Review Article

Lung Oxidative Damage by Hypoxia

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Received 14 May 2012; Accepted 11 July 2012

Academic Editor: Vincent Pialoux

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One of the most important functions of lungs is to maintain an adequate oxygenation in the organism. This organ can be affected by hypoxia facing both physiological and pathological situations. Exposure to this condition favors the increase of reactive oxygen species from mitochondria, as from NADPH oxidase, xanthine oxidase/reductase, and nitric oxide synthase enzymes, as well as establishing an inflammatory process. In lungs, hypoxia also modifies the levels of antioxidant substances causing pulmonary oxidative damage. Imbalance of redox state in lungs induced by hypoxia has been suggested as a participant in the changes observed in lung function in the hypoxic context, such as hypoxic vasoconstriction and pulmonary edema, in addition to vascular remodeling and chronic pulmonary hypertension. In this work, experimental evidence that shows the implied mechanisms in pulmonary redox state by hypoxia is reviewed. Herein, studies of cultures of different lung cells and complete isolated lung and tests conducted in vivo in the different forms of hypoxia, conducted in both animal models and humans, are described.

1. Introduction

Lung's main function is the exchange of gases, hence, it is the organ which makes contact with the higher pressure of oxygen in our body. This is especially significant in the light of the known toxic effect of this gas, although there are precedents of lung malfunction under low oxygen pressure conditions or hypoxia.

The generation of hypoxia occurs when staying at high-altitude environments or when receiving mixtures of contaminated gases. Thus, this condition is also a factor in various lung pathological processes like in obstructive sleep apnea (OSA), acute lung injury, asthma attacks, atelectasis, chronic obstructive pulmonary disease, and idiopathic pulmonary hypertension. Lung hypoxia is related, in acute form, to the increase of the pulmonary artery pressure [1], epithelial malfunction [2, 3], edema [4, 5], and lung inflammation [6, 7]. Chronic hypoxia is related to vascular proliferation [8], increase of vascular reactivity [9], chronic pulmonary hypertension, and right heart failure [10–12]. Different lung diseases have identified the participation of reactive oxygen species (ROS) in their pathogenesis [13–16].

Historically, the study of oxidative damage has been linked to the increase of O₂ content in both environment and organs; one of the first times where the oxidative damage of an organ was probably studied may have been when the effect of hyperoxia on the lung tissue was reported [17–19]. Logic tells us that there is a need of O₂ presence for the generation of ROS and oxidative damage; likewise, it would be unlikely that oxidative damage occurs when this element is less available [20]. Currently, this paradigm has been changed by identifying hypoxia as the generator of ROS and oxidative damage for systems as well as specific organs. Although oxidative damage associated with hypoxia on the organism has been the subject of several studies [21–24], the knowledge of the effects on lung tissue is relatively poor, particularly in humans. The latter is probably because of the difficulty in obtaining samples of this organ.

The decrease of pO₂ is monitored by the pulmonary arteries smooth muscle cells (PASMCs); these cells react
to hypoxia favoring bronchial vasoconstriction [25]. An opposite effect occurs in the systemic circulation [26], where hypoxia favors vasodilatation of both arteries and veins [27, 28]. Pulmonary arterial vasoconstriction caused by hypoxia appears to be more effective when hypoxia is present in alveolar lumen regarding this stimulus in the lumen of arterioles and venules. This response to the decrease of alveolar pO2 in ventilation (V) attempts to improve the diffusion of O2 into the blood through changes in perfusion (Q), a specific feature of the lungs [29]. Alterations in the V/Q relationship will trigger compensatory hypoxic vasoconstriction, which will attempt to normalize the V/Q relationship [29]. This effect will be accompanied by an increase in alveolar pCO2 and respiratory acidosis [29]. In this context, the maximum steady state of hypoxic pulmonary vasoconstriction occurs between 25 and 50 mmHg of alveolar pO2; therefore, the blood redistribution is maximum at these levels. However, the magnitude of hypoxic pulmonary vasoconstriction can decrease with levels <25 mmHg resulting in a reduced blood flow redistribution, deteriorating the V/Q, and worsening hypoxemia [26]. This phenomenon is favored with the existence of a lung disease or exposure to altitude. Now, the increasing of ROS in hypoxic lung has an important role in stimulating PASMC contraction in addition to enhancing inflammation and to establishing oxidative damage as the case may be. This will depend on the severity and duration that hypoxia has with a wide range of effects from the absence of tissue damage to the dysfunction on epithelial and muscle cells associated with vascular remodeling and proliferation (see Figure 1). Current evidence suggests that the primary sensor of hypoxia for the development of pulmonary vasoconstriction is the PASMC mitochondria, which increases the production of ROS at low pressures 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 will 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. The probability that lower concentrations of oxygen in lungs are sufficient to produce similar effects to those observed in other tissues at higher concentrations is true [26].

In this paper, the proposed mechanisms that explain the redox state changes induced by hypoxia are described (see Figure 1). Hypoxia is herein approached, in its different forms, as for exposure time and its varieties: normobaric, hypobaric, hypercapnic hypoxia or in the ischemia and reperfusion process. Furthermore, pharmacological substances [30–32] and measures (transgenic animals) that have been used to describe the participation of ROS in pulmonary function abnormalities for this condition are included, as well as in the search for strategies to mitigate the effects of the hypoxic pulmonary oxidative damage (see Figure 2). This paper also includes protocols of hypoxia performed in different types of lung cells, homogenized lung cells, pieces of tissue, vascular rings, experimental model of isolated-perfused lung, use of KO animals and noninvasive methods such as the analysis of directly exhaled air and exhaled breath condensate (EBC).

2. ROS

Living under aerobic conditions involves the formation of ROS. These substances have among their more commonly formed constituents superoxide, hydrogen peroxide, hydroxyl, and peroxy, as well as nitric oxide and peroxynitrite. Some of these ROS contain unpaired electrons (free radicals) and may damage the different types of biomolecules that constitute us: lipids, proteins, carbohydrates, and nucleic acids. The interaction of biomolecules with ROS may occur in the physiological context as well as promoting cellular malfunction, so they have been involved with different diseases [33, 34]. The formation of ROS in the hypoxic context has been a subject of wide-ranging debate in both theory and reported results; the latter is probably influenced by used experimental models and methods by which ROS
have been determined. Evidence of oxidative damage by hypoxia, in the lungs, is consistent; however, the mechanisms about how it is produced are still being discussed. In this regard, several sources for the generation of ROS in hypoxia have been proposed as detailed below.

