Idh2 Deficiency Exacerbates Acrolein-Induced Lung Injury through Mitochondrial Redox Environment Deterioration

Jung Hyun Park,1 Hyeong Jun Ku,2 Jin Hyup Lee,1 and Jeen-Woo Park2

1Department of Food and Biotechnology, Korea University, Sejong, Republic of Korea
2School of Life Sciences and Biotechnology, BK21 Plus KNU Creative BioResearch Group, College of Natural Sciences, Kyungpook National University, Taegu, Republic of Korea

Correspondence should be addressed to Jin Hyup Lee; jinhyuplee@korea.ac.kr and Jeen-Woo Park; parkjw@knu.ac.kr

1. Introduction

Acrolein is a ubiquitous environmental pollutant that arises from cigarette smoke, incomplete combustion of plastic materials, and pyrolyzed animal and vegetable; it is also endogenously produced during inflammation or oxidation of unsaturated lipids [1]. Acrolein inhalation results in the induction of gene regulation, inflammation, and lung cell apoptosis and necrosis [1]. It has been reported that exposure to acrolein leads to acute lung injury, disruption of alveolar capillary barrier integrity, pulmonary edema, and chronic obstructive pulmonary disease [2, 3].

It has been reported that acrolein causes oxidative stress by inducing, directly or indirectly, the production of excessive reactive oxygen species (ROS) that promote cellular apoptosis [4, 5]. ROS play a particularly important role in acrolein-induced cellular damage because acrolein is one of the most reactive \(\alpha,\beta\)-unsaturated aldehyde products of lipid peroxidation [6, 7]. As an \(\alpha,\beta\)-unsaturated aldehyde, acrolein contains a highly reactive carbonyl group and an electrophilic \(\alpha\)-carbon that is highly reactive to cellular nucleophiles, such as proteins, DNA, and RNA [7]. Acrolein readily targets and reacts with the sulfhydryl group of cysteines to form thioether adducts via a Michael addition mechanism [8]. Depletion of cellular reduced glutathione (GSH) by the formation of GS-acrolein conjugates results in increased oxidative stress [9–11]. Acrolein can also deplete protein thiols, such as thioredoxin and glutaredoxin, which are important antioxidant proteins [12, 13].

Acrolein has also been reported as a mitochondrial toxicant, suggesting that it participates in mitochondrial dysfunction [14]. The mitochondria are one of the most important organelles involved in the production of ROS because the respiratory chain in the mitochondria is one of...
major sources of ROS production [15]. Additionally, these are also the main targets of ROS and oxidative stress-induced damage, as observed in various pathological states [16, 17]. Under normal physiological conditions, cell viability and function are critically dependent on the continued balance between mitochondrial ROS formation and removal [18]. ROS can be eliminated by antioxidant enzymes, such as superoxide dismutases, catalase, glutathione peroxidase, and peroxiredoxins (Prxs) [19]. In this regard, knockdown or inhibition of antioxidant enzymes can disrupt redox balance and exacerbate ROS-induced cell death. To maintain GSH-dependent mitochondrial antioxidant defense systems, the availability of the mitochondrial NADPH pool is critical [20]. In addition, the mitochondrial thioredoxin system, which includes thioredoxin 2 (TRX2) and thioredoxin reductase 2 (TRXR2), provides a disulfide reductase activity that is required for maintaining mitochondrial proteins in their reduced state. The mitochondrial thioredoxin system can interact with Prx3, exclusively detected in the mitochondria. Reduced TRX2 is regenerated by TRXR2 at the expense of NADPH [18]. The major enzyme to generate mitochondrial NADPH is the mitochondrial isoenzyme of NADP+-dependent isocitrate dehydrogenase (IDH2) [21, 22]. Thus, suppression of IDH2 activity may induce an imbalance of the mitochondrial redox state that subsequently increases the vulnerability of lung cells and tissues to acrolein-based modulation of the redox status.

