Endothelial PINK1 Mediates the Protective Effects of NLRP3 Deficiency during Lethal Oxidant Injury

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Endothelial PINK1 Mediates the Protective Effects of NLRP3 Deficiency during Lethal Oxidant Injury

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High levels of inspired oxygen, hyperoxia, are frequently used in patients with acute respiratory failure. Hyperoxia can exacerbate acute respiratory failure, which has high mortality and no specific therapies. We identified novel roles for PTEN-induced putative kinase 1 (PINK1), a mitochondrial protein, and the cytosolic innate immune protein NLRP3 in the lung and endothelium. We generated double knockouts (PINK1<sup>−/−</sup>/NLRP3<sup>−/−</sup>), as well as cell-targeted PINK1 silencing and lung-targeted overexpression constructs, to specifically show that PINK1 mediates cytoprotection in wild-type and NLRP3<sup>−/−</sup> mice. The ability to resist hyperoxia is proportional to PINK1 expression. PINK1<sup>−/−</sup> mice were the most susceptible; wild-type mice, which induced PINK1 after hyperoxia, had intermediate susceptibility; and NLRP3<sup>−/−</sup> mice, which had high basal and hyperoxia-induced PINK1, were the least susceptible. Genetic deletion of PINK1 or PINK1 silencing in the lung endothelium increased susceptibility to hyperoxia via alterations in autophagy/mitophagy, proteasome activation, apoptosis, and oxidant generation. The Journal of Immunology, 2014, 192: 5296–5304.

H igh levels of oxygen are often administered as a necessary and life-saving intervention in critically ill patients. Yet, prolonged oxygen therapy at high concentrations (hyperoxia) has been shown to promote respiratory failure and increase mortality, for which specific therapies do not exist (1). Identifying the molecular pathways involved in promoting and resisting hyperoxia-induced lung injury and mortality is essential for the design of effective therapies against oxidant lung injury.

Targeting the inflammasome subunit, NLRP3 has therapeutic effects in a variety of inflammatory diseases. Recently, Fukumoto et al. (2) reported that NLRP3<sup>−/−</sup> mice were protected from hyperoxia-induced acute lung injury, but the precise mechanism remained unclear, and survival was not presented. We show significant survival differences in NLRP3<sup>−/−</sup>, Asc<sup>−/−</sup>, and Caspase 1/11-knockout mice, as well as identify PTEN-induced putative kinase 1 (PINK1) to be a novel mechanism by which NLRP3 deficiency protects against lethal lung injury.

PINK1 helped to maintain mitochondrial homeostasis by inducing autophagy of damaged mitochondria, a process called mitophagy (3). To our knowledge, we identified for the first time a critical cytoprotective role for lung endothelial PINK1 during lethal hyperoxia. Furthermore, we show that PINK1 mediates the protective effects of NLRP3 deficiency by regulating proteasome activity, apoptosis, and oxidant production in lung endothelial cells and tissues during hyperoxia. These results offer new insights into PINK1 and inflammasome biology, as well as establish previously unrecognized links with autophagy/mitophagy and proteasome activation.

Materials and Methods

Mice

NLRP3<sup>−/−</sup>, Asc<sup>−/−</sup>, and Caspase 1/11<sup>−/−</sup> mice were provided by Dr. Richard Flavell (Yale University) (4, 5). PINK1<sup>−/−</sup> mice were provided by Dr. Jack Elias (Brown University, Providence, RI) and Dr. Jie Shen (Harvard University, Cambridge, MA) (6). All of the mice were backcrossed for >10 generations onto a C57BL/6J background. NLRP3<sup>−/−</sup>/PINK1<sup>−/−</sup> mice were generated by crossing NLRP3<sup>−/−</sup> mice with PINK1<sup>−/−</sup> mice for >10 generations. Mice were bred and exposed to hyperoxia as described previously (7). All protocols were reviewed and approved by the Animal Care and Use Committee at Yale University.

Isolation of primary murine lung endothelial cells and hyperoxia exposure

Isolation of murine lung endothelial cells (MLECs) from mouse lungs and hyperoxia exposure were described previously (8).

