal systems. Lead can impair cognitive function during developmental stage of life and subsequently leads to learning disabilities and behavioral crisis [8]. Acute lead exposure results in encephalopathy, severe abdominal pain, vomiting, diarrhea, seizures, coma, and in some cases even death. Chronic lead exposure can also cause weakness, prolonged abdominal pain, anemia, nausea, weight loss, fatigue, headache, and loss of cognition. Chronic, low-level lead exposure remains as non symptomatic until renal functions deteriorates [8, 30]. Lead alters the hematological functions. It is capable to induce microcytic and hypochromic anemia primarily due to reduction of hemoglobin synthesis curbing erythrocyte lifespan. Lead suppresses the erythropoietin level that regulates erythrocyte formation by making a negative impact on the reserve capacity for erythrpoiesis [7, 31]. In many countries occupational lead poisoning and its clinical symptomatic outcomes are common in nature. Lead also interferes with DNA during transcription in cell nucleus, Vitamin D synthesizing enzymes, and enzymes that regulate the cell membrane integrity. Lead induces anemia which may occur due to greater fragility of red blood cell membrane during lead exposure [32]. Bone and cartilage metabolism are interfered by lead exposure that causes alteration of the vascular permeability and collagen formation. Lead may also be harmful against immune system, causing excessive production of inflammatory proteins which may trigger asthmatic attack in children. Lead exposed reduction of immune cell like polymorphonuclear leukocytes activity has already been reported [30]. Lead also interferes with in the cells. In rodents lead is found to be a potent nephro carcinogenic. Histopathology of kidney shows dense, homogeneous eosinophilic intranuclear inclusions considered as non specific biomarker for renal disorders due to lead exposure [33]. Lead is a immunosuppressive toxic metal [34]. Lead induces experimental gametotoxicities in both male and female albino rats. A relationship between occupational lead exposure and cancer of the lung and brain has already been established from various epidemiological studies. Organic lead compound, tetraethyl lead (TEL) was routinely used since long time in gasoline. Although the production of TEL has been completely stopped in most of the developed countries but many underdeveloped countries still it is produced. Zhu Z and Thiele DJ (1996) confirmed that Tetraethyl lead is metabolized by CYP450 to trimethyl lead (TML). Mechanisms of its toxicity include cell membrane damage, alteration in energy metabolism and direct interference with neurotransmitter synthesis. Symptoms of its toxicity include nausea, vomiting, diarrhea associated with irritability, headache and restlessness. Long term inhalation of leaded gasoline results in signs of dementia and encephalopathy, with clinical cerebellar and corticospinal symptoms [35]. Bouton CM and Pevsner J (2000) further elaborated that Lead primarily acts by competing with endogenous cations on protein binding sites and can substitute calcium and zinc both in numerous proteins as divalent cation. Lead substituted protein can alter the normal functioning of the protein and thus can alter the cellular pathways and induce abnormal gene transcription [36]. Lead exposure induces mRNA expression of N-methyl D-aspartate (NMDA) subunit genes and further disturbs phosphokinase C (PKC) activities that regulate altered transcription of numerous mRNA transcripts including fos and jun [37]. Bouton *et al*. (2000) also demonstrated that annexin A5, a calcium binding protein is capable to remain in binding and being activated by nanomolar concentration of lead [36]. A few stress response gene like GFAP, microsomal glutathione S-transferase, mitochondrial 10KDa heat shock protein, and HSP70 were found to be up regulated in the cell due to lead exposure. It has been observed that Daphnia hemoglobin gene expressed greatly following lead exposure. Bouton *et al*. (2000) further concluded that hemoglobin gene expression may be used as a biomarker of lead toxicity [36]. Zhu Z and Thiele DJ (1996) demonstrated that PC12 cells exposed to lead show significant increase in c-fos, c-jun and egr-1 but not NGF1B mRNA. As it appears that lead induces the expression of immediate early genes by a mechanism that requires protein kinase C hence further studies on lead induced gene expression will be important from public health point of view and will shade the light on molecular mechanism to understand lead toxicities [35]. Occupational exposure of lead is not at all monitored in many developing countries and very poorly monitored in developed countries [38]. The factors which influence the degree of lead poisoning are age, the duration and quantity of exposure, nutritional status and the general health. Iron deficient or malnourished individual, are more susceptible to increased lead absorption [9]. It is expected that houses constructed prior to 1978 is more likely to have lead –based paint or lead contaminated household dusts Even soil of the outside of these houses may also be polluted with lead and be a source of exposure. According to the USA Centre for Disease Control & Prevention (CDC) about 24 million housing units in the United States have lead containing paint and lead-contaminated house dust. Of these, about 4 million homes have one or more young children living in them. Adult lead exposure is usually related to occupational or recreational (hobby) exposure. Children and spouses may become exposed to lead contamination through the clothing of the employees who are exposed to occupational lead exposure [8].

