

Protectin DX Relieve Hyperoxia-induced Lung Injury by Protecting Pulmonary Endothelial Glycocalyx

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Background: Bronchopulmonary dysplasia (BPD) is a common chronic lung disease in premature infants with limited treatments and poor prognosis. Damaged endothelial glycocalyx leads to vascular permeability, lung edema and inflammation. However, whether hyperoxia increases neonatal pulmonary microvascular permeability by degrading the endothelial glycocalyx remains unknown.

Methods: Newborn mice were maintained in 60–70% O₂ for 7 days. Protectin DX (PDX), an endogenous lipid mediator, was injected intraperitoneally on postnatal d 0, 2, 4 and 6. Lung samples and bronchoalveolar lavage fluid were taken at the end of the study. Primary human umbilical vein endothelial cells (HUVECs) were cultured in 80%O₂.

Results: Hyperoxia exposure for 7 days led to neonatal mice alveolar simplification with less radial alveolar count (RAC), mean linear intercept (MLI) and mean alveolar diameter (MAD) compared to the control group. Hyperoxia exposure increased lung vascular permeability with more fluid and proteins and inflammatory factors, including TNF- α and IL-1 β , in bronchoalveolar lavage fluid while reducing the heparan sulfate (HS), the most abundant component of the endothelial glycocalyx, in the pulmonary endothelial cells. PDX relieve these changes. PDX attenuated hyperoxia-induced high expression of heparanase (HPA), the endoglycosidase that shed endothelial glycocalyx, p-P65, P65, and low expression of SIRT1. BOC-2 and EX527 abolished the affection of PDX both in vivo and intro.

Conclusion: In summary, our findings indicate that PDX treatment relieves hyperoxia-induced alveolar simplification, vascular leakage and lung inflammation by attenuating pulmonary endothelial glycocalyx injury via the SIRT1/NF- κ B/ HPA pathway.

Keywords: bronchopulmonary dysplasia, endothelial glycocalyx, inflammation, protectin DX

Introduction

Bronchopulmonary dysplasia (BPD) is a common chronic lung disease in premature infants, especially those with extremely low birth weight or/and extremely preterm.¹ BPD infants usually require months or even years of oxygen therapy. Although BPD has been clinically prevented and treated including lung protective ventilation strategies, glucocorticoid and fluid restriction, the therapeutic effect and prognosis of BPD are still not satisfying.^{2,3} Among survivors it confers a predisposition to chronic respiratory disease, cardiovascular disorder and neurodevelopmental delay.⁴ As a result, developing other innovative and effective preventive or therapeutic measures to reduce mortality rates is urgent for perinatal medicine.

Alveolar simplification and dysmorphic vasculogenesis are the main BPD-associated pathological changes observed in premature lungs that impact effective gas exchange and lung function.¹ The pathogenesis of BPD is complicated; hyperoxia-induced lung injury is the major contributor to BPD.⁵ Hyperoxia-induced lung injury increases pulmonary microvascular permeability, which leads to the transudation of fluid and proteins across the alveolar wall. Alveolar type II

cells (ATII) are responsible for postnatal clearance of excess fluid from the airspaces by moving Na^+ ions from the apical to the basolateral side, which creates a measurable electrochemical gradient as an osmotic gradient driving water movement in the same direction.⁶ Meanwhile, hyperoxia upregulates the expression of epithelial Na^+ channels in both neonatal rat lung tissues and ATII cells.⁷ Dysfunctional transport of Na^+ is not responsible for pulmonary edema of BPD.

The pulmonary endothelium cells regulate the pathophysiology of lung injury.^{8,9} The glycocalyx coats the vascular endothelial surface, which confers essential functions in circulatory homeostasis and lung infection.^{10–12} Damaged glycocalyx increases the permeability of proteins and fluids, causing interstitial leakage and lung edema.¹³ Endothelial glycocalyx dysfunction contributes to severe inflammatory vasculopathy, including infection, sepsis, acute respiratory distress syndrome (ARDS) and trauma.¹⁰ Heparan sulfate proteoglycans (HS) are the most abundant component of the endothelial glycocalyx.¹⁴ The vascular endothelial growth factor and fibroblast growth factor-2, which binding to HS, are critical for vascular and angiogenesis in blood vessel formation and growth.¹⁴ Heparanase (HPA) is an endoglycosidase which cleaves heparan sulfate proteoglycans into heparan sulfate side chains and core proteoglycans, leading to endothelial glycocalyx disruption.¹⁵ However, whether hyperoxia increases neonatal pulmonary microvascular permeability through degradation of the endothelial glycocalyx remains unknown.

SIRT1 is involved in a variety of biological processes and cellular processes, including inflammation, oxidative stress, apoptosis, and mitochondrial function.¹⁶ SIRT1 is widely expressed in the vasculature, including endothelial and smooth muscle cells.¹⁷ Because of its anti-inflammatory and antioxidative properties, many studies focused on the role of SIRT1 in endothelial cell biology.¹⁸ The NF- κ B transcription factors are central regulators of inflammation and angiogenesis.^{19,20} NF- κ B pathway is involved in the regulation of HPA expression.²¹ SIRT1/NF- κ B pathway may play an important role in pulmonary endothelial glycocalyx degradation during lung development.

