Redox Regulation of the Superoxide Dismutases SOD3 and SOD2 in the Pulmonary Circulation

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1 Introduction

When evaluating the role of redox-regulating signaling in pulmonary vascular diseases, it is intriguing to consider the modulation of key antioxidant enzymes like superoxide dismutase (SOD) because SOD isoforms are regulated by redox reactions, and, in turn, modulate downstream redox sensitive processes. The emerging field of redox biology is built upon understanding the regulation and consequences of tightly controlled and specific reduction–oxidation reactions that are critical for diverse cellular processes including cell signaling. Of relevance, both the site of production of specific reactive oxygen and nitrogen species and the site of the antioxidant defenses are highly compartmentalized within the cell. For example, superoxide is generated during oxidative phosphorylation in the mitochondria as well as by a number of enzymatic sources within the cytosol and at the cell membrane. In the pulmonary circulation, these sources include the mitochondrial electron transport chain, NADPH oxidases (NOX1–4, Duox1,2), nitric oxide synthases, and xanthine oxidase; this important topic has been thoroughly reviewed recently [1]. In parallel with these different cellular sites of superoxide production, the three SOD isoforms are also specifically localized to the cytosol (SOD1), mitochondria (SOD2) or extracellular compartment (SOD3). This chapter focuses on the role of redox mechanisms regulating SOD2 and SOD3, with an emphasis on these processes in the setting of pulmonary hypertension.

The superoxide dismutases are an important family of antioxidant enzymes that catalyze the rapid dismutation of superoxide ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2) and molecular oxygen (~6.7×10^{-9} M^{-1} s^{-1}). Three human isoforms, cytosolic CuZnSOD (SOD1), mitochondrial MnSOD (SOD2) and extracellular EC-SOD (SOD3), have been identified and characterized. The first SOD isoform, SOD1 was discovered by Fridovich and McCord in 1969 [2]. However, while SOD1 is essential for cellular homeostasis and there is a body of literature investigating the use of recombinant human SOD1 in the treatment of pulmonary hypertension, the level and activity of SOD1 has not been found significantly perturbed in human pulmonary hypertension [3–5].

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This chapter focuses on the regulation of SOD3 and SOD2 activity since accumulating evidence suggests that impaired activity of these enzymes contribute to pulmonary hypertension.

2 The Extracellular SOD3 Enzyme

2.1 Characterization of SOD3

The eukaryotic superoxide dismutase enzyme SOD3 was first detected in human serum and subsequently isolated from human lung [6–8]. Human SOD3 exists as a tetrameric protein comprised of four identical monomers, producing a mature enzyme of approximately 132 kDa. One key feature of SOD3 is its extracellular matrix (ECM) binding region in the C-terminal part of each subunit that allows it to bind to the ECM and accounts for its high expression in the vasculature. Although the exact molecular interactions have not yet been described, Antonyuk et al. have reported detailed high-resolution structural analysis of SOD3 including two grooves that are likely to represent binding sites for heparan sulfate and collagen [9]. Unlike many other organs, SOD3 is the most abundant SOD isoform in the vasculature, accounting for 60–70% of total SOD activity in human systemic and pulmonary arteries. The ECM binding region is susceptible to cleavage by proteolytic enzymes including furin, which changes the distribution of SOD3, releasing cleaved SOD3 into the extracellular fluids [10–13]. The formation of tetramers containing different combinations of cleaved and uncleaved SOD3 monomers leads to three different classes of SOD3 defined by their heparin-binding affinity [7, 14, 15]. A known polymorphism in human *SOD3* within the ECM binding region, R213G, decreases ECM binding affinity and results in a redistribution of SOD3 from the tissue into extracellular fluids [16]. SOD3 expression and activity is decreased in multiple animal models of PH and in the lungs of individuals with end-stage IPAH [4, 5, 12, 17].

2.2 Redox Regulation of SOD3

The mature secreted SOD3 monomer contains 222 amino acid residues and combines to generate a homotetramer of approximately 132 kDa [7, 18]. Posttranslational modifications include an N-linked glycosylation and the formation of intra- and inter-subunit disulfide bonds. Important regions of SOD3 include an N-terminal region responsible for the formation of the tetramer, an enzymatic domain coordinating zinc and copper ions and a positively charged ECM binding region in the C-terminal. These features of SOD3 are essential to its structure, function, and localization. Specific aspects of SOD3 biology, including disulfide bond formation, proteolytic cleavage of the ECM binding region, regulation of enzyme activity, and regulation of gene expression are influenced by the redox state.