4. HYPOXIA, LEAD (PB) TOXICITIES AND OXIDATIVE STRESS – MOLECULAR INTERACTIONS

Hypoxia-induced factor HIF-1 governs appropriate intracellular oxygen homeostasis by controlling expression of several genes, including heme oxygenase1 and vascular endothelial growth factor. There are various reports on some heavy metals like lead, nickel or chromium which are known to activate HIF-1 [12, 39]. Some heavy metals are found to cause severe organ damage due to inducing cellular hypoxia by inhibition of enzyme cytochrome C oxidase of mitochondria [40, 41]. It has been found that chronic intoxication of lead under hypoxic conditions induced growth retardation in growing rats and damages on femoral and mandibular bones that predispose to fractures [42]. Adonaylo VN., Oteiza, PI (1999) stated that Pb is capable to interact with negatively charged phospholipids in cell membranes and through the induction of changes in membrane's physical properties could facilitate the propagation of lipid oxidation [43]. Some biochemical mechanisms of lead toxicity hypothesized that lead's toxic effect on the component of antioxidant defense systems possibly affects oxidant and antioxidant delicate balance of cells, resulting in oxidative damage. Individual resistance against hypoxia provides an individual reaction of mitochondrial respiratory chain functioning, mitochondrial ion transport, properties of mitochondrial enzymes, and energy metabolism, monooxigenase system activity, biotransformation of genobiotics and drug metabolizing system [44].

Lead induced oxidative stress in humans and experimental animal models showed increase lipid oxidation and alterations in antioxidant defense mechanism [45]. Pb-induced oxidative damages occured by several mechanisms. Adonaylo VN., Oteiza, PI (199B) stated that Pb could also act as a pro-oxidant through the formation of highly polarized Pb2 þ-O2 with higher oxidizing capacity than O^{2-} *per se*. The well described interactions of Pb with glutamate could also potentiate oxidant-mediated damage [42-46].

It has been observed that free radicals are produced from cell during the exposure of heavy metals like lead which activate some of the signaling pathways during hypoxia [47].

Both hypoxia and lead exposure induce generation of ROS (reactive oxygen species) increase expression of p53, NF-kβ, AP-1, MAPK and HIF-1α. The increased expression of all these transcription factors leads to either cellular adaptation or cell death (Fig. **3**). 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 (Fig. **3**). 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.

As the process of ROS induced cell adaptation and cell death is under molecular control, there is potential for active intervention and its description is therefore useful. Heavy metals related to carcinogenesis are possibly due to the hypoxia signaling pathways involving generation of reactive oxygen species and oxidative stress [13]. Although ROS has been shown to have some critical functional role on expression of HIF-1 but it is still not clear whether ROS levels are elevated or reduced while in hypoxia. Contradictory results may appear due to cellular characteristics, mode of generating hypoxia, oxygen saturation and assay procedures to measure ROS [48]. Fig. **4** shows the mechanism by which

