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Genipin attenuates hyperoxia-induced lung injury and pulmonary hypertension via targeting glycogen synthase kinase-3 β in neonatal rats

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ABSTRACT

Objectives: Bronchopulmonary dysplasia is the most common chronic lung disease of infancy and is associated with pulmonary hypertension (PH). Inhibition of glycogen synthase kinase (GSK)-3 β has been shown to attenuate lung injury and PH in hyperoxia-exposed newborn rats. Genipin has been widely used for the treatment of inflammatory diseases. The aim of this study was to show that genipin decreased the expression of GSK-3 β in lung tissues of hyperoxia-exposed rat pups.
Methods: We established models of hyperoxia-exposed rat pups, evaluated lung injury and pulmonary hypertension and detected the mRNA and protein expression of key molecules.
Results: Hyperoxia resulted in the reduction of survival rate and histologic injury of lung tissues; an increase of the messenger RNA (mRNA) expression of transforming growth factor-β1, extracellular matrix proteins collagen-I and fibronectin, and α-smooth muscle actin; an increase of right ventricular (RV) systolic pressure and the weight ratio of RV to left ventriclar (LV) plus septum (S) (RV/LV + S) were inhibited by genipin. Genipin also decreased the levels of tumor necrosis factor-α, interleukin-1 β, and interleukin-6 in both bronchoalveolar lavage fluid and lung tissues after hyperoxia exposure. In addition, genipin inhibited p65 nuclear factor-κB nuclear translocation and matrix metalloproteinase-2 and -9 expression. Moreover, hyperoxia resulted in an increase of methane dicarboxylic aldehyde content and a decrease of superoxide dismutase activity, catalytic subunit of glutamate-cysteine ligase, modified subunit of glutamate-cysteine ligase, and nuclear factor erythroid 2-related factor 2 expression were inhibited by genipin. All these effects induced by genipin were blocked by upregulation of GSK-3 β. Genipin downregulated GSK-3 β expression, decreased nuclear factor-κB translocation, increased nuclear factor erythroid 2-related factor 2 expression, attenuated inflammation and oxidative stress, leading to amelioration of lung injury and PH in hyperoxia-exposed rat pups.
Conclusion: Overall, genipin may provide a novel therapeutic option for preventing and treating infants with bronchopulmonary dysplasia.

Introduction

About 50% of very low birth weight infants with a gestational age are affected by bronchopulmonary dysplasia (BPD) [1], also known as chronic lung disease of prematurity. BPD is the most common chronic lung disease of infancy in industrialized countries [2]. The survival rate of premature infants has been significantly enhanced because of the advances in mechanical ventilation and oxygen supplementation [3], followed by a high morbidity of BPD [4]. BPD results in serial rehospitalizations during the first year of life, long-term pulmonary damages, and delay of neurodevelopment [2]. Nearly 10% to 15% of infants suffering from BPD die of respiratory failure characterized by serious alveolar epithelial cell impairment, alveolarization inhibition, and fibrosis. At present, the mechanism of BPD remains unclear and few interventions have been demonstrated to significantly reduce rates and ameliorate its progression. However, hyperoxia exposure in the presence of the placebo increased glycogen synthase kinase (GSK)-3 β phosphorylation, which was correlated with increased inflammation, decreased alveolarization and angiogenesis, and increased pulmonary vascular remodeling and pulmonary hypertension (PH) [5]. Treatment with the GSK-3 β inhibitor decreased phosphorylation of

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nuclear factor (NF)-κB p65, expression of monocyte chemotactic protein-1, and lung inflammation; improved alveolarization and angiogenesis; and decreased pulmonary vascular remodeling and PH. The data indicated that GSK-3 β may be a novel target to prevent and treat infants with BPD.

PH is common in BPD and is associated with increased mortality and morbidity [6]. PH is now recognized as one of the pulmonary vascular diseases responsible for mortality in susceptible populations, including infants. It is characterized by elevated pulmonary vascular pressure and resistance. PH mainly results from four pathologic conditions: congenital heart disease, acute or chronic hypoxia, neonatal developmental defect, and idiopathic forms [7,8]. It has been observed that airway inflammation plays an important role in the development of both BPD and PH [9–11].

Genipin, an aglycone derived from an iridoid glycoside called geniposide, is a major component of the fruit of Gardenia jasminoides. In traditional medicine, Gardenia jasminoides has been widely used for centuries as a folk medicine for the treatment of inflammatory diseases and hepatic disorders [12]. It has been reported to have antimicrobial, antitumor, antioxidative, and antiinflammatory effects [13–18]. Genipin inhibits allergic responses in ovalbumin-induced asthmatic mice [19]. Moreover, genipin alleviates lipopolysaccharide-induced acute lung injury by inhibiting NF-κB and ACHT, leucine-rich repeat, and pyrin domains containing protein 3 signaling pathways [20]. Genipin also ameliorates hypertension-induced renal damage via the angiotensin II-TLR/MyD88/MAPK pathway [21]. Furthermore, our preliminary findings revealed that genipin decreased the expression of GSK-3 β in lung tissues. However, whether genipin protects against BPD and PH is not known.

We investigated the effect of genipin on BPD in an experimental, acute lung injury rat model exposed to high oxygen concentrations that closely resembles BPD in premature infants [22]. We evaluated the effect of genipin on lung injury, PH, inflammation, and oxidative stress and further examined the role of GSK-3 β on these effects.

