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Mechanistic role of cytochrome P450 (CYP)1B1 in oxygen-mediated toxicity in pulmonary cells: a novel target for prevention of hyperoxic lung injury

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Abstract

Supplemental oxygen, which is routinely administered to preterm infants with pulmonary insufficiency, contributes to bronchopulmonary dysplasia (BPD) in these infants. Hyperoxia also contributes to the development of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) in adults. The mechanisms of oxygen-mediated pulmonary toxicity are not completely understood. Recent studies have suggested an important role for cytochrome P450 (CYP)1A1/1A2 in the protection against hyperoxic lung injury. The role of CYP1B1 in oxygen-mediated pulmonary toxicity has not been studied. In this investigation, we tested the hypothesis that CYP1B1 plays a mechanistic role in oxygen toxicity in pulmonary cells *in vitro*. In human bronchial epithelial cell line BEAS-2B, hyperoxic treatment for 1–3 days led to decreased cell viability by about 50–80%. Hyperoxic cytotoxicity was accompanied by an increase in levels of reactive oxygen species (ROS) by up to 110%, and an increase of TUNEL-positive cells by up to 4.8-fold. Western blot analysis showed hyperoxia to significantly down-regulated CYP1B1 protein level. Also, there was a decrease of CYP1B1 mRNA by up to 38% and *Cyp1b1* promoter activity by up to 65%. On the other hand, CYP1B1 siRNA appeared to rescue the cell viability under hyperoxia stress, and overexpression of CYP1B1 significantly attenuated hyperoxic cytotoxicity after 48 h of incubation. In immortalized lung endothelial cells derived from *Cyp1b1*-null and wild-type mice, hyperoxia increased caspase 3/7 activities in a time-dependent manner, but endothelial cells lacking the *Cyp1b1* gene showed significantly decreased caspase 3/7 activities after 48 and 72 h of incubation, implying that CYP1B1 might promote apoptosis in wild type lung endothelial cells under hyperoxic stress. In conclusion, our results support the hypothesis that

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CYP1B1 plays a mechanistic role in pulmonary oxygen toxicity, and CYP1B1-mediated apoptosis could be one of the mechanisms of oxygen toxicity. Thus, CYP1B1 could be a novel target for preventative and/or therapeutic interventions against BPD in infants and ALI/ARDS in adults.

Keywords

Cytochrome P450; CYP1B1; hyperoxia; lung; endothelial cells

1. Introduction

Supplemental oxygen, which is frequently administered to premature infants with pulmonary insufficiency, is one of the major risk factors for the development of bronchopulmonary dysplasia (BPD) in premature infants [1]. Hyperoxia also contributes to the development of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) in adults. Infants with BPD often are re-hospitalized before 1 year of age, and have worse neurological outcomes with significantly higher rates of cerebral palsy, spastic dyplegia, and lower cognitive and language scores [2]. BPD has a multifactorial etiology, including genetic predisposition, prematurity, mechanical ventilation, oxygen exposure, and inflammation [3].

Supplemental oxygen, which is often a life-saving therapy for premature neonates and ALI/ARDS patients, may lead to hyperoxia, which in turn is a major risk factor for the development of lung injury that is associated with increased pulmonary permeability, increased inflammatory cell count, and injuries to endothelial and epithelial cells [4]. Multiple studies have shown an involvement of CYP enzymes CYP1A (i.e. CYP1A1 and CYP1A2) in oxygen toxicity [5–8]. CYP is a family of heme-containing proteins that are involved in the metabolism of numerous endogenous substrates and xenobiotics [9]. Induction of CYP1A by β-naphtoflavone (BNF) or 3-methylcholanthrene (MC) prior to hyperoxia protects mice and rats against the toxic effects of oxygen exposure [10, 11]. Meanwhile, pre-treatment of rats with a CYP1A inhibitor, aminobenzotriazole, followed by exposure to 95% O_2 leads to severe inflammation, pleural effusions, and severe lung injury [12]. During the initial 48 h of hyperoxia exposure, pulmonary and hepatic CYP1A upregulation is observed [12, 13]. Between 48–60 h, the animals develop severe respiratory distress, accompanied with downregulation of CYP1A [6, 12]. Aryl hydrocarbon receptor (AhR) is a key regulatory transcription factor of many CYP proteins and other important developmental genes, including CYP1A1/2 [14, 15]. Experiments with AhR null mice indicated that the protective effect of CYP1A1/2 on hyperoxic toxicity is dependent on the AhR in the lung [16].

CYP1B1 is the newest member of CYP1 family, described in 1994, after being cloned from a 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-treated human keratinocyte cell line [17], and shares only 40% homology with CYP1A1 and CYP1A2 [17]. CYP1B1 activates PAH in human lung [18] and inactivates benzo[a]pyrene (BP) in mouse aortic smooth muscle cells [19]. CYP1B1 is endogenously expressed in the lung and other tissues, but not hepatocytes [20]. BNF induces CYP1B1 mRNA, but to a lesser extent than that of CYP1A1 [21, 22].