Protectin DX (10 (S), 17 (S)-dihydroxy-4Z, 7Z, 11E, 13Z, 15E, 19Z-docosahexaenoic acid) (PDX), an endogenous lipid mediator produced via sequential lipoxygenation of docosahexaenoic acid, exerts potent anti-inflammatory and pro-resolution bioactions. We previously reported that PDX regulates inflammatory cell infiltration in lipopolysaccharide-induced lung injury via resident macrophage²² and promotes alveolar fluid clearance through stimulating alveolar epithelial sodium channels and Na, K-ATPase.²³ However, the effect of PDX on hyperoxia-induced lung injury remains unknown.

Our study aimed to elucidate the mechanisms of hyperoxia-increased pulmonary microvascular permeability and whether PDX is an endothelial glycocalyx targeting treatment strategy. We hypothesized that hyperoxia induces heparanase activation with consequent degradation of the pulmonary endothelial glycocalyx and vascular leakage. We also investigated the regulatory role of PDX on heparinase and the underlying mechanism in vivo and in vitro.

Materials and Methods

Animals and Experimental Groups

Specific pathogen-free (SPF) C57BL/6 mice were obtained from Charles River (Charles River, China). The mice were housed in an SPF lab on a day-night cycle under controlled temperature and humidity conditions and were allowed food and water ad libitum. All animal procedures were performed in accordance with the policies and guidelines of the Ethics Committee of Wenzhou Medical University.

On day 22 of pregnancy, the dams are delivered naturally. The pups were pooled, randomized, and returned to the nursing dams. Experimentally, the pups were divided into four groups ($n = 12–14$): control group, PDX group, O_2 group, and $\text{O}_2 + \text{PDX}$ group. The mice in the O_2 and $\text{O}_2 + \text{PDX}$ groups were maintained at 60–70% O_2 for 7 days while the others were held in room air (21% O_2). Nursing dams were rotated between hyperoxia and room air every 24 h to prevent O_2 toxicity. Continuous hyperoxia exposure was achieved in a Plexiglass chamber flow-through system. The O_2 level inside the Plexiglass chamber was monitored with an O_2 analyzer. Each mouse in the PDX group and $\text{O}_2 + \text{PDX}$ group was intraperitoneally injected with 100 ng PDX on postnatal d 0, 2, 4 and 6 for four injections. PDX was obtained from Cayman Chemical (Ann Arbor, MI). The mice in the control group and O_2 group were injected with an equivalent volume of normal saline (NS). All mice were anesthetized with 1% pentobarbital and sacrificed at postnatal day 7. The lung samples were extracted for further analysis.

Cell Culture and Experimental Groups

Primary human umbilical vein endothelial cells (HUVECs, purchased from iCell Bioscience Inc., Shanghai) were isolated from the human umbilical vein and were grown adherently with Endothelial Cell Medium kit purchased from ScienceCell. Then the cells were treated with O₂ (80%), PDX (100 nM), BOC-2 (10 µM) or EX527 (10 µM). BOC-2 and EX527 were added 24 hr before the addition of PDX. EX527 (SIRT1 inhibitor) was purchased from MedChemExpress (Shanghai, China). BOC-2 (ALX inhibitor) was obtained from Biomol-Enzo Life Sciences (Farmingdale, NY).

Pulmonary Histopathology Evaluation

Following euthanasia, the mice's right bronchus was ligated. The left lungs were perfused with 4% paraformaldehyde (PFA) at 20 cm H₂O pressure via an intravenous needle inserted into the trachea. Then the left lungs were fixed in 4% paraformaldehyde and embedded in Tissue Tek OCT compound (Electron Microscopy Sciences, Japan). The radial alveolar count (RAC), mean linear intercept (MLI) and mean alveolar diameter (MAD) were measured to quantify lung development, as reported in previous studies.^{24,25} MAD was calculated as the average alveolar diameter. MLI was calculated as the volume-to-surface ratio of alveoli and performed by drawing five lines in each field and dividing the length of each line by the number of alveolar intercepts for that line. RAC was obtained by drawing a line from the centre of terminal bronchioles to the nearest connective tissue septum and counting the number of the alveoli on this line.

Lung Vascular Permeability Assay

Lung vascular permeability was measured by wet-to-dry (W/D) lung weight ratio and the Evans blue dye (EBD, Sigma-Aldrich, USA) leakage method. The lung tissues were weighed, and homogenized in formamide. Following overnight extraction, the tissue fluid was centrifuged at 12,000 × g for 10 min. The EBD concentration of the supernatant was evaluated at 620 nm absorbance by a microplate reader.

Lung Inflammation Analysis

Bronchoalveolar lavage fluid (BALF) was collected and centrifuged at 500×g for 6 min at 4 °C. The protein concentration of supernatants were measured by BCA Protein Assay Kit (23225, Thermo Fisher Scientific, USA). The concentration of TNF-α and IL-1β in BALF supernatant were measured by ELISA kit (Boyun, Shanghai, China) according to the manufacturer's protocol.

Western Blot Analysis

The protein extraction and Western blot analysis were performed as previously described. The following antibodies were applied: anti-mouse monoclonal anti- SIRT1 was from Proteintech (60303, Proteintech, China), rabbit polyclonal anti-Phospho-NF-κB P65 (Ser536) was from Affinity Biosciences (AF2006, Affinity Biosciences, USA), rabbit polyclonal anti-NF-κB P65 was from Affinity Biosciences (AF5006, Affinity Biosciences, USA), rabbit polyclonal Anti-Heparanase 1 was from HuaBio (ER1910-48, HuaAn Biotechnology, China), β-tubulin was from Affinity Biosciences (T0023, Affinity Biosciences, USA).