2.2.1 Intrasubunit Disulfide Bonds

Five of the six native cysteine residues in SOD3 (Cys45, Cys107, Cys189, Cys190, and Cys195) participate in intra-subunit disulfide bond formation and protein folding that results in active (aEC-SOD) and inactive (iEC-SOD) forms whereas Cys219 is involved in the formation of an inter-subunit disulfide bond [19]. The active isoenzyme is stabilized by two disulfide bonds within the subunit between Cys45-Cys190 and Cys107-Cys189, whereas the inactive counterpart forms two disulfide bridges between Cys107-Cys195 and Cys189-Cys190 [20]. The active SOD3 enzyme disulfide pattern is homologous to that of SOD1 [21], critical for active-site conformation in this SOD isoform as well. Dimers formed by these two different SOD3 states in vivo lead to three different combinations of SOD3 dimers (homodimers of aa and ii, heterodimers of ai) with discreet enzyme activity [22]. These dimers assemble into tetramers of various SOD activity synthesized intracellularly before secretion [23]. While Cys195 participates in the disulfide bond formation and protein folding of human iEC-SOD monomer, the absence of this cysteine residue in the primary sequences of SOD3 in mouse [6], rat [24], and rabbit [20, 25] accounts for the exclu-

sive formation of the active SOD3 form in these species. The critical role of Cys195 in determining active vs. inactive states of SOD3 was confirmed by an elegant study in which an expression vector encoding human SOD3 was transfected into mouse cells that do not normally synthesize the inactive enzyme, showing that these cells were now able to synthesize both active and inactive SOD3. Moreover, when mouse SOD3 that lacks Cys195 was expressed in a human cell line that do not generate human SOD3, the resulting product derived from the mouse SOD3 was exclusively aEC-SOD [26]. Collectively, these studies suggest that the interplay between the reduction and oxidation of disulfide bonds in a given subunit may provide a redox-sensitive switch to determine SOD3 activity.

2.2.2 Intersubunit Disulfide Bond

The C-terminus of SOD3 contains a cluster of six basic amino acid residues [18] essential for binding of SOD3 to heparan sulfate [27, 28], collagen type I [29], and other cell surface-associated sulfated glucosaminoglycans [30] within the ECM. The conserved cysteine, Cys219 located within the C-terminus, forms an inter-subunit linkage between two full-length SOD3 monomers, bringing the two ECM binding regions in close proximity. The interaction of the positively charged ECM binding region in SOD3 with the negatively charged matrix components is responsible for the abundant localization of SOD3 to the lung and pulmonary vasculature [31, 32]. Recent data has also provided new insight into the redox-sensitive intracellular equilibrium between monomeric and dimeric SOD3, and confirmed that Cys219 impacts the susceptibility of the C-terminus to proteolytic degradation. Briefly, Cys219 can undergo disulfide bond formation to form dimers or react via cysteinylation or another yet unidentified protein interaction to maintain SOD3 as a monomer. Once secreted, the SOD3 dimers are resistant to proteolysis, while the monomeric form of SOD3 is susceptible to proteolysis in the setting of oxidative stress [33]. These data collectively demonstrate the importance of the cysteine residues and their redox reactions on the structure and localization of SOD3.

2.2.3 Proteolytic Cleavage of SOD3

Redox reactions not only can impact SOD3 directly by influencing cysteine disulfide bond formation, as discussed above, but also can modulate other key processes that in turn influence SOD3 structure or gene expression. Proteolysis is a major regulator of SOD3 biodistribution and contributes to the loss of bound SOD3 in various lung diseases [11–13]. Though the proteolytic processing of SOD3 has not been directly studied in PH, a number of proteolytic enzymes are increased in human PAH and animal models of PH. For example, among the complex vascular abnormalities involved in establishing PAH, there is an imbalance of matrix metalloproteases, adamalysins, serine elastases, and their inhibitors are involved in the excessive ECM synthesis and abnormal degradation during the remodeling process. Thus, there is a potential link between redox-regulated signaling and loss of SOD3 in the pulmonary vasculature in addition to the lung parenchyma [34].