Materials and methods

Animal treatment and lentivirus delivery

All animal experiments were in accordance with the National Institutes of Health guide for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use committee of The First Affiliated Hospital of Xinxiang Medical University. On postnatal day (PND) two, 100 newborn Sprague-Dawley (SD) rat pups were randomly divided into four groups: normoxia (21% O_2) plus placebo (Dimethyl sulfoxide [DMSO]) and lentivirus (LV)-normal control (NC); hyperoxia (90% O_2) plus placebo and LV-NC; hyperoxia plus genipin and LV-NC; and hyperoxia plus genipin and LV-GSK-3 β. Rat pups received genipin (50 mg/kg) or DMSO (equal volume) daily by intraperitoneal injection. LV particles expressing GSK-3 β were generated. LV-GSK-3 β or LV-NC viruses (5×10^9 TU/kg) were injected via the temporal vein before hyperoxic exposure [23]. Treatment of hyperoxia was performed as previously described [5,24]. In brief, pups and surrogate mothers were placed in either a hyperoxic chamber or a normal air chamber. Oxygen level was maintained at 90% and carbon dioxide level was maintained at <0.5%. The chambers were opened once a day for 1 h to replace water and food. Mothers were rotated between oxygen-exposed and room air–exposed litters every day to avoid oxygen toxicity. The experimental period was 2 wk. Survival rate in different groups was recorded.

Histology

Pups were anesthetized with an intraperitoneal injection of ketamine (25 mg/kg body weight [BW]), xylazine (50 mg/kg BW), and heparin (100 units). After 5 min, pups were sacrificed by transection of the abdominal blood vessels, and the trachea was cannulated for perfusion fixation of the lungs with 4% paraformaldehyde for 6 min. The lung tissue was removed and fixed (additionally) in cold formaldehyde at 4°C for 24 h. Fixed lung tissues were dehydrated using a series of ethanol washes, embedded with paraffin and cross-sectioned into 5-μm slices. For hematoxylin and eosin (H&E) staining, sections were immersed in hematoxylin for 5 min and counterstained with eosin for 3 min.

Inflammation

After the experiment, rats were intratracheal injected with sterile phosphate-buffered saline (PBS) three times to acquire bronchoalveolar lavage (BAL) fluid. BAL fluids were then centrifuged at 800 g at 4°C for 10 min to obtain the infiltrated cells and collect supernatant. The cells were resuspended in 0.5 mL cold PBS, stained with Giemsa reagent (JianCheng Bioengineering Institute, Nanjing, China), analyzed, and counted under a microscope (Olympus, Japan). The levels of tumor necrosis factor-α, interleukin (IL)-1 β and IL-6 in the BAL fluids were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits (Cusabio Technology, Wuhan, China) according-the manufacturer's protocols.

Assessment of pulmonary hypertension

PH was assessed by right ventricular (RV) systolic pressure (RVSP) and RV to left ventricle (LV) plus septum (S) weight ratio [5,25]. For RVSP measurement, a 25-gauge needle was inserted into the RV and fitted to a pressure transducer. Levels of pressure were recorded using a Gould polygraph (model TA-400; Gould Instruments, Cleveland, OH, USA). After that, hearts were dissected for RV free wall separation from LV + S for RV/LV + S ratio assessment.

Measurement of oxidative stress

Lung tissues were homogenized at 4°C in 50 mM PBS (pH 7.4, 1/10 g/mL) containing 0.2 mM phenylmethanesulfonyl fluoride, 1 mM EDTA, and 1 mM leupeptin. Homogenates were centrifuged at 10 000*g* for 5 min to obtain the clear upper supernatant fluid. Malonaldehyde (MDA) content, superoxide dismutase (SOD) activity, and glutathione (GSH) level were determined using assay kits obtained from Cayman Chemical (Ann Arbor, MI, USA)

Quantitative real-time polymerase chain reaction

Lung tissue was homogenized in TRIzol (Invitrogen, Carlsbad, CA, USA), and total Ribonucleic acid (RNA) was extracted according to the manufacturer's protocol. Residual genomic deoxyribonucleic acid (DNA) was removed using DNase I (Invitrogen) and the quality of RNA was examined using a Nanodrop ND-1000 spectrophotometer. To synthesize complimentary DNA (cDNA), 500 ng total RNA was used was using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on the CFX96 Real-Time System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using SYBR green real-time PCR SuperMix kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. Amplification reactions were as follows: one cycle of 94°C for 2 min, 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; one cycle of 72°C for 7 min. Relative expression was expressed as fold-changes of template copy numbers of a sample as per negative control after normalizing with their respective control β-actin.

Western blot

Lung samples were homogenized in ice-cold lysis buffer containing 50 mM Tris·HCl (pH 7.4), ethylenediaminetetraacetic acid (EDTA) (1 mM), egtazic acid (EGTA) (1 mM), 0.1% 2-mercaptoethanol, 4-(2-aminoethyl)-benzenenesulfoyl fluoride (1 mM), leupeptin (1 μM), and pepstatin A (1 μM). The cell lysates were centrifuged at 20 000g for 20 min at 4°C to remove cellular debris. Protein content in the supernatant was determined by BCA assay (Thermofisher Scientific, Waltham, MA, USA). Briefly, 20 μg of protein sample per lane was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and proteins from the gel were transferred to polyvinylidene difluoride membrane. Membranes were blocked in 5% non-fat milk in tris-buffered saline with 0.1% Tween 20 (TBST) at 37°C for 1 h afterward. The blots were incubated overnight at 4°C with primary antibodies (β-actin: Santa Cruz Biotechnology [Dallas, TX] 1: 500; GSK-3 β: Cell Signaling Technology, 1: 1000; P65 NF-κB: Cell Signaling Technology, 1: 1000; histone H3: Cell Signaling Technology, 1: 1000). After washing with TBST, blots were then incubated for 1 h at 37 C with horseradish peroxidase antibody (1: 5000 dilution; Thermofisher Scientific). Bands were visualized by enhanced chemiluminescence (ECL Advance kit; Thermofisher Scientific) and captured by a chemiluminescence detector (Bio-Rad).