The present study demonstrates that acrolein exposure promotes the inhibition of idh2 expression, lowers the cell reduction potential, and increases ROS levels. Suppression of idh2 expression led to disruption of mitochondrial redox status, induction of apoptosis, and acute injury in the lung of idh2-deficient (idh2−/−) mice and idh2 short hairpin RNA- (shRNA-) transfected cells. These results suggest that attenuation or deficiency of idh2 leads to increased mitochondrial ROS levels that causes acrolein-mediated apoptosis of Lewis lung carcinoma (LLC) cells and acrolein-induced lung injury in idh2−/− mice. The findings of the present study support a significant role for increased ROS resulting from disruption of mitochondrial antioxidant defense via suppression of IDH2 expression in acrolein-induced acute lung injury.

2. Materials and Methods

2.1. Materials. Propidium iodide (PI), 5,5′-dithio-bis(2-nitrobenzoic acid), 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT), anti-rabbit IgG tetramethylrhodamine isothiocyanate- (TRITC-) conjugated secondary antibodies, Alexa Fluor 647 and Alexa Fluor 594 conjugated secondary antibodies, Cy3 and Cy5 conjugated secondary antibodies, 5-6-carboxyfluorescein diacetate (CFDA), dihydro-2′,7′-dichlorofluorescein diacetate (DCFH-DA), diphenyl-1-pyrenylphosphine (DPPP), 3′-tetaethylbenzimidazolocarbocyanine iodide (JC-1), 5-chloromethylfluorescein diacetate (CMFDA), and MitoSox were purchased from Invitrogen (Eugene, OR). The antibodies used in this study were as follows: β-actin, mouse monoclonal for β-actin; rabbit IgG polyclonal for actin; and rabbit polyclonal for BAX, cleaved caspase-3, cleaved caspase-9, and horseradish peroxidase- (HRP-) conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA); apoptosis regulator BAX (Calbiochem, San Diego, CA); acrolein adducts (Abcam, Cambridge, MA); and oxidized Prx (Prx-SO3) (Abfrontier, Seoul, Korea). A peptide containing the 16 N-terminal amino acids of mouse IDH2 (ADRKIKVAKPVVEMPQ) was used to prepare polyclonal anti-IDH2 antibodies.

2.2. Cell Culture. The LLC cell line was purchased from the Japanese Collection of Research Bioresources Cell Bank (JCRB Cell Bank, Osaka, Japan). Cells were cultured using Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin in a humidified atmosphere at 37°C and 5% CO2. The LLC cells were grown on glass coverslips and stained with ethidium bromide.

2.3. Idh2 shRNA Knockdown. Idh2 shRNA and nontarget shRNA MISSION® lentiviral transduction particles were purchased from Sigma-Aldrich. LLC cells were transduced with a final concentration of 8 μg/mL hexadimethrine bromide, according to the manufacturer’s protocol. Transduced cells were selected as single colonies in a medium containing 5 μg/mL puromycin (Clontech, Mountain View, CA) and maintained in a medium containing 1 μg/mL puromycin.

2.4. RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR). The RNA was extracted from LLC cells using an RNeasy kit (Qiagen, Hilden, Germany) in accordance with the manufacturer’s instructions. RNA was reverse transcribed to cDNA using a first-strand cDNA synthesis kit (Invitrogen), according to the manufacturer’s protocol. cDNAs were PCR-amplified. Sequences of the primers used were as follows: β-actin, forward: 5′-TCTACA ATGAGCTCGTGTTG-3′, reverse: 5′-ATCTCCCTCCTG CATCCT-GTC-3′ and idh2, forward: 5′-ATCAAGAGAA GC-TCACTCCTG-3′, reverse: 5′-TCTGTGAGCCTTGACT GGTGCG-3′. β-Actin was used as an internal control. The amplified DNA products were resolved on a 1% agarose gel and stained with ethidium bromide.

2.5. Flow Cytometric Analysis. Cells were collected at 2000g for 5 min and washed twice with cold PBS. Annexin V and PI staining were performed with the Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit, according to the manufacturer’s protocol. The stained cells were analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ).

2.6. Assessment of Cellular Redox Status. Intracellular peroxide levels were measured using the ferric-sensitive dye xylol orange and DCFH-DA as previously described [21]. Protein oxidation was assessed by immunoblot analysis using anti-Prx-SO3 antibody. Intracellular GSH levels were...
measured using a GSH-sensitive fluorescent dye, CMFDA. Cells were stained with 5 μM CMFDA for 30 min at 37°C.  

