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MULTI-AUTHOR REVIEW

# NOX enzymes: potential target for the treatment of acute lung injury

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**Abstract** Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), is characterized by acute inflammation, disruption of the alveolar-capillary barrier, and in the organizing stage by alveolar pneumocytes hyperplasia and extensive lung fibrosis. The cellular and molecular mechanisms leading to the development of ALI/ARDS are not completely understood, but there is evidence that reactive oxygen species (ROS) generated by inflammatory cells as well as epithelial and endothelial cells are responsible for inflammatory response, lung damage, and abnormal repair. Among all ROS-producing enzymes, the members of NADPH oxidases (NOXs), which are widely expressed in different lung cell types, have been shown to participate in cellular processes involved in the maintenance of lung integrity. It is not surprising that change in NOXs' expression and function is involved in the development of ALI/ARDS. In this context, the use of NOX inhibitors could be a possible therapeutic perspective in the management of this syndrome. In this article, we summarize the current knowledge concerning some cellular aspects of NOXs localization and function in the lungs, consider their contribution in the development of ALI/ARDS and discuss the place of NOX inhibitors as potential therapeutical target.

**Keywords** NOX enzymes · Acute lung injury · Acute respiratory distress syndrome

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**Abbreviations**

| | |
|---|---|
| ALI | Acute lung injury |
| ARDS | Acute respiratory distress syndrome |
| BAL | Bronchoalveolar lavage |
| ROS | Reactive oxygen species |
| HM | Hyaline membranes |
| LPS | Lipopolysaccharide |
| TNF-$\alpha$ | Tumor necrosis factor-$\alpha$ |
| ICAM-1 | Intracellular adhesion molecule-1 |
| EC | Endothelial cells |
| TGF-$\beta$1 | Transforming growth factor-$\beta$1 |
| IPF | Idiopathic pulmonary fibrosis |
| IRF-3 | Interferon regulatory factor-3 |
| AP1 | Activator protein 1 |
| NF-$\kappa$B | Nucleor factor $\kappa$B |
| MCP-1 | Monocyte chemotactic protein-1 |
| MV | Mechanical ventilation |
| N.D. | Not determined |

## Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), which is the most severe form, is associated with a high mortality (50–80 %). ALI/ARDS affects a large number of patients entering intensive care units and is defined by bilateral pulmonary infiltrates on chest radiograph, hypoxemic respiratory failure measured by a partial pressure of arterial oxygen ($PaO_2$)/fraction of inspired oxygen ($FiO_2$) ratio ($PaO_2/FiO_2 < 300$ mmHg for ALI and <200 mmHg for ARDS) with normal hydrostatic pressure corresponding to the absence of left heart failure. Acute respiratory distress syndrome can occur with several diseases either associated with those causing direct lung injury such as pneumonia, gastric aspiration or toxic

inhalation, or indirect injury such as sepsis or severe burn. The heterogeneity of causes and the complexity of clinical histopathological and radiographic manifestations make the study of ARDS pathogenesis and the test of new therapeutics difficult. Indeed, ARDS follows most often a progressive course characterized by two distinct stages. The acute (or exudative) stage includes the disruption of the alveolar-capillary barrier, pulmonary edema, accumulation of protein-rich fluid into the interstitium, alveolar space, and diffuse inflammation. The later organizing stage occurs with the resolution of pulmonary edema and lung inflammation. During this phase, alveolar pneumocytes hyperplasia and fibroblast proliferation lead to disordered collagen deposition and extensive lung fibrosis.

Although the elucidation of the cellular and molecular mechanisms involved in the pathogenesis of ALI/ARDS remain unclear and complex, there is evidence that reactive oxygen species (ROS) contribute to the initiation of endothelial damage characteristic of ARDS and are responsible for most of clinical symptoms of this syndrome [1]. Indeed, a large amount of ROS, which are found in broncho alveolar lavages (BAL) of ARDS patients are produced mainly by alveolar macrophages, neutrophils, lung endothelial, and epithelial cells. These ROS can alter gene and protein function. Among several ROS-producing enzymes, NADPH oxidase (NOX) enzymes, which are membrane-bound complexes catalyzing the reduction of molecular oxygen ($O_2$) to superoxide ($O_2^-$) [2], are involved in principal clinical manifestations of ALI/ARDS [1, 3]. The first NOX has been described in phagocytes and is a complex that includes a catalytic subunit $gp91^{phox}$ called NOX2 associated with $p22^{phox}$ and cytosolic regulatory subunits such $p47^{phox}$, $p67^{phox}$ and small GTPase (RAC1 or RAC 2), required for NOX activation and generation of superoxide [2, 4]. Recently, structural homologues of the phagocyte NOX enzyme were identified, such as NOX1-3-4-5, DUOX1, and DUOX2. Despite their similar structure and enzymatic function, NOX enzymes differ in their mechanisms of activation, which depend on the recruitment of membrane or/and cytosolic regulatory subunits such as $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, NOXO1, NOXA1, and RAC [2]. The NOX isoforms, which are expressed in a variety of lung cell types [5], participate in several cellular processes [6] and are involved in lung pathological situations such as ALI/ARDS, cancer, fibrosis, pulmonary hypertension, and obstructive lung disorders such as emphysema, asthma, and cystic fibrosis [5, 7–10].