3. Mitochondria

This organelle is one of the sites of constant ROS production, particularly superoxide anion. This process specifically occurs in the mitochondrial electron transport chain (ETC), in complexes I and III [35, 36]. Some early reports showed a decrease in intracellular ROS formation by hypoxia, hence, Paky et al. [37], found a decrease in ROS formation and those came from the intracellular environment when the lucigenin assay was applied to a preparation with isolated perfused lung in anoxia (95% N2 and 5% CO2). Archer et al. [38] found an ROS decrease in isolated and perfused lungs by using luminol, when 2.5% O2 during 6 min was applied. The same result was found when rotenone and antimycin A were applied, both ETC inhibitors in complexes I and III, respectively. A similar result was found in rat aortic rings at 36 mmHg PO2, by using lucigenin [39]. In pulmonary artery rings, endothelium-denuded preparations were carried out and a decrease in ROS formation regarding normoxia (analyzed by lucigenin, 2′,7′-dichlorodihydrofluorescein diacetate (DCF), and Amplex Red) in hypoxia (40 mmHg PO2) was found when rotenone and antimycin A were applied. This effect was not observed when cyanide was applied, an inhibitor of mitochondrial complex IV [40].

Another group of investigators have reported increases in ROS production in hypoxia. Grishko et al. [41], in cultures of pulmonary artery endothelial cells (PAECs), incubated at 25 mmHg O2 for 15 min and found ROS increases with DCF. Waypa et al. [42] found an ROS increase in pulmonary artery smooth muscle cells (PASMCs) exposed to 2% O2; this increase was inhibited by myxothiazol (blocker of mitochondrial complex III). Similar results were found by other authors; however, the use of lucigenin and DCF was questioned with regard to its properties to determine ROS properly [43]. Probably the use of more specific methodologies to measure ROS in the last few years, based on proteins that are sensitive to oxidation like HSP-FRET and roGFP, weighed the balance towards hypoxia, as a condition where ROS and oxidation increase [44–48]. Therefore, a decrease of the GSH/GSSG ratio was observed in cultures of PASMCs exposed to 1.5% O2, which was inhibited by myxothiazol. Moreover, increase of cytoplasmatic oxidation by hypoxia was found using HSP-FRET. This change of oxidation by hypoxia was inhibited by nonenzymatic antioxidants like pyrrolidine dithiocarbamate and N-acetylcysteine (NAC). The same effect was gained with the overexpression of mitochondrial catalase through a viral vector. Overexpression of SOD and the use of cyanide did not change the ROS increase by hypoxia [49]. By using the roGFP protein indicator in PASMCs, Waypa et al. [50] after administering 1.5% O2 for 30 min found ROS increases in the mitochondrial intermembrane space and at a cytoplasm level, and ROS decreases in the mitochondrial matrix. Overexpression in these cells to cytoplasmic catalase
inhibited the ROS increases in this compartment [50]. In a subsequent report using rat lungs slices, Desireddi et al. [51], found an increase in cytosolic ROS by hypoxia (1.5% O₂). This increase was attenuated by the catalase overexpression induced by viral vectors [51]. The complete description of the ROS generation at a mitochondrial level was recently revised by Schumacker [52].

4. Nitric Oxide Synthase (NOS)

This enzyme is capable of forming free radical nitric oxide from L-arginine. This molecule has been involved in multiple processes both physiological (vasodilation and bronchodilation) and pathological (inflammation and oxidative damage) in lungs [15, 53]. In addition, it has been used as a drug [54]. NOS is presented in three isoforms, endothelial (eNOS), neuronal (nNOS), and inducible or iNOS. The expression of the two first ones depends on the activity of the calcium/calmodulin system, whereas the expression of iNOS is mainly stimulated in the context of an inflammatory process. Another recognized stimulating factor to the expression/activity of this enzyme is hypoxia. Xue et al. [10] found the increase of the expression of NOS in endothelium of small pulmonary vessels and vascular smooth muscle after exposing rats during 2 to 4 weeks at a 10% O₂. Shaul et al. [55] found increases of the expression of the nNOS and eNOS after 7 and 21 d of hypoxia, while Fagan et al. [56] described increases of the expression of eNOS and iNOS in lung homogenates of rats exposed during 6 weeks at 5,200 m. Probably in hypoxia the more active isoform that is related to vascular remodeling, observed in pulmonary hypertension, corresponds to the iNOS, which in normoxia has a poor role in the production of NO; however, it is increased in hypoxia as previously suggested [10, 57].

Rus et al. [58] subjected rats to hypobaric hypoxia at a barometric pressure of 225 mmHg during 30 min and subsequently to reoxygenation times from 0 to 5 d, finding in lung homogenates higher oxidative damage to proteins, lipids, and apoptosis increase. The administration of a selective inhibitor of iNOS, the most abundant isoform in the airways, N-(3-(aiminomethyl) benzyl) acetamidine (1400 W), resulted in a decrease of nitrite and nitrate, besides decreasing the lipid peroxidation and oxidative damage to proteins. The inhibition of iNOS by 1400 W resulted in the production of NO; however, it is increased in hypoxia as previously suggested [50, 59].

In a model of rats subjected to hypoxia (10% O₂) and hypercapnia (6.5% CO₂) an increase of iNOS (in both mRNA and proteins) was found. In addition, lower quantities of mRNA, proteins and activity of soluble guanylate cyclase (NO effector) were found. This process is probably involved in the development of pulmonary hypertension by hypoxia; this report may be significant in those pathologies where these changes of gases’ pressures coexist like in chronic obstructive pulmonary disease and sleep apnea.

5. NADPH Oxidase (NOX)

Its function is to form free radicals, particularly superoxide; the formation of this free radical begins with the transfer of electrons from NADPH to molecular oxygen [60–62]. This enzyme has a great functional relevance since it decreases the levels of nitric oxide when it reacts with it and forms peroxynitrite [63]. There are NOX isoforms, from 1 to 5, and DUOX 1 and 2 [61, 64]. From the structural composition point of view, lung tissue is formed by several cell types that give rise to the presence of more than one variety of NOX in this organ; endothelial cells express NOX1 and NOX5 but predominantly NOX2 and NOX4. Alveolar macrophages express NOX2, fibroblasts and vascular smooth muscles express NOX4, ciliated epithelial cells of the airways express DUOX1 and DUOX2, and type II alveolar cells express NOX1 [65, 66]. The expression of the different NOX isoforms in cells can be modified according to the conditions they are exposed to just as in the case of the increase of NOX4 in some tumor tissues [67]. Notwithstanding to a greater or lesser extent any of the isoforms present in the described cell types can participate by forming free radicals, and, in the case of lung tissue, it is known that the major source of free radicals derived from endothelium is originated in the NOX [68, 69].