Statistical analysis

Data were expressed as mean ± SEM and statistical analyses were performed using GraphPad Prism 5.0. A normal distribution of the data was confirmed using the normality test. One-way analysis of variance was conducted for statistical analysis of multiple groups followed by post hoc Bonferroni tests. Differences were considered to be statistically significant when $P < 0.05$.

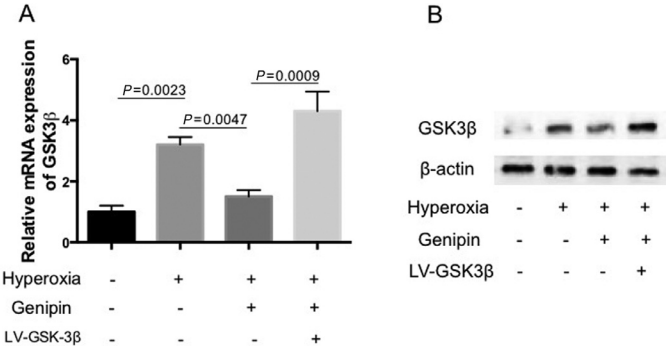

Fig. 1. Effect of genipin on GSK-3 β expression in lung tissues in hyperoxia-exposed neonatal rats. (A) mRNA expression of GSK-3 β (N = 6). (B) $P = 0.0021$ compared to normoxia (N = 6). GSK, glycogen synthase kinase; LV-GSK, left ventricular glycogen synthase kinase;

Results

Genipin inhibits a hyperoxia-induced increase of GSK-3 β expression in lung tissue in neonatal rat pups.

We examined the effect of genipin on GSK-3 β expression during the progression of BPD in infant rats. The results revealed that hyperoxia resulted in a significant increase of GSK-3 β mRNA and protein expression, which was inhibited by the administration of genipin (Fig. 1A, B). Injection of LV-GSK-3 β significantly increased the expression of LV-GSK-3 β even in the presence of genipin in the lung tissues during BPD (Fig. 1A, B).

Genipin attenuates hyperoxia-induced death and histologic injury in the lungs of neonatal rat pups

Figure 2A shows that the survival rate in hyperoxia group was 80%. Genipin increased the survival rate to 92%, which was decreased to 84% in the presence of LV-GSK-3 β. As shown in the H&E-stained images, in the rat pups of control group, lung tissues showed normal histoarchitecture (Fig. 2B). However, hyperoxia resulted in marked edema-like presentations, congestion, and inflammatory cell infiltration in the lungs of rat pups (Fig. 2B). A thickening of the alveolar wall also was observed in hyperoxia group (Fig. 2B). The administration of genipin significantly attenuated hyperoxia-induced histologic lung injury (Fig. 2B), reflected by amelioration of congestion and inflammatory cell infiltration and reduction of the thickness of the alveolar wall. However, the injection of LV-GSK-3 β markedly inhibited the protective effects of genipin on histologic changes of lung tissue in rat pups (Fig. 2B).

Genipin attenuates hyperoxia-induced fibrosis in the lung of neonatal rat pups

To test the effect of genipin on fibrosis in lung tissue, expressions of fibrosis-associated factors also were detected. As illustrated in Figure 3, qRT-PCR showed that the mRNA expressions of profibrotic factor transforming growth factor-β1, extracellular matrix proteins collagen-I (Col-I), fibronectin, and epithelial-mesenchymal transition marker protein α-smooth muscle actin were significantly increased by hyperoxia exposure. All these changes in fibrosis-associated factors were decreased after treatment with genipin (Fig. 3). Upregulation of GSK-3 β could suppress the effect of genipin on the expression of fibrosis-associated factors after hyperoxia exposure (Fig. 3).

