The Triterpenoid CDDO-Imidazolide Confers Potent Protection against Hyperoxic Acute Lung Injury in Mice

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***Rationale*: Oxygen supplementation (e.g., hyperoxia) is used to support critically ill patients with noninfectious and infectious acute lung injury (ALI); however, hyperoxia exposure can potentially further contribute to and/or perpetuate preexisting ALI. Thus, developing novel therapeutic agents to minimize the side effects of hyperoxia is essential to improve the health of patients with severe ALI and respiratory dysfunction. We have previously shown that mice with a genetic disruption of the Nrf2 transcription factor, which squelches cellular stress by up-regulating the induction of several antioxidant enzymes and proteins, have greater susceptibility to hyperoxic lung injury. Moreover, we have recently demonstrated that Nrf2-deficiency impairs the resolution of lung injury and inflammation after nonlethal hyperoxia exposure.**

***Objectives*: To test the hypothesis that amplification of endogenous Nrf2 activity would prevent or dampen ALI induced by hyperoxia.**

***Methods*: Here, we tested our hypothesis using a synthetic triterpenoid compound CDDO-imidazole (CDDO-Im) (1-[2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl] imidazole) in Nrf2-sufficient and Nrf2-deficient mice subjected to hyperoxia-induced ALI.**

***Measurements and Main Results*: We demonstrate that oral administration of CDDO-Im at a dose of 30 μmol/kg body weight during the hyperoxic exposure is sufficient to markedly attenuate hyperoxia-induced ALI in Nrf2-sufficient but not Nrf2-deficient mice. This protection by the CDDO-Im against hyperoxic insult was accompanied by increased levels of Nrf2-regulated cytoprotective gene expression and reduced levels of DNA damage in the lung.**

***Conclusions*: These results suggest that up-regulation of Nrf2 signaling by CDDO-Im or its analogs may provide a novel therapeutic strategy to minimize the adverse effects of hyperoxia.**

Keywords: Nrf2; Keap1; antioxidants; stress response

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Although oxygen supplementation and ventilation are used as therapy to improve oxygenation and the morbidity and mortality in patients with acute lung injury (ALI), oxidant stress generated by hyperoxic ventilation or the initial injurious process itself is believed to play a major role in perpetuating ALI and preventing some patients from recovery. Direct antioxidant strategies to stoichiometrically scavenge the reactive intermediates present in the lungs of patients with ALI or generated by hyperoxic ventilation therapy remain of uncertain benefit.

What This Study Adds to the Field

The results of the present study suggest that targeting Nrf2-ARE signaling with an indirect antioxidant triterpenoid, CDDO-imidazolide, which potently up-regulates several antioxidative and stress-response pathways, intermittently during the hyperoxia exposure may provide an effective therapy to limit the potential adverse effects of oxygen supplementation in clinical settings.

Acute lung injury (ALI), or its most severe form, acute respiratory distress syndrome (ARDS), affects up to 200,000 patients per year in the United States alone, with reported mortality rates of 35 to 40% (1, 2). Many patients with ALI/ARDS require oxygen supplementation (leading to hyperoxia) to maintain adequate tissue oxygenation. However, exposure to hyperoxia results in pathological features, such as lung inflammation and edema accompanied by epithelial and endothelial cell death (3), suggesting that oxygen supplementation, although beneficial, may potentially further perpetuate or exacerbate ALI (4). Moreover, hyperoxia has been shown to enhance lung susceptibility to subsequent bacterial or viral infections (5). Several studies have shown that oxidative stress, generated mainly in response to hyperoxia, contributes to ALI (6).

The Nrf2 transcription factor up-regulates the expression of genes encoding several antioxidant enzymes and proteins (6). Through a genetic linkage analysis, we first identified Nrf2 as an important candidate gene that regulates hyperoxic lung injury (7). We have further demonstrated that mice with genetic disruption of *Nrf2* have greater susceptibility to hyperoxic lung injury compared with wild-type control mice (8). Recently, we have shown that Nrf2 deficiency impairs the resolution of lung injury and inflammation in response to nonlethal hyperoxic insult (9). Nrf2 deficiency enhances lung alveolar epithelial cell death, and its overexpression in cells confers protection against oxidant-induced proapoptotic stimuli such as hyperoxia (10, 11). These studies underscore an important role for Nrf2-regulated

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gene transcription in conferring protection against hyperoxic insult.

Nrf2 binds to the antioxidant response element (ARE) with a consensus sequence 5′-a/gTGAC/GNNNGCa/g-3′ commonly found in the regulatory regions (promoter and/or enhancers) of genes encoding several cytoprotective enzymes and proteins, and up-regulates their transcription in response to a variety of stimuli (12). In unstressed cells, Nrf2 is predominantly localized in the cytoplasm in association with Kelch-like ECH-associated protein 1 (Keap1), which promotes proteasomal degradation of Nrf2 (13). Keap1 sequesters Nrf2 in the cytoplasm by binding to the N-terminal Neh2 domain, thus preventing nuclear accumulation of Nrf2 (14). Oxidant or toxic insults may perturb the tertiary structure of Keap1, thereby impeding Nrf2 degradation and facilitating Nrf2 nuclear accumulation and subsequent ARE-mediated transcription (15). We have previously demonstrated that a synthetic triterpenoid compound CDDO-imidazole (CDDO-Im) (1-[2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl] imidazole) disrupts the cytosolic Keap1:Nrf2 interactions and robustly induces cytoprotective gene expression via Nrf2-ARE signaling in several tissues, including the lung (16–18). In this study, we have investigated whether targeting the activation of Nrf2-dependent ARE-regulated gene transcription by CDDO-Im would provide a therapeutic strategy to attenuate ALI *in vivo*. Here, we provide experimental evidence demonstrating that administration of CDDO-Im during the period of exposure to hyperoxia protects against ALI suggesting that this compound may represent a new therapeutic strategy to minimize the adverse effects of hyperoxia in the treatment of patients with ALI/ARDS.