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Thakur et al. [23] have reported that maternal treatment of BP, followed by 7-days of postnatal hyperoxia exposure leads to upregulation of CYP1B1 [23].

CYP1B1 has a conserved DNA sequence and appears in the early stages in ontogenesis [24]. It may play a role in evolution and development. In this investigation, we tested the hypothesis that CYP1B1, which is also regulated by the AHR, plays a mechanistic role in oxygen toxicity in pulmonary cells *in vitro*.

2. Materials and methods

2.1. Cell culture

BEAS-2B and H358 cells (ATCC, Manassas, VA) were maintained in RPMI 1640 or high-glucose DMEM containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were maintained in room air (RA) or exposed to hyperoxia (95% O2 and 5% CO2), as described earlier [25].

Lung endothelial cells were isolated from wild-type and C*yp1b1*-null mice as previously described [26, 27]. The EC growth medium was DMEM containing 10% FBS, 2 mM L-glutamine, 2 mM sodium pyruvate, 20 mM HEPES, 1% nonessential amino acids, 100 μg/ml streptomycin, 100 U/ml penicillin, freshly added heparin at 55 U/ml (Sigma), 100 μg/ml endothelial growth supplement (Sigma), and murine recombinant interferon-γ (R&D Systems, Minneapolis, MN) at 44 U/ml. Cells were incubated at 33°C with 5% CO2 and progressively passaged to larger plates. Cells were normally maintained in 60-mm dishes coated with 1% gelatin prepared in phosphate-buffered saline (PBS) [27].

To establish the CYP1B1-overexpressed cell line, total RNA was isolated from MC-treated BEAS-2B cells using RNeasy Mini Kit (Qiagen, Germantown, MD), and was subjected to reverse transcription (Bio-Rad, Hercules, CA). RT-PCR was performed using 5'-ATGCTAGCGCCGCCACCATGGGCACCAGCCTCAG-3' and 5'-TAGGTACCCTTATTGGCAAGTTTCCTTGG-3' as the cloning primers. The open reading frame of human CYP1B1 cDNA was subcloned into pcDNA3.1 between the NheI/KpnI sites. The insert sequence of pCD-CYP1B1 was verified by DNA sequencing. To obtain the stable overexpressed cells, pCD-CYP1B1 or pcDNA3.1 was transfected into H358 cells using SuperFect (Qiagen), and maintained in 500 μg/ml geneticin (Life Technologies). Clones that overexpressed CYP1B1 were screened by CYP1B1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) using immunofluorescence staining, and verified by real-time RT-PCR (data not shown).

2.2. Cell proliferation assays

The conventional trypan blue exclusion assay was performed using 0.4% trypan blue as previously described [28]. 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) (MTT) assay was performed according to the manufacturer's instruction (ATCC) as previously described [15].

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2.3. Intracellular ROS

Intracellular ROS level was quantified according to the manufacturer's instruction (Life Technologies) as previously described [15].

2.4. TUNEL fluorescein assay

TUNEL assay was performed using Click-iT TUNEL Alexa-Fluor Imaging Assay Kit according to the manufacturer's instruction (Life Technologies). Briefly, cells in 96-well black-walled plates (BD Biosciences) were fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100, incubated with 50 μl/well of TdT reaction buffer at RT for 10 min, incubated with 50 μl/well of TdT reaction cocktail at 37°C for 60 min, washed twice with 3% BSA in PBS, and incubated with 50 μl/well of the Click-iT reaction cocktail at RT for 30 min. After the final wash with PBS containing 3% BSA, fluorescence was measured with excitation at 495 nm and emission at 519 nm.

2.5. Western blot analysis of CYP1B1 apoprotein

Cell lysates (30 μg protein per well) were resolved in SDS-PAGE and detected with CYP1B1 antibody (Santa Cruz Biotechnology) or β-actin (Actb) antibody (Sigma-Aldrich) by Western blot analyses, as described previously [13, 28].

2.6. qPCR

Total RNA was extracted from the cell lysates using RNeasy Mini Kit (Qiagen). Message RNA was quantified with SuperScript III Two-Step qRT-PCR Kit (Life Technologies). The primer sequences were 5’-CACTGCCAACACCTCTGTCTT-3’ and 5’-CAAGGAGCTCCATGGACTCT-3’ for CYP1B1 [29], 5’-AAACGCATTAACTGGCGAAC-3’ and 5’-GAACTCCAGGAGAACTGCAAA-3’ for human ornithine decarboxylase antizyme OAZ1 [29]. The SYBR Green (Bio-Rad) qPCR condition was 95°C fo r 5 s (denaturation), 65°C for 10 s (annealing), and 72°C for 20 s (extension) for CYP1 B1, and was 95°C for 5 s, 60°C for 10 s, and 72°C for 20 s for OAZ1. The PCR specificity was confirmed with the melting curves. The PCR efficiency was 95.7% for CYP1B1 and 96.5% for OAZ1.