Early studies reported that tetrameric SOD3 existed in multiple states that are characterized by different binding affinity to heparin. Three different classes of SOD3 are determined by the ratio of monomers containing the C-terminal ECM binding region (intact; Trp1-Ala222) vs. those lacking this ECM binding region (cleaved; Trp1-Glu209). To identify if the heterogeneity in heparin affinity is important for tissue biodistribution, the first studies showed that SOD3 could undergo limited proteolysis by endoprotease LysC, trypsin, and plasmin in vitro with significant loss of heparin binding affinity [7, 14, 15, 27, 35]. Subsequently, it was demonstrated that proteolytic processing of SOD3 occurs intracellularly in a two-step mechanism, which first involves the cleavage of Arg215-Glu216 bond by the endoprotease furin after passage through the Golgi compartment followed by the trimming of remaining basic amino acid residues back to Glu209 by a carboxypeptidase [10, 36, 37]. Cys219, discussed above, is not within the target site for furin, but instead the bond formation of Cys219 determines the susceptibility of SOD3 to furin and other carboxypeptidases to cleave the ECM binding region and release SOD3 from its

bound state within the matrix. Interestingly, a common human single nucleotide polymorphism in SOD3 results in a substitution of the Arg213 for glycine (R213G). This form of SOD3 can still be cleaved by furin but is resistant to complete processing by the carboxypeptidases, generating a mature C-terminus at Gly213 [37]. Recently, Morales et al. reported that neutrophil -derived elastase and cathepsin G cleave the N-terminal part of SOD3, which dissociates SOD3 and results in the formation of monomers that can diffuse into tissue [38]. Though the presence of cleaved vs. uncleaved forms of SOD3 has not been specifically tested in pulmonary vascular diseases, these data cumulatively suggest that the upregulation of proteases in the pulmonary circulation may regulate SOD3 distribution.

2.3 Regulation of SOD3 Activity

Multiple studies have demonstrated that SOD1 and SOD2 enzyme activity can be modulated by posttranslational modifications including nitration, phosphorylation, oxidation, glutathionylation, or glycation [39]. In contrast, there is very limited data on the posttranslational modifications that regulate SOD3 [39]. For example, in SOD1, a series of histidine residues involved in the coordination of catalytic metal ions, Cu and Zn, is oxidized by H_2O_2 [40, 41]. Since the amino acid sequence in the enzymatic region of SOD1 and SOD3 are highly homologous [18], and share a similar structural fold [9, 21], it is plausible that the same residues that are targets in SOD1 will also be subject to oxidative modifications in SOD3. Though a comprehensive evaluation of specific amino acid residues susceptible to protein modifications have not been performed for SOD3, it has been shown to be modified by hypochlorous acid (HOCl) and H_2O_2 oxidation that alters its biodistribution and enzymatic activity, respectively [38, 42–44]. In one study, neutrophil-derived HOCl at the inflammatory site of lung injury has been shown to target SOD3 for protein folding destabilization by oxidative modification of the N-terminal region and the formation of intermolecular crosslinking, which the authors speculate increase its susceptibility for proteolysis by neutrophil-secreted proteases. The resultant proteolyzed SOD3 is thought to lead to its change in its biodistribution from the ECM to BALF [38]. In an another study, H_2O_2 has been shown to inhibit/disrupt SOD3 enzyme activity in a peroxidase reaction involving the oxidation of histidine residues in the copper-containing catalytic core [11] similar to that described for SOD1 [45]. The inhibition of SOD3 enzymatic activity has also been demonstrated in a lamb model of persistent pulmonary hypertension of the newborn (PPHN) by H_2O_2 and this activity was restored by intratracheal administration of PEG-catalase [44]. We also previously found that the loss of SOD3 activity immunoprecipitated from lungs of hyperoxia exposed neonatal rats showed a higher nitrotyrosine content [46]. Another level of regulation of SOD3 is via the redox-sensitive membrane bound copper transport protein, Menkes ATPase, ATP7A. This copper ATPase is critical for providing the copper to the active site of SOD3 and its function is impaired by oxidation of cysteine residues [47]. These data suggest that SOD3 activity, similar to the other SODs, may be regulated by these specific redox-dependent protein modifications.