METHODS

Mice and Hyperoxia Exposure

All experimental animal protocols were performed in accordance with guidelines approved by the animal care use committee at the Johns Hopkins University. The Nrf2-deficient (*Nrf2*$^{-/-}$) and wild-type (*Nrf2*$^{+/+}$) CD1/ICR mice (7–8 wk old, ~ 30 g) (19) were exposed to hyperoxia as previously described (9). Type II cells from lungs of *Nrf2*$^{-/-}$ and *Nrf2*$^{+/+}$ mice were isolated and cultured as detailed in our previous publication (11). (*See* online supplement for details.)

Supplementation of Pharmacologic Inhibitors

The synthetic oleanolic triterpenoid CDDO-Im, which is known to disrupt the cytosolic Keap1:Nrf2 interaction, was synthesized as detailed elsewhere (17). CDDO-Im was freshly dissolved in phosphate-buffered saline (PBS) containing 10% dimethyl sulfoxide (DMSO) and 10% Cremophor-EL and then briefly sonicated to prepare a uniform suspension. To assess the effects of CDDO-Im on hyperoxia-induced pulmonary permeability and inflammation, mice received by gavage CDDO-Im (30 μmol/kg body weight) in 200 μL volume every 24 hours before and during hyperoxia exposure as outlined in the Figures. Control mice were gavaged with 200 μl of vehicle (PBS with 10% DMSO and 10% Cremophor-EL). Mice exposed to room air also received CDDO-Im or vehicle every 24 hours to measure baseline differences. Our pharmacodynamic studies showed that the induction of Nrf2 target gene expression by CDDO-Im reached a plateau at doses of 10 to 30 μmol/kg in the lung (18). We therefore chose an optimal dose of 30 μmol/kg body weight for the present study.

Assessment of Lung Permeability and Inflammation

To measure lung injury and inflammation, animals were administered a lethal dose of the anesthetic agent pentobarbital at the end of experiments. The right lung was instilled with 1.5 ml (0.75 ml each time, twice) of sterile PBS, and cells in the bronchoalveolar (BAL) fluid were counted using a hemocytometer as previously described (9). Differential cell counts were assessed with Diff-Quik stain set (Dade-Behring, Newark, DE). BAL protein concentration was measured by BCA Protein Assay kit (Pierce Chemical Co., Rockford, IL). The left lung was fixed in formalin and tissue sections were cut and stained with hematoxylin and eosin.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling Assay

DNA damage was assessed using In Situ Cell Death Detection Kit (Roche, Applied Science, Indianapolis, IN) as per manufacturer's instructions. The number of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells in tissue sections (15 fields/lung section) were counted and expressed as mean ± SE (n = 3 per group) as detailed elsewhere (9).

Real-Time Reverse Transcriptase–Polymerase Chain Reaction Analyses

For RNA isolation, the left lung was quickly frozen and stored in RNAlater. Total RNA was isolated and reverse transcribed using superscript first strand cDNA synthesis system (Invitrogen Corp., San Diego, CA), and real-time reverse transcriptase–polymerase chain reactions were performed using TaqMan gene expression assays for *Nqo1*, *Gclc*, *Gclm*, *Ccnd1*, *p21*, and *Il-6* (Applied Biosystems, Foster City, CA).

Statistical Analysis

Data were expressed as the mean ± SD. One-way analysis of variance followed by Newman-Kuels *post hoc* analysis was performed for multiple group comparisons using Graphpad Prism software (GraphPad Software, Inc., La Jolla, CA). For comparing the interaction between hyperoxia exposure and CDDO-Im treatment, two-way analysis of variance with a Bonferroni correction was used. Student two-tailed *t* test was used to determine the significant differences between different experimental groups in real-time polymerase chain reaction and TUNEL assays. *P* values 0.05 or less were considered as statistically significant for all experimental groups.

RESULTS

Supplementation of Mice with CDDO-Im before and during Hyperoxia Dampens ALI

The wild-type (*Nrf2*$^{+/+}$) CD1 mice were dosed with CDDO-Im at 48, 24, and 0 hours, as outlined in the schematic (Figure 1A) and were then exposed to 95% oxygen for 72 hours. This regimen of hyperoxia exposure was selected because it is known to cause significant and reproducible ALI in several mouse strains (20, 21). During hyperoxia exposure, mice were treated additionally with CDDO-Im at 24 and 48 hours. Animals were killed at 72 hours into the hyperoxia exposure. The left lung was used for tissue fixation and the right lung was used for BAL fluid collection. As shown in Figure 1B, lung histology revealed greater levels of hemorrhage and accumulation of proteinaceous edema in the lung tissue of vehicle-treated mice (*top panel*) compared with mice treated with CDDO-Im (*bottom panel*) after hyperoxia (*see* Figure E1 in online supplement for enlarged images). Lung alveolar protein leakage and leukocyte infiltration are the hallmarks of ALI/ARDS. Thus, the effects of CDDO-Im on hyperoxia-induced lung alveolar permeability and neutrophil accumulation in the BAL fluid were assessed (Figures 1C and 1D). Consistent with previous results, we found a significant increase in total protein accumulation in vehicle-treated mice after 72-hour hyperoxia exposure (Figure 1C, *open bar*). However, protein leakage in the BAL fluid of CDDO-Im–treated mice subjected to hyperoxia was significantly decreased as compared with counterpart vehicle-treated mice (Figure 1C, *solid bar*). Likewise, the total number of inflammatory cells present in the BAL fluid after hyperoxia was lower in CDDO-Im–treated mice compared with vehicle-treated control mice (compare *open* and *solid bars*, Figure 1D). Differential cell count analysis revealed lower numbers of neutrophils and