2.7. Cyp1b1 promoter assay

The dual-luciferase assay (Promega, Madison, WI) was performed in BEAS-2B cells double transfected with 2 plasmid constructs using Qiagen SuperFect. The firefly luciferase construct p1.1 contained the rat *Cyp1b1* 5’-UTR and 1.0 Kb of the proximal 5’-flanking sequence. The renilla luciferase construct was pRL-TK (Promega). *Cyp1b1* promoter activities were determined by the dual-luciferase assay, which entailed normalizing the firefly luciferase activities against those of renilla luciferase.

2.8. *CYP1B1* siRNA knockdown

Cells were transfected with either ON-TARGETplus hCYP1B1 siRNA or the non-targeting control (Dharmacon, Chicago, IL) using Lipofectamine 2000 (Life Technologies). The siRNA effect was examined by qPCR as described in *Materials and methods*.

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2.9. ApoTox-Glo Triplex Assay for cytotoxicity and caspase 3/7 activities

Cytotoxicity and caspase 3/7 activities were determined using ApoTox-Glo™ Triplex Assay (Promega) according to the manufacturer's instruction. Briefly, cells in 96-well black-walled plates were incubated with 20 μl of Cytotoxicity Reagent at 37°C for 30 min. Cytot oxicity was determined by fluorescence (excitation at 485 nm and emission at 520 nm). The cells were then incubated with 100 μl of Caspase-Glo 3/7 Reagent at RT for 30 min. The caspase 3/7 activities were determined by bioluminescence.

3. Results

3.1 Cytotoxicity of hyperoxia to BEAS-2B cells

Hyperoxia impairs lung development in premature babies, as well as in newborn mice and other animals [30, 31]. *In vitro* experiments consistently demonstrated hyperoxic toxicities to pulmonary cell lines such as H358 (unpublished data), H441, and A549 [25]. In BEAS-2B cells, trypan blue exclusion assay showed that the number of live cells increased by about 60% per day under RA conditions (RA) (Figure 1A). Hyperoxia (95% O_2 plus 5% CO_2) [28] showed no effect on cell proliferation during the first 24 h, but exhibited 44 and 81% inhibition at 48 and 72 h, respectively, based on cell numbers (Figure 1A). The MTT cell proliferation assay measures the activity of NAD(P)H-dependent oxidoreductases which represents the metabolic rate of entire cell population, live and dead, in each well. Hyperoxia decreased the A_{570nm} in the MTT assay of BEAS-2B cells by 14%, 24%, and 51% at 24, 48, and 72 h, respectively (Figure 1B).

According to the literature, hyperoxic cytotoxicity is associated with increased production of ROS [32]. We measured the effect of hyperoxia on intracellular ROS in BEAS-2B cells using CM-H_2DCFDA as the probe. ROS converts the fluorescent probe into 5-(and 6-)chloromethyl-2′,7′-dichlorofluorescin (CM-DCF). As anticipated, we found that hyperoxia increased the CM-DCF fluorescence or intracellular ROS by 26% at 48 h and 110% at 72 h (Figure 1C).

Since hyperoxia caused cell death (Figure 1A), we performed TUNEL apoptosis assay, a method based on terminal deoxynucleotidyl transferase (TdT)-associated incorporation of dUTPs at the 3’-OH groups of fragmented DNA. Hyperoxia increased dUTP incorporation in the BEAS-2B cells by 1.5-, 2.7-, and 4.8-fold at 24, 48, and 72 h, respectively (Figure 1D), indicating the involvement of apoptosis in the ROS-associated hyperoxic cytotoxicity.

3.2 Hyperoxia downregulated CYP1B1 in BEAS-2B cells

Previous reports indicate that hyperoxia induces CYP1A1 in the lung or cultured pulmonary cells [5, 25]. When BEAS-2B pulmonary cells were exposed to hyperoxia, CYP1B1 apoprotein was significantly downregulated at 24 and 48 h in Western blot analysis (Figure 2A). qPCR indicated that hyperoxia decreased CYP1B1 mRNA level by 38%, 21%, and 19% at 24, 48, and 72 h, respectively (Figure 2B). The reference gene OAZ1 was not affected by hyperoxia, consistent with our previous publication on the effect of hyperoxia in H441 cells [30]. CYP1A1 mRNA was induced by 94% by a 24 h hyperoxic treatment (not shown), consistent with previous observations [25].

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In order to investigate the mechanisms of downregulation of *Cyp1b1*a 1.1 Kbp of rat *Cyp1b1* promoter sequence was subcloned into pGL3 luciferase reporter system (see *Materials and methods*). Dual luciferase assay of the p1.1/pRL double transfected BEAS-2B cells indicated that *Cyp1b1* promoter activity was down-regulated by hyperoxia by 45–65% at 24, 48, and 72 h (Figure 2C).