2. Cellular Oxidative Damage. Thiobarbituric acid-reactive substances (TBARS) were used for measurement of lipid peroxidation. Cell extracts were mixed with 1 mL TBA solution [0.375% thiobarbituric acid in 0.25 N HCl containing 15% (w/w) trichloroacetic acid] [21]. Lipid peroxidation was also detected by using a fluorescent DPPP probe [24]. The levels of 8-hydroxy-2′-deoxyguanosine (8-OH-dG) in LLC cells were measured with a fluorescent binding assay as described previously [25]. Cells were fixed and permeabilized with ice-cold methanol for 15 min. DNA damage was visualized with avidin-conjugated TRITC (1:200 dilution) using a fluorescence microscope. The comet assay was also performed using the Comet Assay Kit (Cell Biolabs Inc., San Diego, CA). Cell extracts were washed with cold PBS and centrifuged. The cell pellet was mixed with Comet Agarose at 1:10 ratio (v/v) and pipetted onto the Comet assay slide. Slides were dried at 4°C in the dark for 15 min and incubated in chilled lysis solution at 4°C in the dark for another 15 min. After washing with TBE buffer (50 mM Tris, 50 mM boric acid, and 0.2 mM EDTA), the samples were subjected to electrophoresis and stained with Vista Green DNA Dye. Images were obtained with a microscope. The percentage of tail DNA of the cells in each slide was measured and quantified.  

2.8. Measurement of Mitochondrial Redox Status and Damage. Healthy mitochondrial membrane potentials were detected using the fluorescent probe JC-1 (Invitrogen). Cells were incubated at 37°C with 5 μM JC-1 for 30 min. The ratio of the intensity of green/red fluorescence is directly proportional to the mitochondrial membrane potential [26]. The mitochondrial membrane permeability transition (MPT) was visualized using the fluorescent probe Rh-123, and mitochondrial ROS level was measured using MitoSox. Cells were grown in 100 mm plates containing a slide glass coated with poly-L-lysine and treated with acrolein or PBS and were treated for 30 min with 5 μM each of Rh-123 and MitoSox. Slides with cells on top were washed in warm PBS and covered with a glass cover slip. Rh-123 fluorescence (excitation/emission: 500/536 nm) and oxidized MitoSox red fluorescence (excitation/emission: 510/580 nm) were imaged on a Zeiss Axiovert 200 inverted microscope and a Zeiss LSM700 confocal laser scanning microscope, respectively.  

2.9. Immunoblot Analysis. Total protein extracts were separated on 10–15% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with specific primary antibodies overnight at 4°C, and the immunoreactive antigen was recognized using HRP-conjugated secondary antibodies and an enhanced chemiluminescence detection kit (GE Healthcare, Buckinghamshire, UK).  

2.10. Animals. All animal experiments were reviewed and approved by the Kyungpook National University Institutional Animal Care and Use Committee. Experiments were performed using 8-week-old male C57BL/6 mice with different genotypes, including wild-type (WT) idh2+/+ and knockout idh2−/− mice generated by breeding and identified by PCR genotyping, as previously described [27]. The mice were housed in microisolator rodent cages at 22°C with a 12 h light/dark cycle and allowed free access to water and standard mouse chow. Mice were divided into five groups, with 6–10 mice per group (WT, WT + acrolein, KO, KO + acrolein, and KO + acrolein + NAC). Mice were subjected to acute acrolein inhalation (10 ppm for 12 h), where NAC was intraperitoneally administered (500 mg/kg) 2 h before acrolein exposure.  

2.11. Histological Analysis. For histological analysis, the lung tissues were isolated from mice after acrolein treatment and fixed in 4% formalin. Paraffin lung sections (5 μm) were stained with hematoxylin and eosin (H&E) stain. Slides containing the lung sections were stained sequentially with hematoxylin, eosin number 3, bluing solution, and eosin Y by gently shaking at room temperature. To determine the airspace enlargement in the lungs [28], airspace areas were measured using ImageJ software. Lung injury was graded from 0 (normal) to 4 (severe) in four categories: interstitial inflammation, neutrophil infiltration, congestion, and edema [29]. Lung-injury score was calculated by adding the individual scores for each category. Grading was performed by an observer unaware of the treatment groups.  

