ROS Signaling in the Pathogenesis of Acute Lung Injury (ALI) and Acute Respiratory Distress Syndrome (ARDS)

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1 Pathophysiology

Acute respiratory distress syndrome (ARDS) is a critical noncardiogenic syndrome caused by heterogeneous pathologic factors, and is characterized by acute development of respiratory failure, bilateral diffuse lung infiltrations, and severe hypoxemia. The severity of ARDS is associated with poor prognosis and higher mortality. The first description of ARDS was published in 1967 by Ashbaugh et al. [1] where it was defined as acute lung injury developed after various traumas, drug ingestion, aspiration, bacterial or viral pneumonia, sepsis, etc. Thus, ARDS is a syndrome characterized by tachypnea, hypoxemia and loss of lung compliance and diffuse alveolar infiltrations that does not respond to ordinary methods of respiratory therapy, closely resembling infantile respiratory distress syndrome [1]. The new ARDS definition, formulated in 2012 (the Berlin definition), divided ARDS into three categories using hypoxemia as one of main diagnostic parameters. According to the Berlin definition, hypoxemia is defined as decreased arterial blood oxygen tension (PaO_2) to fraction of inspired oxygen (FiO_2) ratio with 201–300 mm Hg for mild ARDS, 101–200 mm Hg for moderate ARDS, and ≤100 mm Hg for severe ARDS [2]. Acute lung injury (ALI), which is similar to mild ARDS, has been excluded from the new definition. Under normal conditions, the dynamic equilibrium between fluid formation and clearance across lung epithelium is strictly regulated [3, 4] and the pulmonary edema associated with ARDS is the result of a loss of the barrier functions of the lung capillary endothelium and alveolar epithelium resulting in vascular leakage and extravascular water accumulation in the lung. Thus, impaired alveolar liquid clearance is characteristic for the majority of patients with lung injury [5]. Similarly, injury to the lung endothelium also leads to fluid hyperpermeability, increased production of pro-inflammatory factors and increased expression of the adhesion molecules needed for leukocyte recruitment and neutrophil migration across the endothelium into the lung. These activated neutrophils induce tissue damage by secreting cytotoxic agents such as granular enzymes, pro-inflammatory cytokines, ROS, and bioactive lipids. The variety of pathologic stimuli leading to ARDS is indicative of the existence of numerous independent risk factors associated with the syndrome. These factors may have either a direct effect on ARDS severity or may potentiate complications by activating inflammatory processes or impairing lung function. However, sepsis is the main risk factor,

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Y.-X. Wang (ed.), *Pulmonary Vasculature Redox Signaling in Health and Disease*,
Advances in Experimental Medicine and Biology 967, DOI 10.1007/978-3-319-63245-2_8

although not all severe sepsis patients suffer from ARDS. Although it should be noted that ARDS developed in sepsis patients is associated with fourfold higher risk of mortality (14 vs. 60%) [6]. Comparative analysis of clinical data shows a good correlation between the Berlin definition of ARDS severity and mortality rates (27%, 35%, and 45%, for mild, moderate, and severe ARDS, respectively), increased lung weight by CT scan, and duration of mechanical ventilation in survivors (6, 12, and 19 days) [2]. Studies have also revealed a correlation between ARDS severity categories and the volume of extravascular liquid accumulated in the lungs (16.1, 17.2, and 19.1 ml/kg) [7]. Early detection is the best way to attenuate the development of ALI/ARDS. However, standard chest X-ray diagnostics may be insensitive for the early phase of ARDS when pulmonary edema is hard to detect and other imaging systems such as lung ultrasound, CT scan, and positron emission tomography are being evaluated [8–12]. Other approaches are investigating the possibility of using biomarkers to identify early signs of ARDS. Several diagnostic methods based on a clearance of isotope-labeled low-molecular weight compounds or pulmonary vessel leakage assessment [13], evaluation of the levels of VEGF, interleukin-2 (IL-2), interleukin-8 (IL-8), and other pro-inflammatory markers in BALF and plasma samples [14–16] have been proposed. Further, a thorough comparative study of biomarkers of inflammation, fibroblast activation, proteolytic injury, lung endothelial and epithelial injury in severe sepsis patients with or without ARDS has identified at least five biomarkers characteristic for ARDS that appear to be suitable for further diagnostics in plasma or bronchoalveolar lavage fluid (BALF) samples: surfactant protein-D (SP-D), receptor for advanced glycation end-products (RAGE), IL-8, club cell secretory protein (CC-16), and interleukin-6 (IL-6) [16]. Indeed, a critical care randomized trial demonstrated that early alveolar damage can be identified by the presence of SP-D in blood. Significantly increased levels of SP-D is a strong independent predictor that the patient suffers from ARDS who will not recover [17]. Due to heterogeneity of ALI/ARDS etiology and complexity of the syndrome, there is no efficient therapy available. Mechanical lung ventilation as a supportive clinical approach for oxygenation of healing lungs is a standard therapeutic method for ARDS patients. However, mechanical ventilation itself can lead to biotrauma increasing lung inflammation and worsening clinical condition due to ventilation-induced lung injury (VILI) [18–20]. Recent studies have demonstrated that injured lungs can be optimally supported by low-tidal volume ventilation and that this requires personalization of the settings [20–23]. Lung ventilation can also be supplemented by anti-inflammatory medicine. First applied for ARDS treatment by Ashbaugh and colleagues [1], corticosteroids are still considered valuable for the treatment [24], as a major anti-inflammatory agent.

Excessive ROS generated by the injured endothelium/epithelium as well as recruited leukocytes plays a major role in ARDS progression and lung damage. Oxidative stress can be a cause of the endothelial and epithelial barrier dysfunctions resulting in massive neutrophil penetration across the barriers followed by secretion of cytotoxic agents (Fig. 1). ROS upregulate the expression of pro-inflammatory cytokines and adhesion molecules amplifying the tissue damage and pulmonary edema. Thus, a proper oxidant–antioxidant balance is critical for vasculature homeostasis. Therefore, the systems responsible for excessive ROS production can be therapeutic targets in ARDS treatment. The following sections summarize our current knowledge regarding ROS generation and their effects on ARDS development and discuss possible approaches to prevent or minimize ROS-induced pulmonary damage.

2 Biological Origins of ROS in Vasculature

2.1 Cellular Free Radicals

Free radical intermediates generated during the reduction–oxidation (redox) reaction involving the conversion of molecular O_2 to water are called

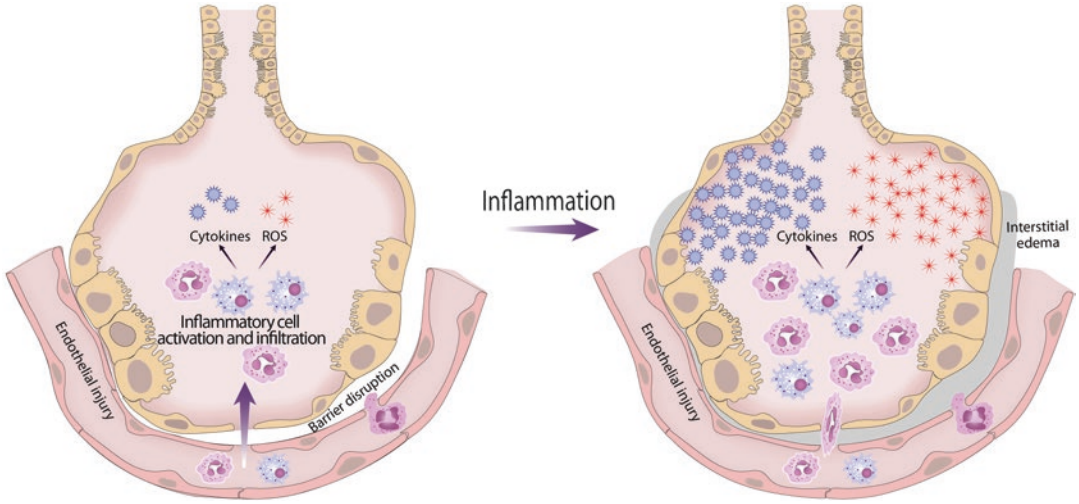

Fig. 1 Dysfunction of microvascular endothelium and alveolar epithelium in ARDS. Polymorphonuclear leukocytes (PMNs) and macrophages infiltrate the inflamed region through the microvascular blood vessels releasing cytotoxic factors such as pro-inflammatory cytokines and ROS. Theese cytokines and ROS contribute to the endothelial and epithelial dysfunction resulting in leakage of fluids from circulation into the interstitial space and alveoli. This results in pulmonary edema and impaired gas exchange. Sources of inflammation range from bacterial infections to mechanical ventilation

ROS. Molecular oxygen (O_2) as such is a free radical having two unpaired electrons with the same spin quantum number or parallel spin. When O_2 tries to oxidize a non-radical by accepting a pair of electrons with antiparallel spin, these electrons do not match the spin number in O_2. Therefore, the reaction of O_2 with non-radicals is thermodynamically unfavorable. However, O_2 can readily accept single electron transfers from other free radicals [25]. For example, during aerobic respiration, cytochrome oxidase of mitochondria catalyzes four such single electron transfers to molecular O_2 from two reduced heme (Fe^{2+}) and two copper (Cu^{+}) ions coupled with proton translocation resulting into molecular water, energy, and oxidized cytochrome [26, 27]. The cytochrome oxidase enzyme operates under severe constraints to prevent release of partially reduced, toxic, and high energy oxygen free radicals. Cells can efficiently reduce almost 95% of molecular oxygen that we consume to water by aerobic respiration. When a single electron is transferred to molecular O_2, the resulting product is a superoxide ($O_2^{\bullet-}$) free radical. Another electron transfer to $O_2^{\bullet-}$ results into peroxide (O_2^{2-}) ion. This reaction may be spontaneous dismutation or catalyzed by superoxide dismutase (SOD) [28]. Hydrogen peroxide when completely reduced is converted to molecular water and O_2. Partially reduced hydrogen peroxide in the presence of transition metals yield the most potent hydroxyl ($OH^{\cdot}$) free radical [29]. Together, these oxygen free radicals are called ROS. The other important free radical in the vasculature is nitric oxide ($NO^{\cdot}$). Under physiological conditions, $O_2^{\bullet-}$ reacts with $NO^{\cdot}$ to form a highly oxidative, reactive nitrogen species (RNS), peroxynitrite ($ONOO^{-}$). These oxidizing and nitrating free radicals can severely damage cellular macromolecules such as lipids, proteins and DNA even under normal physiological conditions [30]. Therefore, antioxidant mechanisms that neutralize the highly oxidative free radicals are critical for cell survival.