Immunofluorescence

Immunofluorescence was performed with pulmonary tissues and HUVECs. Samples were fixed in 4% paraformaldehyde, blocked with donkey serum (Solarbio, Beijing), and incubated with anti-HSPG2 (Abcam, cat. no. ab2501) and anti-vWF (von Willebrand factor, Sigma-Aldrich, cat. no. AB7356). After incubating with a second antibody (Alexa Fluor[®] 488 or 594) (1:200) and 4',6-diamidino-2-phenylindole (Abcam), we observed them with confocal microscopy (Nikon).

Statistical Analysis

The data are presented as the mean ± SD unless otherwise indicated. Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. PASW Statistics version 18.0 (SPSS Inc., Chicago,

IL), GraphPad Prism 6.0 (GraphPad, San Diego, CA) and BioRender.com were used for analyses and graphs. Results with a value of $p < 0.05$ were considered statistically significant.

Results

PDX Mitigate Hyperoxia-induced Alveolar Simplification

Alveolar simplification is the main BPD-associated pathological change observed in the lungs that impact effective gas exchange and lung function. As shown in Figure 1A, hyperoxia treatment induced alveolar simplification. PDX treatment mitigated these hyperoxia-induced pathological changes. We calculated the RAC, MLI and MAD to quantify the lung development. As shown in Figure 1B–D, hyperoxia exposure led to less RAC and longer MAD and MLI than the control group. The chemical structure of PDX is shown in Figure 1E. PDX treatment relieved these hyperoxia-induced changes.

PDX Inhibits Hyperoxia-induced Endothelial Glycocalyx Damage and Lung Inflammation in vivo

Heparin sulphate (HS) and syndecan-1 (SDC-1) are the main components of the endothelial glycocalyx. The HS expression is both in the pulmonary endothelial and epithelial cell (Figure 2A). The vWF-positive cells indicate the pulmonary endothelial cells. Hyperoxia exposure reduced the HS expression in the pulmonary endothelial cells compared with that in the control group. PDX treatment significantly increased HS expression. To determine the effect of PDX on hyperoxia-induced pulmonary edema, we performed the W/D weight ratio test (Figure 2B). Compared with the control group, the W/D lung weight ratio increased significantly in the hyperoxia group and was reduced in the O_2 +PDX group.