In the present article, we will focus on the contribution of NOX enzymes in the development of ALI/ARDS. We will first briefly describe some cellular aspects of NOX localization and function in the lungs. In the second part, we will review the current knowledge concerning the role of NOX-dependent ROS production in the pathogenesis of ALI/ARDS and particularly its involvement in some clinical aspects of this disease. In the third part, we will discuss their therapeutic potential in the management of ARDS/ALI.

## Cellular expression and function of NOX enzymes in lungs

The lung, of which the principal function is to deliver oxygen to tissues, is widely exposed to deleterious environmental factors including virus, bacteria, irritants and allergens, and possesses a potent innate defense system. This defense system not only uses the phagocyte NOX system to eliminate these dangers through oxidative killing [6, 11] but also in regulating cell-signaling pathways involved in host defense mechanisms, cell proliferation, migration, and/or differentiation [4, 12]. Several studies have shown that NOX enzymes are expressed in lungs, both in mice and in humans. The amount of the different NOX isoforms depends on the cell types and also on the species. A high amount of NOX2 [13, 14] and DUOX1/2 mRNA [15, 16] as well as NOX1 [17, 18] and NOX4 [7, 8, 13, 18] are detected in lungs. In addition to the expression of NOX2 in alveolar macrophages and other inflammatory cell types, NOX isoforms have been detected in different lung cell types such as alveolar epithelial and endothelial cells, fibroblasts, smooth muscle cells, and airway epithelial cells [19, 20]. The cell-type-dependent NOX expression in lungs suggests their specific participation in some aspect of physiological and pathological functions including host defenses, proliferation, migration, and/or differentiation. The specific lung expression and function of NOX enzymes are summarized in the Table 1.

Thus, according to the physiological contribution of NOX enzymes in tissue repair and/or remodeling, we could envisage that the modulation of their expression and activation in different lung cell types contributes to the development of lung diseases such as ALI/ARDS. We will first describe the pathogenesis of ALI/ARDS and then the role of ROS-dependent NOX enzymes in different animal models of ARDS/ALI.

## Histopathology and pathogenesis of ALI/ARDS

In spite of the scarce knowledge concerning the mechanisms involved in the pathogenesis of ALI/ARDS, histological analysis of lung sections from ARDS at different stages suggests that lung modifications occurring during this disease follow a scheduled time course and can be divided into three time-dependent phases: acute (or exudative), proliferative, and fibrosis [61].

**Table 1** Summary of NOX enzymes localization, activation, and function in lung cells

| NOX isoforms | Expression | Stimuli | Function | Species | References |
|---|---|---|---|---|---|
| NOX1 | Endothelial cells | FGF-$\beta$, VEGF | Vascular cell growth | M, H | [21] |
| | | FGF-$\beta$, VEGF | Angiogenesis | M, H | [21] |
| | | Hyperoxia | Cell death | M | [17] |
| | Alveolar epithelial cells | TNF-$\alpha$, hyperoxia | Cell death | M | [17, 22] |
| | | Hypoxia | HIF-$\alpha$ signaling | H | [23, 24] |
| | | Growth factors, HIPK2 depletion | Proliferation | M, H | [25, 26] |
| | Fibroblasts | N.D | N.D | M | Personal data |
| | Vascular smooth muscle cells | – | N.D | R | [27] |
| NOX2 | Endothelial cells | Hyperoxia | Cell migration | H | [28, 29] |
| | | Ischemia and High K+, hypoxia | Oxygen sensing | B, M | [30–33] |
| | | LPS, TNF-$\alpha$ | TLR2 crosstalk | M | [34] |
| | Neuro-epithelial cells | Hypoxia | Chemoreceptor $O_2$ sensing | H, R, Ra | [35] |
| | Macrophages/ neutrophils | TNF-$\alpha$, LPS influenza A virus | Anti-microbial host defense/innate immune response | M, H | [34, 36–41] |
| | | Chronic fine particulate | TLR4 crosstalk, NF-$\kappa$B activation | M | [42] |
| NOX3 | Endothelial cells | – | TLR4 crosstalk | M | [10] |
| | | Hyperoxia | Cell integrity | M | [43] |
| NOX4 | Endothelial cells | Hyperoxia | Cell migration | H | [28, 44] |
| | Alveolar epithelial cells | Bleomycin, TGF-$\beta$1, fine particles | Cell death | M, H | [7, 45] |
| | Smooth muscle cells | TGF-$\beta$1 | Proliferation | M, H | [18, 46, 47] |
| | | | Differentiation | R | [27] |
| | Fibroblasts/ myofibroblasts | Bleomycin, TGF-$\beta$1, radiation | Differentiation/activation | M, H | [7, 8, 48, 49] |
| | | Hypoxia | Proliferation | H | [50] |
| DUOX1 | Bronchial cells | *Pseudomonas aeruginosa*, LPO, IL-4, IL-13 cytokines, and cigarette smoke | Host defense | H | [15, 19, 20, 51–54] |
| | | PMA, human neutrophil elastase | Mucin expression | H | [55] |
| | | ATP | Cellular migration | H | [56] |
| | | – | $H^+$ production and secretion | H | [57] |
| | | LPS | Cell proliferation | M | [58] |
| | Alveolar epithelial cells | Hormone mixture | Differentiation | H | [59] |
| DUOX2 | Bronchial cells | IFN-$\gamma$ | Host defense | H | [19, 60] |
| | | – | $H^+$ production and secretion | H | [57] |
| | Alveolar epithelial cells | – | N.D | M | Personal data |