PAECs obtained from bovines were exposed to a protocol of ischemia in vitro (24 h at a 3% O₂) in which increases of extracellular hydrogen peroxide were found. The production of this prooxidant was inhibited by diphenyleneiodonium (DPI) [70]. Marshall et al. [71] obtained, in rings of pulmonary arteries of cats, that the application of DPI favored the relaxation of hypoxic vasoconstriction induced by three series of gases in hypoxia (95% N₂, 5% CO₂). Thompson et al. [72], in pieces of rats pulmonary arteries, found that in one hour of hypoxia (95% N₂, 5% CO₂) an increase of the pressure within these vessels was observed and that this phenomenon was diminished by the application of DPI. Jones et al. [73] found that a relaxation of arteries was produced by DPI, in a similar preparation of rats arteries, to the same percentages of gases supplied in the previous study.

In preparations of isolated-perfused lungs of rabbits, exposure to series of 10 min at 3% of O₂, application of 4-(2-Aminoethyl) benzenesulfonyl fluoride (AEBSF), an inhibitor of the NADPH oxidase, resulted in a decrease of hypoxic vasoconstriction [74]. Archer et al. [39] found a large decrease of radicals’ generation in perfused lungs of rats that lost the gp91phox subunit, after applying 2.5% O₂ during 30 min; however, the maintenance of the vasoconstrictor phenomenon to hypoxia was observed.

Weissmann et al. [75] reported in perfused, ventilated, and isolated lungs a decrease of the release of superoxide radicals measured by spin resonance by using the 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine marker in deficient mice in p47phox subunit; however, when they were exposed for 30 min at 1% of O₂, an increase of superoxide in the perfusion solution was observed.
In models of chronic exposure, it has been reported that in rats exposed at 10% O₂ during 21 d an increase of NOX4 mRNA was found, while no differences for NOX2 mRNA were observed [11].

In a model of rats exposed during 3 weeks at 10% O₂, an increase of the production of superoxide from intrapulmonary artery rings was found, which was related to gp91phox subunit of NADPH oxidase. In addition, there was vascular remodeling and pulmonary hypertension that were not observed in KO rats for gp91phox subunit [76]. Rats were exposed to simulated altitude in hypobaric chamber at 380 mmHg during 21 d where an increase in the ROS production and an increase in the vascular reactivity of intrapulmonary vessels segments were found; these results were not observed in KO rats for gp91phox subunit [79]. Nisbet et al. [80] demonstrated an increase of NOX4 mRNA and protein, besides an increase in the generation of superoxide anion and vascular remodeling with hypertrophy of the pulmonary artery in rats that were exposed to 10% O₂ during 3 weeks. The administration of PPARγ ligand, rosiglitazone, prevented these changes induced by hypoxia. It is likely that this animal model explains, in part, what happens in humans who suffer idiopathic pulmonary hypertension, since normal human PASMCs increased their NOX4 mRNA levels when they were exposed to 1% O₂ during 24 h and lung tissue of patients who suffer idiopathic pulmonary hypertension showed an increase of the expression of this isoform [11].

Nisbet et al. [80] performed a chronic intermittent hypoxia protocol in rats that simulated the desaturations observed in patients with OSA; for this purpose, the O₂ from 21% to 10% every 90 s during 8 weeks was decreased. After this treatment, an increase of the expression of NOX4 and p22phox in lung homogenates was observed. KO rats to gp91phox which were exposed to the simulated OSA protocol, did not show any changes in the expression of NOX4. These findings support the hypothesis that NOX is involved in vascular remodeling by hypoxia.

6. Xanthine Oxidase/Dehydrogenase

This enzyme is present in all endothelial cells of the organism and also in pulmonary vessels, participating in hypoxanthine and xanthine degradation to uric acid [81, 82]. Throughout this process, superoxide and hydrogen peroxide are formed. Dehydrogenase (XD) form, predominant in normal conditions, can be susceptible to oxidation and proteolysis transforming in oxidase form (XO) [83, 84]. In bovine PAECs exposed to 48 h of hypoxia (95% N₂ and 5% CO₂, balanced with ambient air), XO and XD activity increases was found. Also, an increase in superoxide formation to the medium was found, which was decreased by the use of allopurinol and tungsten, both of them inhibitors of the enzyme. Furthermore, enzymatic activity was inversely related to the O₂ pressure they were exposed to [85]. Hassoun et al. [86] found increases in XO activity, using in vitro bovine PAECs at 3% O₂ for 48 h. In the same study, a variation of XO/XD expression between species was reported [86]. Afterwards, the same increase of the XO and XD activity at 3% O₂ for 48 h in PAECs in vitro was found. In addition, rats were exposed to 380 mmHg for 5 days, finding an increase in XO activity and the XO/XR ratio. The supplementation of L-arginine decreased the XO activity [87]. Hassoun et al. [88] found an increase in XO activity in lung homogenate in rats exposed to 380 mmHg for 24 h. This change was related to the increase of fluid content in the lungs of the animals. Rats exposed to 10% O₂ for 21 days showed an increase in XO activity from the first day that remained throughout the period. Also, greater lipid peroxidation in homogenates was observed. The use of allopurinol avoided the pulmonary hypertension and right ventricular remodeling [89].

After endothelial cells were exposed to hypoxia for 24 h (1% O₂), they showed a great increase in intracellular ROS production measured with CDF and that was related to an increase in XO concentration. In the same experimental series, transfected cells of the same type of cells overexpressing intracellular SOD did not show any increase in the ROS production or changes in the XO amount [90]. In a in vivo study, rats were exposed to 10% O₂ for 10 d and an increase in total leucocyte in BALF and in ROS production and XO amount in lung tissue was found. In transgenic rats overexpressing extracellular SOD, no evidence of this phenomenon was found [90]. In a model of chronic exposure to hypoxia (13% O₂) for up to 14 d in newborn rats, vascular proliferation increased and also vascular reactivity increased oxidative injury to lipids and proteins. The administration of allopurinol decreased at the fourth day the increase of XO, consequently decreasing oxidative tissue injury; the same process happened when administering Tempol, an analogous of SOD and U74389G, and a synthetic nonenzymatic antioxidant [91].