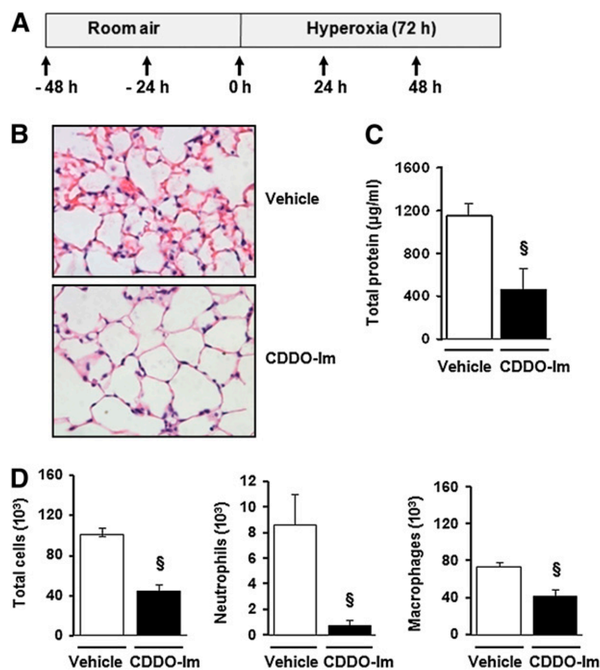

Figure 1. The effects of prior and concurrent treatment of CDDO-imidazole (CDDO-Im) on hyperoxia-induced acute lung injury. (*A*) The wild-type (*Nrf2*$^{+/+}$) mice were administered CDDO-Im or vehicle at 24-hour intervals before and during hyperoxia exposure as outlined in the schematic. Mice were exposed to 95% oxygen for 72 hours, at which time lungs were harvested. The left lung was fixed and stained and the right lung was used for bronchoalveolar lavage (BAL) fluid collection for measurements of total cell counts and total protein, as markers of lung inflammation and lung injury (permeability), respectively. (*B*) Hyperoxia-induced lung histopathology of mice administered vehicle or CDDO-Im. A representative image (×40) of three mice is shown (*see* Figure E1 for enlarged images). Compared with CDDO-Im–treated group, the lungs of mice dosed with vehicle showed the presence of alveolar proteinaceous edema, diffuse septal thickening, and hemorrhage. (*C*) Total protein in the BAL fluid of mice treated with vehicle or CDDO-Im. (*D*) Total and inflammatory cells in the BAL fluid of two experimental groups of mice. Data in *C* and *D* represent the mean values of five mice with SD. *Open columns* = vehicle; *solid columns* = CDDO-Im. One-way analysis of variance followed by Newman-Kuels *post hoc* analysis was performed and *P* values ≤ 0.05 were considered as statistically significant. $^{§}P \leq 0.05$ vehicle versus CDDO-Im.

macrophages in the BAL fluid of mice supplemented with CDDO-Im as compared with vehicle-treated animals after hyperoxic insult. These results suggest that administration of CDDO-Im before and during the exposure attenuates hyperoxia-induced ALI.

Pretreatment of Mice with CDDO-Im Does Not Prevent ALI

To determine whether CDDO-Im pretreatment alone is sufficient to confer protection against hyperoxic lung injury, mice were fed with CDDO-Im at 48, 24, and 0 hours, and then exposed to 95% oxygen for 72 hours as outlined in the schematic (Figure 2A). Mice were not treated with CDDO-Im during the hyperoxic exposure. As expected, there was a greater level of lung hemorrhage and edema in mice exposed to hyperoxia compared with room air control mice (Figure 2B; *see* Figure E2 for enlarged images). We found no striking difference in lung histopathology between the CDDO-Im– and vehicle-treated mice after hyperoxia. The histologic staining showed a comparable level of hemorrhage and edema in the lung tissue of mice pretreated with CDDO-Im or vehicle after hyperoxic insult. Consistent with this result, the BAL protein analysis revealed no significant difference in hyperoxia-induced lung alveolar permeability between mice pretreated with CDDO-Im and vehicle (Figure 2C). Hyperoxia-induced neutrophil accumulation in the BAL fluid did not differ significantly between the two experimental groups (Figure 2D). However, we found modest but statistically significant differences in the levels of macrophages and total cells between CDDO-Im–treated and vehicle-treated hyperoxia experimental groups under this dosing regimen. These results suggest that pretreatment with CDDO-Im does not confer protection against hyperoxic lung injury or neutrophilic inflammation.

CDDO-Im Treatment Solely during Hyperoxia Confers Potent Protection against ALI

To determine the potential beneficial effects of CDDO-Im therapy in the clinical setting, in this set of experiments, *Nrf2*$^{+/+}$ mice were administered CDDO-Im at 0 hours and then immediately exposed to 95% oxygen as outlined in the schematic (Figure 3A). Likewise, we dosed *Nrf2*$^{-/-}$ mice with CDDO-Im to determine whether this treatment confers protection through a Nrf2-mediated transcriptional response. *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice were redosed with CDDO-Im at 24 and 48 hours into the exposure. Lungs from *Nrf2*$^{+/+}$ mice were harvested at 72 hours and BAL was collected from the right lung to measure total cells and protein, whereas the left lung was fixed. *Nrf2*$^{-/-}$ mice were killed at 62 hours into the hyperoxia exposure, as 72-hour exposure causes greater injury in these mice than the similarly exposed *Nrf2*$^{+/+}$ mice and results in increased mortality in *Nrf2*$^{-/-}$ mice during recovery from hyperoxia (9). There were striking differences in lung histopathology between the CDDO-Im– and vehicle-treated *Nrf2*$^{+/+}$ mice after hyperoxia (Figure 3B, *see* Figure E3 for enlarged images). The histologic staining showed a diminished level of hemorrhage and edema in the lung tissue of mice treated with CDDO-Im compared with vehicle-treated mice. In agreement with lung histology, both lung injury and inflammatory (neutrophil and macrophages) cell accumulation in the BAL fluid of hyperoxia-exposed *Nrf2*$^{+/+}$ mice administered with CDDO-Im were significantly lower than that of vehicle-treated counterparts (Figure 3B, *left panel*). However, there was no significant difference in lung injury and inflammatory cell accumulation between vehicle- (*open bars*) and CDDO-Im– (*solid bars*) treated *Nrf2*$^{-/-}$ mice. Histopathology revealed that hyperoxia induced alveolar damage, hemorrhage, and septal thickness. This injury was nearly comparable between CDDO-Im and vehicle-treated *Nrf2*$^{-/-}$ mice (Figure 3B, *right panel*). Consistent with these results, hyperoxia induced protein leakage (Figure 3C) and total number of inflammatory cells (Figure 3D) were also comparable between CDDO-Im– and vehicle-treated *Nrf2*$^{-/-}$ mice. Collectively, these results suggest that continuous administration of CDDO-Im concomitant with the hyperoxic exposure profoundly attenuates hyperoxia-induced lung alveolar permeability and inflammation (neutrophil accumulation) via Nrf2-regulated transcriptional response.