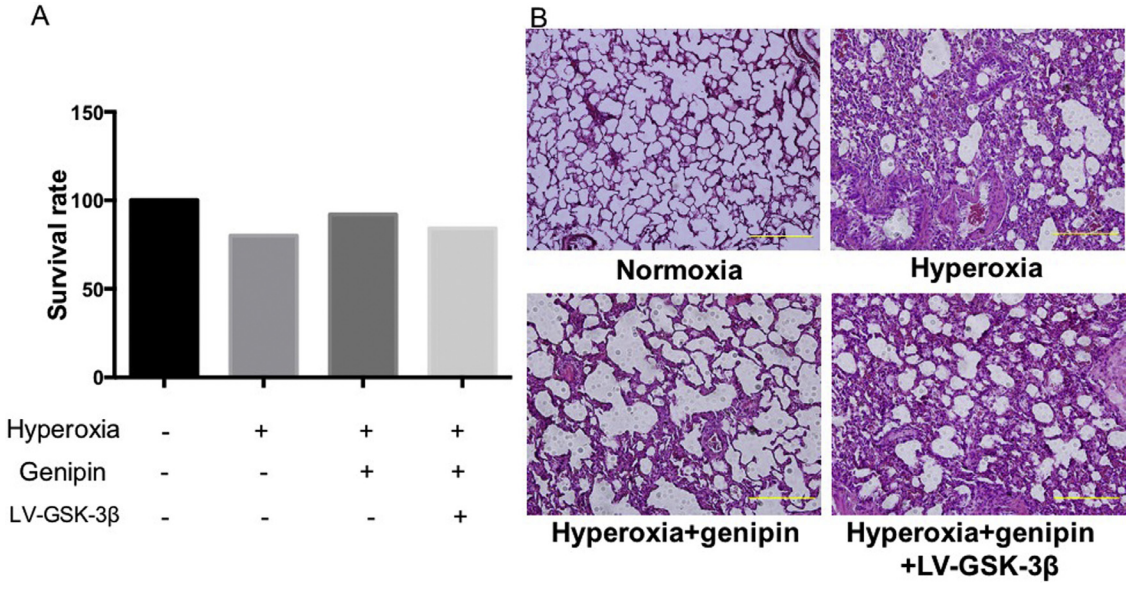

Fig. 2. Effects of genipin on survival rate and histologic injury of lung tissues in hyperoxia-exposed neonatal rats. (A) Survival rate (N = 25). (B) Histologic injury of lung tissues was examined using hematoxylin and eosin staining (N = 6). Representative images were shown.

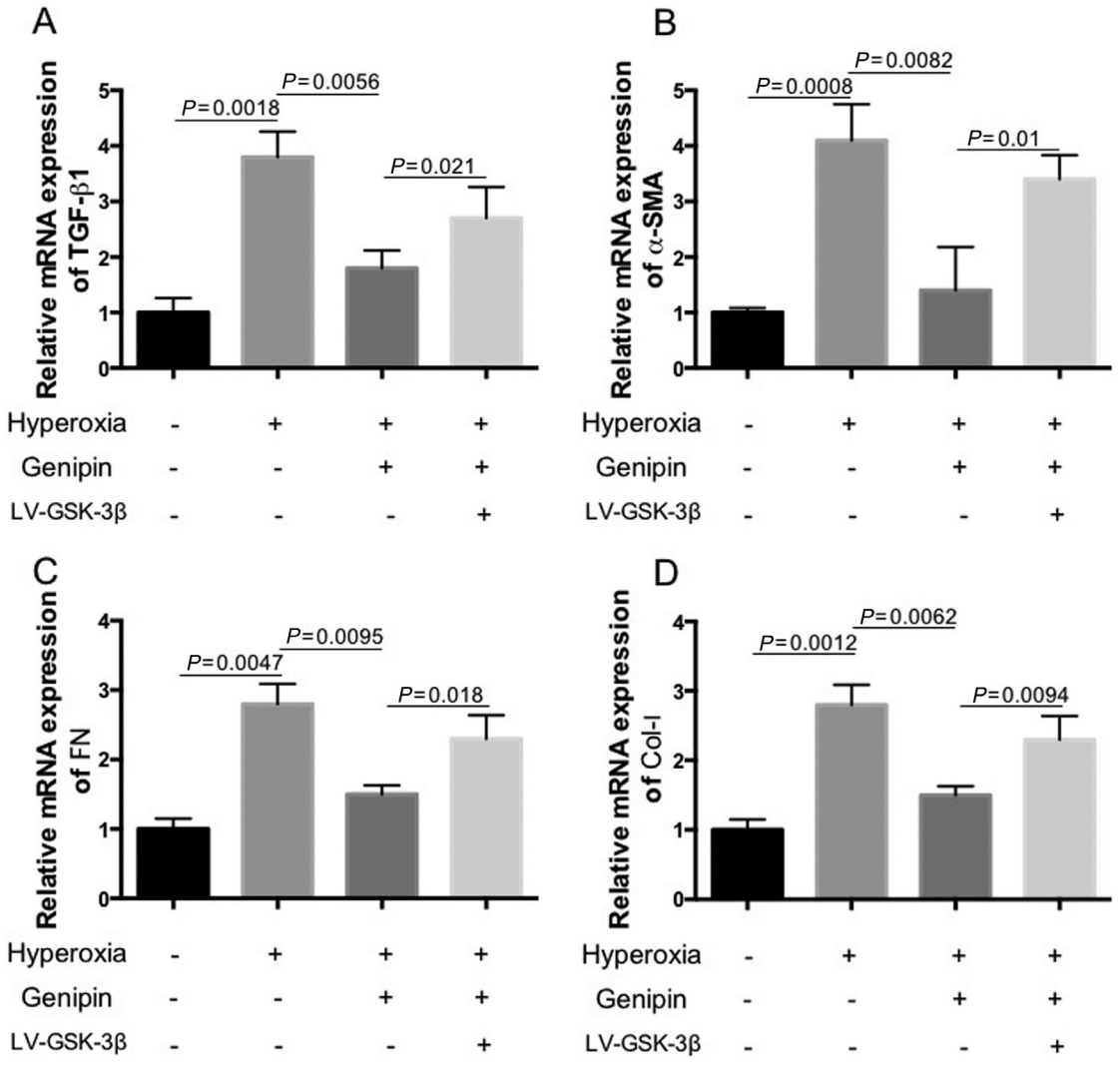

Fig. 3. Effects of genipin on fibrosis of lung tissues in hyperoxia-exposed neonatal rats. mRNA expressions of TGF-β1 (A), Col-I (B), FN (C), and α-SMA (D) were detected using qRT-PCR (N = 6). α-SMA, α-smooth muscle actin; Col-I, collagen-I; FN, fibronectin; LV-GSK, left ventricular glycogen synthase kinase; qRT-PCR, quantitative real-time polymerase chain reaction; TGF, transforming growth factor.