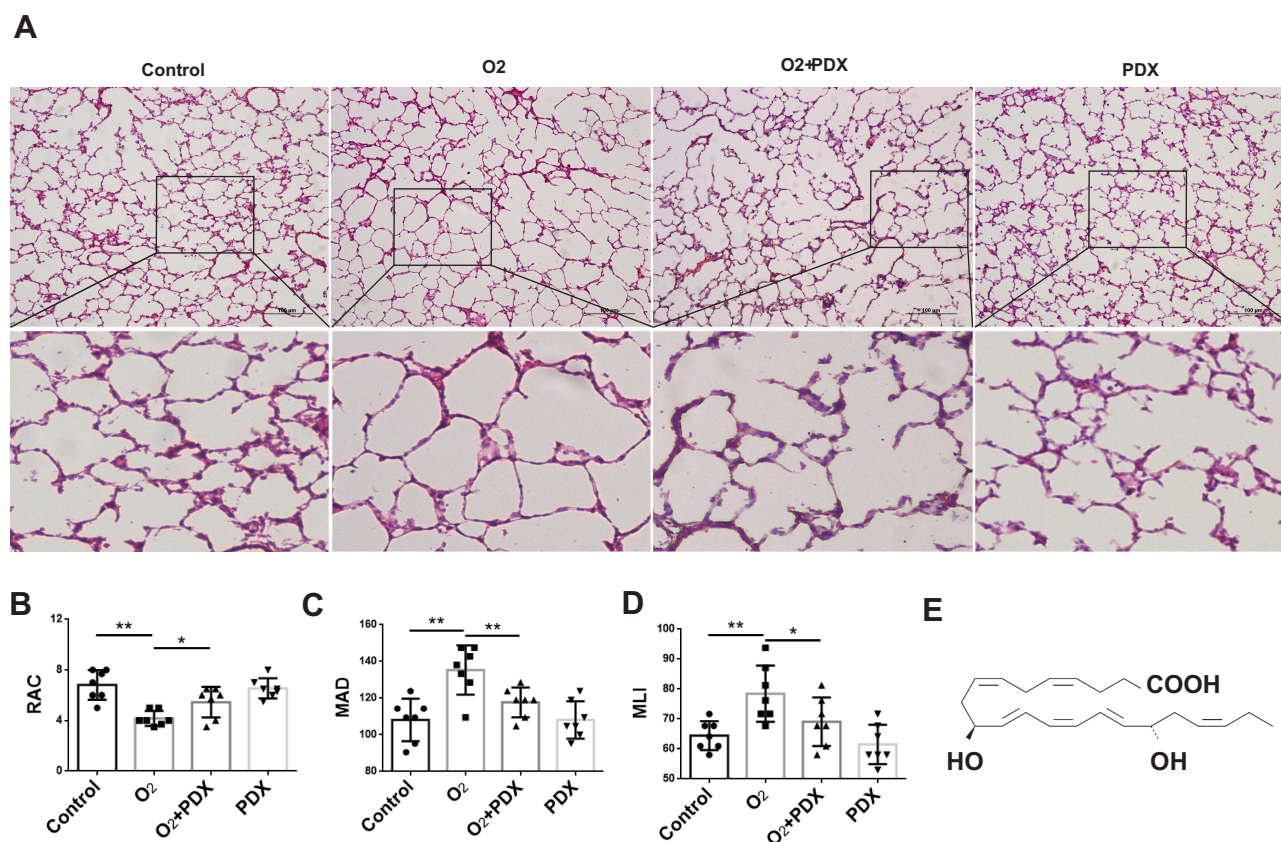


Figure 1 PDX mitigated hyperoxia-induced alveolar simplification. Neonatal mice were maintained in 60–70% O_2 for 7 days and treated with PDX. Representative lung tissue sections were stained with hematoxylin-eosin at a magnification of $\times 100$ (A). Calibration bars=100 μm . The RAC, MLI and MAD were used to quantify the lung development (B–D). (E) The chemical structure of PDX. * $p < 0.05$, ** $p < 0.01$, $n = 7$.

Abbreviations: PDX, Protectin DX; RAC, radial alveolar count; MLI, mean linear intercept; MAD, mean alveolar diameter.

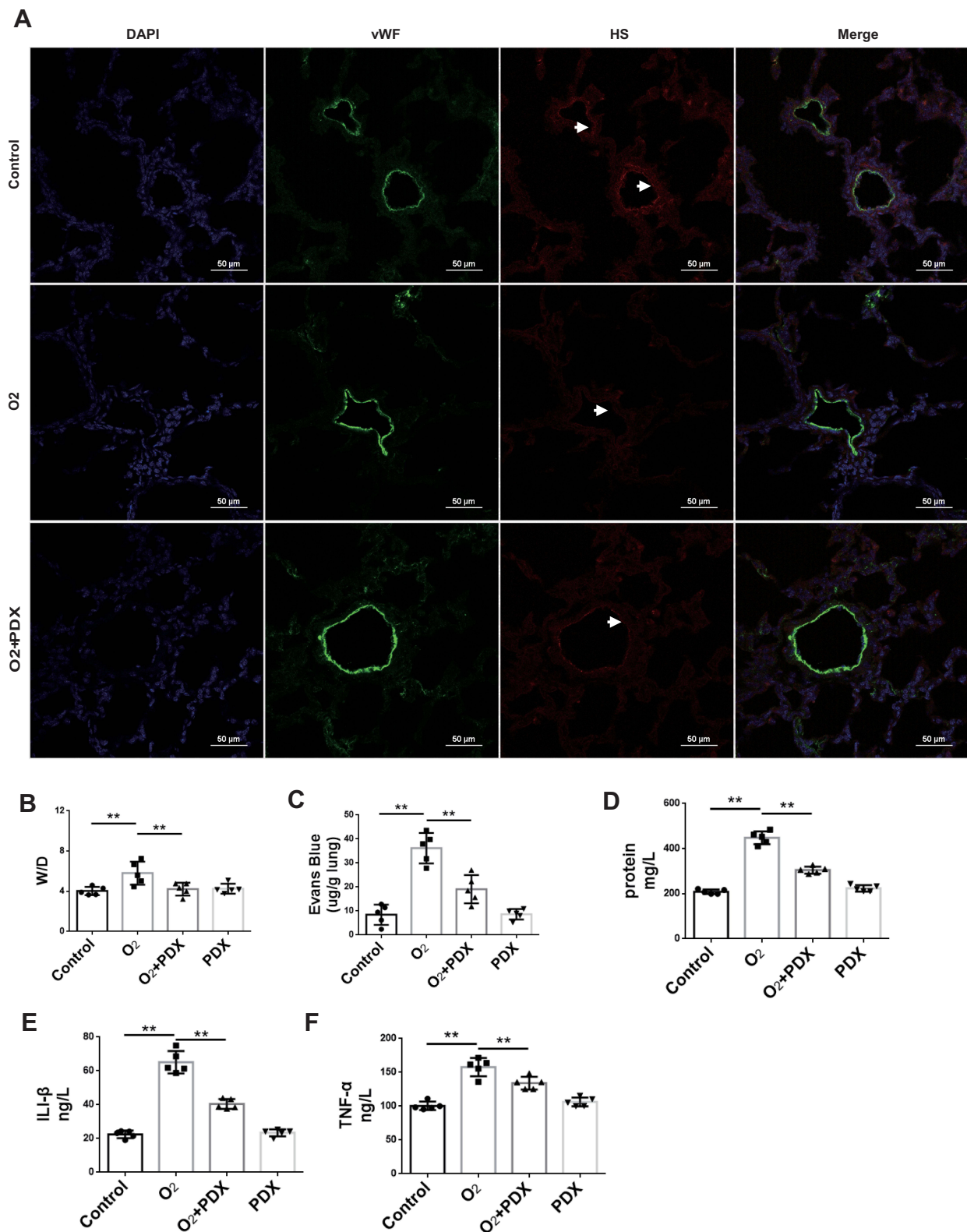


Figure 2 PDX inhibits hyperoxia-induced endothelial glycocalyx damage in vivo. Neonatal mice were maintained in 60–70% O₂ for 7 days and treated with PDX. Representative lung tissue sections were stained with immunofluorescence of HS (red) and vWF (green) at a magnification of $\times 200$ (A). Calibration bars=50 μ m. White arrow indicated vascular. Lung tissue were processed for vascular permeability measurement by wet weight to dry weight ratio (B) and Evans blue dye (C). The concentrations of protein, TNF- α and IL-1 β in BALF supernatant were measured by BCA Protein Assay Kit or ELISA kit (D–F). ** $p < 0.01$, $n = 5$.