*M* mouse, *H* human, *R* rat, *Ra* rabbit, *B* bovine, *PMA* phorbol 12-myristate 13-acetate, *HIPK2* homeo domain-interacting protein kinase-2, *LPO* lactoperoxidase, *ATP* adenosine triphosphate, *ANPH* atrial natriuretic peptide hormone, *N.D* not determined

The acute phase begins after the initial injury; most cells composing the alveolar septa undergo either apoptosis or necrosis, and inflammatory cells mainly represented by the neutrophils, invade alveolar walls and lumens. The number of neutrophils increases fast as they travel via the interstitium into the airspaces. This is facilitated by the disruption of the vascular structures, which occurs when neutrophils and fibrin plugs occlude the capillaries (Fig. 1a, b). Hyaline membranes (HM) made of fibrin, proteins and cellular debris; accumulate along the alveolar walls (Fig. 1a, b). By electron microscopy, all stages of epithelial degeneration can be observed from slight cytoplasmic swelling to huge blister formation and total destruction of the epithelial lining [62]. In parallel, the endothelial cell layer is often irregular because of cytoplasmic swelling and large vacuoles. Endothelial defects

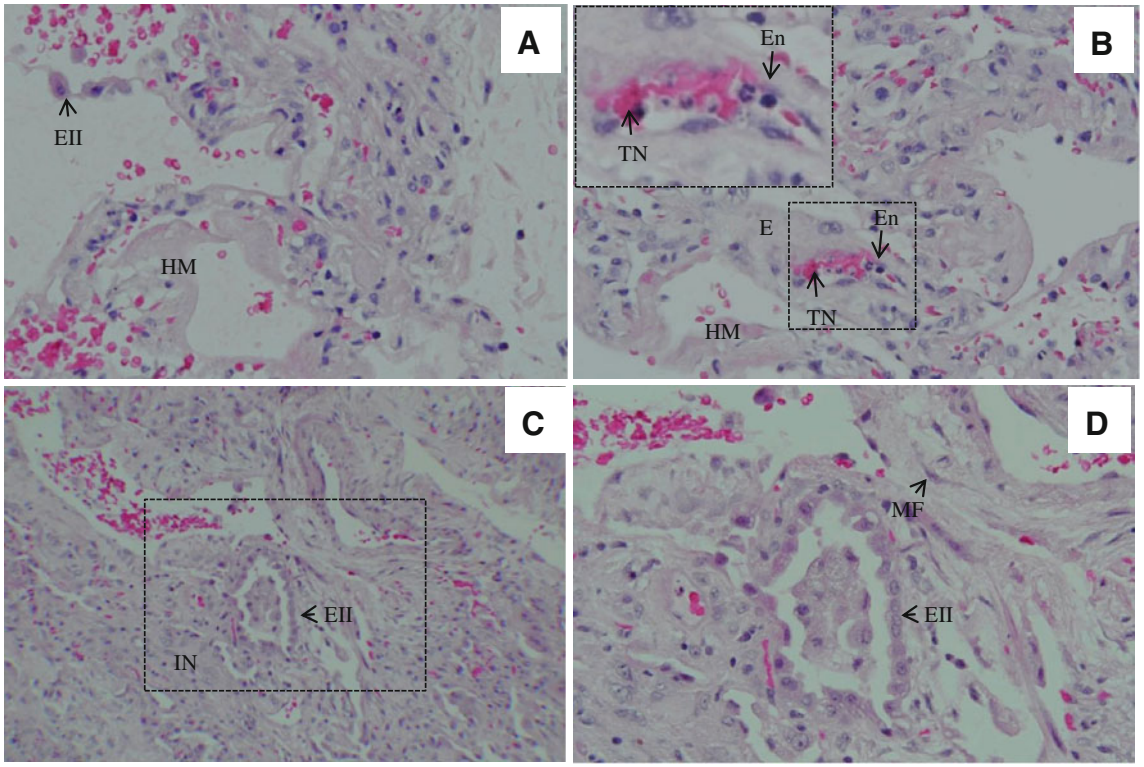


**Fig. 1** Histological hallmarks of acute respiratory distress syndrome during the exudative and the proliferative phases. **a**, **b** Lung sections stained with hematoxylin and eosin (H&E) obtained from biopsy of ARDS subjects during the exudative phase. **a** Deposition of hyaline membranes (HM) on the epithelial side of the basement membrane. At this stage, the presence of detached epithelial type II cells from the alveolar wall (EII) is also apparent. **b** The presence of the interstitial edema (E). The necrosis of endothelial cells (En) and the formation of thrombus associated with the margination of neutrophil (TN) are also obvious at this stage. Original magnification ×400 (**a** and **b**), ×500 for enlarged insert. **c**, **d** Lung sections stained with (H&E) obtained from biopsy of ARDS subjects during the proliferative phase. **c**, **d** The evident hyperplasia of epithelial type II cells (EII) and an extended zone of interstitial (IN) fibro-proliferation. Note the presence of myofibroblasts in the parenchyma (MF). Original magnifications ×200 (**c**), ×400 (**d**)

are covered by fibrin or microthrombi, which completely obliterate the capillaries (Fig. 1b, enlarged insert). Concerning the late exudative stage, the epithelial lining is thin and covered by laminated paucicellular HM. The alveolar septa are enlarged and contain numerous inflammatory cells.