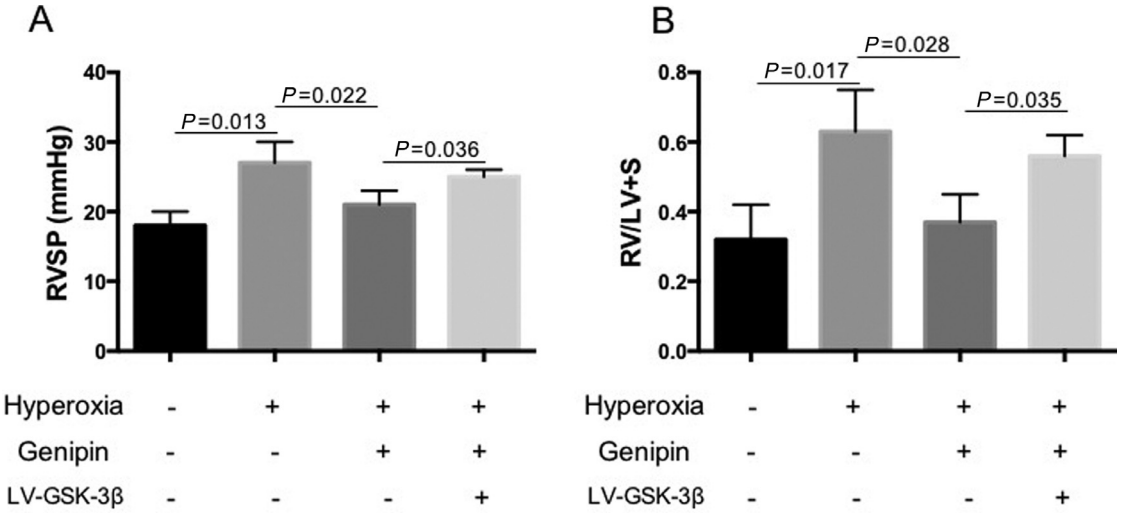

Fig. 4. Effects of genipin on PH in hyperoxia-exposed neonatal rats. RVSP (A) and RV/LV+S (B) were measured (N = 8). PH, pulmonary hypertension; RV/LV+S, right ventricular to left ventriclar plus septum; RVSP, right ventricular systolic pressure.

Genipin inhibits PH in hyperoxia-exposed neonatal rats

RVSP and right ventricular hypertrophy (RVH) were measured to assess the effect of genipin on PH. Rat pups exposed to hyperoxia showed a significant increase in RVSP and RV/LV + S, which was attenuated by genipin (Fig. 4A, B). Injection of LV-GSK-3 β remarkably inhibited the attenuation of RVSP and RV/LV + S induced by genipin (Fig. 4A, B).

