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Role of Nrf2 and Autophagy in Acute Lung Injury

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Abstract Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are the clinical manifestations of severe lung damage and respiratory failure. Characterized by severe inflammation and compromised lung function, ALI/ARDS result in very high mortality of affected individuals. Currently, there are no effective treatments for ALI/ARDS, and ironically, therapies intended to aid patients (specifically mechanical ventilation, MV) may aggravate the symptoms. Key events contributing to the development of ALI/ARDS are increased oxidative and proteotoxic stresses, unresolved inflammation, and compromised alveolar-capillary barrier function. Since the airways and lung tissues are constantly exposed to gaseous oxygen and airborne toxicants, the bronchial and alveolar epithelial cells are under higher oxidative stress than other tissues. Cellular protection against oxidative stress and xenobiotics is mainly conferred by Nrf2, a transcription factor that promotes the expression of genes that regulate oxidative stress, xenobiotic metabolism and excretion, inflammation, apoptosis, autophagy, and cellular bioenergetics. Numerous studies have demonstrated the importance of Nrf2 activation in the protection against ALI/ARDS, as pharmacological activation of Nrf2 prevents the occurrence or mitigates the severity of ALI/ARDS. Another promising new therapeutic strategy in the prevention and treatment of ALI/ARDS is the activation of autophagy, a bulk protein and organelle degradation pathway. In this review, we will discuss the strategy of concerted activation of Nrf2 and autophagy as a preventive and therapeutic intervention to ameliorate ALI/ARDS.

Keywords Acute lung injury · Oxidative stress · Nrf2 · Autophagy

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Acute Lung Injury and Acute Respiratory Distress Syndrome

Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), are severe inflammatory diseases of the lung caused by disruption of the lung endothelial (capillaries) and epithelial (alveoli) barriers. ARDS was first described by Ashbaugh et al. in [3], but it was not until 1994 that a consensus definition and description of ALI/ARDS was achieved by the American-European Consensus Conference (AECC) [3]. The clinical criteria that define ALI/ARDS are the following: (1) acute onset of respiratory symptoms, (2) chest radiographs with bilateral infiltrates, (3) a ratio of partial pressure of oxygen in arterial blood (PaO_2) to

fraction of inspired oxygen (FiO_2) of <200 mmHg for ARDS and <300 mmHg for ALI, (4) pulmonary capillary wedge pressure (PCWP) of less than 18 mmHg, and (5) the absence of clinical evidence of primary left atrial hypertension [7].

It is estimated that 64.2–78.9 per 100,000 persons per year develop ALI, which has a mortality rate of 38.5 % [95]. Based on these data, approximately 190,600 people in the USA each year will develop ALI, and 74,500 people will not survive [82, 95]. Some survivors recover completely [35, 95]; however, others may have lasting damage to their lungs and develop additional health problems [62, 95].

Pathogenesis of ALI/ARDS

As ALI/ARDS is a syndrome, or a collection of signs and symptoms, and not a specific disease, there are many insults that can produce lung injury directly or indirectly and cause ALI/ARDS [7]. Direct pulmonary injury involves a disease process that begins in the lungs by causing primarily lung damage, such as lung contusions, diffuse pulmonary infections (bacterial, viral, and fungal pneumonia), aspiration of gastric contents, near drowning, inhalation of toxic substances, or hyperoxia [7]. ALI/ARDS can be induced or aggravated by mechanical ventilation (MV), a therapeutic intervention used to assist patients with compromised respiratory functions due to edema, trauma, or general anesthesia [101]. MV causes ventilation-induced lung injury (VILI) due to different types of lung injury, including (1) volutrauma, from lung over-distension; (2) barotrauma, from the direct effect of high pressure on the lung; (3) atelectrauma, from the shear stress of repetitive opening and closing of alveoli; and (4) biotrauma, from the generation of cytokines and inflammatory cascades [86]. Indirect pulmonary injury is a systemic disease process that affects the lungs secondarily, as in sepsis syndrome and systemic inflammatory response syndrome (SIRS), severe non-thoracic trauma, shock, acute pancreatitis, cardiopulmonary bypass, transfusion-related ALI (TRALI), disseminated intravascular coagulation (DIC), and burns [7].