After approximately 7 days, the organizing or proliferative stage is observed with increased interstitial cellularity. The number of large cuboidal cells, which resemble epithelial type II cells and might represent a stem cell population of the lung (Fig. 1c and d), increases strikingly. The composition of the interstitial cells changes; neutrophils are partly replaced by macrophages, lymphocytes, and plasma cells. The interstitium is organized by the proliferation of connective tissue, persisting edema, and convolutes of loose fibrous tissue without capillaries. There is a strong diminution of the microvasculature that is sometimes compressed by the surrounding tissue. Myofibroblasts that express vimentin and α-smooth muscle actin (α-SMA) are progressively observed in interstitium and then in airspaces, with a maximum in the early proliferative stage [63]. The late proliferative phase shows easily identifiable proliferating intra-septal and/or intra-alveolar myofibroblasts (airspace fibroplasia) and production of new matrix substances with the doubling of lung collagen in 2 weeks.

After a few more days, the fibrotic phase shows wide connective tissue area interspread between alveolar septa. Bulk tissue masses formed by folded up septa and collapsed alveoli surround unusually wide airspaces, which originated mostly from widened alveolar ducts or respiratory bronchioles. Some airspace is enlarged due to tissue destruction. The histology is characterized by enlarged fibrotic septa and laminated intra-alveolar fibrosis.

ROS are increasingly considered key substances in the initiation of endothelial damage characteristic of ARDS and are responsible for most of the clinical symptoms of this syndrome. There are several causes that increase oxidative stress in ARDS such as breathing high inspiratory oxygen concentration. However, the large majority of oxidants are generated by phagocytic cells transmigrating into the lungs. Neutrophils are crucial since they appear early in histological specimens and are strongly increased in the BAL. They release many inflammatory mediators that include chemokines, cytokines, and proteases [61].

The activation of the neutrophils may occur at remote sites and/or by circulating cytokines, resulting in ROS release and increased pulmonary vascular permeability. However, neutrophils are not mandatory for the development of ARDS, because it can occur in neutropenic patients. Other key initiators of the pulmonary inflammation in ARDS are the circulating inflammatory mediators (e.g., TNF-$\alpha$, IL-1$\beta$, IL-6, IL-8, leukotrienes), as well as changes in the coagulation system. Alveolar edema resulting from endothelial dysfunction and loss of epithelial integrity reduces the barrier function. The edema is even increased by the loss of type II pneumocytes that normally promote fluid transport out of the alveolus through their apical sodium pumps. Early loss of surfactant is explained by the damage of type II epithelial cells, which produce surfactant and by its neutralization by protein-rich edema fluid. This contributes to alveolar collapse, intrapulmonary shunt, and hypoxemia. The hypoxic pulmonary vasoconstriction is impaired by endothelial and smooth muscle cell dysfunction. This may, with the association of microthrombi, contribute to the development of secondary pulmonary hypertension.

## Role of NOX enzymes in ALI/ARDS

Whereas the mechanisms that initiate ALI/ARDS remain unclear, there is some evidence that ROS generated by NOX enzymes participate in the pathogenesis of this syndrome. The study of NOX enzyme contribution in the patho-mechanisms of ALI/ARDS in human is difficult due to the heterogeneity of causes and the paucity of biological samples (biopsies, autopsies, BALF). Animal models reproduce major clinical features of ALI/ARDS observed in humans including the loss of the alveolar-capillary barrier with the damage of both epithelial and endothelial cells and the inflammatory cell influx. In this way, all these models provide key elements to study the role of the NOX family during ALI/ARDS. In the next part, we will examine their involvement in different mouse models of ALI/ARDS that mimic neutrophilic infiltration and lung injury in sepsis-like models and the damage of the alveolar-capillary barrier.

### Lung inflammation and injury

Histological analysis of lung sections from ARDS patients as well as BAL obtained in the acute phase of the disease show massive accumulation of neutrophils [64] and most acute lung injuries induced in animal models are neutrophil-dependent [65–67]. These inflammatory cells produce high levels of ROS, which are thought to increase the inflammatory processes and tissue injury in septic shock syndrome [68–70]. In addition, ROS participates in the modulation of cell-signaling pathways that activate transcription factor of redox-sensitive pro-inflammatory mediators such as NF-$\kappa$B [71, 72]. The studies concerning the effect of ROS generated by NOX enzymes in acute inflammatory responses and lung injury following *Escherichia coli* and lipopolysaccharide (LPS) challenges in mice are controversial [37, 71, 73, 74]. Indeed, some studies have shown that the absence of $p47^{phox}$ (a regulatory subunit essential for NOX2 activation) did not contribute to LPS-induced lung damage, vascular leakage, and infiltration of neutrophils and monocytes in mice [75]. Swain et al. [76] did not observe any improvement of pulmonary lung injury in $gp91^{phox}$-deficient mice during pneumocystis pneumonia. By contrast, it has been demonstrated that LPS-induced inflammation and lung injury was inhibited but also in some cases increased in NOX-deficient mouse models. The absence of $p47^{phox}$ and $gp91^{phox}$ has been associated with enhanced inflammatory gene expression, lung neutrophil recruitment, and mouse survival after LPS challenge [77, 78]. On the other hand, LPS-induced lung inflammation was reduced in mice deficient for Nrf2, a regulator of antioxidant defenses, in absence of $p47^{phox}$ or $gp91^{phox}$ [37]. Moreover, ROS production restricted to macrophages from Nrf2-deficient mice was blunted by the absence of $gp91^{phox}$ after LPS challenge [37]. Similarly, inflammatory response induced by live *Escherichia coli* or LPS was reduced in lung tissues of $p47^{phox}$- and $gp91^{phox}$-deficient mice [71, 73]. Sadikot et al. [74] has reported that NF-$\kappa$B activation and TNF-$\alpha$ levels were decreased in $p47^{phox}$-deficient mice after *Pseudomonas aeruginosa* infection and a recent study showed that ROS generated by NOX2 in neutrophils were involved in TNF-$\alpha$-induced acute lung injury [38] and participated in inflammatory response through the activation of NF-$\kappa$B [36]. These results support the notion that ROS generated by NOX2 play a critical role in the induction of inflammatory responses and tissue injury in sepsis.