Abbreviations: HS, heparan sulfate; vWF, von Willebrand factor; BALF, bronchoalveolar lavage fluid.

We performed the Evans blue dye leakage test to evaluate the pulmonary vascular permeability. As shown in Figure 2C, hyperoxia increase Evans blue dye leakage and PDX treatment reduce this leakage.

The inflammatory cytokines concentration in BALF were measured by ELISA. The concentration of protein, TNF- α and IL-1 β in BALF supernatant were decreased by PDX treatment compared with hyperoxia exposure (Figure 2D–F). These results demonstrate that PDX mitigated hyperoxia-induced alveolar simplification, vascular leakage and lung inflammation.

PDX Decreases HPA Expression via the SIRT1/NF- κ B p65 Pathway in vivo

Endothelial glycocalyx is mainly degraded by HPA. The expression of HPA was increased after hyperoxia exposure (Figure 3A and B). PDX treatment significantly reduced HPA expression. The expression of *sirt1* was decreased in the hyperoxia group, and PDX treatment significantly increased the SIRT1 expression (Figure 3A and C). Interestingly, the expression of p-P65 and P65 was higher in the hyperoxia group than that in the control group (Figure 3A, D and E). PDX treatment relieves this change.

We use EX527 and BOC-2 to inhibit SIRT1 enzymatic activity and ALX respectively. Both EX527 and BOC-2 abrogated the effects of PDX on lung tissue histology and vascular HS expression (Figure 4A–C). They also partially suppressed PDX induced decreases in HPA, P65 and p-P65 expression (Figure 3F–I). Together, these data suggest that PDX decreases HPA expression via the SIRT1/NF- κ B p65 pathway.

PDX Protects the Endothelial Glycocalyx in vitro

To further evaluated the effect of PDX on endothelial glycocalyx in vitro, we performed the hyperoxia-induced HUVEC damage experiment. As shown in Figure 5A–E, hyperoxia exposure decreased SIRT1 expression, and enhanced the HPA, p-P65 and P65 in HUVECs, whereas PDX treatment reversed these changes. EX527 and BOC-2 suppressed PDX induced decreases in HPA, P65 and p-P65 expression (Figure 5F–I). HS expressed both in extracellular matrix and cellular membrane (Figure 5J). EX527 and BOC-2 abolished the effects of PDX on cellular HS expression. Together, these data suggest that PDX decreases HPA expression via the SIRT1/NF- κ B p65 pathway in vitro.

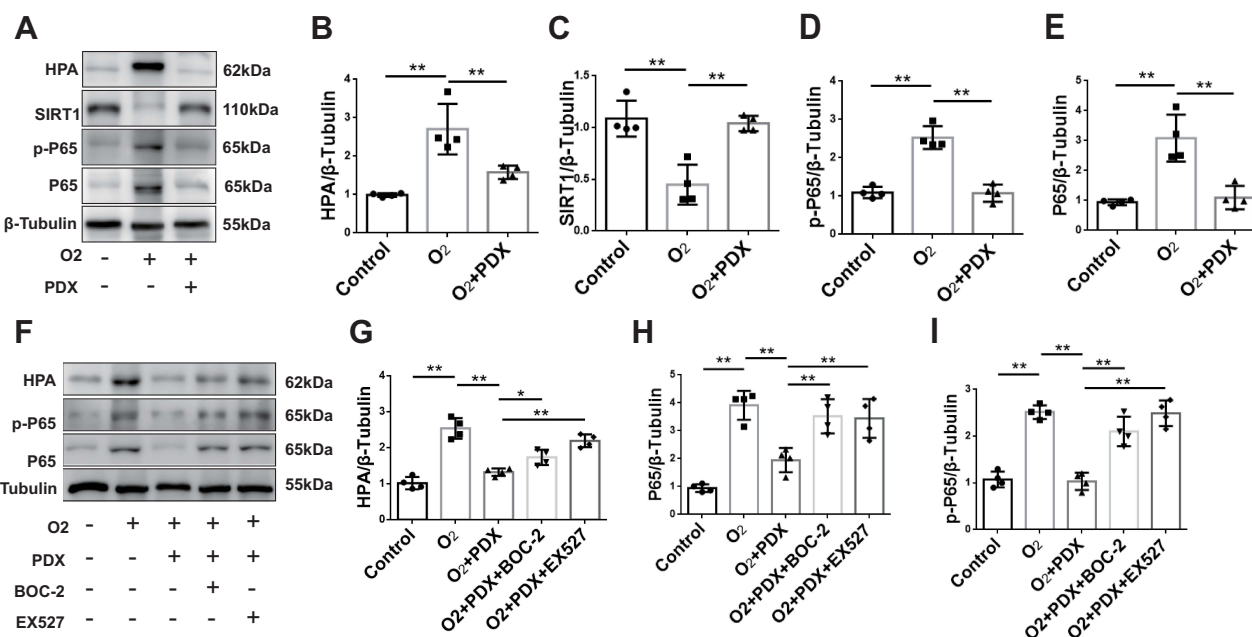


Figure 3 PDX decreases HPA expression via the SIRT1/NF- κ B p65 pathway in vivo. Neonatal mice were maintained in 60–70% O₂ for 7 days and treated with PDX. The protein levels of HPA, SIRT1, p-P65 and P65 in the lung tissue were measured by Western blot (A–E). EX527 and BOC-2 were used to inhibit SIRT1 enzymatic activity and ALX respectively. The protein levels of HPA, p-P65 and P65 were measured (F–I). n = 4. *p < 0.05, **p < 0.01.