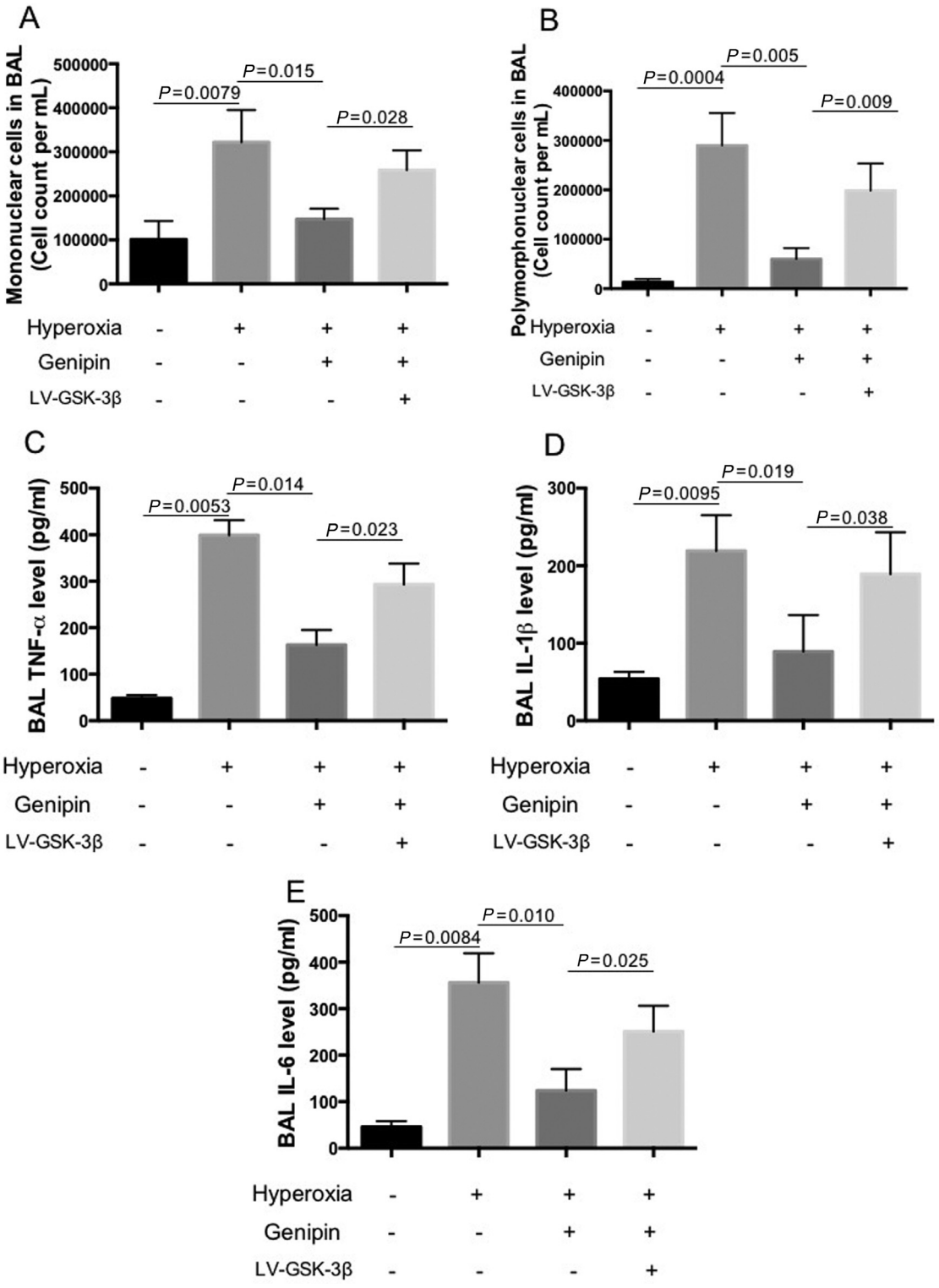

Fig. 5. Effect of genipin on inflammation in BAL fluid in hyperoxia-exposed neonatal rats. Mononuclear (A) and polymorphonuclear cells (B) in BAL fluid were counted (N = 8). Levels of TNF-α (C), IL-1 β (D) and IL-6 (E) in BAL fluid were measured using enzyme-linked immunosorbent assay kits (n = 8). BAL, bronchoalveolar lavage; IL, interleukin; LV-GSK, left ventricular glycogen synthase kinase; TNF, tumor necrosis factor.

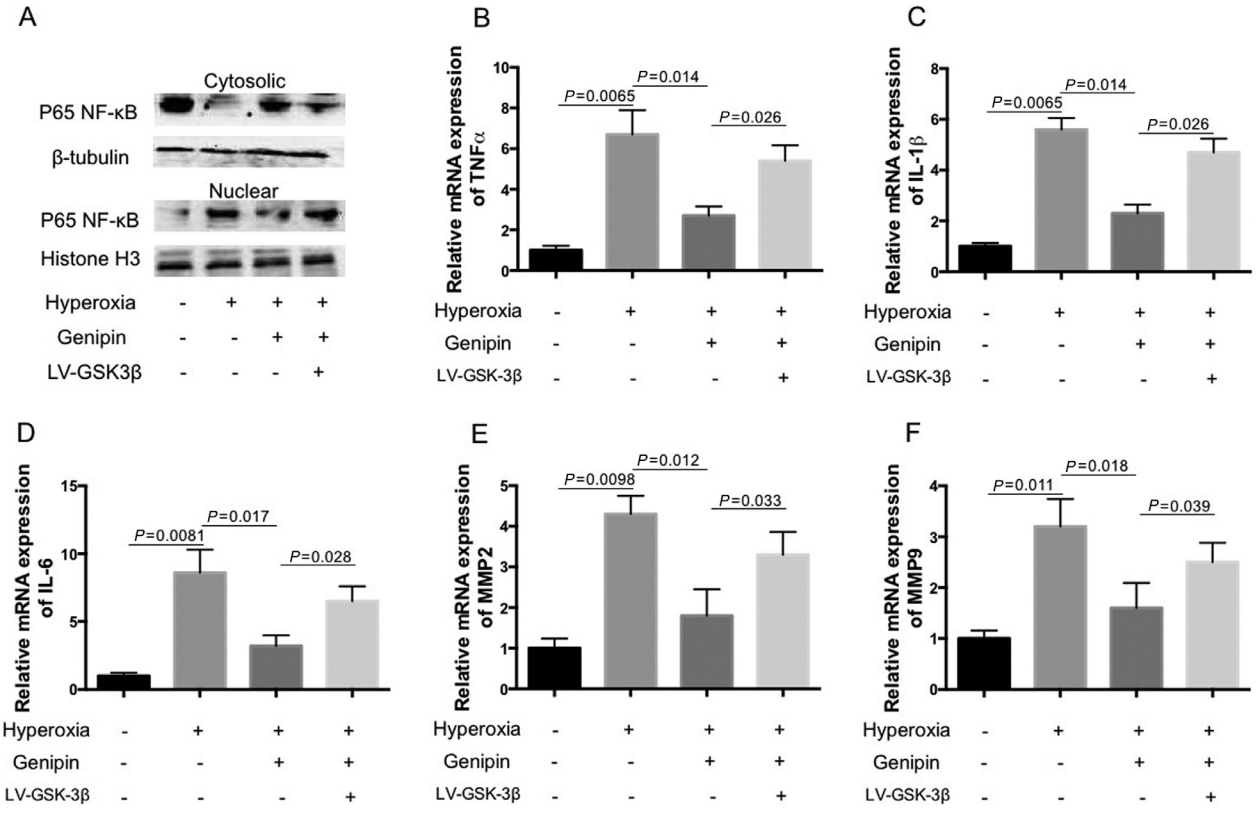

Fig. 6. Effect of genipin on inflammation in lung tissues in hyperoxia-exposed neonatal rats. Cytosolic and nuclear expressions of NF-κB in lung tissues were examined by Western blot (A) (N = 6). mRNA expression of TNF-α (B), IL-1 β (C), IL-6 (D), MMP2 (E) and MMP9 (F) were detected using qRT-PCR (N = 6). IL, interleukin; LV-GSK, left ventricular glycogen synthase kinase; MMP, matrix metalloproteinase; NF, nuclear factor; qRT-PCR, quantitative real-time polymerase chain reaction; TNF, tumor necrosis factor.