2.4 Regulation of SOD3 Gene Expression

One unexplored area of redox-dependent regulation is the transcriptional regulation of *SOD3* through the modulation of transcription factors that bind to the promoter region, or epigenetic mechanisms including DNA methylation, histone acetylation, and miRNA. The initial physiologic response to oxidative stress is an adaptive increase in antioxidant enzyme activity. However, unlike *SOD1* and *SOD2*, the *SOD3* gene does not appear to be regulated directly by the transcription factors, Nrf2 (nuclear factor E2-related factor), a classic redox-regulated transcription factor, nor hypoxia inducible factor HIF-1α. However, *SOD3* transcription is induced by the Sp1/Sp3 transcription factors as well as a number of cytokines including TNF-α, that are induced by other redox sensitive transcription factors in the setting of inflammation and oxidative stress [48]. On the other hand, the cell specific expres-

sion of *SOD3* in the pulmonary circulation and lung, and the loss of *SOD3* in cancers and pulmonary artery smooth muscle cells from individuals with IPAH can be regulated by DNA methylation or histone acetylation [49]. We recently reported that in IPAH human pulmonary artery smooth muscle cells, *SOD3* expression can be increased by molecular silencing or pharmacologic inhibition of Class I HDAC3 [5, 50–52]. There is also indirect evidence that miR21 could regulate *SOD3* expression in PAH, as this specific miRNA is increased in PAH and found to inhibit *SOD3* expression in lung epithelial cells [53–55]. The specific mechanisms responsible for these processes, both redox-regulated transcription factor activation and epigenetic regulation, require further investigation.

2.5 Redox Regulation by SOD3 in Pulmonary Hypertension

The biologic impact of SOD3 has been attributed to its ability to limit reactions of extracellular $O_2^{\bullet-}$ and preserve nitric oxide bioactivity in blood vessels [4, 56]. Thus, alterations in vascular SOD3 directly affect the redox state of the pulmonary circulation and modulate redox sensitive signaling pathways. This was demonstrated in chronically hypoxic mice in which overexpression of lung SOD3 prevented the hypoxia-induced decrease in lung GSH/GSSG [17]. Our lab and others have found that augmenting lung SOD3 either in genetically engineered mice with lung specific overexpression of SOD3 or using adenoviral SOD3 gene delivery protects in rodent models of chronic hypoxia, monocrotaline or bleomycin induced pulmonary hypertension [17, 57, 58]. Several studies also demonstrate that lack of total body SOD3 or vascular SOD3 augments chronic hypoxia or monocrotaline induced PH [59, 60]. In these models, several redox sensitive targets are modulated in the setting of increased or decreased SOD3. In the mouse model of chronic hypoxic PH, overexpression of lung SOD3 prevented the hypoxic induction of the transcription factor early growth response-1 (Egr-1), and its downstream target tissue factor [17]. We observed a similar attenuation in the induction of Egr-1 as well as TGF-β gene expression in the mice overexpressing lung SOD3 in the bleomycin-induced model of lung fibrosis and PH [58]. Conversely, in pulmonary artery smooth muscle cells, knock down of SOD3 increased Egr-1 and enhanced cell proliferation [61]. Furthermore, in mice with decreased vascular SOD3 due to an inducible knock out of SOD3 in smooth muscle cells, we observed a decrease in eNOS and GTP cyclohydrolase-1, the rate-limiting enzyme in the synthesis of tetrahydrobiopterin [59]. Collectively, redox-regulated processes are key to both the regulation of SOD3 and effects of low vascular SOD3 in pulmonary hypertension and substantial work is needed to more fully understand this process and harness this information to improve therapies.

3 The Mitochondrial SOD2 Enzyme

3.1 Characterization of SOD2

After the isolation of SOD2 from prokaryotic cells [62] and later from eukaryotic cells [2, 63], Barra and coworkers described the primary structure of human SOD2 [64]. The native enzyme was suggested to be composed of two identical polypeptide subunits containing 196 amino acid residues each. One feature that distinguished SOD2 of eukaryotic sources was the presence of cysteine residues which are absent in prokaryotic SOD2. When comparing different species, there was less sequence homology between SOD2s that observed for SOD1 between species. Also, no sequence homology was found between human SOD2 and SOD1. However, the predicted secondary structure and the optical characteristics of human SOD2 are similar to SOD2 isolated from *S. cerevisiae* as well as from prokaryotes including *E. coli* and *B. stearothermophilus* [65]. The overall structure of SOD2 is composed of a mixed α-helix and β-sheet in agreement with the described for the crystalized form of SOD2 from *T. thermophilus* [66].