Abbreviations: SIRT1, sirtuin 1; NF- κ B, Nuclear transcription factor kappaB; HPA, heparinase; EX527, 6-chloro-2, 3, 4, 9-tetrahydro-1H-carbazole-1-carboxamide, selective SIRT1 inhibitor; BOC-2, butoxycarbonyl-Phe-Leu-Phe-Leu-Phe, ALX antagonist; ALX, lipoxin A4 receptor.

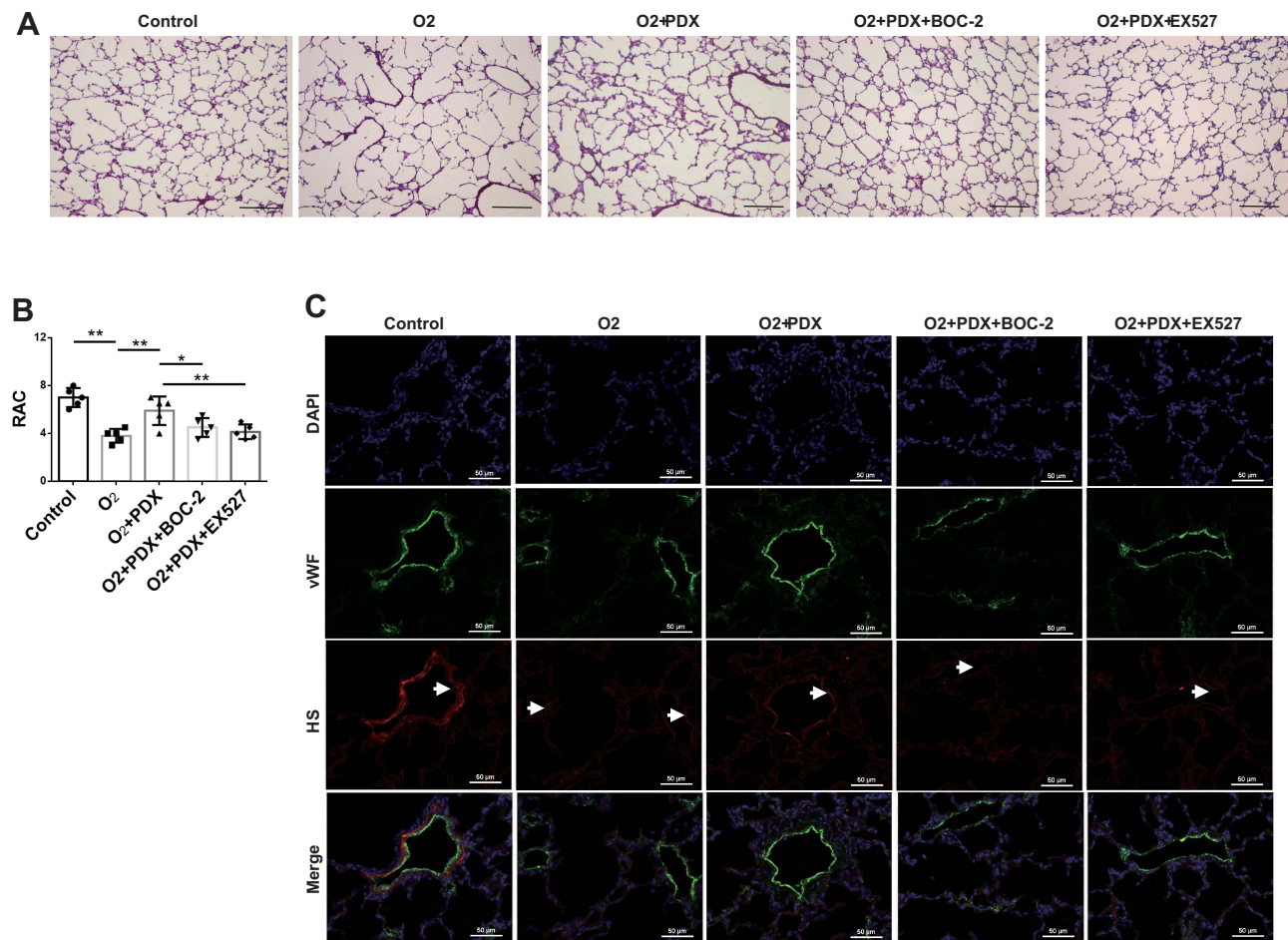


Figure 4 BOC-2 and EX527 abolished the affection of PDX in vivo. Neonatal mice were maintained in 60–70% O₂ for 7 days and treated with PDX. EX527 and BOC-2 were used to inhibit SIRT1 enzymatic activity and ALX respectively. Representative lung sections were stained with hematoxylin-eosin at a magnification of $\times 100$ (A). Calibration bars=100 μ m. The RAC was used to quantify the lung development (B). $n = 5$. Representative lung tissue sections were stained with immunofluorescence of HS (red) and vWF (green) at a magnification of $\times 200$ (C). Calibration bars=50 μ m. White arrow indicated vascular. * $p < 0.05$, ** $p < 0.01$.