2.2 ROS in Vascular Tissue

The generation of ROS is an unavoidable consequence of living in an oxygen rich environment and organisms have evolved with elaborate

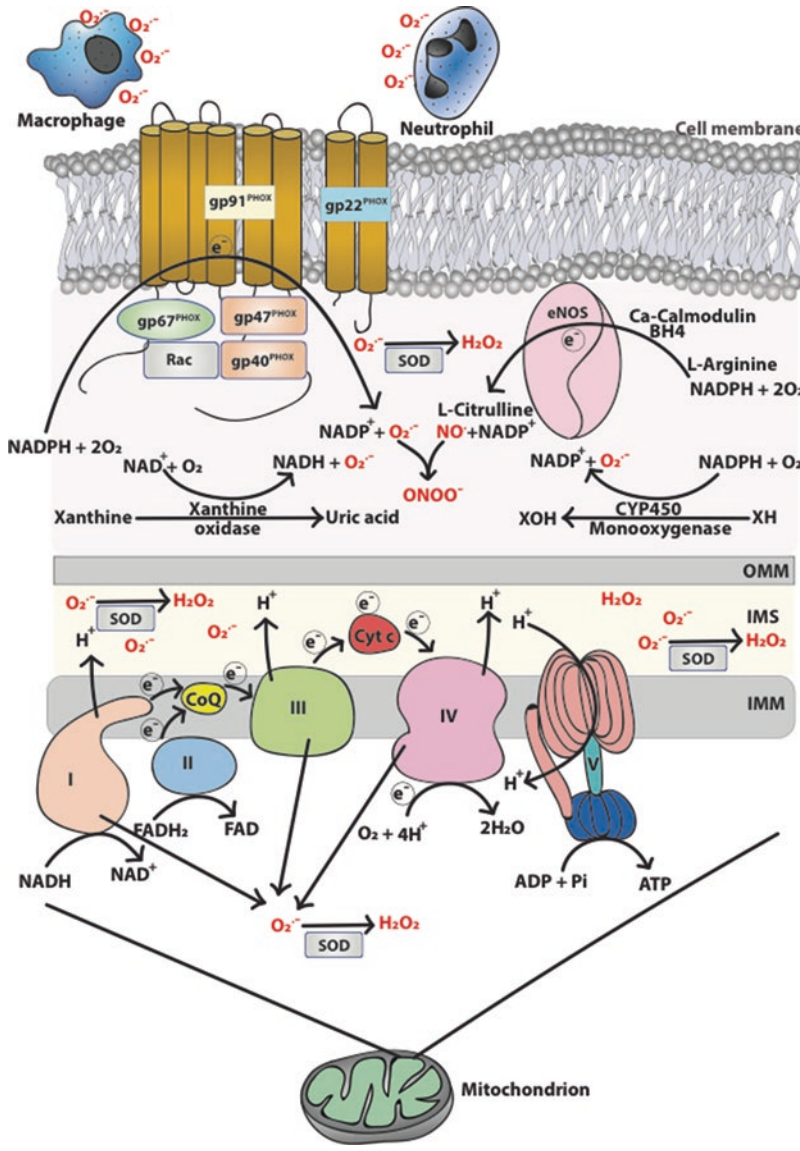

Fig. 2 Sources of reactive oxygen species. Mitochondria, NADPH oxidase, xanthine oxidase, and eNOS are the major contributors of ROS in cells of vasculature during active metabolism. NADPH oxidase in phagocytic cells such as macrophages and neutrophils that are resident in blood vessels contribute to a significant amount of superoxide ($O_2^{\cdot-}$). Endothelial NOS (eNOS) generates NO free radicals that interact with $O_2^{\cdot-}$ to generate peroxynitrite. Peroxynitrite induces nitrasative stress on cells by nitrating proteins and altering signaling pathways. When eNOS is uncoupled, it can generate superoxide. Oxidative phosphorylations in mitochondria are a source of $O_2^{\cdot-}$. Especially complexes I, III, and IV generate $O_2^{\cdot-}$ when there is a leak of electrons at subsequent transfer stages. $O_2^{\cdot-}$ generated in mitochondria is often immediately dismutated to H_2O_2 by SOD which can cross mitochondrial membrane as well as cell membranes. Other lesser sources of ROS are cytochrome P450 enzymes which often generate $O_2^{\cdot-}$ during detoxification of xenobiotics and they are predominantly expressed in hepatic tissue. *ADP* adenosine diphosphate, *ATP* adenosine triphosphate, BH_4 tetrahydrobiopterin, *Ca-Calmodulin* calcium and calmodulin, *CoQ* coenzyme Q, *Cyt c* cytochrome c, *eNOS* endothelial nitric oxide synthase, $FADH_2$ flavin adenine dinucleotide, H_2O_2 hydrogen peroxide, *IMM* inner mitochondrial membrane, *IMS* inter-mitochondrial membrane space, *NADH* nicotinamide adenine dinucleotide, *NADPH* nicotinamide adenine dinucleotide phosphate, NO nitric oxide, *OMM* outer mitochondrial membrane, *Pi* inorganic phosphate, O_2^- superoxide free radical, $ONOO^-$ peroxynitrite free radical, *SOD* superoxide dismutase, complex I—NADH oxidoreductase (I), complex II—succinate dehydrogenase (II), complex III—cytochrome *c* reductase (III), complex IV—cytochrome *c* oxidase (IV), complex V—ATP synthase (V), *XH* xenobiotic, *XOH* alcohol/aldehyde form of xenobiotic

mechanisms to detoxify these ROS. As shown in Fig. 2 both enzymatic (NAPDH oxidase [NOX], xanthine oxidase, and uncoupled nitric oxide sythase [NOS]) and nonenzymatic (mitochondrial) sources can be major ROS producers in endothelium. The endothelium is more than just a single layer of cells lining the lumen of blood vessels. It is intimately involved in maintaining the homeostasis of vascular tissue. ROS can influence many functions of the endothelium. The endothelium acts as a barrier to prevent leakage of the contents of circulation. It also generates NO which mediates vasorelaxation. Endothelial cells are susceptible to increased ROS generation under various pathological conditions. For example, ROS dependent expression of adhesion molecules such as ICAM-1 and VCAM-1 by endothelial cells can recruit immune cells that themselves express enzymes that generate high levels of ROS. Neutrophils and macrophages recruited to the site of inflammation generate free radicals that contribute to the pool of ROS causing oxidative stress (Figs. 1 and 2). It has been noted that neutrophils can kill endothelial cells by generating ROS [31]. In the lungs multiple cell types including, endothelial cells, neutrophils, eosinophils, alveolar macrophages, and alveolar epithelial cells are major ROS generators. Fibroblasts, perivascular adipocytes and vascular smooth muscle cells are also significant sources of ROS in the vasculature. Within these cells, several enzymes are involved in generating ROS. These include NOX, uncoupled NOS, dysfunctional mitochondria, and xanthine oxidase. There is evidence that all these systems may be involved in the oxidative stress associated with ALI/ARDS and antioxidants have been shown to reduce the severity of ALI/ARDS in multiple mouse models including lipopolysaccharide (LPS) [32–34], influenza A [35], hyperoxia [36], toxic gas [37], ischemia–reperfusion (I/R) [38], sepsis [39, 40], acid aspiration [41], burn and smoke inhalation [42] as well as high tidal mechanical ventilation [43–45]. Figure 2 illustrates the biological origins of ROS in a cell. The following sections will deal with the major ROS generating systems in the pulmonary vasculature and how they are involved in the pathogenesis of ARDS.

2.3 Xanthine Oxidase

Xanthine oxidoreductase (XOR) belongs to the molybdoenzyme family with two interconvertible forms, O_2 dependent type O xanthine oxidase (XO) and NAD-dependent type D xanthine dehydrogenase (XDH). It catalyzes the oxidation of hypoxanthine to xanthine and uric acid in purine metabolism [46, 47]. XOR is a homodimer, and each monomer consists of three domains each harboring cofactors molybdopterin (Mo–Co), two iron–sulfur centers [2Fe-2S], and flavin adenine dinucleotide (FAD) arranged linearly in the order of their redox potentials [48]. In the process of purine metabolism, XO generates ROS, $O_2^{\cdot-}$ and hydrogen peroxide. Early studies using isolated rabbit lungs perfused with XO increased the permeability of pulmonary microvascular endothelial cells implicating the role of XO in lung injury [49]. Reperfusion of rabbit lungs were with XO inhibitor allopurinol or superoxide scavenger, SOD decreased the lung injury [50]. In a VILI animal model, application of high tidal volume mechanical ventilation (HTMV) activated XOR and increased the pulmonary capillary permeability [51]. Treatment of endothelial cells directly with ROS or with XO decreases the transendothelial electrical resistance (TEER) and increases the permeability of macromolecules [52]. Oxidative stress is known to induce apoptosis of epithelial cells during VILI [53]. VILI also induces p38 MAPK mediated inflammatory lung injury [54] and activation of p38 increases XOR enzymatic activity. Pharmacological inhibition of p38-XOR attenuates VILI induced lung injury [55]. These studies indicate a significant role of XOR in ROS mediated lung injury.