CDDO-Im Activates Nrf2-dependent Gene Transcription in the Lung

We have previously reported that CDDO-Im at low doses enhances Nrf2-regulated gene expression in multiple tissues, including the lung (18). *Gclc*, *Nqo1*, and *Gpx2* are prototypic transcriptional targets of Nrf2 and are also known to attenuate oxidative stress (6). Herein, we analyzed the levels of mRNA expression of these genes in lungs of *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice

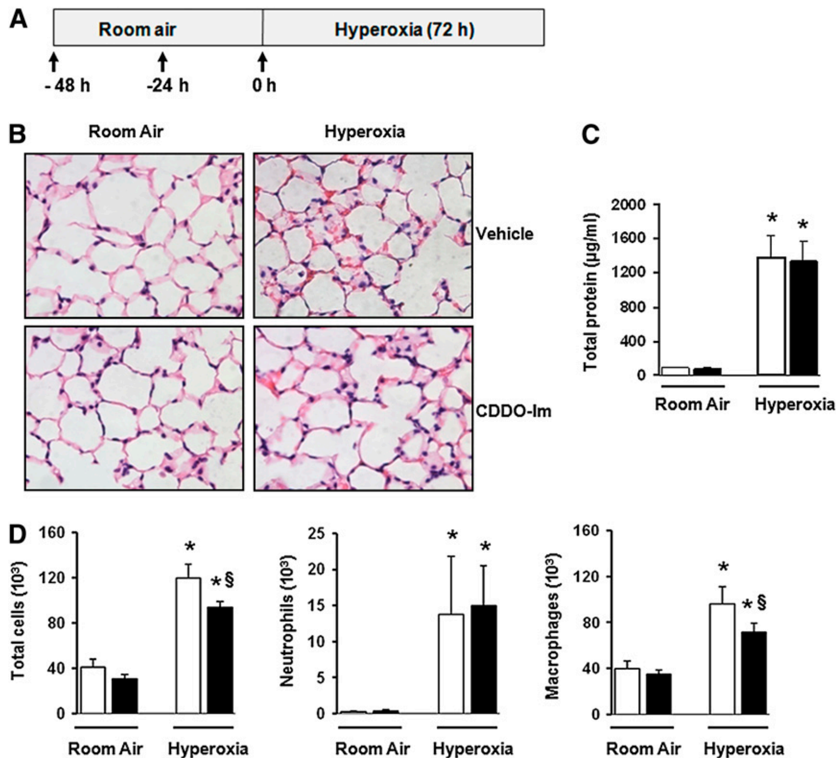

Figure 2. Pretreatment of mice with CDDO-imidazole (CDDO-Im) does not prevent hyperoxia-induced acute lung injury. The wild-type ($Nrf2^{+/+}$) mice (n = 5) were treated with CDDO-Im or with vehicle at 48, 24, and 0 hours, and then exposed to 95% oxygen for 72 hours as outlined in the schematic. (*A*) Lungs were harvested immediately after hyperoxia exposure at 72 hours, and bronchoalveolar lavage (BAL) fluid was collected to measure lung inflammation and lung alveolar permeability. (*B*) Hyperoxia-induced lung histopathology of mice administered vehicle or CDDO-Im. A representative image (×40) of three mice is shown (*see* Figure E2 for enlarged images). The lungs of mice dosed with vehicle or CDDO-Im showed the presence of alveolar proteinaceous edema, septal thickening, and hemorrhage. (*C*) Total protein, and (*D*) total cells, neutrophils, and macrophages present in the BAL fluid. *Open columns* = vehicle; *solid columns* = CDDO-Im. One-way analysis of variance (ANOVA) followed by Newman-Kuels *post hoc* analysis was performed for multiple group comparisons, whereas two-way ANOVA with a Bonferroni correction was used for comparing the interaction between hyperoxia exposure and CDDO-Im treatment. In both cases, *P* values ⩽ 0.05 were considered as statistically significant. $^*P \leq 0.05$ room air versus hyperoxia; $^§P \leq 0.05$, vehicle versus CDDO-Im.

dosed with and without CDDO-Im as in Figure 3. As shown in Figure 4, CDDO-Im significantly elevated the expression levels of *Gclc*, *Gpx2*, and *Nqo1* (*bar 2*) in the lungs of $Nrf2^{+/+}$ mice, when compared with vehicle control (*bar 1*). Hyperoxia significantly stimulated *Gclc*, *Gpx2*, and *Nqo1* expression (*bar 5*). However, induction of *Gclc* and *Gpx2* expression by hyperoxia was more pronounced in $Nrf2^{+/+}$ mice administered with CDDO-Im (*bar 6*) than in vehicle-treated mice (*bar 5*). In contrast to the wild-type mice, hyperoxia or CDDO-Im did not significantly induce *Gclc*, *Gpx2*, and *Nqo1* expression in