Abbreviation: RAC, radial alveolar count.

Discussion

Pulmonary edema is one of the main features of BPD, resulting from increased microvascular permeability which leads to the transudation of fluid and proteins across the alveolar wall.¹ Excessive alveolar edema impairs the efficiency of gas exchange and delayed lung development. This study revealed that PDX treatment effectively relieves hyperoxia-induced alveolar simplification, vascular leakage and lung inflammation. PDX inhibits hyperoxia-induced endothelial glycocalyx damage by increasing HS expression in the pulmonary endothelial cells. PDX decreases the endothelial glycocalyx degrading enzyme HPA via the SIRT1/NF- κ B p65 pathway (Figure 6).

Endothelial glycocalyx damage contributes to increased vascular permeability. SDC-1 and HS are the most prominent components of the core and side-chain structures of the endothelial glycocalyx, accounting for 50–90% of the total endothelial proteoglycans.²⁶ Meanwhile the alveolar epithelial cells also express glycocalyx. The alveolar epithelial glycocalyx integrity is critical to surfactant function, and the shedding of this layer contributes to lung injury in patients with ARDS.²⁷ We stained the pulmonary vessels with vWF to focus on the endothelial glycocalyx expression. In our study, hyperoxia exposure reduced the expression of HS in the lungs of neonatal mice. PDX treatment relieved the reduction. HPA cleaves heparan sulfate proteoglycans into heparan sulfate side chains and core proteoglycans. Our results indicated that hyperoxia significantly increased the expression of HPA which shed the HS, leading to vascular leakage. PDX treatment relieved these changes.