Construction of lentiviral vectors and administration

Lentivirus miRNA vectors with VE-Cad promoter were described previously (9). PINK1 miRNA (lenti-VE PINK1 miRNA) was designed using target site 1283–1303 (GenBank accession AB053476.1 https://www.ncbi.nlm.nih.gov/). Lentiviral PINK1 overexpression (lenti-PINK1 with CMV promoter), pReceiver-Lv158, was purchased (GeneCopoeia). Lentivirus production, titer measurement, and intranasal administration were described previously (9).

Measurement of lung injury markers

Bronchoalveolar lavage fluid (BALF) and protein quantification were described previously (9).

Amplex Red assay

An Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) was used to check H<sub>2</sub>O<sub>2</sub> released from mouse lung in BALF.

IL-1β ELISA

Mice BALF was assessed for IL-1β by ELISA (BD Biosciences).
Western blot analysis
Lung or MLEC protein analyses were performed as previously described (7) using LC3B, p62, caspase 3, autophagy/cytoskeleton-associated protein 3 (ATG3), ATG7, Beclin-1 (Cell Signaling Technology), PINK1 (Millipore; clone N4/15), lysosome-associated membrane protein 2, subunit A (LAMP2A), mitofusin (MFN1, MFN2, optic atrophy type 1, dynamin-related protein 1 (DRP1) (Abcam), proteasome 20S α6 subunit (Enzo Life Sciences), PARIS (Millipore; clone N196/16), caspase 1, IL-1β, Parkin, PGC-1α, and β-actin (Santa Cruz Biotechnology) Abs.

Preparation of small interfering RNA and transfection of small interfering RNA
Preparation of small interfering RNA and transfection of small interfering RNA (siRNA) was purchased from Santa Cruz Biotechnology. Nonspecific siRNA scrambled duplex probes and transfection of small interfering RNA (siRNA) were performed as described previously (9).

Immunofluorescence microscopy
Formalin-fixed, paraffin-embedded lung tissue sections were deparaffinized with xylene, dehydrated gradually with graded alcohol solutions, and then washed with deionized water. After heat-induced Ag retrieval with target retrieval solution (pH 6; Dako, Carpinteria, CA) in a microwave pressure cooker, sections were blocked with serum-free protein block buffer (Dako) for 1 h. Sections were incubated with a 1:200 dilution of anti-PINK1 (Millipore; clone N4/15) and anti-CD31 (Santa Cruz Biotechnology) Ab at 4°C overnight. After washing with PBS, sections were incubated with secondary Ab (Alexa Fluor 488 goat anti-rabbit IgG or Alexa Fluor 594 goat anti-mouse IgG; 1:100 dilution; Invitrogen) and DAPI (Invitrogen) at room temperature for 1 h. Samples were washed three times by immersing in PBS for 5 min and then mounted with Prolong gold mounting medium with DAPI (Invitrogen). Sections were observed under a dark field in independent fluorescence channels using an automated Olympus BX-61 microscope (20×, objective lens, NA 0.50; Olympus Imaging America, Center Valley, PA) equipped with a cooled CCD camera (Q-Color 5; Olympus) and QCapture Pro 6.0 software (QImaging, Surrey, BC, Canada). After surveying the entire lung fields of six to eight mice in each experimental condition, we selected representative sections that showed airway, vessel, and alveolus. As described previously (9), ≥15–20 nonoverlapping and nonaneucleated images were captured at 200× prior to selection. Images were cropped and prepared by Photoshop (Adobe Systems, San Jose, CA) for the visualization.

Oxidant assays
CM-H2DCFDA (Invitrogen) was used to determine levels of reactive oxygen species (ROS) in endothelial cells, as described previously (9).

Proteasome activity
Protein was extracted from lungs or MLECs, and 10 µg was processed for 20S Proteasome activity (Millipore). The free AMC fluorescence was quantified at 380/460 nM in a fluorometer (SpectraMAX Gemini XS; Molecular Devices). MLECs were treated with proteasome inhibitor MG-132 (10 µM; EMD Millipore Chemicals) for 30 min prior to hyperoxia.

Statistics
Data are expressed as mean ± SD and were analyzed by the Mann–Whitney U test or Student t test. A significant difference was accepted at p < 0.05. Survival studies were evaluated using two tests, and a significant difference was accepted at p < 0.05.