Genipin inhibits inflammatory responses in hyperoxic lungs of neonatal rats

To explore the mechanism of genipin-exhibited attenuation of lung injury and PH in hyperoxia-exposed neonatal rats, we assessed inflammation, which was believed to play an important role in the pathogenesis of BPD. We observed a significant increase in the number of mononuclear and polymorphonuclear cells and levels of proinflammatory cytokines TNF-α, IL-1 β, and IL-6 in BAL fluid (Fig. 5). The treatment of genipin attenuated the hyperoxia-induced increase of inflammation markers in BAL fluid (Fig. 5). Genipin-induced attenuation of inflammatory response in BAL fluid was inhibited by the injection of LV-GSK-3 β (Fig. 5).

Moreover, we tested the effect of genipin on inflammation in lung tissues. After the exposure of hyperoxia, cytosolic expression of p65 NF-κB was reduced, but nuclear expression of p65 NF-κB was increased, indicating the enhancement of nuclear translocation of p65 NF-κB (Fig. 6A). Hyperoxia-induced nuclear translocation of of p65 NF-κB was inhibited by genipin treatment (Fig. 6A). The effect of genipin on p65 NF-κB nuclear translocation was blocked by LV-GSK-3 β injection (Fig. 6A). The mRNA expressions of TNF-α, IL-1 β and IL-6, matrix metalloproteinase (MMP) 2, and MMP9 were significantly increased by the exposure to hyperoxia and were inhibited by genipin (Fig. 6B–F). The effect of genipin on the mRNA expressions of TNF-α, IL-1 β and IL-6, and MMP2 and MMP9 was suppressed by LV-GSK-3 β injection (Fig. 6B–F).

Genipin inhibits oxidative stress in hyperoxic lungs of neonatal rats

The effect of genipin on oxidative stress in lung tissues of rat pups exposed to hyperoxia also was evaluated. Hyperoxia resulted in a marked increase of MDA content, decrease of SOD activity and GSH level in lung homogenates, and reduction in mRNA expressions of glutamate-cysteine ligase, catalytic subunit (GCLC) and glutamate-cysteine ligase, modified subunit (GCLM) and nuclear factor erythroid 2-related factor 2 (Nrf2) in lung tissues (Fig. 7), indicating the occurrence of oxidative stress. Hyperoxia-induced oxidative stress and reduction of key transcription factor and antioxidant enzyme expression were inhibited by genipin (Fig. 7). LV-GSK-3 β injection significantly blocked the effect of genipin on oxidative stress and the expression of Nrf2, GCLC, and GCLM (Fig. 7).

Discussion

BPD is characterized by histologic lung injury and PH. In the present study, using a rat pup model of BPD, we investigated the effect of genipin on lung injury and PH. We revealed that genipin significantly attenuated BPD-associated lung injury and PH, as reflected by improvement of histologic lung injury and reduction of RVSP and RV/LV + S. The findings suggest that genipin could protect against the progression of BPD.

Inflammation is a hallmark in the progression of BPD and increase of proinflammatory cytokines has been confirmed by a battery of studies in hyperoxia-exposed neonatal rodents