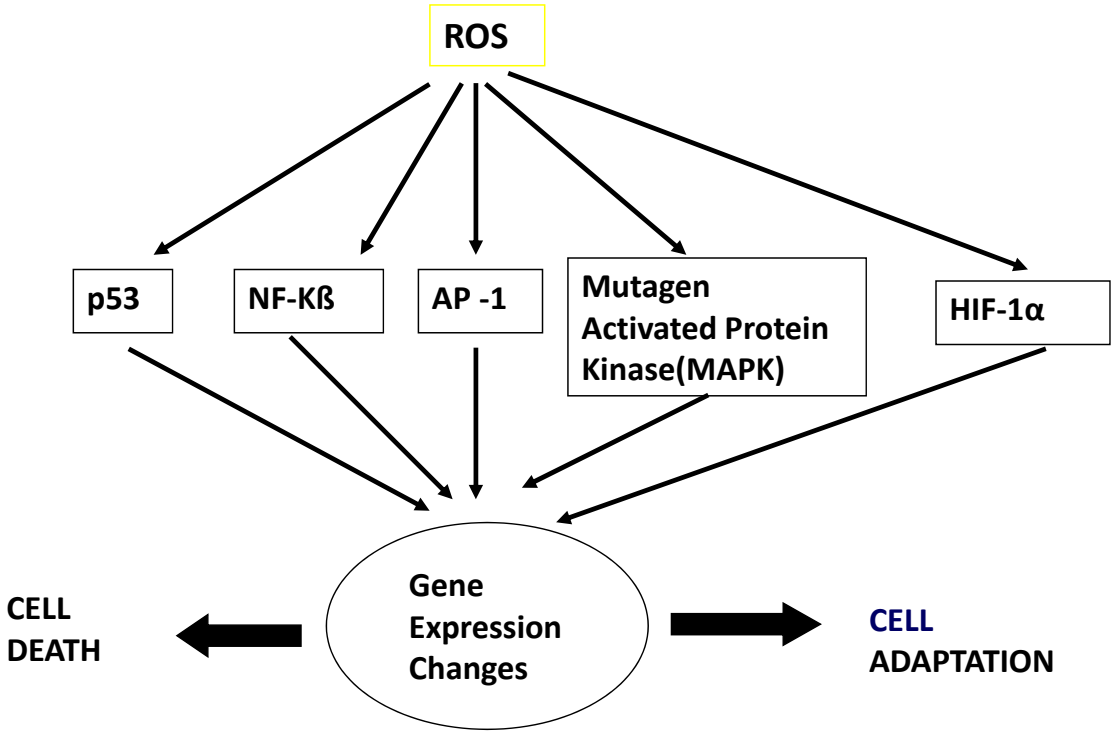

Fig. (3). Potential signaling pathways sensitive to ROS.

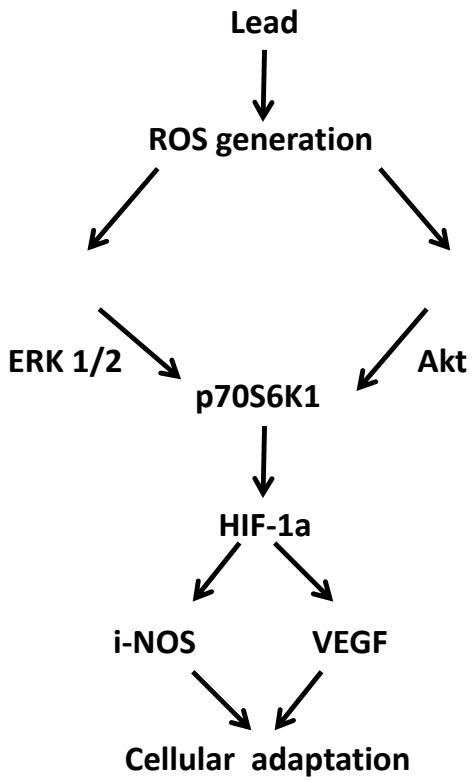

Fig. (4). Lead (Pb) induced generation of ROS and subsequently HIF-1α and VEGF. Gene expression to lead cellular adaptation. ERK 1/2, extracellular-signal-regulated kinases; Akt, the serine–threonine kinase; P70S6K1, P70S6 kinase.

lead induces hypoxia adaptability through ROS. This is a hypoxia independent mechanism to develop cellular adaptability through the expression of VEGF and i-NOS gene.