Results
Inflammasome-knockout mice are more resistant to lethal hyperoxia
We subjected NLRP3−/−, Asc−/−, and caspase 11−/− knockout mice to continuous hyperoxia. Knockout mice had significantly improved survival during hyperoxia compared with wild-type (WT) mice (Fig. 1A). We previously reported 72 h of continuous hyperoxia as a time point of maximal lung injury, which we used as a representative time point (7). NLRP3−/−, Asc−/−, and Caspase 11−/− mice exhibited significantly decreased lung injury (as assessed by BALF cell counts, protein, and lactate dehydrogenase [LDH]), decreased ROS generation (H2O2 release detected by Amplex red assay), decreased lung cell death (assessed by TUNEL), and decreased IL-1β secretion compared with WT mice (Fig. 1B–G). Given that NLRP3 is a major component of the inflammasome complex and our recent findings that lung endothelial responses can determine survival in hyperoxia (9), we focused subsequent studies on NLRP3−/− mice and lung endothelial cells. Consistent with the survival data, we found that primary MLECs from NLRP3−/− mice also exhibited a significantly protected phenotype compared with WT MLECs (data not shown). In summary, we show that deletion of specific components of the inflammasome results in decreased lung injury, oxidant production, and, ultimately, increased survival.

NLRP3−/− mice and MLECs have less caspase 3 activation, increased autophagy, and increased PINK1 expression
NLRP3−/− mouse lungs and MLECs had less basal, as well as hyperoxia-induced, caspase 3 activation (Fig. 2A). Autophagy, a process of lysosomal turnover of organelles and proteins, has been implicated in oxidative stress and linked to caspase-mediated cell death (12, 13). We found that NLRP3−/− lungs and MLECs had higher LC3B ratios at baseline and during hyperoxia than did the WT types, which indicated that NLRP3 deficiency resulted in activated autophagy (Fig. 2A). The LC3B results were consistent with p62 (SQSTM1/sequestosome 1) expression, a selective substrate of autophagy. NLRP3−/− lungs and MLECs had higher LC3B ratios and lower p62 protein expression at baseline and during hyperoxia compared with their WT counterparts, indicating that autophagy is activated in the setting of NLRP3 deficiency (Fig. 2A). Next, we measured PINK1, an initiator of mitophagy. We found that NLRP3−/− lungs and MLECs had higher baseline and hyperoxia-induced PINK1 levels compared with WT types, indicating that mitophagy is induced in the setting of NLRP3 deficiency (Fig. 2A).

PINK1 deletion reverses the phenotype of NLRP3−/− mice
To determine whether PINK1 mediated the protective effects of NLRP3 deficiency in vivo, we generated double-knockout mice (NLRP3−/−/PINK1−/−). As expected, NLRP3−/− mice had increased survival during hyperoxia compared with WT mice, but this was reversed in the absence of PINK1 (i.e., NLRP3−/−/PINK1−/− mice). Of note, survival of the double knockouts was even worse than that of WT mice (Fig. 2B). Lung injury parameters (BALF protein content, LDH, oxidant production, and apoptosis) were consistent with the survival data. PINK1−/− mice had the worst lung injury, NLRP3−/− mice had the least injury, and the double knockouts had intermediate levels of lung injury after 72 h of hyperoxia (Fig. 2C–F). We reproduced the injury and survival data in MLECs using PINK1 siRNA and/or NLRP3 siRNA (data not shown). Collectively, these data show that PINK1 mediates anti-inflammatory, anti-injury, and prosurvival effects in WT and NLRP3−/− mice. NLRP3 deficiency resulted in higher basal, as well as hyperoxia-induced, PINK1 in lungs and cells; however, in the absence of PINK1 the benefits of NLRP3 deficiency were lost. We investigated specific molecules involved in cell survival, death, and organelle turnover. NLRP3−/− lungs also had significantly decreased activated caspase 3 expression in their lungs during hyperoxia, which was reversed in the setting of PINK1 deletion (Fig. 2G). The induction of autophagy and mitophagy in the setting of NLRP3 deficiency suggested that there may be increased efficiency in removing damaged organelles.
performed on lung sections; the number of TUNEL+ cells was quantitated and expressed as a percentage of the total number of lung cells counted on
protein content. (0.05 versus WT mice (were exposed to continuous hyperoxia (0.05 versus hyperoxia WT mice, # PINK1 knockdown had cytotoxic effects; interestingly, however, blocking proteasome activity with specific inhibitor MG-132 augmented the deleterious effects of PINK1 silencing in WT and NLRP3−/− MLECs (Fig. 4B–F). We also determined levels of proteasome activity in MLECs and lungs during hyperoxia. Hyperoxia induced proteasome activation in WT MLECs and lungs; however, in the setting of NLRP3 deficiency, proteasome activity is even higher (Fig. 4B, 4C). The silencing or loss of PINK1 significantly decreased proteasome activity in NLRP3−/− MLECs and mice, suggesting that PINK1-mediated protective effects are partially mediated by proteasome activation. Taken together, these results