Direct or indirect injury to the lung parenchyma leads to damage of type I and type II epithelial cells of the alveoli, and of endothelial cells of the pulmonary capillaries, resulting in loss of the alveolar-capillary barrier [6]. Without this barrier, fluid from the capillaries leaks into the interstitium and the alveoli, causing pulmonary edema, collapse of the lungs (atelectasis), and respiratory failure [6]. These classic histological manifestations of ARDS in the lung are called diffuse alveolar damage (DAD) [6]. The pathogenesis of DAD begins with an acute/exudative stage (≤7 days after insult), characterized by damage to the alveolar-capillary barrier, resulting in the formation of an exudate in alveolar spaces and interstitium, activation of resident alveolar macrophages, and release of pro-inflammatory cytokines to promote neutrophil infiltration. Macrophages and neutrophils secrete various proteases and oxidants that further injure alveolar endothelial and epithelial cells and digest the alveolar stroma. As a result, type II alveolar epithelial cells and fibroblasts proliferate in an attempt to repair the lung injury during the organizing/fibroproliferative stage [6]. DAD may gradually resolve within 6–12 months; however, the most common outcome is a fibrotic stage that results in chronic respiratory compromise [6, 64].

Experimental Models for Studying ALI/ARDS In Vivo

Based on the diverse etiology of ALI/ARDS, many different modeling strategies have been developed in animals in an attempt to reproduce the features of human ALI/ARDS. Direct lung injury in animals can be induced by (1) the intratracheal or intranasal administration of bacteria or bacterial products, such as lipopolysaccharide (LPS) from the outer cell wall of gram negative bacteria, or peptidoglycan and lipoteichoic acid from gram positive bacteria, to reproduce diffuse pneumonia; (2) the administration of hydrochloric acid or gastric particulates to reproduce aspiration; (3) the administration of high inspired fractions of oxygen to induce hyperoxia; or (4) the induction of ischemia/reperfusion by clamping the hilum [65]. Indirect lung injury is based on (1) reproducing sepsis using cecal ligation and puncture, (2) the administration of intravenous bacteria or LPS, or (3) mesenteric ischemia/reperfusion [65]. Animal models of VILI consist of mechanical ventilation with high tidal volumes and low positive end-expiratory pressure [100].

Current Therapeutic and Treatment Options for ALI/ARDS

The treatment for ARDS is mainly supportive and focuses on maintaining ventilation and oxygenation, normal cardiac function, and nutritional support, as well as the administration of nitric oxide (NO) and corticosteroids, and the prevention of further complications [64, 99]. A more appealing strategy would be to treat the underlying cause for the lung injury and respiratory distress [64, 99]. Central to the pathogenesis of ALI/ARDS is the generation of reactive oxygen species (ROS) that cause oxidative stress. Multiple cell types in the lungs, including endothelial cells, neutrophils, eosinophils, alveolar macrophages, and alveolar epithelial cells, are major ROS generators [67]. ROS may be produced by inflammatory cells (macrophages and neutrophils) trying to fight infections, or by dysfunctional mitochondria or enzymatic systems in alveolar epithelial cells and capillary endothelial cells after lung tissue damage by hyperoxia, electrophilic xenobiotics,

and shear stress [8, 9, 79, 97]. Antioxidants have been shown to reduce the severity of ALI/ARDS in multiple mouse models that use different initiating insults, highlighting the central role of ROS in the pathophysiology of ALI/ARDS [2, 10, 36–38, 56, 85, 103, 104, 106, 112].