3.3 CYP1B1 siRNA protected cells from hyperoxic cytotoxicity

BEAS-2B cells were transiently transfected with either non-specific control siRNA or CYP1B1 siRNA. The qPCR analysis indicated that CYP1B1 siRNA significantly knocked down CYP1B1 mRNA in both room air (21.0%) and hyperoxia (85.5%) conditions (Figure 3A). Cells that were transfected with CYP1B1 siRNA showed elevated the cell viability under hyperoxia conditions by 44% (Figure 3B), suggesting that CYP1B1 siRNA rescued cells from oxygen toxicity, albeit this was not statistically significant.

3.4 CYP1B1 overexpression promoted hyperoxic cytotoxicity

CYP1B1-H358 cells were generated by stably transfecting CYP1B1 cDNA into H358 cells using a pCDNA vector. The control cells (pCD-H358 cells) were created by transfecting the H358 cells with the empty vector pCDNA3.1(+). The cells were maintained in room air or subjected to hyperoxia for 24, 48, and 72 h. MTT assay measures the total NAD(P)H-dependent oxidoreductase activity in each well. But the two stable cell lines exhibit a different growth rate, resulting in different amount of cells in each well. Therefore, in Figure 3C, we converted the original MTT A_{570nm} readings into cell densities, using a standard curve of MTT A_{570nm} readings versus cell count. We found that CYP1B1-H358 was more susceptible to hyperoxic toxicity (Figure 3C). A 48 h hyperoxic treatment caused a greater decrease in cell viability of CYP1B1-H358 cells, as compared to pCD-H358 cells, and this difference was much more pronounced at 72 h (Figure 3C).

3.5 Knockout of Cyp1b1 gene in lung endothelial cells alleviated hyperoxic toxicity

Lung endothelial cell line prepared from wild-type and *Cyp1b1*-null mice were subjected to RA and hyperoxic condition. Hyperoxic toxicity to cell viability was observed in both cell line (Figure 4A). Although the $Cyp1b1^{-/-}$ cells grew faster than the $Cyp1b1^{+/+}$ (WT) cells (Figure 4A), the hyperoxia-induced increase of caspase 3/7 activities was lower in the $Cyp1b1^{-/-}$ cells (Figure 4B). The differences became statistically significant at 72 h (Figure 4B), suggesting *Cyp1b1* expression promoted apoptosis under hyperoxic stress.

DISCUSSION

Lung injury due to prolonged hyperoxia is characterized by increased ROS production [32]. ROS could initiate lung damage by oxidative damage to proteins, lipids, and DNA, which could in turn lead to enhanced expression of pro-inflammatory genes [33]. ROS plays a central role in the subsequent extensive inflammatory response, destruction of the alveolo-capillary barrier, impaired gas exchange, and pulmonary edema [34].

In this study, we focused on the mechanistic role of CYP1B1 in oxygen-mediated pulmonary toxicity using human pulmonary cell lines. Using BEAS-2B cells, we found that

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the onset of hyperoxic cytotoxicity was most noticeable at 48 h. No significant cell death (Figure 1A), ROS production (Figure 1C), or dUTP incorporation-associated apoptosis (Figure 1D) was observed after 24 h exposure of 95% oxygen. This observation was consistent with our previous observations that CYP1A1 was induced by hyperoxia within 24 h, and this may have led to protection of the cells from hyperoxic toxicity in that time frame [25]. At later time points, CYP1A1 expression declined and there was more cytotoxicity, suggesting that CYP1A1 decrease may have contributed to the toxicity mediated by oxygen [25].

The mechanisms by which hyperoxia caused downregulation of CYP1B1 at the protein (Figure. 2A) and mRNA levels (Figure. 2B) is not completely understood, but probably entailed attenuation of CYP1B1 gene at the transcriptional level based on our transient transfection experiments using the luciferase reporter gene (Figure. 2C). However, that fact that CYP1B1 mRNA repression by hyperoxia was modest relative to that of CYP1B1 protein could be explained by a combination of transcriptional and post-transcriptional/ translational mechanisms. It is possible that ROS produced under hyperoxic conditions may have decreased the stability of CYP1B1 protein [35].

The downregulation of CYP1B1 might be a protective mechanism because we found that CYP1B1 promoted hyperoxic cytotoxicity. This idea is supported by our observation that overexpression of CYP1B1 in the cells led to increased cytotoxicity caused by hyperoxic incubation for 48 h or longer (Figure 3C). Furthermore, knock-down of CYP1B1 mRNA rescued the cells from toxicity after 48 h hyperoxia (Figure. 3A&B), although this was statistically significant. Conditional deletion of CYP1B1 in endothelial cells significantly decreased hyperoxia-mediated apoptosis (Figure 4B). The fact that endothelial cells lacking CYP1B1 showed lesser apoptosis and toxicity than wild type cells supports the hypothesis that CYP1B1 in pulmonary endothelial cells *in vivo* contributes to the pro-oxidant effects under hyperoxic conditions. Our recent studies showing decreased hyperoxic lung injury in mice lacking the gene for *Cyp1b1* (Veith et al., 2016, unpublished results) lends further credence to this hypothesis.