2.12. Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL) Staining. To evaluate apoptosis, the lung tissue sections were used for TUNEL staining using the In Situ Cell Death Detection Kit (Roche, Basel, Switzerland), according to the manufacturer’s recommended protocol. TUNEL-stained slides were lightly counterstained with 4′,6-diamidino-2-phenylindole (DAPI) before final mounting. The stained slides were analyzed under an Axiovert 40 CFL microscope (Carl Zeiss AG; Oberkochen, Germany).  

2.13. Statistical Analysis. Results are shown as the mean ± SD. Analyses were performed using a two-tailed t-test. p values < 0.05 were considered statistically significant.
Figure 1: Idh2 knockdown in LLC cells and their vulnerability to acrolein. (a) idh2 protein expression levels were measured by immunoblotting using anti-idh2 antibody. RT-PCR analysis of gene expression in WT and idh2 knockdown (idh2KD) LLC cells. β-Actin was used as an internal control for the experiment. (b) Viability of control and idh2KD LLC cells. Cells were cultured for 2 days at 37°C, exposed to 25 µM acrolein for 1 h, and cell viability was then evaluated using MTT assay. Data are presented as the mean ± SD of four independent experiments. *p<0.05 versus WT cells exposed to acrolein. (c) The ratio of cells undergoing apoptosis was measured by FACS. (d) Apoptosis was measured with FITC-labeled annexin in conjunction with PI. Cells were analyzed by flow cytometry. The lower right quadrants represent apoptotic cells. (e) Evaluation of apoptosis with annexin V by FACS. (f) Immunoblot analysis of apoptosis-related proteins. Control and idh2KD cells were exposed to 25 µM acrolein for 1 h. Cell extracts were electrophoresed on 10–15% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with antibodies against cleaved caspase-3 (c-Casp3), cleaved caspase-9 (c-Casp9), cleaved PARP (c-PARP1), cytochrome c, p53, p-p53, p-p38, JNK, p-JNK, and BAX. β-Actin was used as an internal control.
Figure 2: Continued.
plot of LLC cells that were transfected with control or idh2 shRNA. The number of apoptotic cells was estimated by calculating the number of subdiploid cells in the cell cycle histogram. The number of apoptotic cells was markedly increased among the idh2 shRNA-transfected cells compared to the control cells upon exposure to acrolein. To determine whether apoptosis is responsible for the vulnerability of idh2-silenced LLC cells to acrolein-induced cell damage, apoptotic cells were quantified using flow cytometry and PI/annexin V dual staining. The quantitative results in Figure 1(d) show that most of the acrolein-treated cells were in the early stage of apoptosis (depicted in the lower right quadrants). Similar results were also observed using the Annexin-V-FLUOS staining kit (Roche) with fluorescence-activated cell sorting (FACS) (Figure 1(e)). Idh2-silenced LLC cells were more vulnerable to acrolein-induced apoptotic cell death than their WT counterparts. The effect of idh2 knockdown on the modulation of apoptotic marker proteins was also examined in LLC cells. As shown in Figure 1(f), caspase-3 and caspase-9 cleavages were more pronounced in the LLC cells. As shown in Figure 1(f), caspase-3 and caspase-9 cleavages were more pronounced in the idh2 KD cells compared to WT cells. The formation of fragments indicative of proteolytic PARP cleavage, a proapoptotic marker, was increased in cells that were transfected with idh2 shRNA (Figure 1(f)). The levels of proapoptotic proteins such as BAX were also significantly increased in idh2 shRNA-transfected cells compared to control cells. To further evaluate the effect of idh2 downregulation on the proapoptotic signaling pathway, the activation of p38 and JNK was examined by immunoblot analysis. The levels of phospho-p38 and phospho-JNK increased in cells that were transfected with idh2 shRNA and treated with acrolein (Figure 1(f)). Activation of p38 has been implicated in the induction of apoptosis [30]. As shown in Figure 1(f), the level of phospho-p38 increased in LLC cells transfected with idh2 shRNA upon exposure to acrolein. These findings reveal for the first time that IDH2 plays a vital role in LLC cell function and survival against acrolein toxicity.