Lyosome and proteasome degradation are major mechanisms through which dysfunctional mitochondria and misfolded proteins are removed. We first checked for LAMP2A, a receptor in the lysosomal membrane for chaperone-mediated autophagy (14). LAMP2A expression did not appear to be significantly different between the groups (Fig. 2G). However, 20S Proteasome α6, an α-type subunit in the 20S proteasome core particle, was induced in NLRP3−/− lungs and moderately induced in NLRP3−/−/PINK1−/− mice; this induction was partially reversed in the absence of PINK1. We explored the functional significance of proteasome activation in hyperoxia and in NLRP3−/− mice in subsequent studies.

**Lung endothelial PINK1 is protective in hyperoxia**

We sought to determine the relevant cell type involved in mediating PINK1 effects. Our MLEC data pointed to an important role for endothelial cells. We evaluated lung sections of WT and NLRP3−/− mice after hyperoxia exposure or in room air control (RA) using PINK1 and endothelial-specific Ab CD31. PINK1-immunoreactive cells (red conjugate) were detected specifically in the blood vessels after hyperoxia in WT and NLRP3−/− lungs, but NLRP3−/− mice had greater PINK1 induction compared with WT mice. PINK1+ cells were also detected outside of the blood vessels (e.g., macrophages) in RA (Fig. 3A).

To determine the function of endothelial PINK1, we delivered an intranasal endothelial-specific lentiviral PINK1-silencing RNA (lenti-VE PINK1 miRNA) construct to mice, an approach that we recently reported to be endothelial specific (9). We isolated lung endothelium using anti-CD31 Ab. The level of PINK1 expression in mouse lung endothelium decreased significantly with lenti-VE PINK1 miRNA treatment but not by control lenti-VE negative control (NC) (Fig. 3B). Mice given lenti-VE PINK1 miRNA had significantly increased lung permeability (BALF protein content, LDH, oxidants, and apoptosis) compared with lenti-VE control–treated mice (Fig. 3C–F). Importantly, endothelial PINK1 knockdown reversed the survival advantage of NLRP3−/− mice (Fig. 3G). Even WT mice with endothelial PINK1 knockdown were much more sensitive to lethal hyperoxia. Taken together, the results indicated that, in vivo, endothelial PINK1 confers critical protective effects in WT and NLRP3−/− mice during hyperoxia.

**PINK1 and proteasome activity have synergistic protective effects against hyperoxia**

To determine the relationship between PINK1 and proteasome activity during hyperoxia-induced cell injury and ROS generation, we silenced PINK1 in WT and NLRP3−/− MLECs. The knockdown efficiency of PINK1 siRNA is shown in Fig. 4A. PINK1 knockdown had cytotoxic effects; interestingly, however, blocking proteasome activity with specific inhibitor MG-132 augmented the deleterious effects of PINK1 silencing in WT and NLRP3−/− MLECs (Fig. 4B–F). We also determined levels of proteasome activity in MLECs and lung tissues during hyperoxia. Hyperoxia induced proteasome activation in WT MLECs and lungs; however, in the setting of NLRP3 deficiency, proteasome activity is even higher (Fig. 4B, 4C). The silencing or loss of PINK1 significantly decreased proteasome activity in NLRP3−/− MLECs and mice, suggesting that PINK1-mediated protective effects are partially mediated by proteasome activation. Taken together, these results