Tang *et al.* reported that *Cyp1b1* deficiency in retinal endothelial cells showed increased oxidative stress and protection against abnormal angiogenesis, and this was due increased production of thrombospondin-2 (TSP-2), an inhibitor of angiogenesis [36].

In summary, the results of this investigation support the hypothesis that CYP1B1 plays a mechanistic role in pulmonary oxygen toxicity, and CYP1B1-mediated apoptosis could be one of the mechanisms of oxygen toxicity. CYP1B1 could thus be one of the targets for preventative and/or therapeutic interventions against BPD in infants and ALI/ARDS in adults.

Supplementary Material

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Abbreviations

BPD	bronchopulmonary dysplasia
ALI	acute lung injury
ARDS	acute respiratory distress syndrome
CYP	cytochrome P450
ROS	reactive oxygen species
AHR	arylhydrocarbon receptor
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
BNF	β-naphtoflavone
MC	3-methylcholanthrene
BP	benzo[a]pyrene
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide

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Highlights

- In human lung cells, hyperoxia caused downregulation of CYP1B1 protein and mRNA
- Cells expressing human CYP1B1 displayed greater susceptible to oxygen toxicity
- Lung endothelial cells from mice lacking CYP1B1 showed attenuation oxygen toxicity
- CYP1B1 could be a novel target for prevention of hyperoxic lung injury in humans

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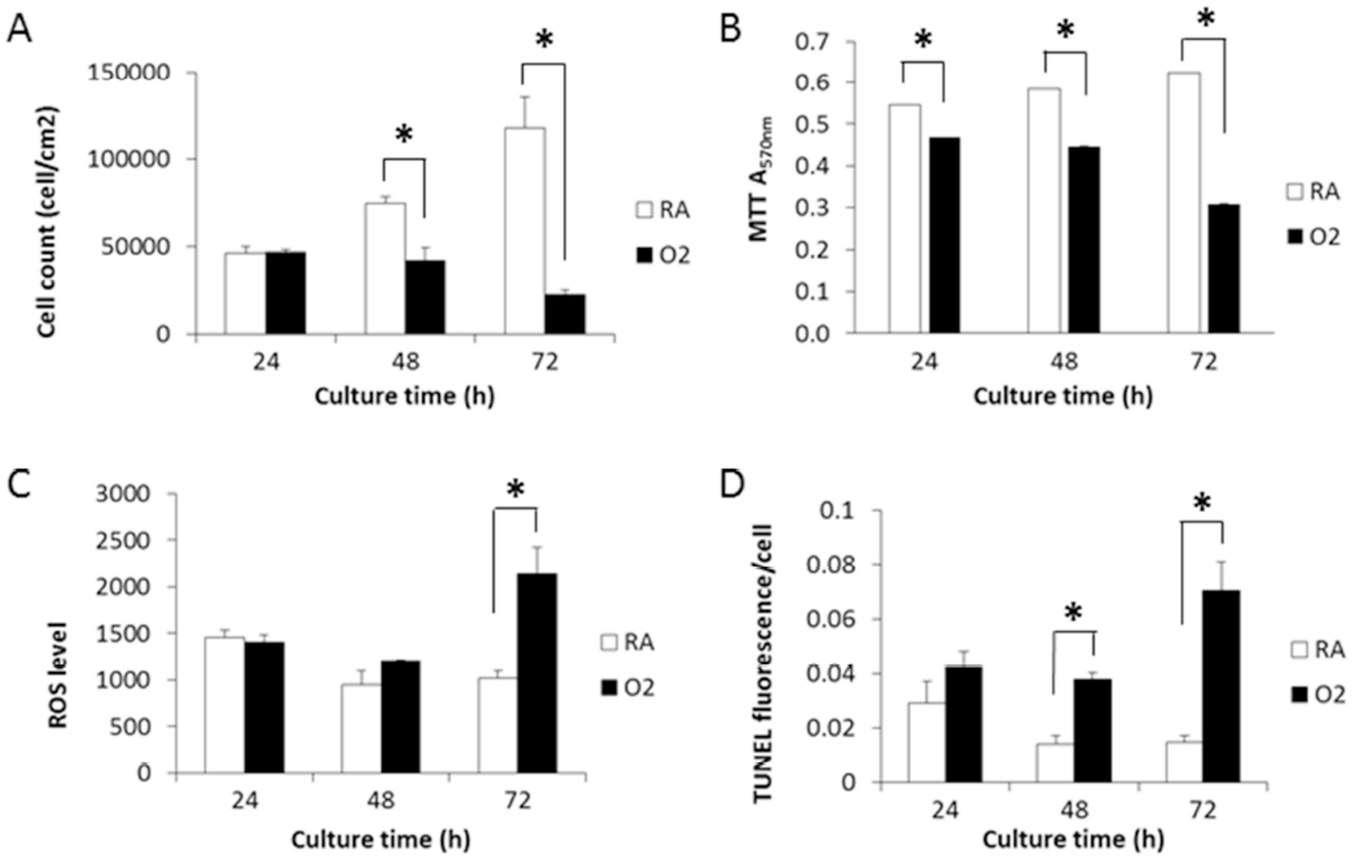

Figure 1.