The family of Toll-like receptors (TLR), which to date contains ten members, recognizes specific molecules conserved among microorganisms and pathogens, and plays an important role in initiating the inflammatory response [79]. Emerging evidence demonstrates that NOX enzymes modulate Toll-like receptor 4 (TLR4) and TLR2 signaling not only in neutrophils and macrophages but also in other cells. Lipopolysaccharide specifically binds to LPS-binding protein (LBP) and forms a complex that activates the TLR4 receptor of macrophages and others cells. This interaction triggers the activation of I$\kappa$b kinase and the mitogen-activated protein kinase kinases (MAPKK), which in turn activate NF-$\kappa$B and AP1, respectively [80]. Activated NF-$\kappa$B and AP-1 translocate into the nucleus where they bind to DNA promoter regions and induce the transcription

of inflammatory genes. It has been shown that in neutrophils, NOX2-derived ROS regulate the TLR4-mediated activation of NF-κB. In addition, in endothelial cells, NOX2 also contributes to TLR2 gene activation in response to LPS [36, 81]. A recent study demonstrated that ROS generated by NOX2 in neutrophils mediates high mobility group box 1 (HMGB1)/TLR4 signaling and tissue damage after hemorrhagic shock/resuscitation in mice [82]. Besides NOX2, NOX4 is able to directly interact with TLR4 and mediate ROS generation and NF-κB activation in HEK293T cells [83]. More recently, NOX4 has been shown to mediate LPS-induced NK-κB-dependent IL8, MCP-1 and ICAM-1 gene expression in human aortic endothelial cells [84] and interferon regulatory factor (IRF)-3 transcription factor activation in U373/CD14 cells [85]. NADPH oxidase4 is also able to activate AP-1 and subsequent CXCR6 expression, after LPS challenge in human aortic smooth muscle cells [86]. Similar to NOX4, the presence of crosstalk between NOX1 and TLR4 was suggested by the observation that LPS derived from *Helicobacter pylori* increases ROS production and NOX1 expression in guinea pig gastric pit cells through a TLR4 signaling pathway [87]. All these results suggest that NOX family plays an important role in the activation of TLR4 signaling pathways (including NF-κB, IRF-3 and AP1) in response to LPS. Nevertheless, the molecular mechanism linking TLR4 to NOX1 remains unclear and the function of NOX1 and NOX4 in TLR-mediated signaling in vivo need to be elucidated using either knock-out mice or RNA interference strategy.

### Endothelial and epithelial targets

Alveolar cell death has been reported extensively in humans and in experimental models of acute lung injury [88]. Indeed, in the acute phase, the presence of edema in the air spaces and hyaline membrane deposits are direct consequences of alveolar-capillary barrier damage. Epithelial type II cells after being initially injured, often by an unknown stimulus, proliferate in order to repair the damaged epithelium [89]. To date, it is not known whether the alveolar-capillary barrier integrity depends preferentially on the endothelial or the epithelial side in acute lung damage and which are the signaling pathways involved in alveolar cell death. Nevertheless, there is evidence that both epithelial and endothelial cells are damaged by ROS-dependent mechanisms and in particular by NOX-dependent ROS generation.

### Endothelial cell target

The injury of endothelial cells is mostly studied in sepsis-like models using systemic injection of LPS or TNF-α. During ALI, the endothelium undergoes large transformations in terms of expression of adhesion molecules, tight junctions, and ROS-producing enzymes [90]. In this context, NOX-derived ROS participates in the damage of endothelial cells either by the direct activation of endothelial signaling or via neutrophils or macrophages.

Lipopolysaccharide can directly increase ROS generation through the modulation of NOX enzymes in endothelial cells [91]. A recent study demonstrates that in these cells, stimulation by LPS leads to the activation of IL8, which in turn regulates the expression and the activity of NOX1 and contributes to the progression of the sepsis cascade. These data suggest that LPS/IL8 signaling is NOX1-dependent in endothelial cells [92]. In addition to NOX1, NOX4, which is also expressed in endothelial cells, is responsible for LPS/TLR4-induced ROS generation and gene expression of chemokines such as IL8, MCP-1, and intracellular adhesion molecule-1 (ICAM-1) in human aortic endothelial cells [84]. The authors also demonstrated that the specific inhibition of NOX4, by siRNA strategy, contributes to the decrease of LPS-induced migration and adhesion of monocytes to endothelial cells [84].