The Nrf2 Signaling Pathway

Nrf2 (nuclear factor erythroid-2 related factor 2) is a transcription factor that regulates an adaptive cellular defense response to various stresses, including oxidative, proteotoxic, and metabolic stresses, as well as inflammation (Fig. 1). Nrf2 heterodimerizes with small Maf (sMaf) proteins and together they bind the antioxidant response elements (AREs) in the regulatory regions of Nrf2 target genes that participate in the regulation of oxidative stress (*HO-1*, *GCLM*, *TXNRD1*), xenobiotic metabolism and excretion (*NQO1*, *AKR1C1*, *GSTA*, *MRP1*), inflammation (*TGF-β*, *NF-κB*), apoptosis (*Bcl-2*, *Bcl-xL*), autophagy (*p62*), and cellular bioenergetics (*G6PD*, *PPARγ*) (Fig. 1) [29, 42, 47]. Even though Nrf2 is ubiquitously expressed in all cell types and tissues, its protein levels are kept low under basal (homeostatic) conditions since it is constantly degraded. The main negative regulator of Nrf2 is Keap1 (Kelch-like ECH associated protein 1), a substrate adaptor for a Cullin 3 (Cul3)-containing E3 ubiquitin ligase complex that ubiquitinates Nrf2 to promote its degradation by the 26S proteasome [48, 94, 108]. Nrf2 binds through its N-terminal ETGE and DLG motifs to two Keap1 proteins at the Kelch domain in what is known as the hinge-and-latch model: the ETGE (hinge) binds to the Kelch domain in Keap1 more strongly than the DLG (latch) motif, which oscillates between open and closed conformations [5, 92, 93]. Nrf2 ubiquitination occurs only when both of its motifs are binding to Keap1 in a closed conformation [5]. Keap1 is a sensor of the intracellular redox status: oxidants and electrophilic chemopreventive compounds covalently modify several important cysteine residues, in particular cysteine 151 (C151), in Keap1. Oxidation or adduction of C151 causes a conformational change in Keap1 that affects the DLG-Kelch interaction, thus preventing Nrf2 ubiquitination and subsequent degradation [109, 110]. Consequently, Nrf2 protein is stabilized, accumulates, and translocates into the nucleus to activate the transcription of its target genes. Many known electrophilic chemopreventive compounds activate Nrf2 signaling through the canonical mechanism, and mutation of cysteine 151 to serine (Keap1-C151S) in Keap1 completely abolishes Nrf2 upregulation by canonical Nrf2 activators [42]. Signal termination occurs when the redox balance is restored, or when the chemopreventive compounds are metabolized and eliminated. At this point, Keap1 shuttles into the nucleus, binds to Nrf2, and brings it back into the cytosol for degradation [87].