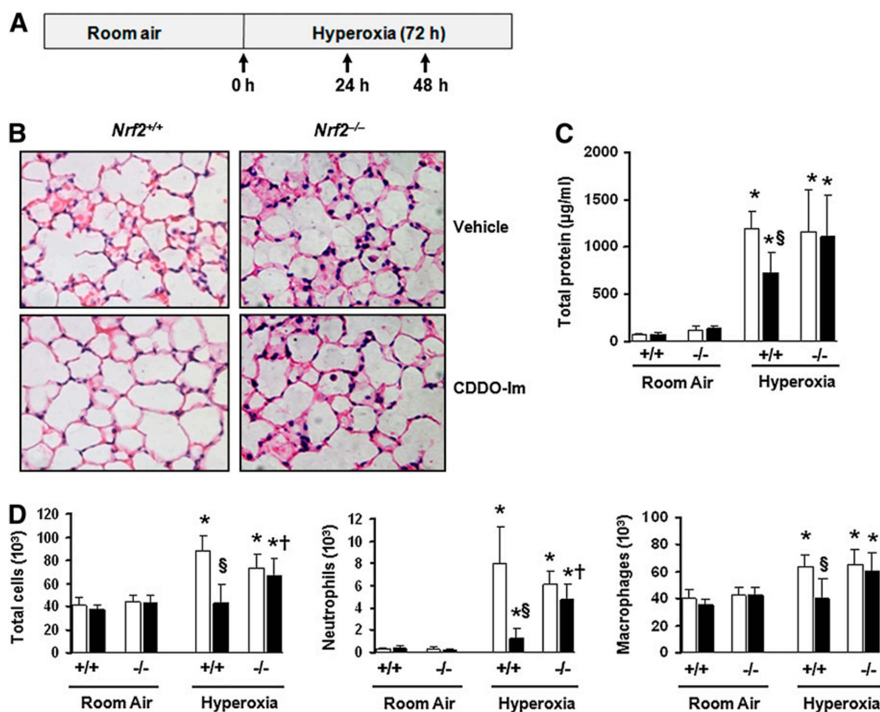

Figure 3. CDDO-imidazole (CDDO-Im) intervention during exposure alone is sufficient to confer protection against hyperoxic insult. (*A*) $Nrf2^{+/+}$ mice (n = 5) were treated with CDDO-Im at 0 hours and then immediately exposed to 95% oxygen for 72 hours as outlined in the schematic. Likewise, $Nrf2^{-/-}$ mice (n = 5) were treated with CDDO-Im but exposed to hyperoxia for 62 hours. Mice received additional doses of vehicle or CDDO-Im at 24 and 48 hours during hyperoxia. Lungs were harvested immediately after hyperoxia; the left lung was fixed and the right lung subjected to bronchoalveolar lavage (BAL) collection. (*B*) Hyperoxia-induced lung histopathology of $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice administered vehicle or CDDO-Im. A representative image (×40) of three mice is shown (*see* Figure E3 for enlarged images). Alveolar proteinaceous edema, septal thickening, and hemorrhage were notably less prominent in the lungs of $Nrf2^{+/+}$ mice dosed with CDDO-Im compared with vehicle control. In contrast, CDDO-Im treatment had no appreciable effect on hyperoxia-induced lung injury indices in $Nrf2^{-/-}$ mice. (*C*) Total protein in the BAL fluid of room air– and hyperoxia-exposed $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice with and without CDDO-Im administration. (*D*) Total inflammatory cells (*left*), neutrophils (*middle*), and epithelial cells (*right*) in the BAL fluid of room air– and hyperoxia-exposed vehicle- and CDDO-Im–treated $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice. *Open columns* = vehicle; *solid columns* = CDDO-Im. One-way analysis of variance (ANOVA) followed by Newman-Kuels *post hoc* analysis was performed for multiple group comparisons, whereas two-way ANOVA with a Bonferroni correction was used for comparing the interaction between hyperoxia exposure and CDDO-Im treatment. In both cases, *P* values ⩽ 0.05 were considered as statistically significant. $^*P \leq 0.05$ room air versus hyperoxia; $^§P \leq 0.05$, vehicle versus CDDO-Im; and $^†P \leq 0.05$, $Nrf2^{+/+}$ versus $Nrf2^{-/-}$ mice.