Hyperoxia exhibited cytotoxicity to BEAS-2B cells, which was accompanied by increase of intracellular ROS level and apoptotic cell population. Cells maintained in RA or hyperoxia (O2) condition for 24, 48, and 72 h were subjected to trypan blue exclusion assay (A), MTT assay (B), CM-H_2DCF-DA based ROS flow cytometry assay (C), and TUNEL Alexa-Fluor imaging assay (D). (n = 3; *, t-test $p < 0.05$) Values represent mean ± SEM of at least 3 independent experiments.

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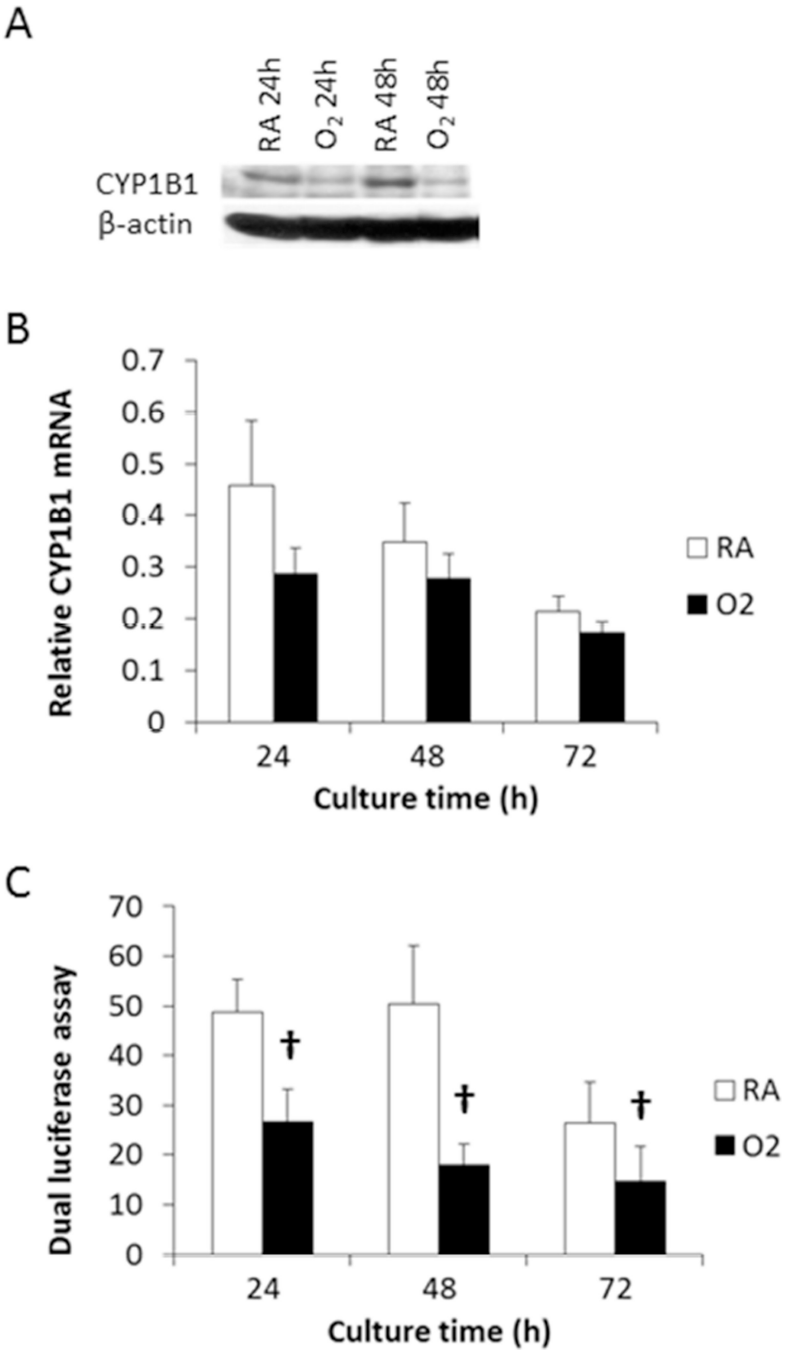

Figure 2.