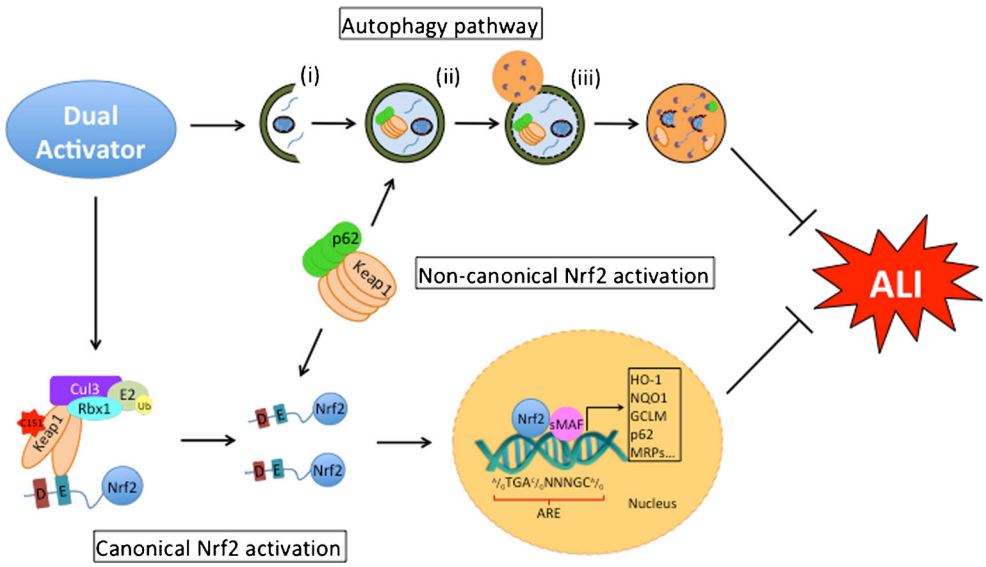

Fig. 1 Nrf2 and autophagy are activated during ALI/ARDS. During ALI, production of reactive oxygen species activates Nrf2 and autophagy. *Nrf2 pathway.* The transcription factor Nrf2 binds to Keap1-Cul3-Rbx1 E3 ubiquitin ligase complex to be ubiquitinated and degraded. During ALI/ARDS, ROS modify cysteine 151 in Keap1 (C151), affecting its interaction with Nrf2. As a result, Nrf2 is no longer ubiquitinated and is stabilized. Newly synthesized Nrf2 accumulates in the cytosol, translocates to the nucleus, dimerizes with small Maf proteins, and binds to the antioxidant response element (ARE) in the regulatory regions of its target genes to promote their transcription (canonical Nrf2 activation). Nrf2 can also be regulated by autophagy during ALI/ARDS. The autophagy substrate protein p62 binds to Keap1, sequestering it into the autophagosome and allowing for Nrf2 accumulation (non-canonical Nrf2 activation). *Autophagy pathway.* ALI/ARDS induce autophagy, a bulk degradation pathway that occurs in three main stages: (*i*) initiation, (*ii*) elongation, and (*iii*) fusion. Initiation involves the formation of the phagophore, which then elongates to encapsulate damaged proteins and organelles in a double membrane-bound autophagosome. The autophagosome then fuses with the lysosome, and the damaged cargo is degraded by lysosomal hydrolases

The p62-Dependent, Non-canonical Mechanism, of Nrf2 Regulation

Recent studies have identified other Nrf2 regulatory protein complexes that participate in other pathophysiological processes [27, 73, 74, 102]. One of these corresponds to the autophagy-related protein p62 (sequestosome 1, SQSTM1), which activates Nrf2 [52]. Autophagy is a bulk degradation pathway that involves the sequestration of damaged cellular components into an autophagosome, which fuses with the lysosome to form an autolysosome that degrades the cargo using lysosomal enzymes (see below for a more detailed description of the pathway and its role in ALI). p62 is an adaptor protein that recognizes the degradation cargo and brings it to the autophagosome; importantly, p62 is itself an autophagy degradation target. Autophagy inhibition at the late stage causes accumulation of autophagosomes and results in pathological activation of Nrf2 signaling [30, 41, 49, 52, 81]. This occurs because p62 contains a pSTGE motif that competes with the ETGE/DLG motifs in Nrf2 for Keap1 binding [39, 41]. When the number of autophagosomes increases, p62 sequesters Keap1 and thus Nrf2 is stabilized (Fig. 1) [44]. Our group called this mode of Nrf2 activation the non-canonical mechanism, as it is Keap1-C151-independent but p62-dependent [52]. For example, arsenic, a human carcinogen, is an Nrf2 activator that induces Nrf2 through blockage of autophagy-lysosomal fusion [53]. This mode of Nrf2 activation arises from a pathological state (autophagy dysregulation) and results in prolonged Nrf2 activation (the "dark side" of Nrf2); therefore, it is not desirable for cellular protection. On the other hand, induction of autophagy (increased autophagy flux) leads to the sequestration of Keap1-p62 complexes into autophagosomes, resulting in lysosomal-mediated degradation of Keap1 and controlled Nrf2 activation. This mode of Nrf2 activation is intermittent and confers protection. Nrf2 also promotes the ARE-driven expression of p62, indicating that autophagy and the Nrf2-mediated antioxidant response work in concert to restore cellular homeostasis [89].

The Protective Role of Nrf2 in ALI

The airway and lung epithelial cells constitute an important interphase between the body and the environment, and as such, these tissues are constantly exposed to oxidative stress and toxicants [22]. Therefore, the expression of intra and extracellular antioxidants in airway epithelial cells and fluids is prominent. Numerous in vitro and in vivo studies have demonstrated the importance of Nrf2 activation to decrease oxidative stress and inflammation in conditions such as pulmonary fibrosis, cystic fibrosis, emphysema, COPD, ALI, asthma, bronchopulmonary dysplasia, and airway infections [12, 23]. In the particular context of ALI/ARDS, the benefits of both basal and induced Nrf2 signaling have been investigated in various cellular and mouse models, as well as in human patient samples, as will be discussed below.