The SOD2 protein has been purified and crystalized from human liver to characterize its subunit association and sulfhydryl reactivity. Matsuda and coworkers [67] described the human SOD2 as a tetrameric enzyme consisting of four identical subunits, and identified four reactive thiols per tetramer under non-denaturating conditions, and eight reactive thiols/tetramer when denaturating conditions were employed. They concluded that one out of the two cysteine residues in the SOD2 monomer was exposed and accessible for reaction with Ellmans' reagent and sensitive to alkylation by iodoacetamide. The remaining cysteine residue (Cys140) was buried and is only accessible for reaction when the protein is completely denaturated. They did not find changes in the enzymatic activity when wild type SOD2 enzyme was treated with thiols.

3.2 Redox Regulation of SOD2

The tetrameric interface of the SOD2 molecule has been analyzed using the wild type and two polymorphic variants at residues Ile58 and Leu60. The Ile58 to Thr58 variant has been found in the SOD2 gene in two out of six human cDNAs libraries [68, 69]. In addition, the coding mutation Leu60 to Phe60, was described in the T-cell leukemia-derived cell line [70]. Both Thr58 and Phe60 SOD2 variants produce a defective protein with modified assembly and activity. Both are located in the four-helix bundle interface. The hydrophobic residue Ile58, is the largest contributor of the buried surface area interacting with six other residues [71]. Replacing Ile58 with the hydrophilic Thr58 destabilizes the four-helix bundle, leading to decreased enzyme activity by a reduction in tetramer thermostability. Heat treatment of wild-type SOD2 at 41.7 °C for 3 hours does not alter its activity, but completely inactivates the Thr58 variant. In addition, the half-life at 37 °C was calculated to be 3.1 years for the wild-type human SOD2, and only 3.2 hours for the variant [72]. The Thr58 and Phe60 variants with weakened dimer–dimer interaction expose the buried Cys140 residue. Both showed thermal instability at 55 °C, and lose activity at 37 °C in the presence of 5 mM *N*-ethylmaleimide, whereas wild type SOD2 does not. When serine was substituted for Cys140 by site directed mutagenesis, both double mutants Ile58Thr/Cys140Ser and Leu60Phe/Cys140Ser lose their increased thiol sensitivity and show no activity loss in the presence of *N*-ethylmaleimide [73]. The authors suggest that cells expressing these SOD2 variants may be compromised at a physiologic body temperature, and may compensate by inducing SOD2 expression.

3.3 Regulation of SOD2 Activity

SOD2 inhibits peroxynitrite formation in mitochondria, and it may be itself inactivated by excess peroxynitrite [74]. In vitro, peroxynitrite-mediated nitration of Tyr34 of human SOD2 results in enzyme inactivation [75–78]. This process has been implicated in renal failure in which, though the quantity of SOD2 protein is increased in rejected human renal allografts, the activity level is impaired. MacMillan-Crow et al. demonstrated that peroxynitrite inactivates SOD2 enzymatic activity by tyrosine nitration and the presence of a nitrated and dysfunctional enzyme in rejected human renal allografts strongly supports the relevance of this process in vivo [79]. Furthermore, decrease in SOD2 activity has been associated with mitochondria dysfunction, over production and leakage of ROS and the consequent redox changes in the cytoplasm. Significant reduction of SOD2 specific activity has been documented in human (IPAH) and rat (FHR) pulmonary smooth muscle cells [80] and in total lungs from PH patients [81]. However, the mechanisms of SOD2 activity inhibition in PH remain to be elucidated.