2.4 Uncoupled Endothelial Nitric Oxide Synthase

Under normal physiological conditions, endothelial nitric oxide synthase (eNOS) functions as a homodimer to produce the vasodilator signaling molecule, NO. NO is a free radical capable of reacting with ROS to generate RNS [56]. eNOS requires molecular O_2 and L-arginine as

substrates along with cofactors NADPH, 6(R)-5,6,7,8-tetrahydrobiopterin (BH4), FAD, and FMN [57] to produce NO and L-citrulline as a by-products. To understand the role of eNOS in ROS generation, we need to first understand the structure and mechanism of action of eNOS. The C-terminus reductase domain of one monomer in the eNOS homodimer is linked to N-terminus oxidase domain of the other monomer. The homodimer is stabilized by a zinc thiolate cluster which include phylogenetically conserved cysteine residues that bind zinc ion in a tetrahedral conformation [58]. The reductase domain binds to NADPH, FMN, and FAD cofactors, and oxygenase domain binds to cofactor BH_4, and substrates L-arginine and O_2 [59]. The oxygenase domain also carries the prosthetic heme cofactor. Calcium–calmodulin binding sequence is located in the center between the reductase and oxygenase domains. Binding of calcium–calmodulin aligns the two domains for an efficient transfer of electrons from reductase domain to the heme on the oxidase domain, therefore making the eNOS homodimer catalytically active [60]. Two electrons donated by NADPH are transported through flavins, FAD and FMN and subsequently to the heme of the oxidase domain to activate O_2. Reduced oxygen is incorporated into the guanidine group of L-arginine in two steps; step one includes hydroxylation of L-arginine to N^{ω}-hydroxy-L-arginine intermediate and in the second step N^{ω}-hydroxy-L-arginine oxidized to L-citrulline and NO [61]. Since NO generation is a tightly regulated process, pathophysiological conditions causing deficiency of any of these cofactors can lead to generation of superoxide $O_2^{\bullet-}$ which is called uncoupling. Under uncoupled conditions, eNOS can synthesize $O_2^{\bullet-}$ at the heme although this requires binding of calcium–calmodulin. $O_2^{\bullet-}$ generation by eNOS appears to be primarily dependent on the availability of BH_4 rather than L-arginine [62]. It is thought that BH_4 couples L-arginine oxidation to NADPH reduction by preventing the disassociation of the ferrous-dioxygen complex of heme [63], suggesting that BH_4 can be used as pharmacological agent to treat vascular diseases. BH_4 has also been implicated in the maintenance of mitochondrial redox balance [64]. The mechanism by which eNOS becomes uncoupled appears to be through increases in the endogenous NOS uncoupler, asymmetric dimethylarginine (ADMA) and the generation of peroxynitrite [65, 66]. Peroxynitrite can add a nitro group ($-NO_2$) to one ortho carbon of tyrosine's phenolic ring to form 3-nitrotyrosine (3-NT), a process called protein nitration. Protein tyrosine nitration dramatically alters the pKa of the tyrosine hydroxyl group producing structural and function changes within affected proteins [67]. Peroxynitrite can also cause uncoupling of eNOS via the oxidation of zinc thiolate clusters and the formation of disulfide bonds between the monomers [68]. The phosphorylation of eNOS at T495, mediated by protein kinase C (PKC), impairs NO production in the endothelial cells [69] and enhances eNOS uncoupling. pT495 eNOS can be translocated to mitochondria [70, 71] where it increases mitochondrial derived ROS. Proteomic studies have begun to identify nitrated proteins that may be important in ALI/ARDS. The proteins identified so far include sphingosine-1-phosphate lyase 1 (SIP lyase 1) [72], Rho-GTPase-activating protein 5 (RHOGAP5) [72] and RhoA itself [73]. However, RhoA is the only nitrated protein validated being involved ALI/ARDS [72, 74]. Uncoupled eNOS has aslo been shown to be involved in the lung injury associated with G^+ bacterial infections [70], smoke inhalation [75], and high tidal mechanical ventilation [76].

2.5 Mitochondrial Respiratory Chain

Mitochondria are the respiratory centers of the cell where ATP is produced by reducing O_2 to water. A series of single electron transfers are performed across four electron transport complexes (ETC) [77]. The four ETC are arranged in the order of increasing redox potential, between -32 V (NADH of complex I) and +39 V (cytochrome *a3* of complex IV). Complex I (NADH dehydrogenase),

complex III (cytochrome *c* reductase), and complex IV (cytochrome *c* oxidase) pump protons H^+ into the mitochondrial intermembrane space which contribute to the mitochondrial membrane potential that will ultimately drive the ATP synthase motor to generate high energy ATP from ADP and inorganic phosphate. The transfer of electrons across these electron transport carriers is usually highly efficient. However, there is 1–2% leak of electrons that react with O_2 to generate $O_2^{\bullet-}$. Complex I and III are the major contributors of $O_2^{\bullet-}$ in mitochondrion [78]. Electrons donated by NADH to complex I are transported through flavin complex, series of Fe–S clusters to ubiquinone Q. Superoxide can be generated at each of these electron transport steps by complex I [79–81]. Furthermore, any blockade in the electron transport downstream of complex I can result in significant generation of $O_2^{\bullet-}$ by complex I. The presence of a higher NADH/NAD^+ ratio can drive more $O_2^{\bullet-}$ generation into the mitochondrial matrix by complex I [81]. Complex I is major source of $O_2^{\bullet-}$ in skeletal and neural cells whereas complex III is the major source of $O_2^{\bullet-}$ in endothelial cells. Therefore, cell type as well as the metabolic state of the cell determines the source of $O_2^{\bullet-}$ in mitochondria. Complex II (succinate dehydrogenase) is not a significant source of $O_2^{\cdot-}$ in mitochondria. Complex III on the other hand generates $O_2^{\cdot-}$ during the Q cycle which involves transfer of electrons from complex I and II to ubiquinone resulting in reduction of ubiquinone to ubiquinol [82, 83] resulting in the release of $O_2^{\bullet-}$ to both sides of the inner mitochondrial membrane [84]. Oxidation of ubiquinol involves donation of two electrons to cytochrome *c* as single electron transfers through reiske iron–sulfur protein and cytochrome *c*1, resulting in unstable intermediate ubisemiquinone. Ubisemiquinone radical donates the single electron to O_2 to generate $O_2^{\cdot-}$ [85]. $O_2^{\cdot-}$ generated by mitochondria is converted to H_2O_2 by manganese SOD (MnSOD) that can cross the mitochondrial membrane into cytoplasmic compartment. $O_2^{\bullet-}$ and H_2O_2 form the pool of mitochondrial ROS (mtROS). Genetic mutations in nuclear and mitochondrial genes encoding the proteins of mitochondrial respiratory complexes can lead dysfunction of specific electron transport complexes. Defects in complexes I and III can lead to significant increase in mtROS and subsequent pathological conditions [86]. Increase in mtROS can alter the signaling of redox sensitive transcription factors such as HIF-1α which can further alter the metabolic state of the cell [87]. Other important aspects of mtROS is regulation of inflammasome [88], activation of caspases [89] and regulation cell death by apoptosis [90]. Therefore, ROS generated by mitochondria have very broad implications on cellular homeostasis. Excessive demand for ATP or damage to any of the ETC components can result in increased ROS leakage by complexes I, III and IV and consume the antioxidant defenses. This can lead to rupture and release of mitochondrial components including mitochondrial DNA (mtDNA) [91]. Release of mtDNA can induce an inflammatory response [92] through activation of TLR9/NLRP3 inflammasome [93]. Excessive ROS can activate pro-apoptotic Bcl-2 family proteins by increasing mitochondrial permeability to drive the MMP, release cytochrome c, mtDNA [94], and pro-apoptotic caspase-3 and -9. This leads to the activation of intrinsic or mitochondrial driven cell death by apoptosis [95]. Mitophagy removes excessive ROS generating mitochondria to avoid cell death by self-destructive inflammatory response [96]. This is evident by the activation of NLRP3 inflammasome when mitophagy is inhibited [97]. Under normal breathing, lung epithelial cells are adapted to cyclic stretch. However, under mechanical ventilated conditions, increased levels of mitochondrial ROS can be generated by epithelial cells when subjected to cyclic stretch as a result of direct distention of mitochondria. This ROS production is dependent upon the time and magnitude of stretch [98]. Excessive ROS generation can lead to loss of mitochondrial function and apoptosis of lung epithelial cells [99]. Viral infections can also alter the mitochondrial dynamics leading to excessive mtROS generation, mitochondrial biogenesis, and altered mitochondrial β-oxidation [100, 101]. Given the central role of mitochondria in cell physiology, antioxidants to combat the deleterious ROS generated by the mitochondrion could be a potential target for developing therapeutic strategies for ALI/ARDS.

2.6 Cytochrome P450

Cytochrome P450 (CYP) belongs to the family of membrane bound heme-thiolate enzymes involved in oxidative metabolism of a variety of hydrophobic endogenous macromolecules and exogenous compounds such drugs, carcinogens, and xenobiotics by monooxygenation reaction [102]. Most CYP enzymes are predominantly expressed in the liver. Some isoforms of CYP such as 2B, 2C8, 2C9, 2C10, 2J2 are expressed in endothelium and vascular smooth muscle cells where they play important roles in arachidonic acid metabolism and in the maintenance of vascular homeostasis and tone. CYP at resting state have a hexa-coordinated low spin heme (LS) having water molecule weakly bound as the sixth axial ligand. The water molecule is then displaced by the substrate resulting in a penta-coordinated high spin (HS) heme. This LS to HS transition of heme increases its redox potential. HS ferric heme is reduced to ferrous heme by accepting electrons donated by a redox partner; in this case, NADPH-dependent cytochrome P450 reductase, a diflavoprotein that contains FAD and FMN. Oxygen binds to ferrous heme followed by a series of oxyferrous intermediates leading to activation of O_2. Second electron transfer from redox partner to oxyferrous intermediate results in a series of intermediates leading to heterolytic cleavage of O–O bond and generation of highly reactive oxyferryl intermediate which is responsible for monooxygenation of substrates [103]. As an unwanted consequence, activated oxygen at the heme of CYP can lead to generation of ROS, leading to uncoupling of CYP. Indeed, the ROS generated from CYP2C in coronary artery endothelial cells has been shown to impair NO mediated vasorelaxation [104]. Also, the activation of endothelial CYP by hemodynamic stimulus such as cyclic stretch also leads to increased production of $O_2^{\cdot-}$ [105], suggesting that CYP activation could be and important factor in the oxidative stress associated HTMV. The increased ROS production in lung epithelial cells exposed to sulfur mustard [106] or environmental pollutants has also been shown to be dependent on increased CYP1A1 enzyme activity [107]. Other CYPs have also been implicated in the oxidative lung injury associated with hyperoxia (CYP1A1 and CYP1A2) [108, 109] and alcohol abuse (CYP2E1) [110]. Therefore, CYP enzymes likely also contribute to the pool of ROS that can mediate lung injury.