Thus, besides the direct effect of LPS on NOX activation in endothelial cells, excessive production of ROS by NOX enzymes located in inflammatory cells has been associated to endothelial cell damage in sepsis. Fan et al. [34, 81] and others demonstrated that in endothelial cells, LPS/TLR4-induced NF-κB and TLR2 gene activation is dependent on NOX2 located in neutrophils. In addition, neutrophilic NOX2 contributes to TNF-α-induced NF-κB-dependent lung inflammation and endothelial cell injury in mice [38] and participates in the activation of NF-κB and the induction of TLR2 in endothelial cells [36]. More recently, Farley et al. [41] reported that co-culture of $p47^{phox}$ and $gp91^{phox}$-deficient macrophages with pulmonary microvascular endothelial cells stimulated with a mix of cytokines such as TNF-α, IL1-β, and IFN-γ led to a significant decrease in endothelial cell injury, supporting the concept that ROS produced by phagocytic NOX2 play a crucial role in the injury of endothelial cells.

### Epithelial cell target

Although the epithelial barrier after being injured by an unknown stimulus is mostly able to repair, the persistence of a lung injury leads to the development of fibrosis. It is considered that epithelial cell death is crucial not only in the weakening of the alveolar-capillary barrier, but also in lung abnormal repair, which leads to pulmonary fibrosis [93]. While the pathogenesis of pulmonary fibrosis, a lethal lung disorder characterized by abnormal lung repair, is unknown, it involves early inflammatory steps and late fibrotic changes with proliferation of fibroblasts and their

differentiation into myofibroblasts [94, 95]. Intratracheal instillation of bleomycin in mice, a well-known characterized model to study initial lung epithelial injury and subsequent fibrosis, mimics ALI/ARDS features occurring during the late proliferative and fibrotic phase. In this model, NOX-dependent ROS are not only responsible for initial epithelial damage but also for the differentiation of fibroblasts into myofibroblasts, the hallmark of the disease. Several studies have shown that TGF-$\beta$1 increases ROS levels by up-regulating NOX4 expression in lung fibroblasts and induces their differentiation into myofibroblasts [8, 96, 97]. Interestingly, in human idiopathic pulmonary fibrosis (IPF), NOX4 has been detected in myofibroblasts of late fibrotic scars, suggesting a possible role of NOX4 in the development of organized fibrosis, and was also detected in the alveolar proliferative epithelium of IPF lungs adjacent to fibroblastic foci. In mice, we demonstrated that NOX4 deficiency as well as acute treatment with NOX inhibitors blunted TGF-$\beta$1-induced alveolar epithelial cell death and prevented subsequent pulmonary fibrosis [7].

The role of NOX2 and NOX1 in bleomycin-induced lung fibrosis has also been investigated in NOX2 and NOX1-deficient mice. Only a moderate protection from bleomycin-induced lung fibrosis was observed in NOX2-deficient mice [98]; however, extrapolation to human IPF is difficult as inflammation might not be as prominent in humans compared to mice. We found that NOX1-deficient mice were not protected from bleomycin-induced fibrosis (personal data). Finally, NOX4 rather than NOX1 and NOX2 could be a good candidate for the treatment of ARDS/ALI patients during both the acute and the proliferative stage.

## Epithelium and endothelium targets

Aspiration of the gastric content is considered to be an important cause of ALI/ARDS. In addition to the low pH, the gastric content contains particulate bacterial material, which contributes to lung injury [99]. The intratracheal instillation of hypochlorite (HCl) is a well-used model for inducing lung injury secondary to gastric acid aspiration in mice. Aspiration-induced lung injury, which depends on neutrophilic influx into the alveolar space, is characterized by the damage of both epithelial and endothelial cells leading to alveolar hemorrhage and edema. Some studies have implicated ROS as a key element in the pathogenesis of ALI/ARDS following gastric content aspiration in mouse models [100], but to date, only one has demonstrated the role of NOX enzyme in this context. Indeed, exposure of $p47^{phox}$-deficient mice to HCl leads to increased pulmonary neutrophilic infiltration, alveolar-capillary barrier leakage, and enhanced level of pro-inflammatory cytokine compared to WT mice [101], suggesting a protective role of NOX2 in HCl-induced lung injury by modulating the inflammatory response.

Mechanical ventilation (MV) is the unique strategy used in patients with acute hypoxemic respiratory failure to improve arterial oxygenation and their survival [102]. However, this therapy provokes tissue injury due to mechanical stretch (MS). Mechanical ventilation associated with alveolar barrier overstretching contributes to neutrophilic infiltration, release of pro-inflammatory cytokines, and lung injury [103]. The cellular mechanisms involved in MV-induced lung injury (MVILI) and -inflammation remain unknown. A high level of ROS is thought to be one potential initiating signal in response to MV following mechanical stress. Indeed, treatment with *N*-acetyl cysteine (NAC) attenuates MV-induced neurophilic influx into alveolar spaces and reduces epithelial cell apoptosis in rats [104, 105]. Besides mitochondrial enzymes, NOXes have been shown to contribute to ROS production in response to mechanical stress in different cells such as endothelial cells, epithelial cells, and vascular smooth muscle cells [106–111]. It has been described that NOX activation was associated with a membrane translocation of $p47^{phox}$ in smooth muscle cells (SMC) [108, 109]. On the other hand, exposure of vascular SMCs to MV leads to $p47^{phox}$ membrane translocation followed by an increased NOX1 mRNA expression and ROS production [108], suggesting a role for NOX1 in MVILI. Some studies also demonstrated that ROS produced by NOX enzymes participated in cyclic stretch-induced vascular remodeling in SMC via matrix metalloproteinase-2 activation [108]. The NOX isoform involved in MV-induced lung injury and the NOX-dependent signal transduction pathways need to be clarified.