3.2. Modulation of Redox Status by idh2 Knockdown in Acrolein Toxicity. Excessive ROS are detrimental because they cause nonspecific oxidative damage to cellular components, DNA, proteins, lipids, and other macromolecules [31, 32]. In addition, ROS modulate redox homeostasis and redox-regulated signaling cascades, thereby causing further damage to tissues and cellular compartments [33]. To determine whether differences in susceptibility to acrolein toxicity between control and idh2 shRNA-transfected cells were associated with ROS formation, the levels of intracellular peroxidases in the cells were measured by FACS using the oxidant-sensitive probe, DCFH-DA. As shown in Figure 2(a), markedly increased ROS levels were observed in idh2 KD cells compared to WT cells. As shown in Figure 2(b), markedly increased ROS levels were observed in idh2 KD cells compared to WT cells. As shown in Figure 2(b), markedly increased ROS levels were observed in idh2 KD cells exposed to acrolein. Furthermore, idh2 knockdown was accompanied by a substantial elevation in intracellular H<sub>2</sub>O<sub>2</sub> level, as measured by xylene orange when cells were exposed to acrolein (Figure 2(b)). Increased
levels of Prx-SO₃, a marker for oxidative damage of the antioxidant enzyme Prx [34], were also found in idh2 shRNA-transfected cells exposed to acrolein in comparison with control cells (Figure 2(c)). The occurrence of oxidative DNA damage, lipid peroxidation, and protein oxidation was evaluated as markers indicative of cellular oxidative damage. Next, the overall DNA fragmentation was measured using the Comet assay to evaluate DNA strand breaks induced by oxidative stress. As shown in Figure 2(d), the induction of DNA damage following acrolein treatment was augmented by knockdown of idh2. In this assay, damaged DNA exhibits the shape of a comet in which the tail length relates to the number of DNA strand breaks. To further confirm DNA damage, the level of 8-OH-dG, an indicator of oxidative DNA damage both in vivo and in vitro [25], was determined. After acrolein treatment, the endogenous DNA levels of 8-OH-dG were significantly increased in idh2 shRNA-transfected cells as compared to control cells (Figure 2(e)). Consistent with the elevation of ROS, there was a considerable increase in the level of malondialdehyde (MDA), a lipid peroxidation marker, in idh2 knockdown cells exposed to acrolein (Figure 2(f)). It has been shown that DPPP is a suitable probe for monitoring lipid peroxidation, specifically within the cell membrane [24]. Upon exposure to acrolein, DPPP fluorescence intensity was markedly increased in idh2 shRNA-transfected cells in comparison with control cells (Figure 2(g)). To determine whether idh2 knockdown increased sensitivity to protein damage, carbonyl contents were measured to evaluate protein oxidation. The carbonyl content of idh2 shRNA-transfected cells was significantly higher than that of control cells (Figure 2(h)). An alternative method for monitoring oxidative stress within cells involves measuring the cellular levels of GSH, which is closely associated with many effects of acrolein on cell death [35, 36], using the GSH-sensitive fluorescent dye CFMDA [37]. Using this technique, GSH levels in idh2 shRNA-transfected cells exposed to acrolein were found to be significantly decreased compared to that in control cells (Figure 2(i)). There are numerous oxidative stress-induced conditions during which the redox status and GSH/GSSG ratio are perturbed [38]. Protein S-glutathionylation is a posttranslational modification of protein sulfhydryl groups that occurs under oxidative stress [39]. The redox status of idh2 shRNA-transfected cells was impaired more than...
Figure 4: Continued.
that of control cells, as reflected by an increase in glutathionylated proteins (Figure 2(j)). NADPH, required for GSH generation by glutathione reductase, is an essential factor for cellular defense against oxidative damage. As expected, knockdown of idh2 in LLC cells significantly decreased NADPH levels, which were further decreased upon exposure to acrolein (Figure 2(k)). Taken together, these results indicate that combination of idh2 knockdown and acrolein exposure markedly elevated ROS generation and subsequently induced oxidative damage in LLC cells.