2.7 NADPH Oxidase (NOX)

An increase in leukocyte respiration was observed when these cells exposed to bacteria as early as 1933 by Baldridge and Gerard. During this respiratory burst, the leukocytes generate ROS, superoxide, hydrogen peroxide and hydroxyl free radicals to kill the phagocytosed pathogens [111, 112]. NOX is the enzyme that catalyzes reduction of oxygen to generate superoxide using NADPH [113]. Therefore, NADPH oxidase is often referred as the "professional ROS producer." The phagocytic NOX (NOX2) is a multicomponent enzyme with two membrane bound subunits ($gp91^{PHOX}$, $p22^{PHOX}$) and three cytosolic subunits ($p67^{PHOX}$, $p47^{PHOX}$, and $p40^{PHOX}$). In addition to these subunits, small GTPase Rac1 or Rac2 may be associated with NAPDH oxidase [114]. Membrane bound subunits $gp91^{PHOX}$, $p22^{PHOX}$ form heterodimeric flavoprotein called cytochrome b_{558}. When the cytosolic components migrate to the membrane, the NOX complex can now accept electrons to transfer to O_2 and generate $O_2^{\cdot-}$ [115]. Initially NOX was thought to be expressed only in phagocytic immune cells (hence the name PHOX), but later other homologues of NOX were discovered in non-phagocytic cells types and were designated as NOX family of NADPH oxidases [116]. Seven different NOX isoforms have been identified: NOX1, NOX2, NOX3, NOX4, NOX5, Duox1, and Duox2. Only NOX1, NOX2 and NOX4 are expressed in vasculature and are all implicated with ROS mediated vascular diseases [117]. When exposed to TNFα, human aorta smooth muscle cells rapidly induced ROS generation which mediated by NF-κB induced upregulation of NOX1 and NOX4 [118]. Aldosterone induced expression of NOX1 and superoxide generation which was mediated through PKC delta in

vascular smooth muscle cells [119]. When rat vascular smooth muscle cells are exposed to cigarette smoke extract, NOX1 derived superoxide causes cellular toxicity [120]. Neutrophils, the majority of circulating white blood cells play an important role host defense mechanism against invading pathogen and NOX2 is responsible for the respiratory burst and superoxide generation. Chronic granulomatous is a disease caused by genetic mutations in NOX2 subunits, especially in $gp91^{PHOX}$ and observed predominantly in males because of the presence of gene on the X chromosome [121]. Phagocytic cells with defective $gp91^{PHOX}$ are unable to produce superoxide and patients are susceptible to severe infections [122]. On the other hand, increased expression of $p22^{PHOX}$ by activation of p38-Erk1/2-MAPKinase pathway resulted in ROS mediated endothelial dysfunction in type 2 diabetes mice [123]. NOX2 and NOX4 are also known to mediate ROS dependent proliferative response in microvascular endothelial cells [124]. Rac1 pharmacological inhibition improved function of endothelial cells obtained from vein grafts of patients who underwent bypass surgery due to severe vascular disease. Rac1 inhibition not only reduced NOX dependent ROS but also increased eNOS function by suppressing ROCK1 which is a negative regulator of eNOS [125]. NOX4 is a constitutive producer of ROS at basal levels unlike NOX1 and NOX2 which are signal activated. However, NOX4 increases ROS generation on demand when cells are exposed to inflammatory stimulus. TGFβ induced expression of NOX4 in vascular smooth muscle cells along with inflammatory phenotype commonly seen in atherosclerosis and aging [126]. NOX4 is mainly involved in maintenance of basal ROS mediated signaling of vasculature. NOX4 knockout mice developed cardiac dilation, contractile dysfunction and cardiac failure due to chronic overload suggesting the importance of NOX4 mediated ROS signaling in cardiac function [127]. Together, NOX isoforms contribute widely being beneficial in innate immunity and basal ROS mediated signaling and deleterious in the development several vascular pathological conditions.

Analysis of lung sections and BALF from patients with ARDS show massive accumulation of PMNs especially neutrophils [128]. These cells produce very high levels of ROS that exacerbates the inflammatory responses in lungs. ROS generated by LPS exposure has been shown to be NOX1 dependent in macrophages [129] and NOX2 dependent in the LPS-challenged lung [130]. LPS activation of TLR4 receptor induced NOX mediated ROS production which subsequently lead to activation of pro-inflammatory NF-κB [131] and TNFα signaling [132, 133]. ROS generated by NADPH oxidase of PMNs during hemorrhagic shock which is a known cause of ARDS, activates NOX of endothelial cells through HMGB1, TLR4, and Rac1 signaling pathway [134]. A significant decrease in hyperoxia mediated ROS production was observed in lung epithelial and capillary endothelial cells of NOX1 knockout mice compared to wild type mice, thereby preserving the alveolocapillary barrier [135]. Epithelial cells under cyclic stretch produce ROS and it is NOX dependent [98]. Knockout of NOX1 prevented lung injury in mice exposed to hyperoxia [136]. Inhibition of NOX4 highly expressed in epithelial cells and fibroblasts can prevent epithelial cell death and prevent ROS mediated epithelial cell deatch, inflammation and lung fibrosis [137]. In a gastric acid aspiration mice model, it was shown that NOX was able to limit lung injury by Nrf2 mediated decrease of PMN airway accumulation [138]. NADPH oxidase can also limit lung injury by activation of redox sensitive anti-inflammatory transcription factor NRF2 [139]. Largely described, above studies significantly implicate the role of NOX in ARDS/ALI. Therefore, inhibitors of NOX may utility in the treatment of ARDS.

3 Cellular Defenses Against Oxidative Stress

Since oxidants have the capacity to react in an indiscriminate manner leading to damage of almost any cellular component, an extensive

range of antioxidant defenses have evolved to protect the cell from the oxidant-induced damage. As shown in Fig. 3, there are several enzyme systems that catalyze reactions to neutralize free radicals and ROS. These form the body's endogenous defense mechanisms to help protect against oxidant induced cell damage. The main cellular antioxidant enzymes and their involvement in protection against ARDS will be discussed below.

3.1 Superoxide Dismutase

The enzyme superoxide dismutase catalyzes the dismutation of superoxide to hydrogen peroxide (H_2O_2) [140]. The H_2O_2 must then be removed by catalase or glutathione peroxidase. There are three forms of superoxide dismutase in mammalian tissues, each with a specific subcellular location and different tissue distribution. Copper zinc superoxide dismutase (CuZnSOD) is found in the cytoplasm and organelles of virtually all mammalian cells. It has two protein subunits, each containing a catalytically active copper and zinc atom. Manganese superoxide dismutase (MnSOD) is found in the mitochondria of almost all cells. It consists of four protein subunits, each containing a single manganese atom. The amino acid sequence of MnSOD is entirely dissimilar to that of CuZnSOD and it is not inhibited by cyanide, allowing MnSOD activity to be distinguished from that of CuZnSOD in mixtures of

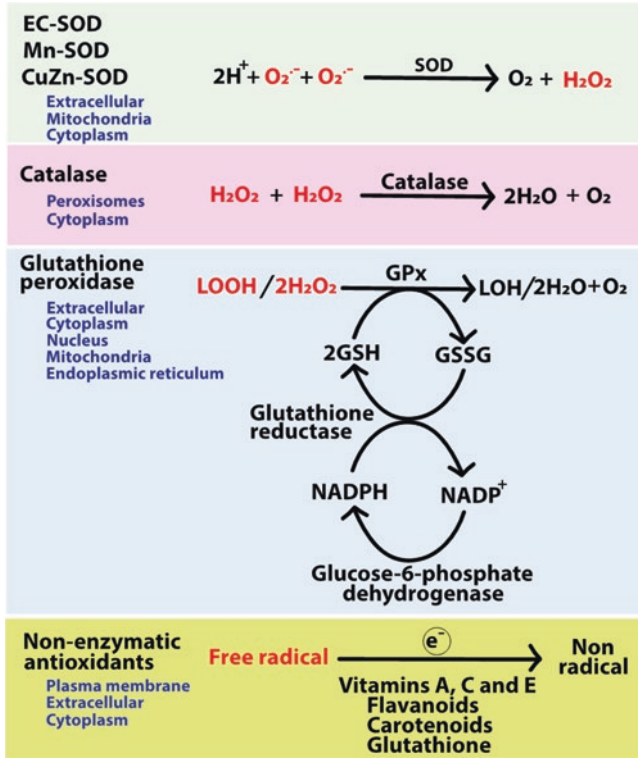

Fig. 3. The antioxidant system in cells. Enzymatic and nonenzymatic antioxidants catalyze reactions to neutralize free radicals by donating electrons. Enzymatic antioxidants catalyze reactions to neutralize specific free radicals such that superoxide dismutase (SOD) dismutates superoxide to hydrogen peroxide (H_2O_2), and catalase and glutathione peroxidase (GPx) convert hydrogen peroxide to water. GPx also converts lipid hyroperoxides (LOOH) to lipid alcohols or aldehydes (LOH). Glutathione reductase replenishes reduced glutathione (GSH) pools from oxidized glutathione (GSSG) using NADPH as reducing equivalents. Nonenzymatic antioxidants such as vitamins, flavonoids and glutathione can also reduce free radicals by donating electrons