3.4 Regulation of SOD2 Gene Transcription

The molecular structure and the organization of the human *SOD2* gene was first described by Wan et al. [82]. Both human and mouse SOD2 have two regulatory regions, promoter and enhancer. The transcription initiation site is local-

ized after a G + C rich (78%) promoter region containing a cluster of seven Sp1 and three AP-2 consensus sequences, with no TATA box or CAAT box. In addition, the 3′ flanking region of the *SOD2* gene contains one NF-κβ consensus sequence. The authors suggested that the presence of such sequences may play a role in the regulation of the expression of the human *SOD2* gene. The Sp1 binding sites in the 5′ region of the *SOD2* gene are necessary for the synergistic induction of SOD2 expression by interacting with the distal enhancer through a DNA loop [83]. There is a sequence containing 11 consecutive guanine nucleotides responsible for the D-loop structure which allow the binding of the RNA-binding protein [84], and thus the complex interaction of Sp1, NF-κβ, and p53 factors to regulate the constitutive and inductive expression of the *SOD2* gene [85]. Previously, it was shown the functional mechanism of basal SOD2 expression regulated by the Sp1/Sp3 ratio, where Sp3 binding have a lower effect in the induction of the *SOD2* gene [86]. Binding of AP-2 to the SOD2 promoter has been proposed as a negative regulator of the expression of the *SOD2* gene through a mechanism involving the Sp1–AP-2 ratio [87]. Also, a FOXO3 binding site located in the 5′ flanking region has been proposed to exert a regulatory role in the transcription of the *SOD2* gene [88, 89]. The promoter region is metabolically controlled, and polymorphisms in three loci of the *SOD2* gene have been associated with the levels of expression of the gene in cancer cell lines [90]. An enhancer element in intron 2 of the gene has been described as well [90, 91]. This region confers inducibility by inflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1 (IL-1), and interferon-γ [92, 93]. This enhancer element is responsive to inflammatory and perhaps hormonal regulators. Also, it is one polymorphism located near the enhancer region of the *SOD2* gene (G1677 T) between two potential AP-1-binding consensus sequences. The activity of the AP-1 binding protein is redox regulated. We identified an association of T allele with a decreased risk of lung cancer [94]. There are several studies regarding the redox regulation of the human *SOD2* gene.

Early studies of SOD2 biosynthesis regulation were done in *E. coli* by Gardner and Fridovich [95]. They show inhibition of SOD2 synthesis and suppression of transcription associated with changes in the redox state of the cells by using thiols such as glutathione, dithiothreitol and *β*-mercaptoethanol. They suggest that thiols directly may reduce a regulatory protein which suppresses transcription in its reduced state, but not in its oxidized one. One protein was isolated from S-30 extracts, which can interact with the palindromic region located near the transcription start site. Another possible option was that thiols can prevent the formation of one "compound" which causes increased transcription of the *SOD2* gene. The candidate was an aminoacyl *t*-RNA synthetase, because it is sensitive to oxidative inactivation, and because it can be protected by thiols. Later it was shown that depletion of glutathione by using thiol-modulating agents, such as *N*-ethylmaleimide, induces an increase of SOD2 activity in human lung fibroblasts [96].

Reactive thiols have been associated with the activation of NF-κβ and the consequent SOD2 expression by cytokines in human lung adenocarcinoma cells [97, 98]. Das and collaborators described how cytokines such as TNF-α or IL-1 enhance the activity of NF-κβ, which results in the activation of SOD2 expression, and how thiols (*N*-acetyl cysteine, dithiothreitol, and *β*-mercaptoethanol) modulate the regulation of SOD2 expression through the cytokine-NF-κβ and/or AP-1 activation. TNF-α and H_2O_2 appear to inhibit the induction of human *SOD2* gene through different pathways [99]. NF-κβ-DNA binding and expression of *SOD2* gen induced by TNF-α is inhibited by pretreatment with *N*-acetyl cysteine. Also, H_2O_2 pretreatment increased expression of SOD2 mRNA, while pretreatment with *N*-acetyl cysteine blocked that induction. Also, addition of diamide, which oxidizes sulfhydryl groups, increases the expression of *SOD2* gene [99]. However, no consistent redox regulation or changes in AP-1-DNA binding by modulated thiols on human *SOD2* gene expression were found [98, 99]. The nuclear factor NF-κβ exists in the cytoplasm in an inactive disulfide form, and its reduction may be required to allow

its DNA binding activity. The p50 subunit of NF-κβ is an disulfide-linked dimer, and mutations in the Cys62 residue alters the DNA binding and thiol sensitivity of NF-κβ [97]. Dhar et al. showed in vivo reduction of SOD2 induction mediated by p50 repression after 12-*O*-tetradecanoylphorbol-13-acetate treatment [100].