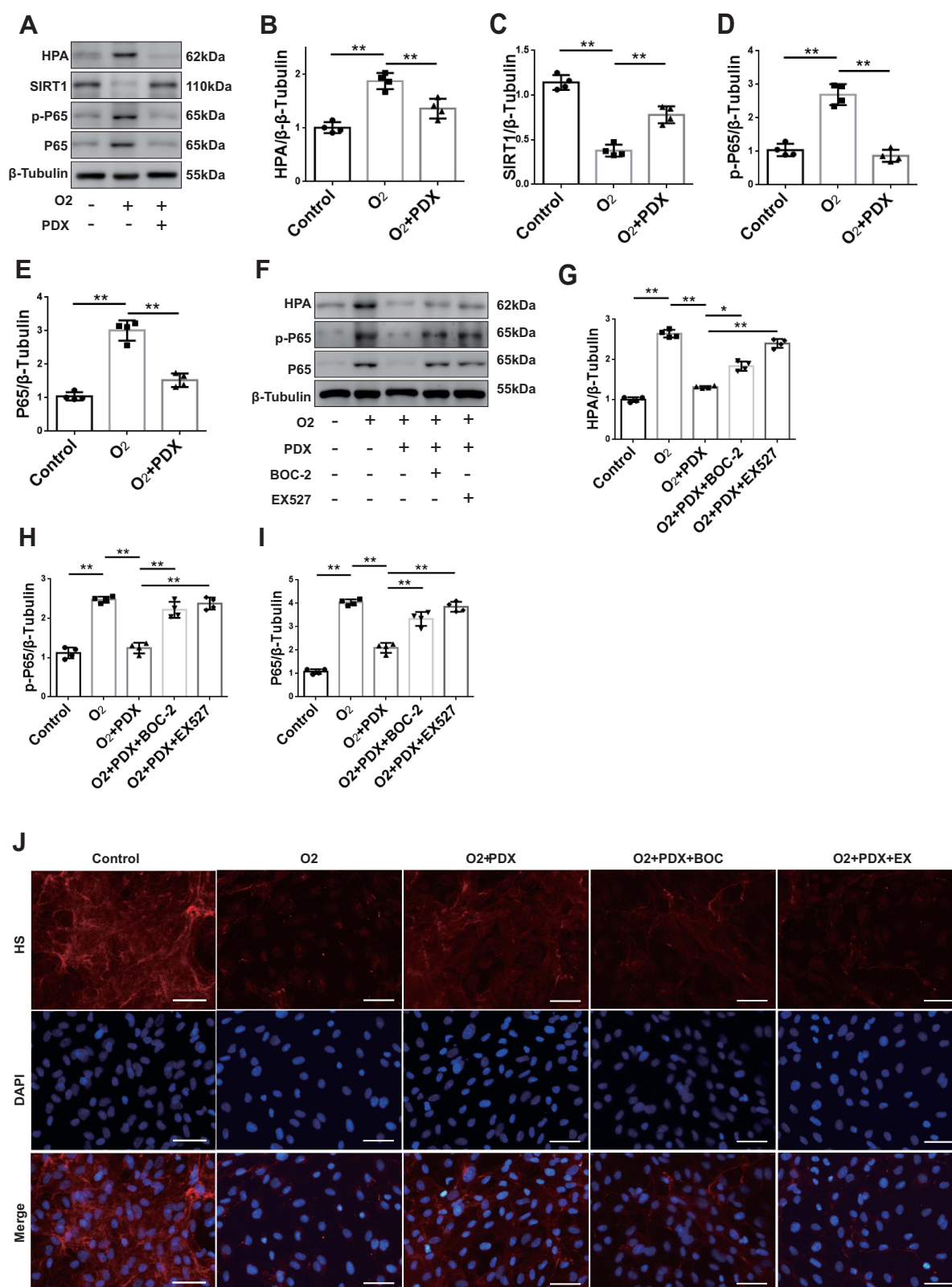


Figure 5 PDX protects the endothelial glycocalyx in vitro. HUVECs were treated with O₂ (80%), PDX (100 nM), BOC-2 (10 μM) or EX527 (10 μM). The protein levels of HPA, SIRT1, p-P65 and P65 in the lung tissue were measured by Western blot (A–E). EX527 and BOC-2 were used to inhibit SIRT1 enzymatic activity and ALX respectively. The protein levels of HPA, p-P65 and P65 were measured (F–I). n = 4. HUVECs were stained with immunofluorescence of HS (red) at a magnification of ×200 (J). Calibration bars=50 μm. *p < 0.05, **p < 0.01.

Abbreviation: HUVECs, human umbilical vein endothelial cells.

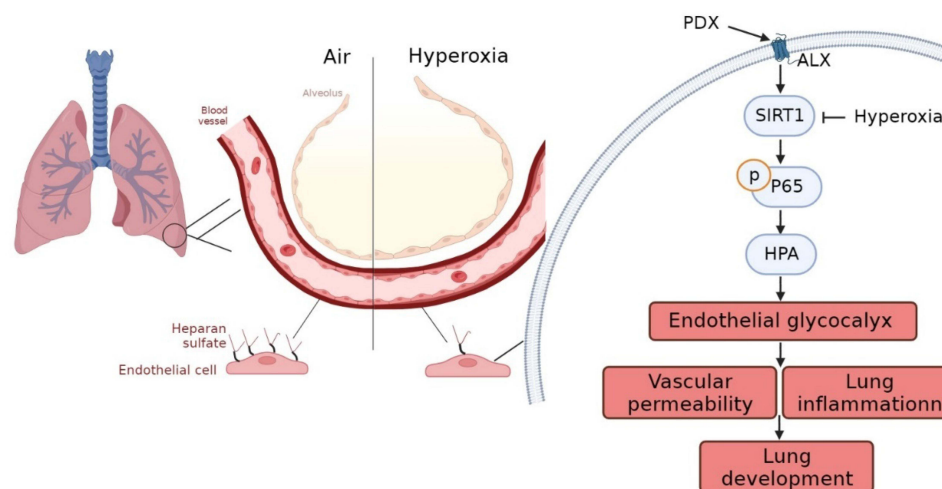


Figure 6 Scheme illustrating how PDX relieves hyperoxia-induced lung injury by protecting pulmonary endothelial glycocalyx.

Abbreviations: PDX, Protectin DX; HPA, heparinase; SIRT1, sirtuin 1.

SDC-1 is primarily expressed in the lung epithelium but not endothelial.²⁸ SDC-1 regulates the inflammatory response and suppresses epithelial apoptosis to attenuate lung injury during virus infection.²⁹ Interestingly, our Western blot analysis showed the SDC-1 expression in the hyperoxia group was higher than that in the control group, with an opposite trend to that of HS (data not shown). The Western blot method analyzed the whole lung protein level. The immunofluorescence experiment may not be suitable as SDC-1 is primarily expressed in the lung epithelium but not endothelial. We will explore a new method to better value the endothelial SDC expression in future.

Many studies have shown that SIRT1 plays a protective role in oxidative stress. Dong et al show that the expression of SIRT1 in BPD infants is obviously reduced along with the severity of the disease.³⁰ Mody et al reported that lower SIRT1 in tracheal aspirate leukocytes is associated with developing BPD or death in premature infants.³¹ In our study, hyperoxia exposure reduced the expression of SIRT1 in the lungs of neonatal mice. PDX treatment relieved the reduction. While the use of a SIRT1 inhibitor abolishes the effects of PDX treatment, suggesting that SIRT1 plays a critical role in these biological processes. It has been reported that SIRT1 negatively regulates NF- κ B-dependent inflammatory responses.³² SIRT1 inhibits NF- κ B signalling to maintain normal skeletal remodelling.³³ The NF- κ B pathway is involved in the regulation of HPA expression. Drugs used in cancer treatment activate the NF- κ B pathway to trigger heparanase expression in tumor cells.²¹ NF- κ B signalling was activated downstream of PI3K/Akt, promoting HPA expression in gastric cancer.³⁴ In our study, SIRT1 downregulated NF- κ B and HPA expression in hyperoxia-exposure mice, which was reversed by PDX treatment.

Conclusions

In conclusion, our study indicates that PDX protects the endothelial glycocalyx during hyperoxia exposure via SIRT1/NF- κ B/HPA signal. The preservation of the endothelial glycocalyx alleviates tissue edema and lung inflammation, further promoting alveolar development. Our findings provide a new treatment for BPD infants. Our study has some limitations. Pulmonary microvascular endothelial cells may better reflect this hyperoxia-induced pulmonary endothelial injury model. New methods should be explored to better value the endothelial SDC expression.

Ethical Approval

All experiments were performed following the Guidelines for the Use of Experimental Animal Care issued by the National Institutes of Health and approved by the Experimental Ethics Committee of Wenzhou Medical University.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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