Down-regulation of CYP1B1 by hyperoxia in BEAS-2B cells. Cells maintained in RA or hyperoxia condition were subjected to Western blot analysis of CYP1B1 and β-actin (A), or qPCR of CYP1B1 and the reference gene OAZ1 (B) as compared to room air levels (C) Cells were transfected with p1.1, a firefly luciferase reporter plasmid containing the rat CYP1B1 promoter sequence, together with the pRL-TK renilla luciferase reporter plasmid. The transiently transfected cells were maintained in RA or hyperoxia condition, and subjected to dual-luciferase assay. Two-way ANOVA analysis indicated that hyperoxia

significantly decreased CYP1B1 promoter activity (n = 3; †, $p < 0.05$) at each time point. Values represent mean ± SEM of at least 3 independent experiments.

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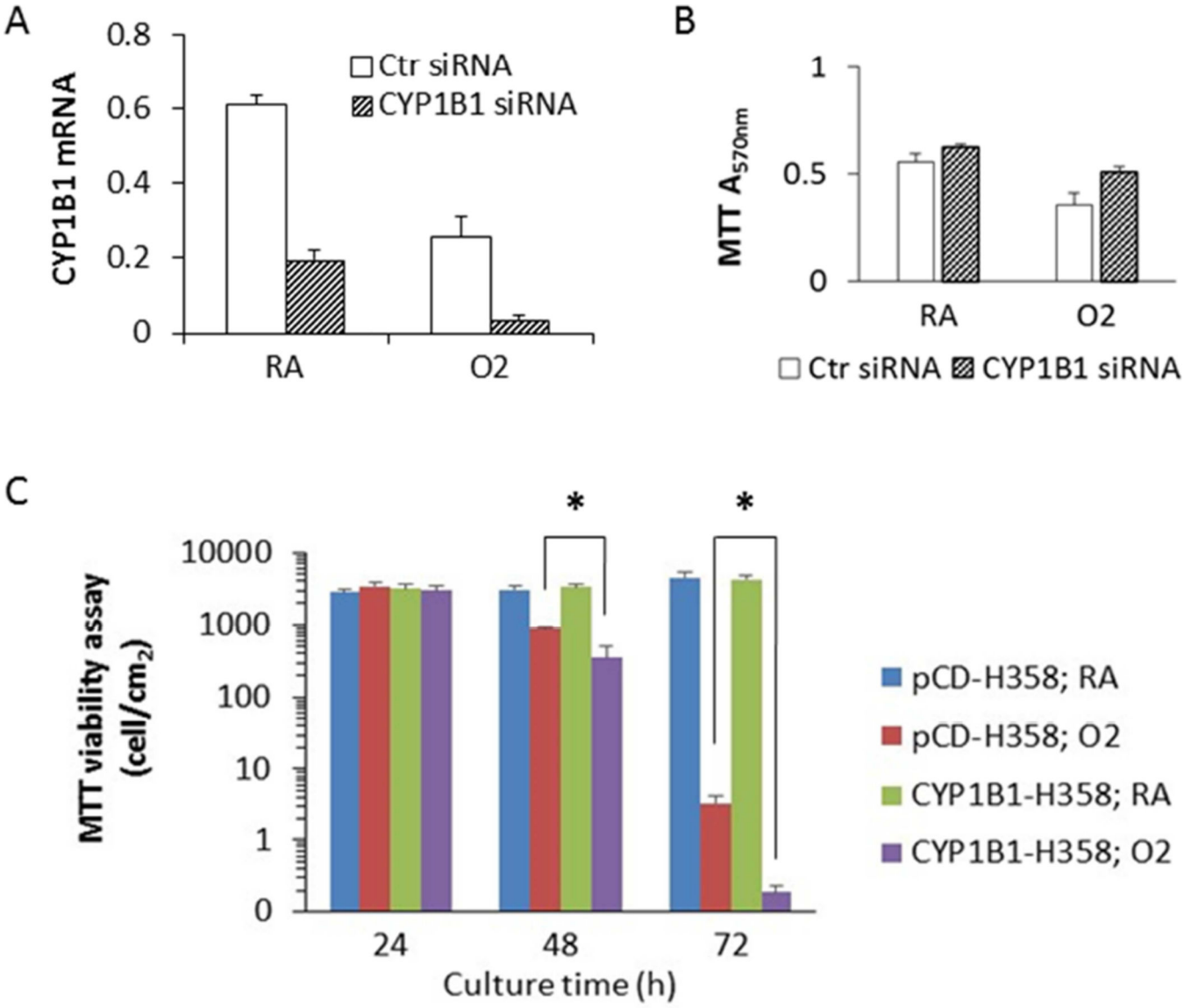

Figure 3.