7. Lung Inflammation

The inflammatory process is one of the factors involved in the increase of ROS’s generation and tissue oxidative damage; in turn, ROS are involved in the NF-κB release, a transcriptional factor that triggers the inflammatory process; HIF1-α has a central role in the response to hypoxia as well [92, 93]. Alveolar hypoxia corresponds to a proven stimulus that triggers inflammation that is first localized and later it becomes systemic; in this point, the alveolar macrophages’
role seems to be essential [6]. It has been previously demonstrated the increase of both the in vitro secretion of monocyte chemoattractant protein (MCP-1) and the tumor necrosis factor-alpha (TNF-α) derived from alveolar macrophages of rats exposed to 5% O2 for 5 h [94]. Gonzalez et al. [95] observed a decrease of the inflammatory response activated by hypoxia after the administration of clodronate liposomes to deplete lung of alveolar macrophages in rats exposed to 10% O2. In hypobaric hypoxia, an increase in number, proinflammatory activation, and release of ROS by alveolar macrophages has been described [96, 97]. Humans exposed to hypobaric chamber at 4,500 m for one hour during seven days evidenced leukocytosis with a predominance of polymorphonuclear neutrophils leukocytes (PMNLs) on the first day of protocol, while this was not observed on the seventh day. In addition, in in vitro PMNLs, which were stimulated by zymosan, the formation of basal superoxide at the end of the seventh day of intermittent altitude was increased and there were no changes in any stage in the formation of ROS by acute exposure [98]. The expression of CD18 and formation of superoxide increased in an exposure to 3,196 m. Training and altitude exposure decreased the capacity to form superoxide by PMNL [99]. One hour at 5% O2 increased inflammatory cell adhesion to alveolar epithelial cells of rats. Thus, Beck-Schimer et al. [100] found an in vitro increase of ICAM-1 and VCAM-1, which was related to a higher PMNL and macrophages adhesion to alveolar cells. In lung homogenates of rats subjected to hypoxia (10% O2) increases of myeloperoxidase (MPO), NF-κB activity, and mRNA of ICAM-1, VCAM-1, HIF-1α, macrophage inflammatory protein beta (MIP-1β), and MCP-1 were found. In BALF, an increase in both proteins and total number of cells was found. These changes were reduced by the depletion of alveolar macrophages using clodronate [94]. In rats exposed to 9.9% O2 for 24 h, an increase in the PMNL number and TNF-α, IL-1 beta and IL-6 and MPO was found. At the same time, an increase in the generation of nitrite, nitrate, and hydroxyl radicals in BALF was evidenced. These changes were associated with the increase of the expression of HSP70 in lungs. The hypoxic preconditioning (18.3% O2 during 5 days) decreased the formation of previously described ROS, nitrites, and, also, pulmonary edema [7]. Shukla et al. [101] exposed rats during 48 h at 7,619 m, observing an increase in ROS formation measured as DCF and lipid peroxidation (TBARs and 4-hydroxynonenal). These changes were associated with increases of TNF-α, interferon gamma and interferon alpha, as well as MCP-1 in BALF. In turn, an increase in mRNA and protein expression of HSP-32 and HSP70 was observed. Furthermore, the expression and activity of HIF-1α increased together with the increase of its targets EPO and GLUT-1. The use of CoCl2, a stabilizer of HIF-1α [102] and an inducer of ischemic preconditioning [103], decreased both oxidative damage and inflammation of this organ by hypoxia.

Exposure of rats during 5 h at 9,142 m favors the generation of pulmonary edema and promotes the release of inflammatory mediators (MCP-1, IL-1, TNF-α, TGF-β, and IFN-γ) and adhesion molecules (ICAM-1, VCAM-1, and P-selectin) in BALF. These changes were associated with increases of NF-κB in lung homogenates. The administration of CoCl2 reduced these effects [5]. Sarada et al. [104] found pulmonary edema and an increase of ROS (DCF), lipid peroxidation (MDA), NF-κB, IL-1, IL-6, and TNF-α and also ICAM, VCAM and P-selectins in lung homogenate of rats exposed at 7,620 m. Preconditioning with curcumin, an inhibitor of the expression of NF-κB, reduced edema and reduced the expression of NF-κB. Rats exposed for 5 h at 9,144 m, in hypobaric chamber, showed an increase of IL-6, IL-10, TNF-α, MCP-1, and VEFG in BALF. The administration of an ethanol extract of Hippophae rhamnoides L. leaves, rich in flavonoids, with known antioxidant and anti-inflammatory effect such as quercetin, epicatechin, and flavonols, or the administration of dexamethasone favored inhibition in the formation of proinflammatory factors, reduced edema, and decreased VEGF, the latter, a known promoter of the endothelial permeability increase. An increase of E-selectin after a cicloergometric exercise at 3,810 m has been reported in the BALF analysis in humans [105]. In the same kind of sample, subjects who had pulmonary edema at a moderate altitude (2,600–3,000 m) increased the amount of alveolar macrophages, lymphocytes, PMNL, and total proteins. An increase of IL-1β, IL-6, IL-8, and TNF-α was found; after recovery, these values returned to normal. A similar result was previously reported in four patients for the same cytokines in BALF at a similar height [106].

8. Pulmonary Antioxidant Defenses

The oxidizing effect of ROS can be countered by reducing agents called antioxidants, preventing their formation or removal and, hence, modeling the cellular redox state. Antioxidants can be classified according to their nature as enzymatic or nonenzymatic antioxidants. In lungs, the main enzymatic reducers are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and thioredoxin (Trx) [107, 108]. Among the nonenzymatic reducers we can find mucin, urate, glutathione (GSH), ascorbate, ceruloplasmin, transferrin, vitamin E, ferritin, and small molecules such as bilirubin [107, 109–113]. Regarding this category, in the central airways in lungs we can find mucopolypeptidic glycoproteins with high molecular weight, released by epithelial cells and glands, which are capable of increasing the amount of mucus. Furthermore, a thin protective film so called epithelial lining fluid (ELF) is deposited on the surface of the airways, which has a significant amount of both enzymatic and nonenzymatic antioxidants among its components [107, 114]. In the ELF, the most important nonenzymatic antioxidant contributions are mucin, urate, ascorbate, and GSH; among the enzymatic antioxidants are SOD, CAT, and GSH-Px [114].