Hyperoxia-induced acute lung injury is one of the most relevant models of oxidative stress and alveolar cell death, which is not closely linked to the magnitude of the inflammatory response. In rodents and in alveolar cell culture, oxygen toxicity (100 % $O_2$) has been used as a well-established model of lesions mimicking the acute phase of ALI/ARDS and for studying direct alveolar damage induced by high levels of oxidants. It was first explored in rats and later extensively characterized in mice [112–114]. During the initiation phase (usually lasting for 48 h), only subtle changes can be detected, such as the arrest of cell replication, and lesions are not evident on light microscopy. This phase is followed by diffuse alveolar damage with hyaline membrane deposition and extensive death of alveolar cells (mainly endothelial and epithelial cells) associated with a generally mild inflammatory response, which can vary according to the species [115]. Alveolar cell death has been shown to be directly related to increased generation of oxidant in hyperoxic

condition [116]. Reactive oxygen species can be generated by mitochondrial chain transport as well as by NADPH oxidase enzymes [117]. In vitro studies have shown that the diphenyleneiodonium (DPI), a non-specific inhibitor of NOX isoforms, was effective in reducing hyperoxia-induced ROS generation in a pulmonary epithelial cell line (MLE-12) and in primary pulmonary type II cells [117–119]. Recently, our laboratory demonstrated that NOX1, which is highly expressed in lungs, plays a crucial role in hyperoxia-induced acute lung injury [17]. NADPH oxidase1-deficient mice exposed to hyperoxia exhibited reduced pulmonary edema, hyaline membrane deposition, and alveolar-capillary damage. Indeed, in situ lung cell death was markedly decreased in NOX1-deficient mice and paralleled with decreased ROS production and cell death in endothelial and epithelial cells. The phosphorylation of both c-Jun *N*-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), as well as caspase-3 activation were decreased in lung homogenates. All these results demonstrate a role for NOX1 in hyperoxia-mediated barrier dysfunction; however, the question of the contribution of NOX1 restricted to the epithelial side or to endothelial side or both in ALI/ARDS and its precise cellular signaling pathway is still open.

As stated above, the integrity of the alveolar-capillary barrier depends not only on the epithelium but also on the endothelium. Recent studies have shown increased NOX1 mRNA expression in mouse lung endothelial cells after hyperoxic condition [17, 28] as well as NOX1 contribution to endothelial cell death [17]. The involvement of NOX4 in hyperoxic cultured endothelial cells (EC) has also been investigated. NADPH oxidase4 mRNA expression is increased in hyperoxia [28] through direct modulation of gene transcription. Indeed, direct interaction between nrf2 transcription factor and NOX4 promoter has been reported. The authors demonstrated that hyperoxia increased the recruitment- and the binding of nfr2 to endogenous NOX4 promoter via antioxidant response element (ARE) in pulmonary endothelial cells [44]. Hyperoxia also regulates NOX activation in part by ERK1/2 and p38 MAPK [117], but also by an Src-dependent tyrosine phosphorylation of $p47^{phox}$ [29]. It has also been proposed that NOX2 could be activated by tyrosine phosphorylation of cortactin and $p47^{phox}$ translocation following hyperoxia in ECs derived from human pulmonary artery [120]. Recently, Pendyala et al. [28] demonstrated the contribution of NOX4 in endothelial dysfunction. They showed that the transfection of a NOX4-specific siRNA in HPAEC attenuated hyperoxia-induced migration and capillary tube formation. Therefore, we have now convincing evidence of NOXs activation (NOX1, 2 and 4) by oxidative stress in murine and human endothelial cells [17, 29, 117, 120, 121]. Although we have less evidence, we could also hypothesize that NOX4 participates in epithelial cell damage in ALI/ARDS. Indeed, we recently reported that NOX4 mRNA was expressed in primary type II epithelial cells and mediated TGF-$\beta$1-induced epithelial cell death [7]. These results suggest that NOX4 contributes not only to the dysfunction or death of endothelial cells, but it might be involved in the alveolar-capillary disruption observed in ALI/ARDS. However, all these experiments were performed essentially in cell cultures and should be confirmed first by using NOX4-deficient mice or/and transfection of NOX4-specific siRNA and then in humans.

Controversial studies concerning the role of NOX2 in hyperoxia-induced lung injury have been reported. Pendyala showed that NOX2-deficient mice exposed to acute hyperoxia developed attenuated pulmonary edema, lung fibrosis, and weak inflammatory response [122]. By contrast, we found that NOX2-deficient mice exposed to hyperoxia were not protected and display a huge neutrophil influx in BAL, alveolar cell death, and lung injury [17], suggesting that NOX2 does not mediate alveolar-capillary disruption in hyperoxia. Indeed, neutrophil or macrophage depletion did not change lung damage in hyperoxic lung injury [123, 124]. Similarly, NOX2-deficient mice exposed to 48 h of hyperoxia following acid aspiration showed a greater amount of neutrophils compared to WT mice, without modification of lung injury [73].