As mentioned above, the main regulation of Nrf2 occurs post-transcriptionally; however, over 500 single nucleotide polymorphisms (SNPs) in the regulatory and coding regions of *Nrf2* have been identified [18]. The first evidence that Nrf2 had a role in ALI came from identification of *Nrf2* as a candidate gene for ALI susceptibility by positional cloning in mice and subsequent analysis of SNPs in human populations and in vitro functional studies [63]. Multiple studies have determined that SNPs in *Nrf2* confer susceptibility to ALI and other respiratory diseases like COPD and asthma [20, 24, 28]. In a hyperoxia-induced ALI mouse model, the functional effects of promoter and coding SNPs revealed that some haplotypes characteristic to certain inbred mouse strains confer increased susceptibility to hyperoxia due to lower *Nrf2* mRNA expression and compromised protein function [20]. These studies will help identify populations at greater risk of developing ALI that would benefit from more effective chemopreventive interventions through Nrf2 upregulation.

Hyperoxia-induced ALI causes oxidative lung damage. $Nrf2^{-/-}$ mice were more susceptible to hyperoxia (95–98 % oxygen, 72 h) and developed more severe ALI-like phenotypes than $Nrf2^{+/+}$ mice, as determined by increased lung permeability, inflammation, and lung epithelial damage [21]. In $Nrf2^{-/-}$ mice, both the basal and induced expression of Nrf2 target genes were lower than in $Nrf2^{+/+}$, demonstrating that Nrf2 transcriptional activity is key in the response to hyperoxia-induced ALI. Furthermore, conditional deletion of Nrf2 in club cells (Clara cells, secretory bronchoepithelial cells) using cell type-specific Cre recombinase (*CCSP-Cre* × $Nrf2^{fl/fl}$) yielded mice more sensitive to hyperoxia-induced ALI, confirming that Nrf2 activity in the airways is necessary for protection in this model system [77]. Additionally, unresolved oxidative stress and inflammation in $Nrf2^{-/-}$ mice increased apoptosis in sublethal hyperoxic exposure (48 h) and compromised tissue repair [76]. These results indicate that Nrf2 has additional roles that go beyond the resolution of oxidative stress, including the regulation of inflammation and tissue remodeling factors [23]. Currently, it is unknown if Nrf2 modulates these processes directly or indirectly, so further detailed mechanistic studies are needed to elucidate these Nrf2-dependent effects. Pharmacological Nrf2 activation as a strategy to prevent and treat hyperoxia-induced ALI has been explored. The Nrf2 activator CDDO-Im (a synthetic triterpenoid compound, CDDO-imidazole) conferred resistance against hyperoxia [78]. In contrast to the clear effects of Nrf2 upregulation, administration of direct antioxidants like N-acetylcysteine (NAC), has limited efficacy in protection against hyperoxia-induced ALI. Administration of antioxidants to quench ROS is a strategy with limited effectiveness due to their limited availability and the fact that once oxidized they are useless, and some antioxidants only

scavenge certain ROS selectively. However, Nrf2 activation promotes sustained expression of endogenous antioxidants, detoxifying/excretion enzymes, repair and degradation proteins, and metabolic reprogramming [29]. Together, these Nrf2 downstream effector proteins remove insults, neutralize ROS, and repair damage to restore cellular homeostasis. In addition, Nrf2 regulates the expression of anti-inflammatory, pro-proliferative, anti-apoptotic, and autophagy-related genes, constituting a more holistic approach to counteract and repair the damage [42]. As such, Nrf2 activation provides not only short-term beneficial effects but also confers medium and long-term protection.

In high tidal volume MV models that induce VILI/ALI, *Nrf2*$^{-/-}$ mice have greater structural damage, increased alveolar-capillary permeability, greater inflammation and oxidative stress than *Nrf2*$^{+/+}$ mice [71]. Supplementation of NAC decreased VILI in *Nrf2*$^{-/-}$ mice, indicating that oxidative stress is a major contributing factor in the pathogenesis of VILI. Subsequent studies have been performed to evaluate the effects of Nrf2 activation in the protection against VILI. Sodium sulfide protects against VILI by upregulating Nrf2 target genes involved in the restoration of redox balance [31]. Our group recently demonstrated that a newly identified Nrf2 activator, the carotenoid and food additive bixin, protects against VILI only in *Nrf2*$^{+/+}$ but not *Nrf2*$^{-/-}$ mice [90].