9. Enzymatic Antioxidants

SOD has an important role in catalyzing the conversion of superoxide to H2O2 and O2. This enzyme has three isofoms: the copper-zinc SOD (CuZnSOD), located in the nucleus,
cytoplasm, and peroxisomes, the manganese SOD (MnSOD) located in the mitochondria, and the extracellular SOD (Ec-SOD), which is outside of the plasmatic membrane of the lungs [115]. In hypoxia, MnSOD seems to be the target of modifications of this enzymatic variety, unlike the other isoforms. In this regard, Nakanishi et al. [116] found a decrease in the activity of MnSOD in rats’ lung homogenates, after 5 d at 5,500 m height in hypobaric chamber. Subsequently, Cantin [117] observed a reduced activity of mitochondrial MnSOD after subjecting rats to 7% O2 during 60 min. Russell and Jackson [118] observed an increase of the MnSOD protein expression and activity in lung homogenates of rabbits by using an in vivo model of hypoxia by lung collapse, during 7 d without subsequent reexpansion. On the other hand, when a group of rats was preconditioned to low concentrations of O2 simulating a height of 5,500 m during 15 h, an increase of the activity and protein level of MnSOD but not of CuZnSOD was produced [119]. Russell et al. [120] did not find any changes in MnSOD mRNA in alveolar type II cells after subjecting in vivo rabbits’ lungs to collapse during 7 d without subsequent reexpansion. Russell et al. [121] did not observe increases in mRNA of mitochondrial MnSOD of the lungs in transgenic rats which overexpressed this enzyme, after being for 7 d at 10% O2 under normobaria. However, when isolated and ventilated rats’ lungs were subjected to subsequent reoxygenation to an anoxia period (10 min to 5% CO2 and 95% N2 and then reoxygenated 10 min to 21% O2), the content of MnSOD mRNA in lung homogenate was increased [122]. These studies confirm the importance of MnSOD as one of the major lines of defense against pulmonary hypoxia. In addition, Ec-SOD isoform is the main extracellular enzymatic antioxidant in lungs [123, 124], with a known protector effect in the lining of the lungs fluids [125, 126] and at interstitial level and a high affinity to the extracellular matrix [127, 128] in rats and humans. In these studies, developed under hypoxia, fibrosis, and hemorrhage conditions [128–131], the protective effects of Ec-SOD against oxidative damage by hypoxia have also been observed. Both this enzymatic antioxidant and GSH-Px are in charge of protecting the vulnerable targets to hypoxia-induced free radicals, such as alveolar epithelial cells and the endothelium of pulmonary capillaries [125]. Nozik-Grayck et al. [132] studied rats with pulmonary hypertension after chronic hypobaric hypoxia. The group of wild-type rats, after 1 d of exposure at a simulated height of 5,486 m, increased the activity of Ec-SOD and then it decreased at day 35. Transgenic rats that overexpressed Ec-SOD did not show any change in the activity of Ec-SOD and they protected the lung against vascular remodeling and hypertension. In another study, Giles et al. [133] observed a decrease in the expression of Ec-SOD at transcriptional and translational level in lung homogenates of rabbits that were subjected to hypobaric hypoxia (4,572 m for 36 h) at 26 d of gestation. As it happened with Ec-SOD, hypoxia limited the expression of pulmonary MnSOD. A recent study [90] has confirmed the potential inhibitor that the Ec-SOD overexpression has in the processes of pulmonary hypertension induced by hypoxia. According to the authors, the concept of ROS reduction could explain the reversibility of this phenomenon. In vivo, the hypoxic chamber animal model (10 d at 76 mmHg O2) showed a significant decrease of ROS, right ventricular systolic pressure, and pulmonary vascular wall thickness in the group of transgenic mice that overexpressed Ec-SOD in comparison with the WT adult mice transected with an empty vector. Other authors have recognized the contribution of Ec-SOD in other oxidative states such as COPD [134], exposure to cigarette smoke [126], and hyperoxia [135]. These results suggest that high concentrations of Ec-SOD present in lung fluids [125] play an essential protective role against the oxidative threat from exposure to external environment.

Catalase is mainly located in peroxisomes of most mammalian cells, but it can also be found in mitochondria and endoplasmic reticulum. This enzyme is responsible for catalyzing the decomposition of H2O2 to O2 and water. In hypobraric hypoxia simulated in chamber, Nakanishi et al. [116] found an increasing trend without significant changes in the activity of CAT in lung homogenates of rats that stayed up to 21 d at 5,500 m. As it happened with MnSOD, Shen et al. [122] demonstrated increases of CAT mRNA subsequent to reoxygenation in a model of isolated lungs of rats subjected to hypoxia and reoxygenation. A recent investigation showed that the activity of CAT was significantly increased with regard to the control in lung homogenate of rats exposed to ischemic preservation. Besides, the increase of CAT was higher as the time of storage increased [136]. Both pregnant and nonpregnant rats exposed to 10% O2 during 4 d did not show any variation of the activity of CAT [137].

CAT and GSH-Px share the function of controlling the concentrations of H2O2; they are the most outstanding cellular mechanisms (to detoxify hydroperoxides) for the neutralization of ROS [138]. Whenever the concentrations of H2O2 are in a high level, CAT becomes more effective than GSH-Px and vice versa [117]. Nevertheless, GSH-Px is capable of removing organic peroxides, such as those derived from lipid peroxidation [139]. To control the concentrations of H2O2, the enzymatic reaction of GSH-Px uses GSH, which acts as a reducer. Oxidized GSH (GSSG) will be reduced by glutathione reductase (GSH) with the help of NADPH. All glutathione peroxidases described in the body contain selenium, which include the following: the classic cytosolic shape present in all cells (cGSH-Px) [140–143], one associated with membrane phospholipids (PHGSH-Px) [144], other associated with gastro intestine (giGSH-Px) [145] and to extracellular (eGSH-Px) [146]. The latter is part of ELF and it is produced and secreted by alveolar epithelial cells and macrophages increasing lung antioxidant defense against oxidative stress as it occurs during hypoxia [147].