the two enzymes. Extracellular superoxide dismutase (EC-SOD) is a secretory copper containing SOD distinct from the CuZnSOD. EC-SOD is synthesized by only a few cell types, including fibroblasts and endothelial cells. A number of studies have shown that exogenously administered antioxidant enzymes, particularly when encapsulated in lipid vesicles (liposomes) or conjugated to polyethylene glycol to prolong biological half-life and aid delivery to cells, can protect against oxidant damage and mitigate the severity of acute pulmonary injury. A synthetic Mn-containing superoxide dismutase mimetic (SODm), M40403, inhibits endotoxin-induced production of TNF-α and IL-6 in alveolar macrophages [141]. MnTMPYP, a superoxide dismutase mimetic, restored the inflammatory responses to LPS challenge including reduced lung myeloperoxidase activity and vascular permeability in mice [142]. In addition to SODs, several other antioxidant agents have been studied in therapeutic applications for lung injury. This includes EUK-8, a synthetic low molecular weight compound with powerful SOD, catalase, and oxyradical scavenging properties. Treatment with EUK-8 ameliorated pulmonary dysfunction in a porcine model of LPS-induced adult respiratory distress syndrome [143]. EUK-8 significantly attenuated many of the features of LPS-induced acute lung injury such as arterial hypoxemia, pulmonary hypertension, decreased dynamic pulmonary compliance and pulmonary edema. The authors concluded that EUK-8 prevents many of the manifestations of LPS-induced adult respiratory distress syndrome by detoxifying reactive oxygen metabolites without affecting the release of other important proinflammatory mediators. In another study, endothelium targeted EUK-134 accumulated in lungs after intravascular injection, providing >60% protection against pulmonary edema in endotoxin-challenged mice [34]. The superoxide scavenger Manganese (III) tetrakis (4-benzoic acid)porphyrin (MnTBAP) played a protective role in alleviating acute inflammatory response and lung injury [144].

3.2 Catalase

Catalase was the first antioxidant enzyme to be characterized; it catalyzes the two-stage conversion of H_2O_2 to water and oxygen. Catalase consists of four protein subunits, each containing a heme group and a molecule of NADPH [145]. The rate constant for the reactions described above is extremely high, implying that it is virtually impossible to saturate the enzyme in vivo. Catalase is largely located within cells in peroxisomes, which also contain most of the enzymes capable of generating H_2O_2. It has been shown that in sheep pretreatment of intraperitoneal injections of catalase attenuated changes in pulmonary arterial pressure, lung lymph flow, and arterial leukocyte counts and oxygen tension after endotoxin infusions [146]. Another study showed that catalase prevents increased lung vascular permeability during air emboli in unanesthetized sheep [147]. In a study targeting catalase to the pulmonary endothelium showed alleviated oxidative stress and reduced acute lung transplantation injury [148]. These studies indicate that H_2O_2 plays a role in the pathogenesis of the acute lung injury and catalase is an important player in development of ALI/ARDS.

3.3 Glutathione and Related Enzymes

Reduced glutathione (GSH) is a major source of thiol groups in the cell [149]. GSH can function directly as an antioxidant, scavenging a variety of radical species, as well as participating in the reactions of glutathione peroxidase. Glutathione peroxidases (GPx) catalyze the oxidation of glutathione at the expense of a hydroperoxide, which might be hydrogen peroxide or another species such as a lipid hydroperoxide (LOOH) [150]. Other peroxides, including LOOH, also act as substrates for these enzymes, which might therefore play a role in repairing damage resulting from lipid peroxidation. GPx require selenium at the active site. Their predominant subcellular distribution is in the cytosol and mitochondria, suggesting that GPx is the main scavenger of H_2O_2 in

subcellular compartments. The activity of the enzyme is dependent on the constant availability of GSH. The ratio of reduced to oxidized glutathione (GSSG) is usually kept very high as a result of the activity of the enzyme glutathione reductase [151]. The NADPH required by this enzyme to replenish the supply of GSH is provided by the pentose phosphate pathway. Glutathione reductase is a flavine nucleotide dependent enzyme and has a similar tissue distribution to GPx. GSH supplementation has been shown to attenuate lipopolysaccharide (LPS)-induced mitochondrial dysfunction in a mouse model of acute lung injury [32]. Selenium, a GPx cofactor, activates GPx in vivo and attenuates lipid peroxidation and lung injury early after paraquat intoxication in rats, but did not affect the survival [152]. Ebselen, an organoselenium compound, mimics GPx activity and showed protective action in animal model of pleurisy [153]. BXT-51072 and BXT-51077, selenium-containing GPx mimics, prevented TNF- and neutrophil-induced endothelial alterations through the downregulation of endothelial proinflammatory responses [154].

3.4 Nonenzymatic Antioxidants

Whenever a free radical interacts with another molecule, secondary radicals may be generated that will further react with the available targets to produce yet more radical species. The classic example of such a chain reaction is lipid peroxidation, and the reaction will continue to propagate until two radicals combine to form a stable product or the radicals are neutralized by an antioxidant. Antioxidants are molecules that can receive an electron from a radical or donate an electron to a radical with the formation of stable by-products. The most important lipid phase antioxidant is probably vitamin E (tocopherol). It quickly reacts with a peroxyl radical to form a relatively stable tocopheroxyl radical, with the excess charge associated with the extra electron being dispersed across the chromanol ring. The dietary supplement γ-tocopherol (γT), a natural form of vitamin E, inhibited LPS-induced increase in BAL fluid total cells, neutrophils, protein, and secreted mucins, along with tissue neutrophil influx [155]. Pretreatment with vitamin E has also been shown to ameliorate acute lung injury induced by burn and smoke inhalation in sheep [156]. The carotenoids are a group of lipid-soluble antioxidants based around an isoprenoid carbon skeleton. The most important of these is beta-carotene, although at least 20 others may be present in membranes and lipoproteins. They are particularly efficient scavengers of singlet oxygen, but also trap peroxyl radicals at low oxygen pressure with an efficiency at least as great as that of alpha-tocopherol. The other important role of certain carotenoids is as precursors of an antioxidant, vitamin A. Flavonoids are a large group of polyphenolic antioxidants found in many fruits, vegetables and beverages such as tea and wine. Over 4000 flavonoids have been identified and they are divided into several groups according to their chemical structure, including flavonols (quercetin and kaempherol), flavanols (the catechins), flavones (apigenin), and isoflavones (genistein). There is evidence that augmenting the intake of flavonoids might improve biochemical indices of oxidative damage and epidemiological studies suggest an inverse relation between flavonoid intake and incidence of chronic diseases [157]. Many flavonoids such as epigallocatechin-3-gallate, xanthohumol, casticin, astilbin, naringenin, apigenin, and baicalin have been shown to protect against ALI [158–164]. Resveratrol, a polyphenolic compound, reduced acute lung injury which was accompanied by activation of Sirtuin1 (Sirt1) and downregulation of NF-κB [165, 166]. α-Lipoic acid (ALA), a cofactor, is essential for energy production and the regulation of carbohydrate and protein metabolism. ALA is synthesized in vivo, however, when ALA is supplemented in the diet, it is readily absorbed and acts as a redox modulator and antioxidant [167]. ALA reduces oxidative stress and prevents against oleic acid-induced ALI [168]. ALA protects against LPS induced acute lung injury (ALI) through activation of heme oxygenase 1 (HO-1) and suppression of NF-κB-mediated inflammatory responses [169]. Qualitatively the most important aqueous phase

antioxidant is vitamin C (ascorbate). Ascorbate has been shown to scavenge superoxide, H_2O_2, hydroxyl radical, hypochlorous acid, aqueous peroxyl radicals and singlet oxygen. Ascorbate undergoes a two electron reduction, initially to the semidehydroascorbyl radical and subsequently to dehydroascorbate during its antioxidant action. Ascorbic acid administered intraperitoneally following lipopolysaccharide infusion attenuated proinflammatory and procoagulant states that induce lung vascular injury in mice model of sepsis [170]. Ascorbic acid (AA) also prevented the toxic effects of zinc oxide nanoparticles (ZnO NPs) inhalation induced acute pulmonary dysfunction including oxidative stress, inflammation, and injury [171].

4 ROS Damaging Effects on the Endothelial Barrier

The pulmonary endothelium, as a semipermeable interface, coordinates the influx and efflux of fluids, solutes, macromolecules and cells from the blood vessel lumen, over the interstitium to the alveolar lumen [172]. Consisting of a thin monolayer of endothelial cells which are connected by a vast amount of junctional proteins, the endothelium forms a tight barrier and lines the entire circulatory system. The pulmonary blood vessel system by itself disposed about a blood surface of 130 m^2. Moreover, by an alveolar-capillary barrier thickness of only slightly more than 0.5 µm, the capillary network forms a dense meshwork close to the alveolar lumen that guarantees an efficient gas exchange [173, 174]. For the exchange of substrates between the blood and alveolar lumen two pathways controlling endothelial barrier have been identified: The transcellular pathway that transports substrates through the body of the cells and the paracellular pathway that transfers material through the intercellular spaces between the cells.

Under normal physiological conditions, fluids and solutes with a molecular radius under 3 nm, utilize the paracellular system to enter the endothelial barrier by a hydrostatic pressure gradient between the intra- and perivascular space. Within the transcellular pathway, aquaporins are the main agents for the transmission of fluids. Macromolecules having molecular radius over 3 nm are actively transcellular transported over caveolae-mediated vesicular carriers [175, 176]. This system ensures that only a highly restricted volume of plasma protein and blood cells can enter into the pulmonary interstitium and thereby avoid alveolar flooding [177]. However, in pathological conditions such as ALI and ARDS, lung endothelial cells are activated or even damaged, which leads to a phenotypic shift with massive functional impairments of the vascular endothelium. Increased vascular permeability combined by an enhanced expression of adhesion molecules creates an excellent environment for trafficking of inflammatory cells and chemotactic substances through the blood barrier [178]. The following section describes the importance and role of ROS as key signaling molecules in the progression of ALI and ARDS by elevated vascular permeability and PMN migration.