## NOX as treatment of ALI/ARDS

As largely described above, NOX inhibitors might have potential in vivo use in ARDS. However, the multiplicity of lung cells combined with the cellular and functional specificity of the different NOX isoforms makes this approach delicate. Moreover, the measurement of NOX activity is often indirect since it is evaluated by the dosage of ROS-derived products using colorimetric or fluorescent probes. One must be aware that measuring ROS or derived products levels might not only be due to ROS production by NOX enzymes but also by other ROS-producing enzymes. Therefore the specific efficacy of NOX inhibition can be difficult to prove.

Today, therapeutic intervention for ALI/ARDS consists of protecting the lung by using adaptive mechanical ventilation and oxygenation and thus limiting mortality [125]. Lung-protective mechanical ventilation with lower tidal volumes in patients not suffering from acute lung injury: a review of clinical studies. This strategy was elaborated according to the results obtained in clinical trials and in experimental animal models [126]. Some studies have suggested that subgroups of patients may benefit from targeted therapeutic interventions. Most promising is the differentiation between patients in early versus late-phase

**Table 2** Summary of potential effect of NOX inhibitors in ALI/ARDS

| NOX isoform inhibitors | ARDS/ALI clinical stages | Target cells | Expected effects | Secondary effects |
|---|---|---|---|---|
| NOX1 | Acute stage: alveolar-capillary barrier disruption | Epithelial and endothelial cells | Decreased cell death (genotoxicity, MAPK signaling, TNF-RI-JNK signaling) | N.D |
| NOX2 | Acute stage: inflammation/ endothelial cell injury | Macrophages/ neutrophils | Decreased inflammatory response, endothelial cell death, crosstalk with TLR4 signaling | Increased susceptibility to infection |
| | | Endothelial cells | Decreased cell death, crosstalk with TLR2 signaling | N.D |
| NOX4 | Acute stage: alveolar-capillary barrier disruption | Epithelial cells | Decreased cell death (genotoxicity) interference with TGF-$\beta$ signaling | N.D |
| | Acute stage: inflammation/ endothelial cell injury | Endothelial cells | Decreased cell death,TLR4 crosstalk signaling | N.D |
| | Fibro-proliferative stage | Myofibroblasts | Decreased proliferation and differentiation, interference with TGF-$\beta$1 signaling | N.D |

ARDS, direct versus indirect lung injury, and patients with altered coagulation. A high dose of corticosteroid administration did not improve mortality, whereas low to moderate doses appear to be harmful if initiated later and are of unclear benefit [127, 128]. Surfactant supplementation was shown to be helpful only in pediatric patients with direct lung injury [129] and anticoagulants may be successful in the subgroup of patients with vascular disease [130]. There is an interest in developing NOX inhibitors, since they can act for some of them in the early phase and for other in the fibro-proliferative phase. Table 2 summarizes the expected effects of the potential NOX inhibitors for the treatment of ARDS.

NADPH oxidase1 inhibition might be useful in the acute phase of ALI/ARDS since it interferes with endothelial and epithelial cell death either by decreasing oxidative stress-induced genotoxicity or by affecting MAPK signaling pathways [17]. However, this study was performed only in mice and evidence in humans is still required. The possibility that NOX1 inhibition can affect TNF activation might also be interesting in case of ARDS due to sepsis [22].

NADPH oxidase2 inhibition effects are more complex and the results are somehow controversial. NADPH oxidase2 is present mainly in phagocytic cells, but also in a great amount in endothelial cells. Indeed, changing phagocyte killing might be dangerous in situations of ARDS due to sepsis or to unknown origin even if ROS-produced by NOX2 in other cells might prevent concomitantly. In this case, a tagged-cell inhibitor would be ideal, but at the present time rather difficult.

More promising would be the inhibition of NOX4, which is present in several lung cells epithelial and fibroblasts. Several studies have shown a very robust effect in decreasing epithelial cell death initiated by TGF-$\beta$1 and myofibroblast differentiation [7, 8]. Moreover, NOX4 action is upstream of the pleiomorph effects of TGF-$\beta$1, and therefore could be more efficient in blocking the TGF-$\beta$1 deleterious cascade. We can also hypothesize, as NOX4 is strongly expressed in epithelial cells and its signaling potentiates cell death induced by TGF-$\beta$1, that NOX4-specific inhibition could prevent the alveolar-capillary disruption in the ARDS early phase [7]. NADPH oxidase4 is also involved in TLR4 signaling mediated by LPS and might therefore participate in endothelial dysfunction [84]. This study has been performed in vitro, and more in vivo data are needed before envisaging therapeutic possibilities.

## Conclusions

NADPH oxidase enzymes, which are widely expressed in different lung cell types, participate not only in the maintenance of physiological processes in lungs but also contribute to the pathogenesis of acute lung diseases such as ALI and ARDS. The multiplicity of the well-characterized animal models mimicking ARDS/ALI, the use of NOX-deficient mice and in vivo siRNA transfection strategies allowed to explore NOX-dependent cellular and molecular mechanisms involved in the development of the disease and finally envisage new therapeutic approaches. Developing NOX inhibitors could therefore be a promising treatment concept for ARDS/ALI. Nevertheless, at the present time, no direct method for measuring specifically NOX-dependent ROS generation has been developed to prove the efficacy of NOX inhibitors and specific inhibitors for one single NOX isoform are not available. Whether in some case it might be useful to target two different isoforms concomitantly, such as in early phase, in other situations such as ARDS induced by sepsis, this could be deleterious due to combined unwarranted secondary effects. Thus, further in vivo studies concerning NOX inhibitors are necessary to prove their clinical utility in the management of ALI/ARDS.

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