Although the increase of ROS was observed after exposure to both metals and hypoxia but it was not clear whether the hypoxic gene expression was due to ROS stimulates**.** Recently, a new human gene, *Cap43* which was found to be over expressed by hypoxia and some heavy metals through HIF-1-dependent pathways [49]. Because these transition metals generate ROS hence it may be interesting to see whether ROS played a role in the activation of hypoxic genes by metals. In the studies in author's laboratory it has been observed that treatment with lead acetate or chronic sustained hypoxia induced oxidative stress which leads to generation of ROS. ROS can stimulate i-NOS pathway *via* NF-Kb or stimulation of HIF-1α transcriptional factor.

Recent evidence suggests that to develop pulmonary vasoconstriction, the fundamental sensor of hypoxia is the PASMC (pulmonary artery smooth muscle cell) mitochondria, which increases the production of ROS at low O_2 tension, probably in the complex III of the electron transport chain system. Perhaps the existence of secondary sensing mechanisms may further contribute to this effect and lead to production of ROS during hypoxia like for an example sarcolemmal NADPH oxidase from pulmonary vasculature. Several reports indicate an elevation in the mitochondrial ROS generation in various tissues including PASMC in reaction to hypoxic condition [50].

Chronic sustained hypoxia also induced HSP90 (heat shock protein 90). Both lead and hypoxia also stimulate VEGF pathways for cellular adaptability. Excessive nitrite as NOx, is generated due to hyper activities of i.NOS or through HIF-1α either by lead acetate or hypoxia exposure or simultaneously exposure of both heavy metal (Pb) and chronic hypoxia. It will cause cytotoxicities by pathological manifestation by alteration of cell normal metabolism. Usually high concentration of intracellular NO also induces a negative feedback mechanism to control HIF-1α transcriptional factor gene [51].

5. ANTIOXIDANT (VITAMIN C) DEFENSE, HYPOXIA AND LEAD (PB)

Vitamin C is a water-soluble antioxidant and found to be the most effective circulatory antioxidant in human system [52]. Ascorbic acid or vitamin C prevents lipid peroxidation, oxidation of low-density lipoproteins and advance oxidation protein products [53]. The respiratory tract lining fluid (RTLF) contains a variety of endogenous antioxidant enzymes like superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase. It also contains vitamins C, E and A and other exogenous antioxidant compounds [54]. Vitamin C may comprise the first line of defense system in RTLF against external pro-oxidative assaults [55]. It has been reported that intracellular depletion of ascorbic acid aggravated some heavy metal (nickel, cobalt etc.) induced carcinogenicity and acute toxicity [55]. In our laboratory we have earlier shown the changes in chemical behavior of l-ascorbic acid alone or in combination of nickel sulfate at different pH solutions [56].

The effect of simultaneously supplemented vitamin C on experimental lead treatment show ascorbic acid is capable to reduce intestinal absorption of lead. The mechanism involves that vitamin C is capable to reduce ferric iron to ferrous iron in the duodenum thus availability of divalent ferrous ion increases which competes with lead (also a divalent cation) for intestinal absorption [41]. Recent reports indicate the capability of ascorbic acid as a regulatory factor may influence gene expression, apoptosis and other cellular functions of living system [57]. In many studies it has been revealed that vitamin C protection against cell death triggered by various stimuli and most significant of this protection has been associated with its antioxidant capability. Studies in our laboratory on experimental rats showed that serum i-NOS activities, serum nitrite, serum HIF-1α and serum VEGF concentration are increased significantly in lead acetate treatment as compared to their respective controls. Simultaneous treatment of l-ascorbic acid or vitamin C showed a significant decrease of serum i-NOS, serum nitrite, serum HIF-1α and serum VEGF concentration when it compared with Pb alone treated rats. Rats exposed to chronic sustained hypoxia showed significant increase of serum i-NOS, serum nitrite, serum HIF-1α and serum VEGF concentration as compared to both control and lead acetate exposed rats. Interestingly in case of rodents treated with lead acetate and exposed to hypoxia simultaneously did not show any significant difference in comparison to only hypoxic animals although their values are significantly