4.1 Increased Vascular Permeability Triggered by ROS

To facilitate structure maintenance by adhesion and transmission of mutual information, endothelial cells continuously interact with the extracellular matrix, the basement membrane, and other surrounding cells via specialized protein complexes. The vascular endothelium does not have a rigid and inflexible architecture. Rather it possesses a dynamic structure defined by moment-to-moment changes in the cytoskeleton, cell–cell, cell–basal membrane, and cell–extracellular matrix interactions, by which the transcellular and paracellular transport of fluids and substrates between blood and alveolar lumen is ensured [175] (Fig. 4).

The transcellular transport mechanism is mediated by water channels and vesicle-dependent uptakes of substrates via endocytosis at the apical endothelial, transport across the interior cell body via transcytosis, and substrate release via exocytosis at the basolateral mem-

brane. Hereby, the endocytosis and exocytosis is carried out with individual vesicles shuttles, or interconnected vesiculo-vacuolar organelles (VVOs) that form channel-like structures [179, 180]. Both, caveolae-mediated vesicle transport options, open up the possibility to transit substantial fluid and substrate volumes of around 15–20% of the total cell volume within a very short time [181, 182]. During situations of high ROS levels such as ALI and ARDS, there are changes in the caveolae-based transport. It has been shown that the primary structural protein caveolin-1 required for caveolae formation is affected in a different manner by various ROS. While O_2- and H_2O_2 downregulate caveolin-1 expression, $OH^{\cdot}$ increases it [183]. Moreover, several studies have been demonstrated that the combination of different ROS generated by increased endogenous ROS production correlates with increased cellular transcytosis. Therefore, Angiotensin-II mediated ROS enhancement increases transcellular permeability. The dithiothreitol (DTT)-mediated attenuation of ROS levels leads to a decrease in transcytosis [184]. LPS caused expression of ROS through multiple mechanisms [74, 185, 186], induces mRNA and protein expression and thereby increase the vascular transcellular permeability [187, 188]. Increased caveolin-1 phosphorylation after LPS treatment has also been associated with increased transcellular permeability [188, 189]. Lungs from caveolin-$1^{-/-}$ mice showed a remarkable attenuation of transcellular permeability after LPS treatment [190]. Even thrombin stimulates the transport of fluorescently labeled albumin transcytosis across a confluent

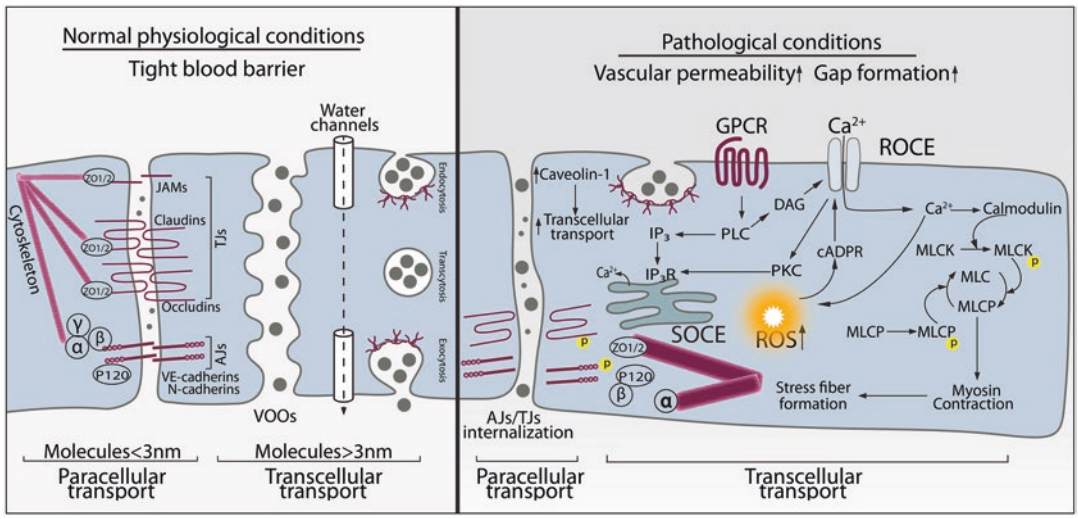

Fig. 4. Comparison of the transcellular and paracellular transport in physiological and pathological conditions. The transport of fluids, solutes, and macromolecules occur over transcellular and paracellular pathways. Under physiological conditions both transcellular and the paracellular transport are highly restricted, whereas under pathological conditions increased vascular permeability can be observed. ROS have several distinct impacts on the endothelial barrier. Initially, within the transcellular pathway caveolin-1 is affected by ROS leading to increased vascular permeability. ROS mainly influence the paracellular pathway through decreased expression and oligomerization of the junctional proteins as well as increases in the phosphorylation of junctional proteins on both serine and tyrosine residues. Both ROCE and SOCE are affected by ROS leading to increased endothelial Ca^{2+} influx. This increases calcium/calmodulin-dependent phosphorylation of myosin light chains leading to myosin contraction. Both ROCE and also SOCE are affected by ROS. *AJs* adherens junctions, *cADPR* cyclic adenosine triphosphate ribose, *DAG* diacylglycerol, *ER* endoplasmatic reticulum, *GPCR* G protein coupled receptor, *IP_3* inositol triphosphate, *JAMs* junctional adhesion molecules, *MLC* myosin light chain, *MLCK* myosin light chain kinase, *MLCP* myosin light chain phosphatase, *PLC* phospholipase C, *PKC* protein kinase C, *ROCE* receptor-operated calcium entry, *ROS* reactive oxygen species, *SOCE* store-operated calcium entry, *TJ* tight junctions, *TRPC/M* transient receptor potential canonical/melastatin, *VOOs* vesiculo-vacuolar organelles, *ZO* zonula occludens

human lung microvascular endothelial cells (HLMVECs) monolayer via enhanced caveolin-1 de novo synthesis [191]. However, caveolin-1 mediated increase of vascular permeability based on increase ROS production is a highly concentration- and time-dependent process [187, 188, 192].

Even through enhanced ROS levels affect the transcellular pathway, under pathophysiological conditions the paracellular transport mechanism is responsible for the predominant amount of blood fluid and proteins passage across the microvascular endothelium. In general, to seal endothelial cells to a tight monolayer together, two different types of intercellular junctions have been characterized as cell-cell adhesive barrier structures: adherens junctions (AJs) and tight junctions (TJs) [175]. Endothelial AJs are mainly composed of two different kinds of transmembrane proteins of the cadherin family, the vascular endothelial cadherin (VE-cadherin) and the neuronal cadherin (N-cadherin). The intercellular protein domains of the transmembrane proteins are connected to the cytoskeleton by α-, β-, γ-, and p120 catenins [193, 194]. The extracellular protein domains of the transmembrane proteins are attached to other extracellular protein domains of adjacent endothelial cells by Ca^{2+}-dependent homotypic interactions. The binding of Ca^{2+} to the negative charged amino acid residues localized on the extracellular cadherin domain causes a change in the protein conformation, which ensures the formation of a tight endothelial monolayer [195]. The structure of endothelial TJs is similar to AJs. However, compared to AJs, TJs consist of transmembrane proteins such as claudins, occludins, and junctional adhesion molecules (JAMs) [175], [196–198]. Claudins and occludins possess four transmembrane domains and two extracellular loops. The intercellular domains of the tight junctions are connected to the cytoskeleton by cytoplasmic adaptor proteins such as zonula occludens (ZO)1, -2 and -3, AF6, or cingullin combined with downstream situated α-catenins. As in AJs, the extracellular loop domains of occludin and claudins form homotypic bindings to extracellular domains of adjacent endothelial cells. JAMs belong to the immunoglobin (Ig) superfamily of proteins. Compared to claudins and occludins, JAMs are composed of only single-pass transmembrane proteins. Currently, three different isoforms of JAMs are known, JAM-A, JAM-B, and JAM-C. The expression pattern of the JAMs varies significantly in different kinds of tissue. JAM-A and JAM-C are predominantly expressed in endothelial cells [199]. The main differences of AJs and TJs exist in pore size or rather potential passing molecule size and appearance. TJs only represent 20% of all existing endothelial junctions and possess a mean pore size of approximately 1 nm. AJs are the most ubiquitous endothelial junctions and have a mean pore size of about 3 nm [175].

In the last decade, an increasing amount of studies described that an enhanced ROS production plays a critical role in initiating the junctional disassembly within ALI and ARDS development [200, 201]. The damage of AJs is mediated by a phosphorylation of serine and tyrosine residues localized in VE-cadherin, β-, and p120-catenin. Only VE-cadherin has five tyrosine residues (Y645, Y658, Y685, Y731, and Y733) and one serine residue (S665) that all can be phosphorylated [202–204]. Vascular endothelial growth factor (VEGF) treatment demonstrated an increase in vascular permeability in both, arteriolar, venular and capillary vessels [205]. Moreover, the treatment of HLMVECs with VEGF leads to an enhanced vascular permeability by VE-cadherin and β-catenin tyrosine phosphorylation. Using N-acetylcysteine as free radical scavenger identified increased ROS production as an essential factor of vascular barrier impairment [206]. Increased endothelial junctional disassembly by enhanced tyrosine phosphorylation of VE-cadherin and the associated proteins β-catenin, γ-catenin, and p120-catenin was also measured after the treatment with thrombin [207]. LPS-induced ROS production enhances endothelial barrier dysfunction via elevated protein tyrosine phosphatase oxidation and an associated decrease in their activity [208]. LPS can then promote the internalization of VE-cadherin from the plasma membrane to intracellular compartments [209]. An increase in TJs

disassembly due to enhanced ROS production has also been demonstrated in several studies. Oxidative stress decreases the expression of TJs significantly [210–213]. In addition to this, oxidative stress reduces the oligomerization of remaining TJs, which leads to an increased vascular permeabilization [214]. One indicator for oxidative stress is the ratio of GSH to GSSG. The oligomerization of occludin itself is regulated by this ratio. Under physiological conditions, GSH is about 30 to 100-fold higher compared to GSSG and similar amounts of occludin monomers and oligomers can be detected [215, 216]. During pathological conditions caused by oxidative stress a dramatically shift to higher GSSG levels and reduced occludin oligomer values occurs [210, 217]. TJs can also undergo serine and tyrosine residue phosphorylation leading to barrier dysfunction [218].