In lungs, antioxidant activity of GSH-Px in ELF is headed by eGSH-Px followed by cGSH-Px. Avisar et al. [147] showed a significant increase of the expression of eGSH-Px and cGSH-Px in a group of bronchial epithelial cell lines and primary human alveolar macrophages when both of them were subjected—in vitro—to oxidizing substances such as ozone (O3). The immediate presence of eGSH-Px in the used medium places these cells and the interstitial cells as potential sources of pulmonary eGSH-Px. In human lungs subjected to oxidizing conditions, as in the case of
smokers, the activity and expression of eGSH-Px in ELF showed some increases [148, 149], the same occurred in asthmatic subjects who were in contact with cigarette smoke [150, 151]. Similar answers have been observed on hypoxia in studies that have quantified pulmonary GSH-Px. In rats placed into a simulated environment at 5,500 m, the activity of GSH-Px in lung homogenate was slightly increased and no significant differences until 21 d of exposure were presented [116]. Likewise, Yeginsu and Ergin [136] published findings about increases of GSH-Px in lungs of rats subjected to 12 h of ischemic preservation in cold solution at 4°C, reaching even lower values than the control group's when preserved at 48 h. On the contrary, Zhao et al. [152] observed a decrease in the activity of GSH-Px in lung homogenate of rabbits exposed to a simulated altitude at 8,500 m in barochamber during 3 h. Pregnant rats exposed to 10% O2 during 4 d showed a decrease of the activity of GSH-Px, and no changes in non-pregnant rats were observed [137].

Comhair and Erzurum [146] claim that the transcription of mRNA, under oxidative pathological conditions, protein expression, and release of eGSH-Px to ELF are the most inducible at epithelial level in humans, just as it occurs in the presence of hypoxia.

TRX is a ubiquitous enzyme with a crucial role in controlling cellular redox environment [153]. The active site of this polypeptide has the -Cys-Gly-Pro-Cys-amino acid sequence. Functionally, TRX participates in redox regulation of numerous proteins such as NF-κB, HIF-1α, c-Fos/c-Jun complex, glucocorticoid receptor, estrogen receptor [154]. TRX is induced by the production of H2O2 demonstrating a potent cytoprotector effect. Likewise, human TRX (hTRx) protects cells from the increase of superoxide during ischemia-reperfusion (IR) in the reaction that converts hypoxanthine to xanthine by xanthine oxidase [155]. Fukuse et al. [156] observed an increase in the protective capacity of recombinant hTRx in vivo in rats of IR in a nonventilated lung during 75 min ischemia. The immediate administration to reperfusion of recombinant hTRx implied higher survival, improved gas exchange, decreased edema, and reduced pulmonary lipid peroxidation [156, 157]. Okubo et al. [158] in a similar protocol of IR administered hTRx, which reduced IR injury in rabbits probably by reduction of ROS. The protective effects in pulmonary IR have been corroborated by several authors. These studies suggest that hTRx could be effective as a radical eliminator in the lung reperfusion treatment, just as it occurs after transplantation [158–160].

10. Nonenzymatic Antioxidants

As previously mentioned, there are many nonenzymatic antioxidants in ELF that are capable of detoxifying ROS [161]. The latter are the first line of defense against inhaled oxidants from environments such as O3, nitrogen oxides, and tobacco smoke or against changes in O2 tension such as hypoxia or hyperoxia. One of them is mucin, which besides acting as an antioxidant regulates the viscosity of mucus [162]. It has been demonstrated that oxidative stress increases the expression and production of mucin, improving the antioxidant condition in the surface of the respiratory tract [163, 164]. Likewise, a decrease of GSH in the respiratory epithelium, just like in hypoxia, will favor the increase of viscosity in mucin. Under oxidative-inflammatory pathological conditions, such as cystic fibrosis and chronic bronchitis, excessive increase of mucus viscosity by production of mucin is considered a negative condition for the airways. Therefore, a projection in the therapeutic use of mucin as antioxidant must be carefully considered.

GSH, an abundant antioxidant in lung epithelial cells and in ELF [165, 166], is the key to modulate the development of inflammatory-oxidative pulmonary injury. Reduction of H2O2 and lipid hydroperoxides is carried out together with GSH-Px or peroxiredoxin. In both cases, GSH is converted in GSSG and quickly reduced by glutathione reductase and NADPH, or it is used in the process of protein folding in the endoplasmic reticulum. In the latter, GSSG is recycled by the protein disulfide isomerase and converted in GSH.

In hypoxia, Jenkinson et al. [167] measured GSSG in isolated perfused rat lungs. Firstly, a period of anoxia (95% N2 and 5% CO2) and subsequent reperfusion (95% O2 and 5% CO2) were performed. GSSG only was increased during reoxygenation in both perfusate and lung tissue. This suggested the presence of H2O2. In another study, Jackson and Veal [168] used a hypoxia and reoxygenation (collapse and reexpansion) model in lungs of rabbits. In this case, GSSG was significantly increased in the alveolar lavage fluid and lung tissue after 2 h of reexpansion subsequent to collapse; besides, a significant decrease of the total glutathione was observed.

On the other hand, a research carried out by White et al. [169] on isolated lungs of rats previously exposed to hypoxia demonstrated that the activity of the glutathione redox cycle was effective on pulmonary cytoprotection after increasing H2O2 by hyperoxia. This effect seems to depend on hexose-monophosphate derivation, since lungs previously exposed to hypoxia and that were perfused with oxidase glycogen had increased the reduction equivalents of H2O2 when compared to lungs that were preexposed to normoxia. This was reflected in the increasing of GSH/GSSG and NADPH/NADPH+ ratios. Therefore, White et al. [169] proposed the existence of an adaptive process to previous hypoxia with more tolerance towards H2O2 by increasing of GSH and NADPH in lung tissue. In ELF, GSH concentrations in humans are at least 100 times greater than those found in plasma (0.5–5 μM) [165, 170, 171]. Low-molecular-weight antioxidants having greater concentrations in upper and lower respiratory tract are ascorbate and urate. GSH is present in ELF only in a significant manner. The latter does not play down the importance of the GSH function in the pulmonary oxidative control, since this is incorporated to one of the main antioxidant enzymatic systems [172].