Table 1. Effect of l-ascorbic acid (50 mg / 100g. b.wt, orally) on lead acetate (25 mg/kg b. wt.,i.p.) and chronic normobaric hypoxia (10% oxygen) induced alteration of serum i-NOS, serum nitrite, serum HIF-1α and serum VEGF in rats.

Parameters	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII	Group VIII	't' value	df	'p' value
Serum i-NOS (nmol/mL)	6.73± 1.11[a]	59.18± 3.56[b]	6.44± 0.86[a]	38.23± 2.64[e]	61.75± 2.36[b]	81.75± 3.44[d]	38.42± 1.90[c]	62.00± 2.48[b]	8.55	7	0.0000
Serum Nitrite (μmol/L)	24.52± 4.04[a]	103.77± 7.60[b]	22.58± 6.14[a]	54.29± 15.67[c]	101.11± 5.75[b]	104.96± 5.22[d]	69.13± 4.13[e]	67.02± 4.33[e]	8.24	7	0.0000
Serum HIF-1α (pg/mL)	152.51± 8.38[a]	351.33± 21.36[b]	160.10± 5.55[a]	159.27± 14.41[a]	648.50± 25.71[e]	655.08± 8.15[c]	333.50± 22.02[b]	530.04± 21.95[d]	17.42	7	0.0000
Serum VEGF (pg/mL)	300.31± 5.65[a]	423± 25.20[b]	303.47± 6.52[a]	324.47± 17.66[c]	617± 35.50[d]	622.17± 15.53[d]	426.83± 12.60[b]	519.83± 18.72[e]	16.42	7	0.0000

Each value is mean ± SEM of six observations in each group. Groups: I- control; II- Pb; III- l-ascorbic acid; IV- Pb + l-ascorbic acid; V- Hypoxia; VI- Pb + Hypoxia; VII- l-ascorbic acid + Hypoxia; VIII- Pb + l-ascorbic acid + Hypoxia; Values with all the superscripts (a,b,c etc.) are statistically significant with each other ($P < 0.05$). i-NOS, inducible nitric oxide synthase; HIF-1α, hypoxia inducible factor 1α; VEGF, vascular endothelial growth factor.

higher than only lead acetate treated rats. A remarkable significant decrease of serum i-NOS, serum nitrite, serum HIF-1α and serum VEGF concentration was noticed in case of hypoxic rats supplemented with l-ascorbic acid when it compared with only hypoxia exposed rats. A significant reduction of serum i-NOS, serum nitrite, serum HIF-1α and serum VEGF concentration was also observed in case of rats exposed with Pb and hypoxia and also supplemented by vitamin C when it was compared with either Pb and Hypoxia combined rats or only hypoxia exposed rats (Table **1**).

The possible mechanism by which vitamin C counteract lead toxicities on HIF-1 α gene transcription is depicted in Fig. **5** which shows how lead (Pb) and hypoxia exposure generate ROS and regulate i-NOS pathway to produce nitric oxide (NO) and generate reactive nitrogen species like nitrite. Vitamin C counteracts HIF-1 α transcription factor expression may be through regulating/inhibiting ROS formation and indirectly controlling over production of reactive nitrogen species.

Studies of the anti-apoptotic activity of vitamin C have suggested a role of vitamin C in modulation of the immune system. Several studies reported the mechanisms by which vitamin C regulates the AP-1 complex that include the Fos and Jun super families and control cell proliferations. Ascorbate treated cells exposed to UV-B irradiation led to a 50% decrease in JNK phosphorylation (which activated AP-1), therefore inhibiting the JNK/AP-1 signaling pathways and regulates apoptosis [57].