**FIGURE 1.** NLRP3−/−, Asc−/−, and Caspase 1/11−/− mice are more resistant to hyperoxia. WT, NLRP3−/−, Asc−/−, and Caspase 1/11−/− mice were exposed to continuous hyperoxia (A) or 72 h of hyperoxia (B–G). (A) Survival proportions were compared to WT within each of the graphs. *p < 0.05 versus WT mice (n = 15–18 mice/group). (B) Lung inflammation was detected by BALF cell counts. (C) Lung permeability was assessed by BALF protein content. (D) LDH activity assay from BALF. (E) Oxidant generation was detected by Amplex red from BALF. (F) TUNEL staining was performed on lung sections; the number of TUNEL+ cells was quantitated and expressed as a percentage of the total number of lung cells counted on each section. (G) IL-1β was detected by ELISA in BALF. Data are mean ± SD. *p < 0.05 versus RA WT mice, #p < 0.05 versus hyperoxia WT mice (n = 5 mice/group).
demonstrated that PINK1 induction and proteasome activity are anti-injury mechanisms during hyperoxia. To our knowledge, these studies are the first to demonstrate that NLRP3, via endothelial PINK1, regulates proteasome activity and that proteasome activation is necessary to prevent hyperoxia-induced injury and death.

Overexpression of PINK1 is protective in hyperoxia in vivo

To demonstrate the impact of PINK1 overexpression in vivo, we treated WT, NLRP3−/−, and NLRP3−/−/PINK1−/− mice with intranasal, and thus lung-targeted, PINK1 overexpression lentivirus prior to 72 h of hyperoxia. We demonstrated the efficacy of intranasal gene delivery in an earlier study (9). We detected the FLAG tag in lungs treated with the PINK1 overexpression construct. FLAG tag acted as overexpression of PINK1 was detected in total lung lysates (Fig. 5E). Lenti-PINK1 treatment improved lung inflammation and injury indices in all groups, but the improvement was the most dramatic in the WT and NLRP3−/−/PINK1−/− mice (Fig. 5A–D).

Effects of NLRP3 deficiency on mitochondrial maintenance and autophagy activation

In normal physiological conditions, PINK1 recruits Parkin to the mitochondria and eliminates abnormal mitochondria through mitophagy. Recently, a novel Parkin-interacting substrate (PARIS)
was identified (15). PARIS is ubiquitinated by Parkin and degraded through the UPS. Because PARIS represses the expression of PGC-1α, a key stimulator of mitochondrial biogenesis, mitochondrial protein expression, degradation of PARIS by Parkin induces PGC-1α–dependent gene expression and promotes mitochondrial biogenesis. As shown in Figs. 2A and 6A, NLRP3−/− lungs had exaggerated induction of mitochondrial fusion proteins MFN1, MFN2, and optic atrophy type 1 during hyperoxia compared with WT lungs, which was blunted in the absence of PINK1 (Fig. 6A). Fission protein DRP1 expression was not significantly different in the groups during hyperoxia, but basal levels appeared lower in NLRP3−/− and PINK1−/− lungs. These data suggest that NLRP3 and PINK1 regulate mitochondrial fusion and fission protein expression at baseline or during hyperoxia.

Based on our earlier observation that NLRP3−/− lungs had increased autophagy marker LC3B (Fig. 2A), we measured other autophagy-associated proteins, such as Beclin-1, which is required for the initiation of autophagosome formation (18), and ATG7 and ATG3, which control the conversion of LC3I to LC3II (19). Beclin-1, ATG7, and ATG3 were decreased by hyperoxia in WT
Proteasome activity was measured in MLECs transfected with PINK1 siRNA (exposed to 72 h of hyperoxia). MLECs treated with proteasome inhibitors (MG-132 at 10 μM) were used as a negative control. Data are mean ± SD. *p < 0.05, versus corresponding WT Ctrl siRNA, **p < 0.05 versus corresponding NLRP3 siRNA in (B); *p < 0.05 versus WT RA. **p < 0.05 versus WT hyperoxia, *p < 0.05, versus NLRP3−/+ hyperoxia (n = 5 mice/group) in (C). (D–F) WT and NLRP3−/+ MLECs were transfected with PINK1 siRNA, treated with proteasome inhibitor MG-132 (10 μM), and exposed to RA or 72 h of hyperoxia. (D) Quantitation of flow cytometry analysis of apoptosis. (E) LDH activity assay from MLEC supernatant. (F) H2O2 generation by CM-H2DCFDA staining. Data are mean ± SD. *p < 0.05, versus corresponding group in no-MG132 WT MLEC, **p < 0.05 versus corresponding Ctrl siRNA in WT, *p < 0.05 versus corresponding Ctrl siRNA in WT, #p < 0.05 versus corresponding group in no-MG132 NLRP3−/+ MLEC, **p < 0.05 versus corresponding Ctrl siRNA in NLRP3−/+ (experiments were performed in triplicates).