Jackson et al. [173] found a decrease in mitochondrial GSH and MnSOD and an increase in the concentrations of lactate dehydrogenase enzyme and H2O2 in lung cell lines subjected to 1% O2. As you can see, the pulmonary antioxidant system of GSH depends on the capacity of all its components, both enzymatic and nonenzymatic, in and out of the cell.
It is well known the importance of urate as a nonenzymatic antioxidant in human respiratory tract [174–177]. Nasal lining fluid has very similar levels as plasma (200–300 μM). Although there is high plasma concentration levels of urate, the theory of urate consumption by the bronchoalveolar or nasal fluid does not seem solved. In a study carried out by Van der Vliet et al. [172] no significant correlation of urate concentrations between plasma and ELF was obtained, which strengthens the presence of local maintenance mechanisms of urate.

Secretion of urate is carried out together with mucin, hence making evident its role in controlling the attacks of oxidant agents in the respiratory tract. Indeed, its main results in the antioxidant defense have been observed in oxidative control to O₃, where urate concentrations in nasal lining fluid significantly decrease after being exposed to this element [175]. With regard to hypoxia, there is no convincing evidence about its effects in the modification of urate concentrations in the respiratory tract. Deaton et al. [178] using an animal model for recurrent bronchial obstruction without inflammation did not find significant modifications in urate concentrations in EBC samples of horses. The lack of evidence makes it difficult to quantify and determine the antioxidant contribution of urate in airways.

Ascorbate (vitamin C) has a double role: sweeping the neutrophil oxidants away and reducing the oxidation of vitamin E [179]. The latter allows returning the antioxidant capacity to the vitamin E. Researchers have described different ascorbate concentrations in plasma and ELF obtained from both nasal region and bronchoalveolar zone [166, 180, 181]. The effects of hypoxia on ascorbate concentrations in lungs are scarce. Deaton et al. [178] observed an inverse correlation between H₂O₂ concentrations in EBC and ascorbate concentrations in ELF from horses affected by inflammatory recurrent airway obstruction.

11. Oxidative Damage in Lungs by Hypoxia

Oxidative stress to lipids is one of the most studied phenomena regarding oxidative stress by hypoxia. In that regard, a 75% increase in TBARs in rats initially exposed to 12% O₂ for 48 h and to 10% for 12.5 d was found in lung homogenates [183]. Kiliç et al. [182] found an increase in TBARs in lung homogenates in newborn rats exposed to 8% O₂ for 3 h. This process was prevented by administering a nitric oxide synthase inhibitor (NG-nitro-L-arginine) and by administering a precursor for its synthesis (L-arginine). Hoshikawa et al. [89] found an increase in lipid peroxidation measured as phosphatidylcholine hydroperoxide in lung homogenates after rats were exposed to 10% O₂ for four days. The administration of both N-acetylcysteine and allopurinol prevented the increase of lipid peroxidation. Minko et al. [4] found an increase in TBARs and conjugated dienes of lung homogenates in rats exposed to 6% O₂ during 120 min. This process was diminished by the application of intratracheal liposomes charged with α-tocopherol. Wilhelm et al. [184] found an increase of aldehydes in lung homogenates after being in a normobaric chamber with 10% O₂ for 5 d. Jankov et al. [91] exposed rats to 13% O₂ for 4, 7, or 14 d, finding an increase in lipid peroxidation measured as 8-isoprostone in lung homogenates. Rats exposed to a moderate altitude (1,500 m) during 8 weeks had increased MDA in their lung homogenates. The administration of polyphenol extract in another experimental group decreased hypoxia-induced lipid peroxidation [185]. After exposition to high altitude simulated in the hypobaric chamber for 5 h at 9,144 m, an MDA increase in lung homogenates of rats was found. This change was avoided in rats receiving dexamethasone or a leaf extract rich in flavonoids [186].

In regard to oxidation to hypoxia-induced protein, Rus et al. [187], subjected rats to hypobaric hypoxia to a 225 mmHg barometric pressure during 30 min and later to reoxygenation from 0 to 5 d, finding an increase in oxidative stress to proteins in lung homogenates measured as the increase of nitrotyrosine. In rats’ lung sections, an increase of nitrotyrosine was also found as from the fourth day at 13% of O₂ [91]. In another report, newborn rats were exposed to the hyperoxia protocol with hypoxic events (65% O₂ with 10 hypoxic events of 8% O₂ for 10 min) during four weeks, finding an increase in carbonyl concentration in lung homogenates [188].

Regarding the oxidative damage to nucleic acids, an increase of oxidative damage to the gene promoter for VEGF and to the DNA recognition area for HIF-1α was reported in culture of PAECs and PASMCs exposed to 2% O₂ from 3 to 48 h [189]. In another similar protocol, PAECs cells remained in a hypoxic medium (2% O₂ and 5% CO₂) from 3 to 48 h. Using the endonuclease fingerprinting and ligation-mediated PCR techniques, the injuries to mitochondrial DNA and nuclear DNA were determined. In nuclear DNA the injury to the gene promoter region for VEGF was found [190].

12. Pulmonary Oxidative Damage by Hypoxia Assessed by Noninvasive Methods

Difficulties to obtain lung tissue samples are the reason of the low number of studies carried out in humans. For this reason, most of the information currently available regarding the effects of hypoxia on lung redox state changes is derived from studies carried out on animal models which have been previously reported. In order to find a solution to these limitations, non-invasive methods are arising and are currently being developed to study pulmonary diseases. By using these methodologies, the effects of hypoxia on ROS formation were studied, as well as the effects this condition generates over lung redox state.

In patients having respiratory distress, an H₂O₂ increase in EBC was found [191]. Similar results were found by Sznaider et al. [192] when describing H₂O₂ increase in patients having hypoxic respiratory failure. Climbers increased its H₂O₂ concentration in EBC after descending a 6,125 m volcano. Another experience was carried out in biathletes that remained training at 2,800 m, and an increase in H₂O₂ concentration was found in the expired air [193]. Patients with OSA showed an H₂O₂ increase [194]. Malakasioti et al.
found an increase of H2O2 concentration in EBC in children with the same disease. Previous studies, although in different contexts, share hypoxia as at least one of the main causes, hence making models with limitations. For this reason, studies developed in more controlled conditions, in animals, are complementary, such as the one carried out by Wilhelm et al. [97] where EBC of rats exposed to normobaric hypoxia for 7 d with 10% O2 was obtained. In this condition, an increase of H2O2 concentration was found. A similar result was reported in subsequent studies with the same species and at the same O2 fraction but under different exposed times: three days [196] and five days [184]. The measurements of H2O2 in EBC show various results that guarantee H2O2 increase in hypoxia; however, the origin of this marker has not been determined yet, due to many different sources that might be involved (NOX, mitochondria, inflammatory cells, and XO).