Hypoxia and lead (Pb) treatment induced HIF-1 α gene transcription actually facilitate VEGF gene expression in endothelial cell to improve adaptability against hypoxia and lead induced cellular hypoxia in physiological system. Vascular endothelial growth factor (VEGF) which was originally known as vascular-permeability factor (VPF) was found to increase cellular permeability. VEGF has faster kinetics and 50,000 times more potency than histamine 110 [58]. Vascular permeability is a precondition for wound healing process although its role on pathophysiology of cancer by promoting the formation of oedema and ascites, and facilitating the distant spread of metastases can not be ignored.

VEGF-induced vessel fenestrations allow leakage of small solutes, but larger molecules are still retained in the system. Bates DO and Harper SJ (2005) describes that VEGF-induced permeability depends on nitric oxide (NO) production, which requires activation of NO synthase (NOS) pathways, either as a consequence of phospholipase C-γ activation and calcium influx, or through phosphorylation of NOS by AKT/protein kinase B (PKB) pathways [59]. Role of vitamin C supplementation is found to be interesting to counteract excessive HIF-1 α transcription factor expression followed by VEGF gene expression (Fig. **5**). Their roles are mainly to decrease ROS production and improve intracellular hypoxic status by stimulating heme biosynthesis and reduction of nitrite levels. Vitamin C also improves intracellular hypoxic status as it is reported that loading cells with vitamin C by dehydroascorbate (DHA) treatment caused a protective mechanism against hypoxia- and hypoxia/ reoxygenation-induced cell death by neutralizing the actions of reactive oxygen species and induce hypoxia adapting capabilities [60]. Kim HN *et al*. (2011) reported that vitamin C can down-regulate VEGF production *via* the modulation of COX-2 expression and p42/44 MAPK that acts as an important signaling mediator in the process of hypoxia gene expression [61]. Further Nespereira B *et al*. (2003) demonstrated that augmented VEGF and VEGFR-2 expression in apoE-/- vasculature can be down regulated by both vitamins C and E, at least partially through oxidative stress reduction. This unique mechanism could highlight the analysis of the beneficial effects of antioxidant vitamins in experimental atherosclerosis [62].

Hence, it can be concluded that exposure to heavy metal (lead, Pb) possibly activates the hypoxia-inducible pathway and facilitates increased transcriptional activity of hypoxia-inducible genes, which may be important in the Pb-induced alteration of cellular metabolic process. Pb might affect oxygen sensing mechanism by either suppressing the sensor by directly inhibiting prolyl hydroxylases (PHD 1-3) or forming ROS and increase adaptability of HIF-1α in the cellular metabolic process reflected. Simultaneous treatment with vitamin C may be beneficial to combat hypoxia or lead toxicities or in combination with both [55, 63].