CYP1B1 promoted hyperoxic cytotoxicity in human lung cell lines. A. CYP1 B1 siRNA knocked down CYP1B1 mRNA level in both RA and hyperoxia conditions (48 h). B. CYP1B1 siRNA appeared to rescue hyperoxic cytotoxicity in BEAS-2B cells, although this was not statistically significant. . C. Overexpression of CYP1B1 in H358 cells augmented hyperoxic cytotoxicity (n = 3; *, t-test $p < 0.05$). , Values represent mean ± SEM of at least 3 independent experiments.

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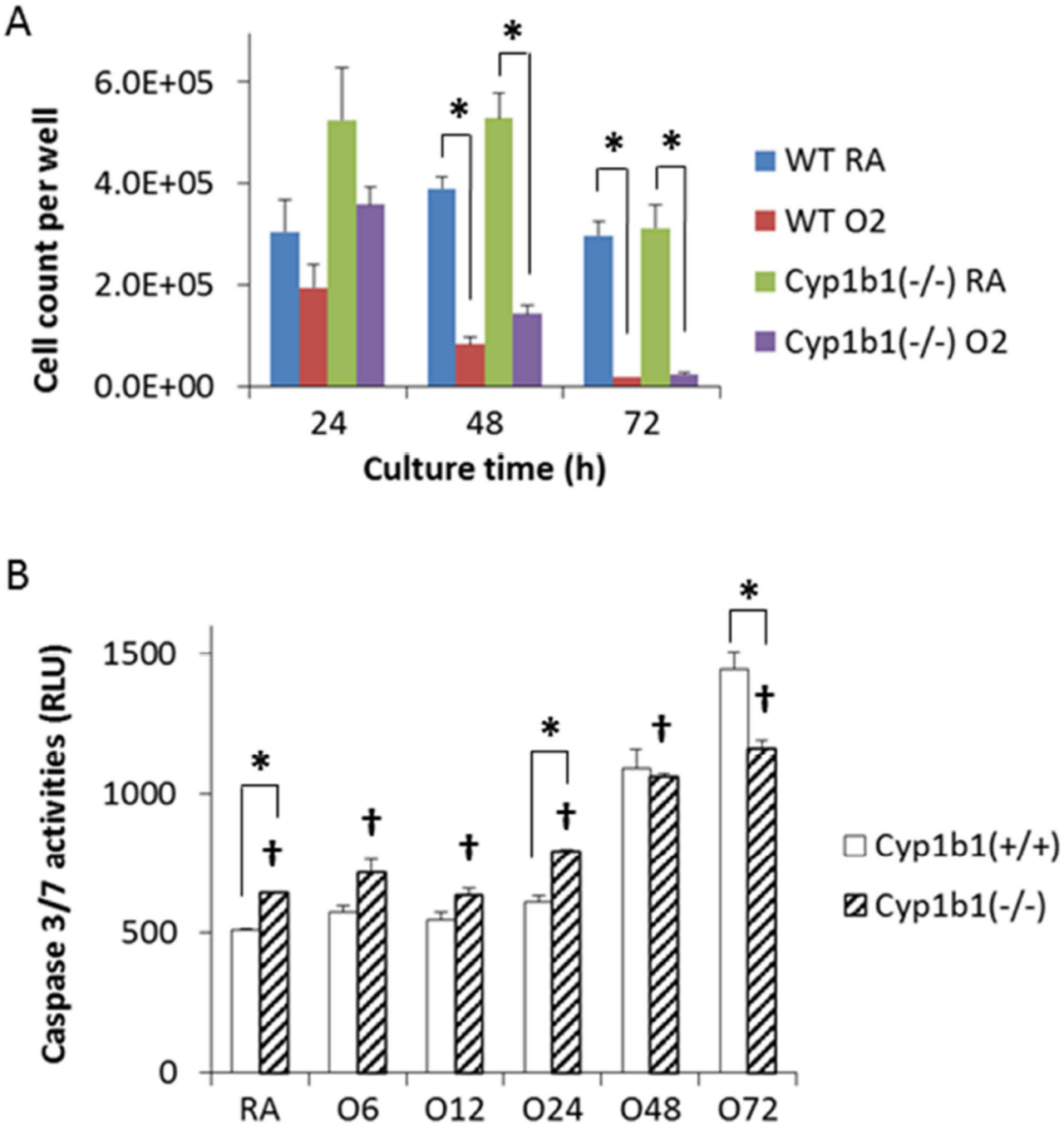

Figure 4.

Lack of *CYP1B1* expression decreased the apoptotic activity after 72 h. Lung endothelial cells prepared from wild-type and C*yp1b1*-null mice were exposed to high oxygen and viability was assessed. A. Trypan blue exclusion assay exhibited hyperoxic toxicities in both *Cyp1b1*$^{+/+}$ (WT) and *Cyp1b1*$^{-/-}$ cells. B. Two-way ANOVA analysis indicated that lack of CYP1B1 expression significantly reduced the intracellular caspase 3/7 activities promoted by hyperoxia (†, $p < 0.05$). (n = 3; *, t-test $p < 0.05$).