Another prooxidant widely determined in humans (at a lung level) is exhaled NO (ENO) [197, 198]. With regard to this marker, results are not even, and there are many studies demonstrating that nitric oxide in exhaled breath increases in relation to hypoxia. Hence, Mansoor et al. [199] found ENO increases with no differences in the concentrations of nitrite plus nitrate in EBC in subjects climbing 4,342 m. The administration of L-arginine did not generate modifications in both types of samples. Macinnis et al. [200] found ENO concentration increases after two hours in subjects remaining with 12% O2 in normobaria. Furthermore, these increases were related to acute mountain sickness incidence, finding that ENO was lower in subjects developing the disease. In adult patients with OSA, an increase in ENO was found and was directly related to the total sleeping time with saturation of hemoglobin by O2 below 90%.

There are other results indicating that ENO decreases or does not modify by hypoxia. In subjects breathing 12% O2, ENO decrease was found after two hours in subjects susceptible to developing high altitude pulmonary edema. Moreover, the percentage change of ENO was correlated inversely with pulmonary artery pressure after 2 h [201]. Under hypobaric hypoxia conditions, Hemmingsson et al. [202] found that subjects had decreased ENO when exposed acutely to 5,000 m in a hypobaric chamber, meanwhile this phenomenon was not observed when carried out in a normobaric hypoxia laboratory (11.3% O2). Donelly et al. [203] carried out ENO measurements in humans until reaching saturation of hemoglobin for 80% O2 in the laboratory and then in a climb to 5,050 m in land conditions finding that ENO decreased in hypobaric hypoxia, meanwhile it remained the same in normobaric. In both situations, pulmonary artery pressure increased; therefore, authors came to the conclusion that ENO probably does not participate in tone regulation of pulmonary artery in hypoxia. In longer permanence in hypoxia as the one carried out by Güzel et al. [204] at 2,300 m for seven days, a decrease in ENO was also found. Miners staying sporadically (3 weeks in altitude versus 3 weeks at 800 m) for more than one year in altitude (between 3,600 m and 4,000 m) were measured at 4,000 m, finding a decrease in ENO. Children exposed chronically to altitude did not show differences in ENO—children from Aymara ethnic group versus children having European descent, although the first ones had lower pulmonary artery pressure. The diversity of the results, increased, decreased, or remaining levels of NO, shall be discussed as for the meaning of this parameter in different scenarios where hypoxia participates. All causes of different results are also not known, nevertheless, to a considerable extent, decreases have been attributed to the lack of O2 (substratum to form NO). Consequently, Schmetter et al. [20] found that 10 min administration of O2 from 10% to 100% in humans, oxygen pressure, and ENO were directly related. Another cause is related to the type of hypoxia, hence, the results of hypobaric are not alike to normobaric [202, 203]. Finally, measurements of ENO have variations attributed to the method and equipment, requiring the modification of measurement methods, as it was suggested by Hemmingsson et al. [202].

Oxidative stress in lungs by hypoxia has also been measured in EBC samples; hence, Araneda et al. [205] found MDA increases measured in EBC after 10 d starting from 3,000 m in subjects climbing 6,125 m. In the same work, MDA concentration increases were found in the same type of samples in subjects that carried out a maximal cycloergometer exercise at 2,160 m altitude, meanwhile this same physical effort did not generate changes at 670 m. In a subsequent study, 8-isoprostane was determined in exhaled breath samples in biathletes that trained at 2,800 m for six weeks, finding an increase tendency of this marker [193]. Patients having OSA showed an 8-isoprostane concentration increase in EBC after using oxygen with continuous positive airway pressure. This parameter returned to normal values [194]. Similar results were found by Carpagnano et al. [206], also in patients with this same disease.

13. Conclusions

Early studies on hypoxia have reported the decrease of ROS. Nowadays, with the development of methodologies based on oxidation-sensitive proteins, it is known that ROS and oxidative damage increase in hypoxia. In lungs, ROS sources by hypoxia are multiple: in the mitochondrial ETC they are mainly formed in the complexes I and III, which increases ROS in the intermembrane space and cell cytoplasm. The enzyme XO/XD has a proven role as ROS generator by hypoxia in lungs, being, probably, one of the most important ROS sources. Another source is NOx, which has a large representation in lungs. Current records point out that, from the various isoforms, the ROS formation from this system is mainly originated in NOX4, this isoform being also associated with vascular remodeling and proliferation. From the three ROS isoforms, the inducible variety, activated in this context by NF-κB, HIF-1α, and ROS, has a clearer role in the ROS formation under hypoxic conditions. The inflammatory process, which is developed by hypoxia, provides alveolar macrophages that secrete proinflammatory substances and ROS, which promote the infiltration of PMNL, ROS secretor activators.
In regard to antioxidant systems, from all the works that have studied this compound, it is possible to conclude that there is great variety of results. SOD is the most studied antioxidant, showing modifications of mRNA, amounts and activity in both mitochondrial and extracellular varieties. Studies on animals overexpressing Ec-SOD are probably the ones that make the role of this isoform in hypoxia clearer for being a protector of the development of pulmonary hypertension by chronic hypoxia. Catalase and glutathione peroxidase have demonstrated a great variety of results, from nonshowing any variation to increases and decreases; although, in cultured cells transfected with these enzymes, a decrease in the formation of ROS by hypoxia has been observed.

In relation to TRx, available results do not allow directly knowing their modifications by hypoxia. We do know that their administration as a recombinant protein has a protector effect against ischemia and reperfusion of this organ. Regarding nonenzymatic antioxidants against hypoxia, there is almost exclusively information about glutathione and the decrease of the relation GSH/GSGG against hypoxia; besides, on the basis of its concentration, its protector role as part of the ELF is speculated.

It is a fact that the great difficulty for studying oxidative damage by hypoxia in lungs, in all species including humans, is because of the difficulties to obtain tissue, the current evidence being mostly obtained from studies conducted whether in cells and pieces of organs or isolated complete organs. In this sense, it is of utmost importance to check and transfer many of the results presented herein to whole bodies and humans, too. In order to accomplish this task, it is necessary to move forward in using and developing methodologies, minimally invasive for the study of this phenomenon, as well as developing new ways that allow direct studies on humans.

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