Superoxide and peroxynitrite have been suggested to regulate the *SOD2* gene expression. Earlier studies in human tracheal epithelial cells showed a 2–3 fold increase in SOD2 message levels when exposed to superoxide produced by the xanthine/xanthine oxidase system [101]. However, no direct mechanism of the gene regulation by superoxide has been described. In contrast, peroxynitrite appears to regulate SOD2 expression in the rat SOD2 promoter in vitro. Experiments using a promoter-luciferase reporter assay driven by the rat SOD2 promoter showed an increase in SOD2 message levels in lung epithelial cells when exposed to peroxynitrite [102]. The authors suggest that such induction of expression may involve a direct activation of intracellular signaling pathways, or indirect activation of an oxidant sensitive nuclear factor.

The SOD2 promoter contains CpG islands, which could be target for methylation and therefore epigenetically regulate SOD expression. Cytosine methylation in the SOD2 promoter has been associated with epigenetic silencing of the *SOD2* gene expression in cancer [103–105]. Likewise, high levels of histone acetylation of the proximal promoter of the *SOD2* gene were associated with decrease of the SOD2 expression in cancer cell lines [106, 107]. The inhibition of methyl transferases in pancreatic carcinoma cell lines reduced the methylation levels in the SOD2 promoter and reestablished the SOD2 transcription levels [108].

3.5 Redox Regulation by SOD2 in Pulmonary Hypertension

Oxidative stress is a product of either the increased production of reactive oxygen species (ROS) and/or the diminishing of antioxidants. The mitochondria are the main sources of $O_2^{\bullet-}$ production, and their damage and consequent changes on the cellular redox balance contribute to the development and worsening of pulmonary hypertension.

Reduction of catalase and SOD2 has been shown in the development of pulmonary hypertension (PH) in a lamb model of increased postnatal pulmonary blood flow [109]. Increased lipid peroxidation and reduced plasma glutathione peroxidase is correlated in patients with cardiac insufficiency and pulmonary hypertension. The use of antioxidants improves the prognosis in patients with PPH [110]. Early studies showed than SOD2 protects pulmonary epithelial cells from oxygen injury [111]. Because of the chronic inflammatory environment created under those circumstances, a vascular endothelial dysfunction is expected to be associated with late complications. It is possible the existence of a unifying mechanism linking mitochondrial dysfunction, reactive oxygen species unbalance, endothelial dysfunction, and unregulated proliferation observed in chronic pathologies such as obesity, type 2 diabetes, cardiovascular disease, and pulmonary arterial hypertension. There are evidences of a redox signaling (O_2-sensitive) pathway involved in the intersection of pulmonary hypertension and cancer. Some new mitochondrial abnormalities, such as activation of a pyruvate dehydrogenase kinase, SOD2 deficiency, and fragmentation and hyperpolarization of the mitochondrial reticulum have been found in PH [112]. The use of anti-inflammatory drugs has shown to decrease superoxide radicals improving the response of pulmonary arteries to vasodilators in a lamb persistent pulmonary hypertension model [113].

Decreased SOD enzymes have been associated with PH. SOD2 deficiency has been identified in the pulmonary arteries and plexiform lesions of PAH [80, 81]. In a fawn-hooded rat model (FHR), it was observed downregulation of SOD2 preceding the development of PAH [80]. Archer's group discovered a selective hypermethylation of the CpG islands on the intron 2 of the *SOD2* gene in pulmonary smooth muscle cells (PASMC), but not in the aortic smooth muscle cells of the fawn hooded rat. Such hypermethylation reduces its expression ~50% compared to

PASMCs from genetically matched consomic rats, which contributes to the proliferative, anti-apoptotic phenotype of the PASMC of the FHR [114]. This study demonstrated that SOD2 methylation is important in the development of PAH and contributes to HIF-1α activation and the development of an apoptotic resistant state with marked proliferation. Previously, it was showed that SOD2 regulate and stabilize HIF-1α via $O_2^{\bullet-}$ availability [115]. Overall, we conclude that both SOD3 and SOD2 are regulated by oxidative stress and redox-dependent signaling mechanisms and impaired activity of these enzymes alters downstream redox-sensitive targets and contributes to the pathogenesis of pulmonary hypertension.

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