ALI-like symptoms can also be chemically induced in mice using agents that cause oxidative damage and inflammation. The phenolic antioxidant butylated hydroxytoluene (BHT) is used as food additive to prevent the oxidation of lipids, and while it is relatively safe in humans, it is toxic when administered to mice. Oral administration of BHT is noxious to alveolar type I epithelial cells, the cells that comprise most of the alveolar structure and are responsible for the gas exchange and barrier functions [98]. BHT causes ALI-like symptoms in the lungs of *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice, which develop massive edema and hemorrhage; however, *Nrf2*$^{-/-}$ mice are much more susceptible [12]. The precise cytotoxic mechanism of BHT in the lung has not been clarified, but oxidative stress may play a central role as *Nrf2*$^{+/+}$ mice are more resistant [25]. The effects of oxidative lung damage have been studied in an ozone (O_3) mouse model. O_3 is a strong oxidant and air pollutant that greatly affects the airways and lungs. Predictably, Nrf2 deficiency in mice exacerbates oxidant-induced lung injury [19]. An important implication of this study is that Nrf2 activation by chemopreventive compounds could be very beneficial for populations living in areas with poor air quality exposed to very high O_3. Carrageenin, a seaweed-derived polysaccharide used as a thickening and emulsifying agent in food and pharmaceuticals, induces acute inflammation and ALI-like symptoms after intratracheal injection [68]. Consistently, *Nrf2*$^{-/-}$ mice are more susceptible to carrageenin-induced ALI than *Nrf2*$^{+/+}$ mice [68]. This study also helped to elucidate the anti-inflammatory functions of Nrf2. Carrageenin-induced inflammation induced COX-2 (cyclooxygenase 2), an enzyme that converts arachidonic acid into prostaglandins, which are mediators of inflammation [80]. In particular, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) activates Nrf2, and pharmacological inhibition of COX-2 induced severe ALI in *Nrf2*$^{+/+}$ mice comparable to that of *Nrf2*$^{-/-}$, which may be due at least in part to decreased expression of Nrf2 target genes [68, 80]. These results indicate that resolution of inflammation and oxidative injury are both important to prevent ALI.

Extensive work has been performed to characterize the biological effects of acute exposure to certain gases that represent particularly important hazards, either for their use as chemical warfare agents or due to common work place exposure. These studies have demonstrated that lung epithelial cells are among the first to be exposed to toxic gases such as chlorine (Cl_2), phosgene, mustard gas, and hydrogen sulfide (H_2S), making these cells a particularly important target for toxicity and for therapeutic intervention [13, 72]. A common feature of these gases is that acute exposure causes airway and lung epithelial cell apoptosis, alveolar-capillary barrier dysfunction, and an exacerbated inflammatory response [66]. Central to the pathophysiological mechanisms of these gases is the generation of ROS [113]. Many studies have proposed the use of antioxidants to diminish the effects of acute exposure to toxic gases as no effective antidotes or countermeasures have yet been discovered [1, 13, 43, 72]. Antioxidants like NAC and the metalloporphyrin AEOL10150 have been used with moderate success to reduce ROS, oxidative damage, and inflammation [43, 66]. Few studies have investigated the beneficial effects of targeted Nrf2 activation to ameliorate toxic gas exposure, but this strategy may prove to be more effective.

Nrf2 also participates in infection-induced ALI. The bacteria *Staphylococcus aureus* causes pneumonia and in extreme cases progresses to ALI due to increased alveolar permeability, neutrophil infiltration, and cytokine production. *Nrf2*$^{-/-}$ mice developed ALI after *S. aureus* inoculation, unlike *Nrf2*$^{+/+}$ mice, due to enhanced inflammation and decreased mitochondrial biogenesis in alveolar cells of Nrf2$^{-/-}$ mice [4]. Mitochondria not only maintain cellular energy production, but when damaged, they can also be a major source of ROS. Autophagy, in particular mitophagy, degrades damaged mitochondria. Therefore, these results suggest that the mitochondrial effects of Nrf2 and possibly its crosstalk with mitophagy are essential for lung adaptation and recovery from injury [29].