3.3. Role of idh2 in the Mitochondrial Status Induced by Acrolein. In addition to the essential role of the mitochondria in energy metabolism, regulation of cell death, which presumably is associated with mitochondrial ROS production, has emerged as another major function of these organelles [40]. ROS play a major role in modulation of MPT, an important event in apoptosis [41]. The lipophilic cationic dye Rh-123 was used to determine changes in MPT in LLC cells exposed to acrolein. Acrolein-induced alteration of MPT, which was reflected by a decrease in Rh-123 fluorescence, was greater in idh2 knockdown cells compared with control cells (Figure 3(a)). IC-1 is a fluorescent dye exhibiting potential-dependent accumulation in the mitochondria, which is commonly employed to detect MPT changes [26]. The ratio of green/red fluorescence also demonstrated that treatment of LLC cells with acrolein resulted in decreased MPT, and this effect was exacerbated by knockdown of idh2 expression (Figure 3(b)). To assess whether changes in MPT were accompanied by alterations in intracellular ROS concentrations, the levels of intracellular peroxides in the mitochondria were evaluated using confocal microscopy and the mitochondrial oxidant-sensitive probe MitoSox. As shown in Figure 3(c), following idh2 silencing, there was a marked increase in MitoSox fluorescence intensity after treatment of idh2 knockout cells with acrolein. In control cells, this increase in fluorescence intensity was less pronounced, indicating that idh2 deficiency promotes production of mitochondrial ROS in acrolein-treated cells. The mitochondrial dysfunction in energy metabolism was determined by measuring ATP synthesis [42]. After exposure to acrolein, a significant decrease in ATP production was observed in idh2 shRNA-transfected cells compared with control cells (Figure 3(d)). Taken together, these results indicate that idh2 knockdown is associated with mitochondrial ROS production and disruption of the mitochondrial redox environment and induction of mitochondrial dysfunction when cells are exposed to acrolein.

3.4. Idh2 Deficiency Promotes Acrolein Toxicity In Vivo. To test the physiological relevance of idh2 deficiency in acrolein toxicity, WT (idh2+/+) mice and mice lacking idh2 (idh2−/−) were exposed to 10 ppm acrolein for 2 h or filtered air (control). As expected, protein expression of IDH2 was not detected in the lung tissues harvested from idh2−/− mice (Figure 4(a)). H&E-stained sections of the lung tissues after acrolein exposure (Figure 4(b)). Immunohistochemical analysis of IDH2 protein expression using an anti-IDH2 antibody. As expected, protein expression of IDH2 was not detected in the lung tissues harvested from idh2−/− mice (Figure 4(b)). Histological analysis of the lung tissue showed that acrolein caused enlargement of alveolar and immune cell infiltration, and these histological characteristics were dramatically enhanced in idh2−/− mice compared with idh2+/+ mice (Figure 4(b)). The significant increase in susceptibility of idh2−/− mice to acrolein-induced lung injury was also reflected by a significant increase in alveolar airspace area (Figure 4(c)) and lung injury score (Figure 4(d)). To gain further insights into the effect of IDH2 on lung damage in acrolein-treated mice, the effect of idh2 deficiency on the process of apoptosis was studied. As shown in Figure 4(e), more TUNEL-stained spots were observed in the lung tissues of idh2−/− mice compared with WT mice. Western blot