Thus, enhanced ROS production leads to increased vascular permeability by reinforced junctional protein disassembly. In addition to this, the stimulation of endothelial cells induces actin cytoskeleton shortening. By the motion of myosin along the actin filaments, pronounced thicker actin cytoskeleton with a huge amount of contractile actin bundles, called the stress fibers, are developed. Thus, an increasing number of inter-endothelial gaps are formed and expanded, leading to the pathologically altered vascular permeability [172, 219]. The dynamic interaction between AJs or TJs and the actin cytoskeleton is disrupted by myosin light chain (MLC) phosphorylation with accompanying cytoskeleton endothelial cell contraction [220]. The MLC kinase (MLCK) phosphorylates MLC in a Ca^{2+}/calmodulin mediated reaction. Small Rho-GTPases such as Rho, Rac, and Cdc42 are key regulators of the actin cytoskeleton by activation of MLCK and other cytoskeletal-remodeling agents [221]. While RhoA mainly regulates stress fiber formation [222], Rac and Cdc42 promote the formation of lamellipodia and filopodia [223, 224]. Together with the Rho kinase (ROCK), small Rho-GTPases can reinforce the effect of MLC by inhibiting of the MLC phosphatase (MLCP) [225]. Rho-GTPase activity is inhibited by guanine nucleotide exchange factors (GEFs), which replace guanine triphosphate (GTP) with guanine diphosphate (GDP). Through the exchange of GDP for GTP, GTPase-activating proteins (GAPs) induce activation [226]. The deletion or inhibition of MLCK210, the endothelial cell-specific MLCK isoform, protects mice from experimental pulmonary edema, inflammation, and death [227]. However, a basal level of MLCK is essential due to physiological vascular permeability. A pathological increase of MLCK leads to an abnormal enhancement of vascular permeability followed by blood barrier disruption. Based on enhanced ROS production, various inflammatory agents such as LPS [228], thrombin [229, 230], or VEGF [231] have been shown to enhance Ca^{2+} influx in the endothelium in two distinct pathways: store-operated calcium entry (SOCE) and receptor-operated calcium entry (ROCE). The initial increase via SOCE is due to a Ca^{2+} release from stores within the endoplasmatic reticulum (ER) by an inositol triphosphate (IP_3)-dependent reaction [232]. At the activated G protein coupled receptors (GPCR) bound phospholipase C (PLC) cleaves phosphatidylinositol-4,5-bisphosphate (PIP_2) into IP_3 and diacylglycerol (DAG). IP_3 binds to an IP_3 receptor on the ER, which releases Ca^{2+} from the ER in the cytosol. DAG, in turn, connects SOCE to ROCE. Within this second mechanism, DAG activates transient receptor potential canonical/melastatin channels (TRPC/TRPM), whereby Ca^{2+} enters into the endothelium and activates calmodulin. Finally, calmodulin triggers the RhoA-dependent myosin contraction via MLC phosphorylation [233]. Furthermore, PKC localized on TRPC/TRPM is activated by phosphorylation and promotes SOCE [234]. Increased cyclic adenosine triphosphate ribose (cADPR) expression caused by enhanced ROS production contributes to ROCE via the activation of TRPC/TRPM [235, 236]. The actin cytoskeleton is not the only cellular structure that contributes to vascular permeability. Microtubules, the filamentous biopolymers formed by the polymerization of α, β-tubulin dimers, also modulate vascular permeability [237–239]. The ROS-dependent disassembly of the microtubule network after LPS treatment has also been reported. The stabilization

of microtubules with epothilone B or the inhibition of the guanine nucleotide exchange factor (GEF)-H1 suppressed LPS-induced barrier disruptive effects in vitro and significantly improve vascular permeability in vivo [240]. Inhibition of microtubule destabilization with taxol leads to barrier improvement during LPS treatment in vivo [241]. Microtubule dynamics also depends on ROS levels, as enhanced dynamics are observed in a ROS-free environment, whereas increased dynamic instability occurs in the presence of ROS [242].

Thus, the overall consensus in the field is that ROS can reduce the tightness of the endothelial barrier by causing the disruption of endothelial cell junctions in combination with enhanced cytoskeleton contraction and microtubule destabilization.

4.2 Transendothelial Leukocyte Migration Affected by Increased ROS Formations

Endothelial cell adhesion followed by transendothelial migration of inflammatory cells play an important role in the pathology of ALI and ARDS. Mainly occurring in postcapillary venules, the process is mediated by six sequential steps: (1) tethering and rolling, (2) activation, (3) adhesion, (4) crawling, (5) transendothelial migration, and finally (6) diapedesis (Fig. 5). The migration of inflammatory cells across the endothelium happens through paracellular or more rarely through transcellular transport [243, 244] and is mediated by adhesive interactions between cell adhesion molecules (CAMs) expressed in both activated endothelial cells and migrating PMNs. Selectins, integrin, and members of the immunoglobulin (Ig) superfamily are the three families of adhesion molecules that are crucial for PMNs transmigration.

Selectins, responsible for the initial tethering and rolling of PMNs on endothelial cells, are membrane glycoproteins which are classified in three different subtypes: P-selectins, L-selectins and E-selectins. P-selectins, also known as granule membrane proteins-140, are expressed in platelets or endothelial cells and stored in Weibel-Palade bodies, and can be rapidly recruited to the cell surface during inflammation. L-selectins formed in activated PMNs, contribute to PMN rolling and are quickly shed from the cell surface, via a protease-dependent mechanism, upon activation. E-selectins are not expressed under baseline conditions. Only cytokine stimulation leads to increased expression of E-selectins in endothelial cells [245–248]. Integrins, are heterodimeric transmembrane receptors consisting of two noncovalently linked transmembrane glycoproteins, one α and one β subunit at a time. Within the mammalian genome, 18 α subunit and 8 β subunit genes have been identified that can assemble into 24 different integrin combinations characterized by distinct binding properties and tissue distributions [249, 250]. The most common integrins in PMNs are β_2-integrins. Integrins are activated by binding of endothelial secreted chemokines like CXCL1 and CXCL8 to PMNs existing chemokine receptors CXCR1 and CXCR2. As a result, integrins change their conformation from bent low-affinity to fully extended high-affinity state, bind to an Ig superfamily member and arrest the PMNs on the endothelial cell surface [251, 252]. Compared to the adhesion by selectins to integrins, the integrins-mediated adhesion is a strong and solid binding to the vascular endothelium and represents an essential factor for PMNs transendothelial migration [253]. All Ig superfamily members like intercellular cell adhesion ICAM-1 and -2, vascular cell adhesion molecule 1 (VCAM-1), JAMs (JAM-A, -B, and -C), platelet endothelial cell adhesion molecule 1 (PECAM-1 or CD31), CD99 antigen-like 2 (CD99L2), and endothelial cell-selective adhesion molecule (ESAM) possess a structural Ig domain and are expressed in endothelial cells, platelets, and PMNs [254–256].

In the last decade, mounting evidence has supported the idea that extravasation of PMNs in response to inflammatory stimuli is regulated by increased oxidative stress. Initially, increased ROS levels produced in the injured tissue can function as chemoattractant for immune cells [248, 257, 258]. Moreover, ROS transmit signals from activated cell surface receptors and act

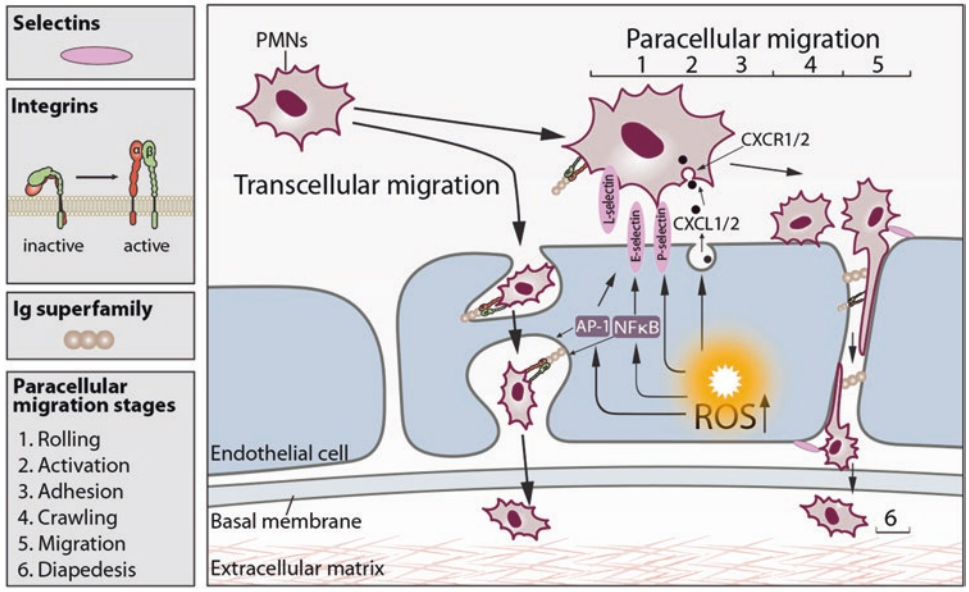

Fig. 5. ROS-mediated endothelial polymorphonuclear leukocyte migration. Over both transcellular and paracellular pathways, polymorphonuclear leukocytes (PMNs) pass through the endothelium to migrate from the blood lumen into the alveolar lumen. In both pathways selectin, integrins, and immunoglobulins (Ig) help facilitate this cellular migration. The transcellular migration of PMNs through the cell body is a rare event. More commonly paracellular migration occurs. This requires a number of migration steps: (1) tethering and rolling; (2) activation; (3) adhesion; (4) crawling; (5) transendothelial migration, and (6) diapedesis. Within each migration steps, varying cell adhesion molecules (CAMs) are needed. Selectins modulate the initially tethering and rolling of PMNs on the inner surface of the blood vessel, whereby PMNs start to slow down. Based on the rolling, endothelial cells are activated to release chemokines (CXCL1 or CXCL8) that transmit and bind to chemokine receptors (CXCLR1 and CXCLR8) localized on the surface of the PMNs. This causes PMN localized integrins to change from a low-affinity state (inactive bent conformation) to a high-affinity state (active fully extended conformation) forming a firm adhesion to CAMs of the Ig superfamily. The ultimate entry of the PMNs into the blood barrier occurs over these established CAMs formations. Increased ROS leads to enhanced invasion of immune cells into the injured tissue. ROS also regulate the expression CAMs both directly and through transcription factors (NF-κB, AP-1) that exert major influences on PMN migration. *NF-κB* nuclear factor-kappa-B, *PMNs* polymorphonuclear leukocytes, *ROS* reactive oxygen species