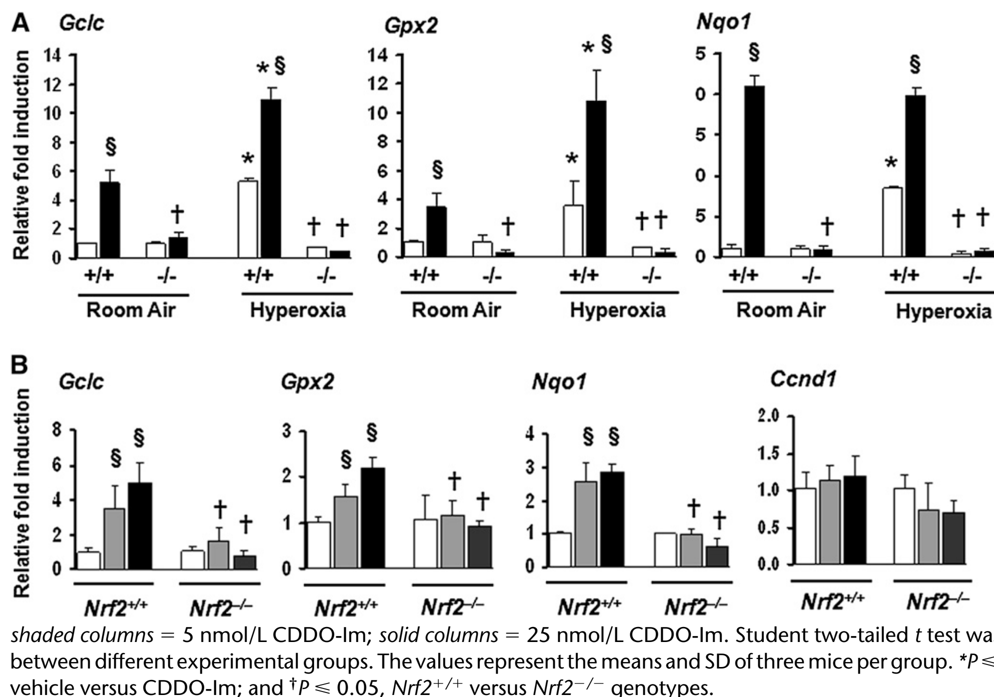

Figure 4. The expression levels of Nrf2 target genes in the lung tissue and type II cells after CDDO-imidazole (CDDO-Im) treatment. (*A*) cDNA was prepared from total RNA isolated from lungs of *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice exposed to either room air or hyperoxia treated with vehicle or CDDO-Im as detailed in Figure 3A. *Open columns* = vehicle; *solid columns* = CDDO-Im. The expression levels of *Gclc*, *Gpx2*, and *Nqo1* were determined using TaqMan real-time probes. (*B*) Type II cells were isolated from the lungs of *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice. After reaching semiconfluence the cells were treated at Day 3 with dimethyl sulfoxide (DMSO) or CDDO-Im at 5 or 25 nmol/L concentrations, and gene expression was analyzed by real-time reverse transcriptase–polymerase chain reaction analysis. *Open columns* = DMSO; *shaded columns* = 5 nmol/L CDDO-Im; *solid columns* = 25 nmol/L CDDO-Im. Student two-tailed *t* test was used to determine statistical significance between different experimental groups. The values represent the means and SD of three mice per group. *$P \leqslant 0.05$ room air versus hyperoxia; $^{§}P < 0.05$, vehicle versus CDDO-Im; and $^{†}P \leqslant 0.05$, *Nrf2*$^{+/+}$ versus *Nrf2*$^{-/-}$ genotypes.

Nrf2$^{-/-}$ mice. These results, in agreement with our previous studies (18), demonstrate a robust induction of Nrf2 target genes by CDDO-Im in the lung.

We have previously shown that the ability of CDDO-Im to induce several antioxidant cytoprotective enzymes and proteins in the lungs of Nrf2-deficient (*Nrf2*$^{-/-}$) mice is markedly impaired (18, 22, 23). To demonstrate that CDDO-Im directly activates antioxidant gene expression in lung cell types, we freshly isolated alveolar type II epithelial cells from lungs of wild-type (*Nrf2*$^{+/+}$) and *Nrf2*$^{-/-}$ mice as previously described (9). After reaching subconfluence (at 3 d), cells were treated with CDDO-Im at two different concentrations (5 and 25 nmol/L) for 6 hours, and gene expression was analyzed by real-time reverse transcriptase–polymerase chain reaction analysis. DMSO was used as vehicle. CDDO-Im treatment provoked a strong induction of *Gclc*, *Nqo1*, and *Gpx2* expression in *Nrf2*$^{+/+}$ but not *Nrf2*$^{-/-}$ cells. In contrast, CDDO-Im failed to stimulate the expression of *Ccnd1* (*cyclin D1*), a nontarget of Nrf2, in both cell types. The induction of antioxidative gene expression in *Nrf2*$^{+/+}$ but not *Nrf2*$^{-/-}$ cells demonstrates that Nrf2 mediates the actions of CDDO-Im in lung epithelial cells.

CDDO-Im Blocks Hyperoxia-induced Lung Cellular DNA Damage

Because hyperoxia causes alveolar epithelial and endothelial cell damage (24), we assessed the extent of cellular injury and the expression levels of *p21*, which regulates DNA repair, in the lungs of *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice exposed to hyperoxia with and without CDDO-Im treatment (Figure 5). As expected, we found a greater number of TUNEL-positive cells ($\sim$ 30/field) in the lungs of hyperoxia-exposed *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice (Figure 5A, *left panel*; *see* Figure E4 for enlarged fluorescent images), whereas TUNEL staining was undetectable or low in the lungs of room air–exposed mice (data not shown). However, the number of hyperoxia-induced TUNEL-positive cells was markedly lower in the lungs of *Nrf2*$^{+/+}$ mice treated with CDDO-Im compared with counterpart *Nrf2*$^{-/-}$ *mice* (Figure 5A, *right panel*). Expression levels of *p21* were elevated fourfold in response to hyperoxic exposure in *Nrf2*$^{+/+}$ mice (Figure 5B). However, the mRNA induction of *p21* by hyperoxia was more pronounced in mice treated with CDDO-Im (*bar 6*) than in vehicle-treated counterparts (*bar 5*). In contrast, CDDO-Im did not induce *p21* expression in *Nrf2*$^{-/-}$ mice in response to hyperoxia when compared with vehicle control (Figure 5B, compare *bar 7* and *bar 8*). We also analyzed the expression levels of *Il-6*, a proinflammatory cytokine, in the lungs of hyperoxia-exposed mice with and without triterpenoid administration (Figure 5C). We found significantly lower levels of hyperoxia-induced *Il-6* expression in *Nrf2*$^{+/+}$ mice administered CDDO-Im (*bar 6*) compared with vehicle (*bar 5*). However, hyperoxia-induced *Il-6* expression remained elevated in *Nrf2*$^{-/-}$ mice administered with CDDO-Im (*bar 8*), and the induction was significantly higher than that of their counterpart *Nrf2*$^{+/+}$ mice (*bar 6*). Taken together, these results suggest that CDDO-Im attenuates hyperoxia-induced DNA damage and inflammatory cytokine expression, while promoting the expression levels of antioxidative enzymes, such as *Gclc* and *Nqo1*, as well as regulators of DNA repair, such as *p21*.