![Image](image.jpg)
analysis indicated that cleaved caspase-3, cleaved caspase-9, and cleaved PARP, which represent apoptotic index, were increased in the lung tissue of idh2−/− mice exposed to acrolein as compared to control. Increased expression of cytochrome c, representative of mitochondrial apoptosis, was also identified in the lung tissue of idh2−/− mice upon acrolein exposure (Figure 4(f)). These results suggest that the difference in severity of acrolein-induced lung damage depends on the presence of idh2, which influences the level of apoptosis. The major enzyme to generate mitochondrial NADPH is the mitochondrial isoenzyme, IDH2 [21]. Thus, deficiency of idh2 may induce an imbalance of redox status in the mitochondria, subsequently increasing the vulnerability of lung cells to acrolein. To determine whether the differences in acrolein-induced lung cell death observed between WT and idh2−/− mice were associated with ROS formation,
the levels of intracellular hydrogen peroxide in the lung tissue were measured using xylene orange. As depicted in Figure 4(g), a significantly higher level of intercellular hydrogen peroxide was observed in the lung tissue of acrolein-treated $idh2^{-/-}$ mice compared to that in WT mice. Increased expression levels of Prx-SO$_3$, a marker of oxidative stress, were also observed in the lung tissue of acrolein-treated $idh2^{-/-}$ mice (Figure 4(h)). Additionally, the levels of MDA (an indicator of lipid peroxidation) and acrolein-adducted proteins, and the level of oxidative protein damage that was measured by determining the number of derivatized carbonyl groups on oxidized proteins by immunoblotting, were significantly higher in the lung tissues of acrolein-treated $idh2^{-/-}$ mice compared to those in control mice (Figures 4(i) and 4(k)). On exposure to acrolein, the redox status of $idh2^{-/-}$ mice was impaired more than that of $idh2^{+/+}$ mice, as reflected by an increase in glutathionylated proteins in the lung tissues (Figure 4(l)). In addition, nitrotyrosine immunoreactivity was found to be relatively stronger in the lung tissue of acrolein-treated $idh2^{-/-}$ mice compared with that in $idh2^{+/+}$ mice, indirectly reflecting the higher level of reactive nitrogen species in $idh2^{-/-}$ mice (Figure 4(m)). Collectively, these results supported the notion that $idh2$ deficiency deteriorates the mitochondrial redox status that aggravates acrolein-induced colitis through apoptosis.

3.5. Protective Effects of NAC against Acrolein Toxicity In Vitro and In Vivo. To confirm the influence of increased oxidative stress on acrolein-induced damage, the effect of the thiol antioxidant NAC on acrolein toxicity was evaluated both in vitro and in vivo. It was previously shown that NAC reduced oxidative stress by improving the thiol redox status [43]. Pretreatment of $idh2$ shRNA-transfected LLC cells with 1 mM NAC efficiently suppressed cell viability loss (Figure 5(a)) and apoptotic cell death (Figure 5(b)) after exposure to acrolein. Cellular oxidative stress, reflected by an increase in DCF fluorescence (Figure 5(c)) and Prx-SO$_3$ level (Figure 5(d)), was significantly attenuated in acrolein-treated $idh2^{-/-}$ knockdown cells pretreated with NAC. Pretreatment of $idh2$ knockdown cells with NAC significantly inhibited acrolein-induced disruption of MPT, reflected by the protective effects of NAC against acrolein-induced lung damage in $idh2^{-/-}$ mice. NAC (500 mg/kg) was intraperitoneally administered to $idh2^{-/-}$ mice 2 h prior acrolein exposure. (a) H&E-stained sections of the lung tissue after acrolein administration. (b) Emphysema in acrolein-treated lung tissues assessed by mean alveolar airspace area ($\mu$m$^2$). (c) Lung injury scores were evaluated after acrolein exposure. (d) Immunoblots comparing the levels of apoptotic marker proteins in the lung tissue extracts from $idh2^{-/-}$ mice. β-Actin was used as an internal control. (e) Immunoblot analysis of Prx-SO$_3$ levels in the lung tissue extract from $idh2^{-/-}$ mice. In (b) and (c), data are shown as the mean ± SD ($n = 3–6$ mice in each group). *$p < 0.05$ versus acrolein-treated and NAC-untreated $idh2^{-/-}$ mice.
functions, we hereby propose that downregulation of IDH2 in the regulation of redox status and in lung tissue chondrial redox status. Having established the importance of increased susceptibility to acrolein-induced toxicity in LLC acrolein toxicity.

Application of NAC in the treatment or prevention of study provides a useful model to investigate the potential model for future lung disease research. Furthermore, our in both in vitro and in vivo systems could be an evidence that results from idh2 deficiency.

4. Conclusion

The present study demonstrates that idh2 deficiency leads to increased susceptibility to acrolein-induced toxicity in LLC cells and lung tissue in mice through disruption of the mitochondrial redox status. Having established the importance of IDH2 in the regulation of redox status and in lung tissue functions, we hereby propose that downregulation of idh2 in both in vitro and in vivo systems could be an effective model for future lung disease research. Furthermore, our study provides a useful model to investigate the potential application of NAC in the treatment or prevention of acrolein toxicity.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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