The Autophagy-Lysosomal Pathway

The autophagy-lysosomal pathway is a key cellular degradation pathway responsible not only for the removal of long-lived proteins, but also damaged or dysfunctional proteins

and organelles, breaking them down to monomeric components for re-use by the cell. Autophagic dysfunction has been linked to the progression of a number of diseases including diabetes, neurodegeneration, cardiovascular disease, pulmonary disease, and cancer, demonstrating the importance of proper autophagic function in preserving normal cellular homeostasis [26]. Three main autophagic pathways have been identified: microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy (hereafter referred to as autophagy). The three main stages of the autophagy pathway are, (i) initiation, (ii) elongation, and (iii) fusion (Fig. 1), and are mediated by a number of tightly regulated protein-protein and protein-lipid interactions. Initiation of autophagy requires formation of the Beclin-1/Vps34/UVRAG/Atg14L complex, which based on its binding partners, can result in either autophagy activation (i.e., bound to AMBRA-1 or Bif-1) or autophagy inhibition (i.e., bound to Rubicon or GAPR-1) [50, 55]. Following initiation, elongation of the phagophore involves the activation of two autophagy-related (Atg) protein-dependent ubiquitin-like conjugation systems, Atg5 conjugation to Atg12 mediated by Atg7 and Atg10, and the conjugation of microtubule-associated protein light chain 3 (LC3) to phosphatidylethanolamine via Atg3 and Atg10 [75]. The elongating phagophore encapsulates its cargo to form a double membrane-bound autophagosome, which then fuses with the lysosome via the interaction between endosomal sorting complexes required for transport (ESCRT) proteins, soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) proteins, as well as Rab7 and a number of vacuolar protein sorting (Vps) proteins [75]. This fused vesicle, termed the autolysosome, can then degrade its contents using a battery of hydrolases, which break down the cargo for re-use by the cell.

Activation of Autophagy During ALI

While the role of autophagy in chronic lung disease and cigarette smoke exposure has been studied [16, 83], very few studies have investigated the role of autophagy in ALI. As mentioned above, ALI is induced by different stimuli, including mechanical injury, exposure to industrial or environmental toxicants, infection, and sepsis. Autophagy has been shown to contribute to the progression of acute viral and bacterial lung infection. *Atg5*$^{fl/fl}$;*LysM-Cre*$^{+}$ mice, which have autophagy deficient macrophages, exhibit increased bacterial burden and inflammation following infection with *Mycobacterium tuberculosis* [11]. Autophagy also plays a key role in alveolar macrophages and mast cells in response to *Pseudomonas aeruginosa* infection, with knockdown of *Beclin1* or *Atg5*, two key autophagy initiation proteins, resulting in decreased clearance and intracellular accumulation of *Pseudomonas* in infected macrophages [107]. These studies indicate that autophagy can play either a protective or detrimental role, depending on the pathogen and cell type affected during lung infection.

One of the main initiators of autophagy in ALI is increased oxidative stress, which has been shown to induce autophagy in a number of lung cell lines and animal models of lung injury [60]. For example, lung endothelial cells of rodents exposed to sulfur mustard or nitrogen mustard exhibit increased oxidative stress and autophagy activation within 6 h of gas exposure [61]. O_3 exposure has also been shown to result in oxidative stress-induced autophagy in the rat lung [88]. Heavy metal exposure, such as arsenic trioxide, can also lead to oxidative stress and autophagy dysregulation in human bronchial epithelial cells [111]. Exposure of H441 lung epithelial cells to chlorine gas (Cl_2) induces mitochondrial dysfunction and increased reactive oxygen species production 1 h after exposure, with increased autophagy being associated with improved bioenergetic function by 6 h post-exposure [46]. While autophagy upregulation is thought to be sufficient for preventing toxic protein aggregation during mild oxidative stress, if the damage is too great and autophagy can no longer compensate, apoptosis is initiated leading to cell death [17]. This may account for why autophagy upregulation is generally observed during lung injury, but is not always sufficient to mitigate or protect against inflammatory damage. Thus, a need for a better understanding of the temporal regulation of autophagy activation during ALI will be required to create therapeutics that maintain the beneficial effects of autophagy upregulation and reduce proteotoxic effects following toxic-gas exposure, and in turn, preserve lung function.