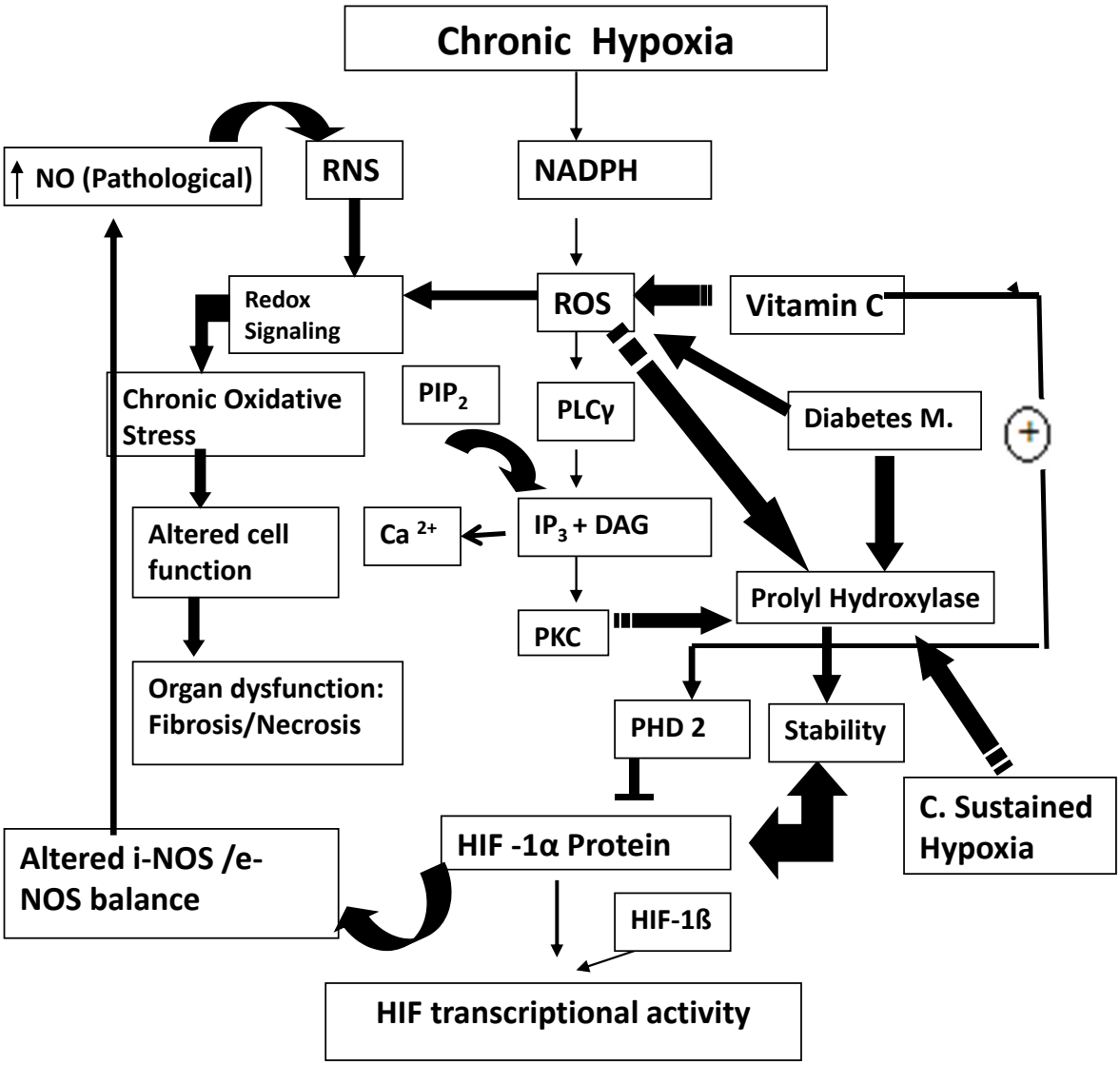

Fig. (5). Possible protective role of vitamin C on chronic sustained hypoxia and lead (Pb) toxicities. RNS, reactive nitrogen species; NO, nitric oxide; PLCɣ, phospholipase C gamma; PHD2, prolyl hydroxylase 2; PIP_2 , phosphatidylinositol bisphosphate; PKC, protein kinase c; i-NOS, inducible nitric oxide synthase; e-NOS, endothelial nitric oxide synthase; HIF, hypoxia inducible factors.

CONCLUSION

Both hypoxia and heavy metal (lead) stimulate HIF-1α pathways for cellular adaptability. Excessive nitrite generations due to over expression of i-NOS through HIF-1α either by lead acetate or hypoxia exposure or simultaneously exposure of both heavy metal (Pb) and chronic hypoxia causes cytotoxicities by alteration of cell metabolism. The supplementation of vitamin C was found beneficial to counteract both and hypoxia and metal (lead) induced cytotoxicities by generation of oxidative and nitrosative stress.

CONFLICT OF INTEREST

The authors stated that there are no conflicts of interest regarding the publication of this article. Research support played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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