intrinsically within migrating cells and the surrounding tissue to promote migration. Thereby, increased ROS production can regulate the expression of endothelial CAMs by a direct transcription-independent activation or by a transcription-dependent mechanism via redox-sensitive transcription factors like nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) [259]. For instance, the exposure of endothelial cells to inflammatory stimuli like H_2O_2 [260], LPS or cytokines [254, 261] leads to a reinforced PMNs migration by increased endothelial CAMs expression. After 1 h treatment with H_2O_2 or t-butylhydroperoxide, in HUVECs there is an increase in transcription-independent surface expression of P-selectin followed by PMNs adhesion. Prior treatment of the cells with anti-P-selectin antibody or an antioxidant decreases the PMNs adhesion significantly [262]. Moreover, the exposure of human umbilical vein endothelial cells (HUVECs) to 60 min anoxia followed by 10 h reoxygenation causes a biphasic early transcription-independent and late transcription-dependent neutrophil adhesion response correlated to endothelial ROS production [263]. In contrast to the ROS-mediated expression of P-selectin, the expression of ICAM-1, VCAM-1, and E-selectin is only regulated on transcriptional level [264]. NF-κB as one of the transcription factors is involved in the expression of many inflammatory and immune response CAMs like E-selectin [265, 266], ICAM-1 [266], and VCAM-1 [267]. The

binding of inducible redox sensitive transcription factors in the promoter regions of the CAMs leads to their increased expression and activation [268, 269]. H_2O_2 increases the ICAM-1 expression while the pretreatment with an antioxidant or the application of an ICAM-1 antibody abolished the H_2O_2-induced PMNs adhesion [260]. VCAM-1 expression can also be induced by NOX associated ROS generation that promotes lymphocyte migration across the endothelial cell layer. The inhibition of NOX with diphenyleneiodonium or apocynin blocked the migration [270]. Rats exposed to hypoxia have increased ROS production and enhanced NF-κB and CAMs expression and the transvascular leakage is abrogated by nifedipine treatment due to reductions in oxidative stress and NF-κB [271].

5 Potential Therapies

5.1 N-Acetylcysteine (NAC)

NAC is a precursor for GSH, an antioxidant present in high levels in the normal lung. Lavage from patients with ALI/ARDS is deficient in GSH, and GSH levels are also below average in some pulmonary fibrotic disorders. Increased intracellular levels of GSH reduce production of pro-inflammatory cytokines like TNFα and IL-1. In addition to promoting GSH production, NAC also has direct antioxidant properties because of its thiol group, and it can scavenge reactive oxidants including hydrogen peroxide, superoxide anion, and hypochlorus acid. Animal studies indicate that NAC has significant protective effects against acute pulmonary injury from hyperoxia, endotoxin, or GSH synthesis inhibition [272–274]. The antioxidant effects of liposomally entrapped NAC is more effective as evaluated in rodents challenged with LPS [273]. In one study the benefits of NAC treatment in the management of ARDS were assessed by measuring patient's intracellular glutathione and plasma antioxidant biomarkers and outcome [275]. Treatment by NAC apart from increasing intracellular glutathione also increased extracellular total antioxidant power, total thiol status, and the outcome of the patients. The authors suggested that patients with ARDS could potentially benefit from NAC supplementation. In a randomized, double-blind, placebo-controlled, prospective clinical trial the levels of glutathione and cysteine in patients with ARDS were determined and the effect of NAC treatment examined [276]. The study concluded that repletion of glutathione may safely be accomplished with NAC in patients with ALI/ARDS which may shorten the duration of lung injury. It has also been shown that NAC protects against H9N2 virus-induced acute lung injury [277]. Repetitive post-treatment of NAC in LPS-exposed attenuates the extent of ALI through the inhibition of NF-κB activation [278]. NAC also improves respiratory function, but not survival, in adults with ALI/ARDS. A double-blind, placebo-controlled study in 48 patients at five centers found that treatment with NAC increased cardiac index and decreased the number of days of ALI without improving mortality [276]. No adverse side effects have been reported from the use of NAC in patients with ALI/ARDS. Given the potential benefits of NAC supplementation studies are needed to investigate the utility of NAC in combination therapies for ALI/ARDS.

5.2 Vasodilator Gases

NO is an important endogenous gaseous mediator in several physiological processes in vivo. One of its most important action is potent vasodilation, which results from decreased calcium in vascular smooth muscle cell cytoplasm following an NO-dependent increase in cyclic-GMP [279]. Inhaled NO affects gas exchange by increasing blood flow in ventilated areas. Administration of NO by inhalation has been shown to acutely improve hypoxemia associated with pulmonary hypertension in humans and animals [280–282]. Inhaled NO results in a transient improvement in oxygenation without any effect on mortality in both adults and children with ARDS [283]. Hydrogen sulfide (H_2S), another signaling gas, is produced by the catalytic conversion of L-cysteine by two enzymes: CBS (cystathionine β-synthase)

and CSE (cystathionine γ-lyase). H_2S reduces inflammation and protects tissues from injury especially in the gastrointestinal tract [284, 285]. Hydrogen sulfide pretreatment aslo exerts protective effects on both hyperoxia and LPS induced ALI [286, 287]. Recent findings suggest that carbon monoxide (CO) can also act as an endogenous defensive gaseous molecule to reduce inflammation and organ injury [288–290]. The endogenous production of CO occurs through the activity of bot the constitutive heme oxygenase 2 (HO-2) and inducible heme oxygenase 1 (HO-1). The therapeutic potential of CO has been shown in models of acid-induced lung injury, HTMV, endotoxin challenge, and cecal ligation and puncture induced-sepsis [291]. The efficacy of NO, H_2S, and CO in humans with ALI/ARDS remains unclear and awaits further controlled clinical studies.

5.3 NADPH Oxidase (NOX) Inhibitors

NOX is the primary generator of $O_2^{-\cdot}$ and is responsible for the initiation of the ROS generation cascade. NOX is a unique target and inhibiting NOX would reduce $O_2^{-\cdot}$ production which would result in less available $O_2^{-\cdot}$ for the generation of H_2O_2 and $ONOO^{-\cdot}$, subsequently reducing OH generation and increasing NO bioavailability as a result. NOX is an important contributor of oxidant production and is an upstream actor in oxidative stress-induced acute lung injury involving JNK and ERK pathways [135, 292]. There are several NOX inhibitors currently being studied, however the most common is apocynin, which is a NOX inhibitor that preferentially blocks NOX-2 at low doses. Apocynin acts by preventing the assembly of the NOX enzyme subunits. The administration of apocynin, a NOX inhibitor, reduces lipid peroxidation, suppresses the NF-κB pathway, attenuates lung injury, and improves survival in rat hemorrhagic shock and LPS models [293, 294]. Similarly, the inhibition of NOX-2 activity ameliorates influenza A virus-induced lung inflammation, indicating that pharmacologically targeting NOX-2 may have therapeutic potential in ALI [295]. The NOX inhibitor diphenyleneiodonium chloride, attenuates oleic acid-induced lung injury [296].

5.4 Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2) Activators

Nrf2 presents a potential target for reducing oxidative stress in ALI/ARDS. Nrf2 is found in the cytoplasm of many mammalian cells and is responsible for the regulation of various antioxidants and cytoprotective genes, acting as a "master switch" for these genes. In response to oxidative stress, Nrf2 translocates to the nucleus and binds to the antioxidant response element (ARE) of target genes, along with other binding factors and cofactors, resulting in the induction of stress response genes. Nrf-2 activators such as andrographolide sulfonate [297, 298] and CDDO-Me [299, 300] have been shown to attenuate acute lung in lung in animal models of ALI.

5.5 Activated Protein C

Protein C is a vitamin K-dependent plasma protein zymogen. Activated protein C (APC) inactivates factors (F) Va and VIIIa and downregulates thrombin generation. The cytoprotective effects of APC involve gene expression profile alterations, anti-inflammatory and anti-apoptotic activity, and endothelial barrier stabilization [301]. However, its utility in ALI/ARDS is still controversial. The infusion of recombinant human activated protein C (rh-APC) in patients with ARDS showed attenuation of pulmonary coagulopathy and injury without any side effects [302]. However, in another randomized, saline-controlled, single-blinded clinical trial with rh-APC did not find any improvement in increased alveolocapillary permeability or the clinical course of ARDS patients [303]. Thus, further studies are required to confirm the role of rh-APC as therapeutic candidate for ALI/ARDS.

6 Conclusion

Studies over the last decade have clearly demonstrated that ROS can increase the permeability of the pulmonary endothelial bed and that this oxidative stress plays a major role in the pathogenesis of ALI and ARDS. The source of the ROS is complex and a number of pathways are involved. However, it is hoped that tackling the overproduction of ROS, and the decreased defense capacity, during ALI/ARDS may open up a new field of therapeutic approaches for a disease that has not seen significant advances, despite 50 years of investigations.

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