DISCUSSION

We have previously reported that disruption of Nrf2-ARE signaling enhances susceptibility to hyperoxic insult (8, 9) and that there is an association between a polymorphism in the *NRF2* promoter and increased susceptibility to ALI in humans (25). The present study establishes that intermittent administration of a synthetic small molecule triterpenoid compound, CDDO-Im, during exposure to hyperoxia confers protection against the development of ALI in mice. Both the influx of alveolar protein leakage and neutrophil accumulation, which are cardinal features in patients with ALI and ARDS (26), were markedly lower in the lungs of mice treated with CDDO-Im compared with vehicle during hyperoxic insult. Thus, it appears that activation of Nrf2-ARE signaling during hyperoxia exposure may provide a viable novel therapeutic strategy in the treatment of ALI/ARDS syndromes and to minimize the clinical side effects of hyperoxia. Importantly, our findings have revealed that continuous administration of CDDO-Im during

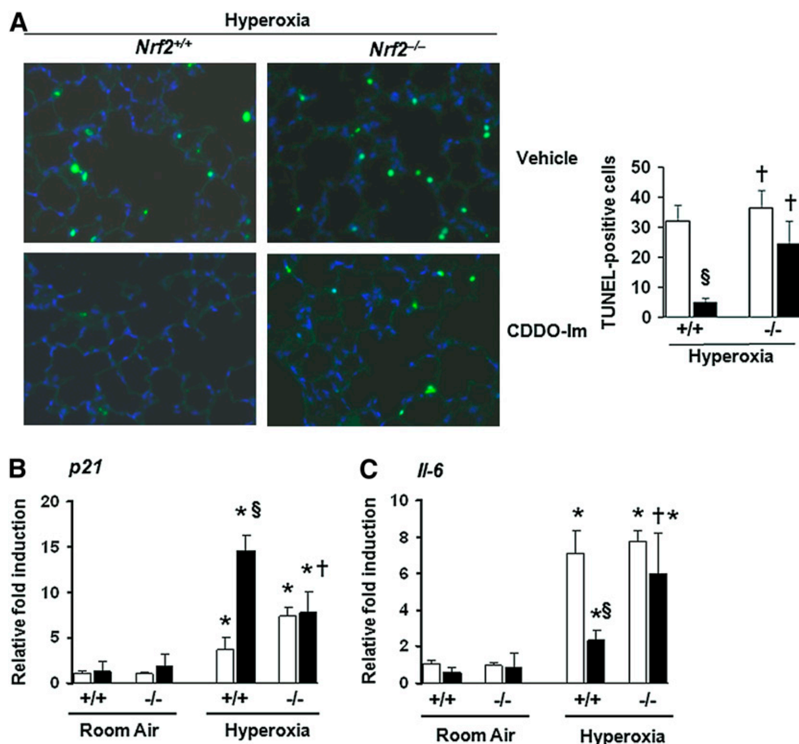

Figure 5. Effects of CDDO-imidazole (CDDO-Im) on markers of lung cellular injury of mice exposed to hyperoxia. (*A*) Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed using In Situ Cell Death Detection Kit on lung tissues of *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice exposed to hyperoxia with and without CDDO-Im intervention as outlined in Figure 3A. Graph represents the percentage of TUNEL-positive cells of five fields per mouse for each group (n = 3). (*See* Figure E4 for enlarged pictures). There are fewer TUNEL-positive cells in CDDO-Im treated *Nrf2*$^{+/+}$ mice than *Nrf2*$^{-/-}$ mice. The relative expression levels of (*B*) *p21* and (*C*) *Il-6* in lung tissues of *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice exposed to hyperoxia with and without CDDO-Im intervention were analyzed by real-time reverse transcriptase–polymerase chain reaction. *Open columns* = vehicle; *solid columns* = CDDO-Im. Student two-tailed *t* test was performed to determine significant differences between different experimental groups. $^{*}P \leq 0.05$ room air versus hyperoxia; $^{\S}P \leq 0.05$, vehicle versus CDDO-Im; $^{\dagger}P \leq 0.05$, *Nrf2*$^{+/+}$ versus *Nrf2*$^{-/-}$ genotypes.

the exposure is essential to effectively dampen hyperoxia-induced ALI. The administration of CDDO-Im before exposure of mice to hyperoxia alone showed limited benefit (Figure 2). The inability of triterpenoid, when administered before exposure, to block lung injury or neutrophil accumulation may reflect a limited duration to the pharmacodynamic effects on activation of Nrf2-ARE–mediated stress-response pathways relative to sustained oxidative stress generated by a 72-hour hyperoxic insult. Studies with activators of Nrf2 signaling indicate that inductive responses are largely attenuated by 48 to 72 hours (27, 28). In contrast, repetitive administration of this compound can result in a sustained activation of Nrf2-ARE signaling to drive the antioxidant gene expression required to mitigate oxidative stress generated by hyperoxia. Apparently, maintenance of a sustained Nrf2 adaptive response is required to mitigate the hyperoxic stress.