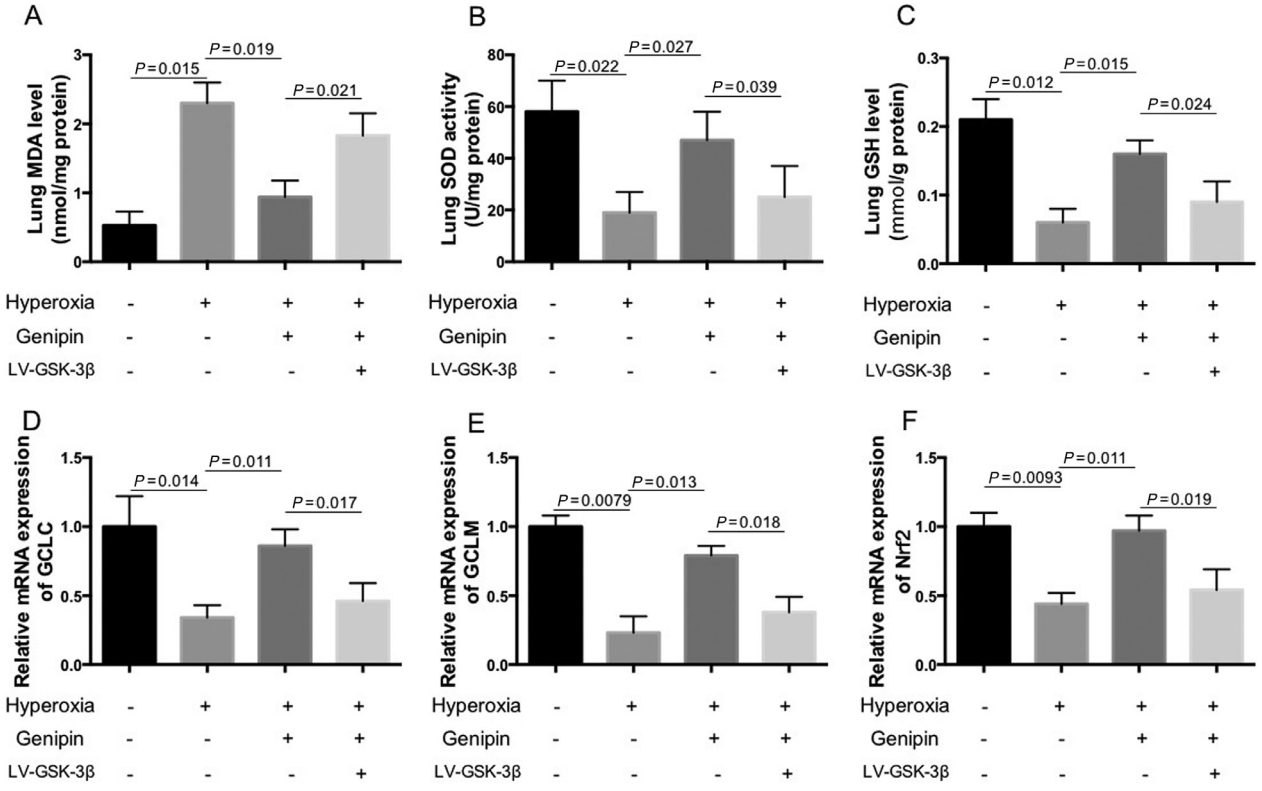

Fig. 7. Effect of genipin on oxidative stress in lung tissues in hyperoxia-exposed neonatal rats. Content of MDA (A), activity of SOD (B), and content of GSH (C) in lung homogenates were determined by commercial kits (N = 8). mRNA expression of GCLC (D), GCLM (E), and *Nrf2* (F) were detected using qRT-PCR (N = 6). GCLC, glutamate-cysteine ligase, catalytic subunit; GCLM, glutamate-cysteine ligase, modified subunit; GSH, glutathione; MDA, malonaldehyde; Nrf2, nuclear factor erythroid 2-related factor 2; qRT-PCR, quantitative real-time polymerase chain reaction; SOD, superoxide dismutase.

and human neonates [26–28]. The transcription factor NF-κB plays a paradoxical role in the development of lung in neonatal rats [29–31]. For the beneficial role, NF-κB is required for angiogenesis and alveolarization. In contrast, NF-κB–mediated inflammation is detrimental to lung function and leads to BPD. Previous studies have shown that genipin could downregulate NF-κB expression and thus attenuate inflammation under several conditions [20,32–34]. In the present study, we confirmed that genipin inhibited hyperoxia-induced NF-κB nuclear translocation and the expression of downstream proinflammatory cytokines, including TNF-α, IL-1 β, and IL-6. Genipin also decreased the expression of MMP2 and MMP9, which were reported to promote inflammation. The results indicate that genipin exhibits a potent anti-inflammatory activity after hyperoxia exposure via regulation of NF-κB nuclear translocation in rat pups.

In addition, oxidative stress was tested to play important roles in hyperoxia-treated neonatal rats [35–37], and deletion of Nrf2, an antioxidant gene, exacerbated hyperoxic lung injury in newborn mice [38]. In the present study, we found that genipin inhibited hyperoxia-induced oxidative stress as evidenced by a decrease of MDA and increases of SOD activity, GSH content, and Nrf2, GCLC, and GCLM expression in lung tissues in neonatal rats. Nrf2 is a crucial transcription factor that is found to regulate the expression of GCLC and GCLM, and many other antioxidant enzymes. The regulation of GCLC and GCLM expression and the consequent GSH level and oxidative state by genipin may be attributed to the modulation of Nrf2 expression. Consistent with our hypothesis, previous reports found genipin-exhibited regulation of Nrf2 under differential conditions [39–41].

Moreover, we focused on the role of GSK-3 β in genipin-exhibited effects against hyperoxia exposure in rat pups. We showed that genipin reduced the expression of GSK-3 β in lung tissues in hyperoxia-exposed rat pups. Upregulation of GSK-3 β significantly inhibited the protective effects of genipin against lung injury, PH, inflammation, and oxidative stress. Moreover, genipin-inhibited NF-κB nuclear translocation was reversed by overexpression of GSK-3 β. Regulation of NF-κB signaling by GSK-3 β was observed in previous research [42,43]. In addition, genipin-induced upregulation of Nrf2 was also suppressed by overexpression of GSK-3 β, indicating a negative relationship between GSK-3 β and Nrf2. These findings suggest that GSK-3 β is key target of genipin that mediates the consequent protective effects.

In addition to the biological role, genipin also functions as an excellent natural crosslinker for proteins, collagen, gelatin, and chitosan. The acute toxicity is low with intravenous lethal dose 50% 382 mg/kg in mice, therefore, much less toxic than glutaraldehyde and many other commonly used synthetic crosslinking reagents. Moreover, genipin can be used as a regulating agent for drug delivery [44]. Although we did not observe significant adverse effects of genipin under the present study, whether the dose selected in the present study had a toxic effect in infants or newborns is not known. Further studies are needed to test the effect of lower doses of genipin on infant lung injuries in animals and to evaluate the safety of genipin in infants or newborns.

Conclusion

Results from the present study clearly demonstrated that genipin possesses beneficial effects against hyperoxia-associated lung injury and PH in neonatal rats. Genipin downregulates GSK-3 β expression,

resulting in the decrease of NF-κB translocation and an increase of Nrf2 expression, contributing to the attenuation of inflammation and oxidative stress. Thus, genipin may provide a novel therapeutic option for preventing and treating infants with BPD.

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