Discussion

We demonstrate a cytoprotective role for PINK1 in lungs and endothelium, identify previously unrecognized functional links between NLRP3 and PINK1, and show that NLRP3, via PINK1, regulates autophagy/mitophagy, as well as proteasome activity. Previous studies showed that PINK1 is expressed primarily in the CNS with some expression in epithelium (20). Our studies point to an important functional role for PINK1 in lung endothelium. NLRP3 deficiency results in higher basal, as well as hyperoxia-induced, PINK1 and less caspase 3–mediated apoptosis and autophagy suppression during hyperoxia. We show that NLRP3 deficiency prevented caspase 3–mediated apoptosis by upregulating autophagy, specifically mitophagy, via PINK1 expression in lungs and MLECs during hyperoxia. PINK1 mediated the protective effects of NLRP3 deficiency by optimizing Parkin–PARIS–PGC-1α interactions, mitochondrial biogenesis, and proteasome/disposal of dysfunctional mitochondria.

Hyperoxia leads to an accumulation of mitochondrial dysfunction, increased ROS, and cell death. IL-1β is a proinflammatory cytokine that mediates diverse sterile inflammatory responses, and it is also found in patients with acute lung injury. NLRP3 inflammasome is implicated in sensing stress caused by ROS. Our studies define previously unrecognized ROS- and NLRP3-regulated mechanisms in a mouse model of acute lung injury. Mitochondrial ROS generation can reflect mitochondrial dysfunction or health. Macrophagocytosis (autophagy) is a lysosomal-dependent cellular pathway for the turnover of organelles and proteins. Autophagy involves the formation of double-membrane vesicles (autophagosomes or autophagic vacuoles) that target and engulf cytosolic material, which may include damaged organelles or denatured proteins (21). We recently re-
ported that hyperoxia induced mitochondrial oxidant generation and inhibition of autophagy in lungs and MLECs (9). We extended our studies to identify a pivotal role for NLPR3 in autophagy. NLRP4 and NLRP3 were found to bind the extracellular domain of Beclin-1, a marker of autophagy, through the NACHT domain of NLR proteins (22, 23). The ASC component of NLRP3 may be polyubiquitinated and bound by p62 protein, thus targeting the inflammasome complex for degradation via LC3-mediated autophagy. Other investigators showed that knockdown of NLRP4 enhanced autophagy and that NLRP4 could inhibit the maturation of autophagosomes (22). We now show that NLRP3 deficiency or silencing activates autophagy at baseline and during stress conditions, pointing to the presence of crosstalk between autophagy and inflammasomes.

PINK1 and its downstream protein Parkin can mediate autophagy of damaged mitochondria in a process called mitophagy. Mitophagy is the selective engulfment of mitochondria by autophagosomes and their subsequent catabolism by lysosomes. We found that PINK1 expression was increased by hyperoxia, and NLRP3 deficiency results in higher basal, as well as hyperoxia-induced, PINK1 in lungs and cells. PINK1 prevents ROS generation both at baseline and during oxidant injury. The PINK1–Parkin pathway promotes mitophagy (24, 25). In the current study, we found that PINK1-mediated Parkin ubiquitination plays a key role in the overall mitochondrial dynamics of the cell via mitophagy and mitochondrial biogenesis. The molecular mechanisms of PINK1 are through its effects on PARIS and PGC-1α; PARIS (ZNF746) is a newly identified Parkin-interacting substrate, whose levels are regulated by the ubiquitin proteasome system by binding to and ubiquitination by Parkin. PARIS represses the expression of PGC-1α and, thereby, inhibits mitochondrial biogenesis (15). We found that NLRP3 deficiency promotes PINK1/Parkin-induced degradation of PARIS, which causes derepression of PGC-1α and increased mitochondrial biogenesis. In the absence of PINK1, these adaptive effects of NLRP3 deficiency are lost.