Exposure to 95% oxygen for 72 hours has been shown to cause lung injury and inflammation, but the severity of hyperoxia-induced ALI varies in different strains of mice. BALB/cJ mice are more susceptible to hyperoxia exposure than 129/Svlm, C57BL/6J, and C3H/HeJ mice (7, 20, 21, 29). Hyperoxia caused a 5-, 4-, 3-, and 2.5-fold increase in BAL protein accumulation in BALB/cJ, 129/Svlm, C57BL/6J, and C3H/HeJ mice, respectively (7, 20, 21, 29). DBA/2J and FVB/NJ mice, which are relatively resistant to hyperoxia, exhibited a twofold increase in BAL protein accumulation. In our experimental conditions, exposure of CD1 mice to hyperoxia for 72 hours caused a significant increase in alveolar protein leak (~ threefold to sixfold increase over room air control). However, disruption of *Nrf2* in CD1 mice increased susceptibility to hyperoxia and caused severe ALI (8, 9). Several studies have shown that hyperoxia causes DNA damage resulting in lung epithelial and endothelial cell death, which in turn leads to dysfunction of the lung vasculature and alveolar barrier as well as respiratory impairment (30). We found that a simultaneous administration of CDDO-Im along with noxious exposure attenuates hyperoxia-induced lung cellular injury, assessed by TUNEL assay in *Nrf2*$^{+/+}$ but not *Nrf2*$^{-/-}$ mice (Figure 5A), suggesting that the protective effects of CDDO-Im against ALI may be mediated through the modulation of cellular damage. Analysis of some selected genes regulated by the Nrf2-ARE pathway demonstrated that CDDO-Im markedly stimulates the expression levels of genes encoding several antioxidant enzymes, such as *Nqo1*, *Gpx2*, and *Gclc* both *in vivo* and *in vitro* (Figure 4). The induction of the prototypic antioxidant proteins was inversely correlated with hyperoxia-induced cellular injury in CDDO-Im–treated mice. Increased levels of proinflammatory cytokine gene expression play fundamental roles in the development or perpetuation of ALI/ARDS (31). We found significantly decreased levels of proinflammatory cytokine (e.g., *Il-6*) gene expression as well as BAL neutrophil accumulation in the lungs of hyperoxia-exposed *Nrf2*$^{+/+}$ but not *Nrf2*$^{-/-}$ mice treated with CDDO-Im as compared with vehicle-treated control group. In contrast, CDDO-Im strongly induced the expression of *p21* in the lungs of hyperoxia-exposed *Nrf2*$^{+/+}$ but not *Nrf2*$^{-/-}$ mice (Figure 5B). Previous studies have shown that increased expression of p21, a cyclin-dependent kinase inhibitor, is critical for promoting cell cycle arrest and the repair of DNA damage after hyperoxic lung injury (32). Although these results suggest that CDDO-Im reduces the extent of lung inflammatory cell accumulation and promotes DNA repair, it is unclear whether this compound regulates these responses by modulating the transcription of inflammatory cytokines and DNA repair enzymes via Nrf2-ARE signaling or indirectly by attenuating oxidative stress and its subsequent damage via Nrf2-dependent antioxidant gene expression. Nrf2 is ubiquitously expressed and activated by stressful stimuli in lung endothelial cells as well as in type II epithelial cells (6). Thus, it is likely that CDDO-Im also potently activates Nrf2-dependent gene expression in endothelial cells (important structural components with epithelial cells of the alveolar capillary membrane), thus providing a protective role in hyperoxia-induced ALI (33).

Most patients with ALI with severe hypoxemia require supplemental oxygen and ventilation therapy for several days

and sometimes weeks (34). Although this indispensable life support can improve oxygenation and decrease morbidity and mortality in patients with ALI, oxidant stress generated by the initial injurious process and exacerbated by hyperoxic ventilation is believed to play a major role in perpetuating ALI and preventing some patients from recovery (35). Although a prominent role for antioxidant enzymes and proteins in conferring protection against oxidative stress generated by hyperoxia or mechanical ventilation has been demonstrated in experimental studies, currently, with the exception of low tidal volume mechanical ventilation (36), there is no effective therapy in the treatment of ALI, and the mortality associated with severe ALI remains significantly high around the world. It is noteworthy that several antioxidant compounds showed beneficial effects in experimental models of lung injury. For example, administration of antioxidants such as N-acetylcysteine (37, 38), tempol (39, 40), superoxide dismutase mimetics (41), and tocopherol (42) confer protection against various stressful stimuli or toxin-induced lung pathogenesis in animal models, including ALI. However, several antioxidant clinical trials performed with direct antioxidants, such as N-acetylcysteine, procysteine, or superoxide dismutase mimetics, yielded either limited or no benefit in improving the mortality or morbidity associated with ALI (*see* review in Reference 35). Thus, it appears that the benefits of these direct antioxidant strategies to stoichiometrically scavenge reactive intermediates present in the lungs of patients with ALI or generated by hyperoxic ventilation therapy remain uncertain at best. However, enhancing Nrf2-ARE signaling with CDDO-Im serves as an indirect antioxidative mechanism through the up-regulation of antioxidant and stress-response pathways. Whether this indirect approach might provide an effective therapy to limit the adverse effects of hyperoxia in clinical settings remains to be determined.

In summary, our data collectively demonstrate that the activation of Nrf2-ARE pathway by CDDO-Im intermittently during exposure is sufficient to confer protection against hyperoxic ALI in mice. These changes are accompanied by increased expression of antioxidant enzymes, which are known to mitigate oxidative stress resulting in decreased lung injury and inflammation. Clinical trials using antioxidant strategies in patients with ALI/ARDS have not consistently demonstrated benefit. Our results suggest that activating specifically the ARE signaling by CDDO-Im may effectively decrease susceptibility to hyperoxia-induced ALI. CDDO-Im analogs, such as CDDO–methyl esters that are currently used in phase III clinical trials (http://clinicaltrials.gov); however, further studies are warranted to determine the therapeutic value of CDDO-Im or its analogs in ALI/ARDS syndromes.

Conflict of Interest Statement: N.M.R. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. V.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.S.Y. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.R.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.M.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.Y. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.T.L. received up to $1,000 from Reata Pharmaceuticals, Inc. in royalties. M.B.S. received more than $100,001 from Reata Pharmaceuticals in industry-sponsored grants and holds a patent from Reata Pharmaceuticals for synthetic triterpenoids and methods of use in the treatment of renal disease. T.W.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.P.R. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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