Protective Role of Autophagy During ALI

Studies have also demonstrated that upregulation of autophagy can be protective during ALI. For example, pharmacological activation of autophagy using rapamycin, a well-established autophagy activator, enhances the clearance of *Pseudomonas* from lung epithelial cells, whereas inhibition of autophagy using the lysosomal inhibitor chloroquine resulted in the accumulation and decreased killing of *Pseudomonas* bacteria [45]. Autophagy is downregulated following cecal ligation and puncture (CLP) injury, a model for sepsis-induced ALI, with rapamycin treatment enhancing autophagic activity, reducing the pro-inflammatory response, and preventing activation of the apoptotic cascade in septic mice [105]. Transgenic mice overexpressing *LC3*, a protein integral for autophagosome formation, also exhibit improved survival following CLP [57]. LPS-induced lung damage is exacerbated by the inhibition of autophagy, and rapamycin treatment decreased LPS-induced inflammatory cytokine production and secretion in lung macrophages [32, 58, 69]. Mice administered trehalose (2 % in the drinking water), an mTOR-independent

autophagy activator, for 6 weeks or aerosolized trehalose administration for 24 h prior to Cl_2 exposure demonstrated improved bioenergetic function and decreased lung inflammation [46]. Rapamycin treatment following paraquat-induced oxidative stress and ALI also significantly reduced lung inflammation and damage in mice via the inhibition of mTOR to activate autophagy [14]. Interestingly, low dose carbon monoxide has also been shown to stimulate oxidative stress, which upregulates autophagy to protect against hyperoxia-induced lung injury [54]. These studies indicate that autophagy activation, both before and after toxic insult, can significantly decrease lung inflammation, improve lung cell bioenergetic function, and mitigate the damage associated with ALI.

Dual Activation of Nrf2 and Autophagy as a Potential Therapy for ALI

As the above studies indicate, proper upregulation of Nrf2 and the autophagy pathway are required to mitigate damage during ALI, and thus represent two key pathways that can be targeted for therapeutic benefit. Since there is crosstalk between autophagy and Nrf2 activation through the Keap1-p62 interaction, it is presumable that dual pathway activators could be used to maximize the therapeutic benefit of Nrf2 and autophagy activation, however, the *mechanism* of activation must be considered.

In recent years, uncontrolled Nrf2 activation, the "dark side" of Nrf2, has been recognized as a contributing factor of cancer chemoresistance and tissue damage [44, 51]. This uncontrolled activation may result in either constitutive or prolonged activation of Nrf2. The constitutive activation of Nrf2 observed in numerous cancer cell lines and patient tumor samples confers them a more malignant phenotype, as assessed by higher proliferative rates, increased migration, and chemo- or radio-resistance [15, 33, 34, 59, 70, 84, 91, 96]. Prolonged Nrf2 activation occurs when autophagy is dysregulated, either by deletion of *Atg5* and *Atg7*, or when autophagosome-lysosome fusion is blocked by arsenic treatment, causing the formation of p62-Keap1 aggregates and Nrf2 activation [44]. Autophagy dysfunction and prolonged Nrf2 activation cause tissue damage, inflammation, fibrosis, and tumorigenesis. In the lungs, inducible disruption of *Atg7* caused bronchiolar epithelial cell edema and hyperresponsiveness of the lungs to a cholinergic stimulus [40]. Interestingly, these $Atg7^{-/-}$ cells had p62 accumulation and increased levels of Nrf2 target genes. On the other hand, basal or pharmacologically induced autophagy, and the subsequent activation of Nrf2, may constitute a good approach for lung protection. This mode of autophagy activation, much like the canonical activation of Nrf2, is oscillatory: activation of autophagy occurs after a stimulus (for example, oxidative stress or rapamycin administration) and elimination of the stimulus (restoration of redox homeostasis or drug metabolism and excretion) restores the pathway to basal levels. Therefore, this controlled dual Nrf2-autophagy activation maintains the cytoprotective properties of both pathways.

Conclusion

ALI/ARDS are lung dysfunction syndromes with high prevalence and mortality, mainly because no therapies against them have been approved. ALI can be induced directly or indirectly by oxidative stress. Airway and lung epithelial cells are protected from oxidative stress by the Nrf2 and autophagy pathways. While extensive research has demonstrated the importance of Nrf2 in the protection against ALI and other lung pathologies, the role of autophagy is still ambiguous, though sufficient evidence supports its role as protective. Controlled dual activation of Nrf2 and autophagy may prove to be a better treatment strategy than single pathway activation due to the existing crosstalk of these pathways and potential synergistic beneficial effects.

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Compliance with Ethical Standards

Conflict of Interest The authors have no conflict of interest to disclose.

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