Our proposed schemata is summarized in Fig. 6C. Mitochondria proliferate from preexisting mitochondria and generate new mitochondria (biogenesis). At baseline conditions, PINK1 recruits Parkin to the mitochondria and eliminates abnormal mitochondria through mitophagy. Parkin ubiquitinates and degrades PARIS through the UPS. At baseline, PARIS represses the expression of PGC-1α, but once PARIS is degraded by Parkin, PGC-1α-dependent gene expression is activated, and mitochondrial biogenesis ensues. Mitochondria then undergo either fission or fusion to enter its life cycle. Unhealthy mitochondria are sequestered in autophagosomes. Beclin-1 is the initiator of autophagosome formation; ATG7 and ATG3 regulate the conversion of LC3I to LC3II. Mitochondrial biogenesis and degradation serve to decrease the pool of dysfunctional mitochondria and increase functional mitochondria. We postulate that NLRP3 is involved in autophagy by binding and repressing Beclin-1. In NLRP3 deficiency, Beclin-1 is derepressed, and autophagy is activated. During acute stress, such as hyperoxia, the net effect of NLRP3 deficiency is a reduction in dysfunctional mitochondria, mitochondrial oxidant production, apoptosis, and tissue injury/death. Loss of PINK1 leads to the deleterious accumulation of unhealthy mito-

**FIGURE 5.** Lung-targeted PINK1 overexpression decreased susceptibility to hyperoxia in vivo. WT, NLRP3<sup>−/−</sup>, and NLRP3<sup>−/−</sup>/PINK1<sup>−/−</sup> mice were administered intranasal lentivirus (lenti-Ctrl or lenti-PINK1) and exposed to 1.0% RA or 72 h of hyperoxia. (A) Lung permeability. (B) LDH activity. (C) Oxidant generation. (D) Percentages of TUNEL<sup>+</sup> cells. Data are mean ± SD. (E) Lysates from mouse lungs were isolated and immunoblotted against Abs as listed. One representative Western blot of three experiments is shown. *p < 0.05 versus RA lenti-Ctrl WT mice, **p < 0.05 versus hyperoxia lenti-Ctrl WT mice, #p < 0.05 versus hyperoxia lenti-PINK1 WT mice (n = 5 mice/group).
chondria due to inadequate mitophagy. In addition, PARIS accumulates and represses PGC-1α, preventing mitochondrial biogenesis from increasing the pool of healthy mitochondria.

At this juncture, the precise relationship between NLRP3 and PINK1 is unclear, but our studies show that a NLRP3–PINK1 axis is a critical determinant of susceptibility to hyperoxia-induced cell and tissue death. PINK1 silencing also exaggerated hyperoxia-induced mitochondrial oxidant generation and inhibition of autophagy, which ultimately increased hyperoxia-induced apoptosis. In the absence of PINK1, the endothelial cell cannot maintain adequate levels of autophagy or optimal protein disposal by the UPS, leading to caspase 3–mediated cell death. To the best of our knowledge, these studies are the first to demonstrate that PINK1 and proteasome activity are important protective mechanisms during oxidant-induced endothelial and lung injury. Understanding the molecular mechanisms of NLRP3-mediated resistance to hyperoxia may lead to new therapeutic strategies in oxygen toxicity and deepen our understanding of the basic biology of oxidative injury.

Disclosures

The authors have no financial conflicts of interest.

FIGURE 6. NLRP3 deficiency promotes mitochondrial maintenance and autophagy. (A) Lysates from mouse lungs were immunoblotted against the listed Abs. (B) WT and NLRP3−/− MLECs were transfected with PINK1 siRNA. Lysates from MLECs were immunoblotted against the listed Abs. β-actin was used as protein loading control. One representative Western blot of three experiments is shown. (C) Proposed schematic. Mitochondria proliferate from preexisting mitochondria and generate new mitochondria (biogenesis). PINK1 recruits Parkin to the mitochondria and eliminates damaged mitochondria through autophagy/mitophagy. Parkin ubiquitinates and degrades PARIS through the USP. In NLRP3 deficiency, PINK1–Parkin is induced, PARIS is degraded, and mitochondrial biogenesis is activated via PGC-1α. NLRP3 deficiency or PINK1 induction leads to reduced apoptosis. The arrows indicate the direction of change for each of the cellular processes; the height of the arrows indicates the relative degree of change. H, hyperoxia exposure.
References


