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PG490-88, a derivative of triptolide, suppresses ischemia/reperfusion-induced lung damage by maintaining tight junction barriers and targeting multiple signaling pathways

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ABSTRACT

Previous studies demonstrated that triptolide (PG490) has many anti-inflammatory and immunosuppressive effects. However, little is known about the effect of PG490-88 (a water-soluble derivative of triptolide) on ischemia/reperfusion (I/R)-induced acute lung injury. We assessed the effects of PG490-88 on I/R-induced acute lung injury in rats and on hypoxia/reoxygenation (H/R) in a line of murine epithelial cells. Isolated perfused rat lungs were subjected to 40 min of ischemia, followed by 60 min of reperfusion to induce I/R injury. Induction of I/R led to lung edema, elevated pulmonary arterial pressure, histological evidence of lung inflammation, oxidative stress, and increased levels of TNF-α and CINC-1 in bronchoalveolar lavage fluid. PG490-88 significantly suppressed all of these responses. Additionally, induction of I/R reduced the expression of claudin-4, occludin, and ZO-1, and increased apoptosis in lung tissue. PG490-88 also significantly suppressed these effects. I/R reduced the levels of IκB-α and MKP-1, and increased the levels of nuclear NF-κB and mitogen-activated protein kinase in lung tissue, and PG490-88 suppressed these effects. *In vitro* studies using mouse lung alveolar epithelial cells indicated that H/R increased the levels of phosphorylated p65 and MIP-2, but decreased the level of IκB-α. PG490-88 also suppressed these effects. In I/R damaged lungs, PG490-88 suppresses the inflammatory response, disruption of tight junction structure, and apoptosis. PG490-88 has the potential as a prophylactic agent to prevent I/R-induced lung injury.

1. Introduction

Previous research reported that extracts of the traditional Chinese herb *Tripterygium wilfordii* Hook F have potent immunosuppressive and anti-inflammatory properties, and that these extracts have the potential to treat many autoimmune and inflammatory diseases, such as rheumatoid arthritis, systemic lupus erythematosus, psoriatic arthritis, and Behcet's disease [1]. Triptolide (PG490), a diterpene triepoxide, is the most abundant and active component of this herb extract, and is responsible for the pharmacological effects of these extracts [2,3]. Recent studies found that triptolide protected against cerebral and hepatic ischemia/reperfusion (I/R) injury and ulcerative colitis, and prolonged the survival of bone marrow, cardiac, renal, and skin allografts [2–5]. PG490-88 is a new semi-synthetic, water-soluble derivative that is more easily formulated and administered. Previous studies of this derivative in rodent and dog models reported it can prevent graft-*versus*-host disease and allograft rejection, and can also protect from cisplatin-induced acute kidney injury [2,6].

Acute lung injury and acute respiratory distress syndrome (ALI/ARDS) are characterized by acute onset of hypoxemia, bilateral lung opacities, and partial arterial pressure of oxygen [PaO_2]/fractional concentration of oxygen in the inspired air [FIO_2] below 300 [7]. Various clinical conditions, such as hemorrhagic shock, septic shock, cardiopulmonary bypass surgery, resuscitation for cardiac arrest, reperfusion after lung transplantation or embolectomy, may lead to I/R-induced ALI/ARDS. The main therapies for ARDS are lung-protective ventilation, conservative fluid therapy, neuromuscular blockage, and maintaining the prone position [7]. However, even with advanced treatment, mortality in patients with ARDS is up to 46% [8].

Many survivors from ARDS have cognitive impairment, mood disorders, and persistent ICU-acquired weakness [9]. No currently available pharmacological intervention for ALI/ARDS can reduce short-term

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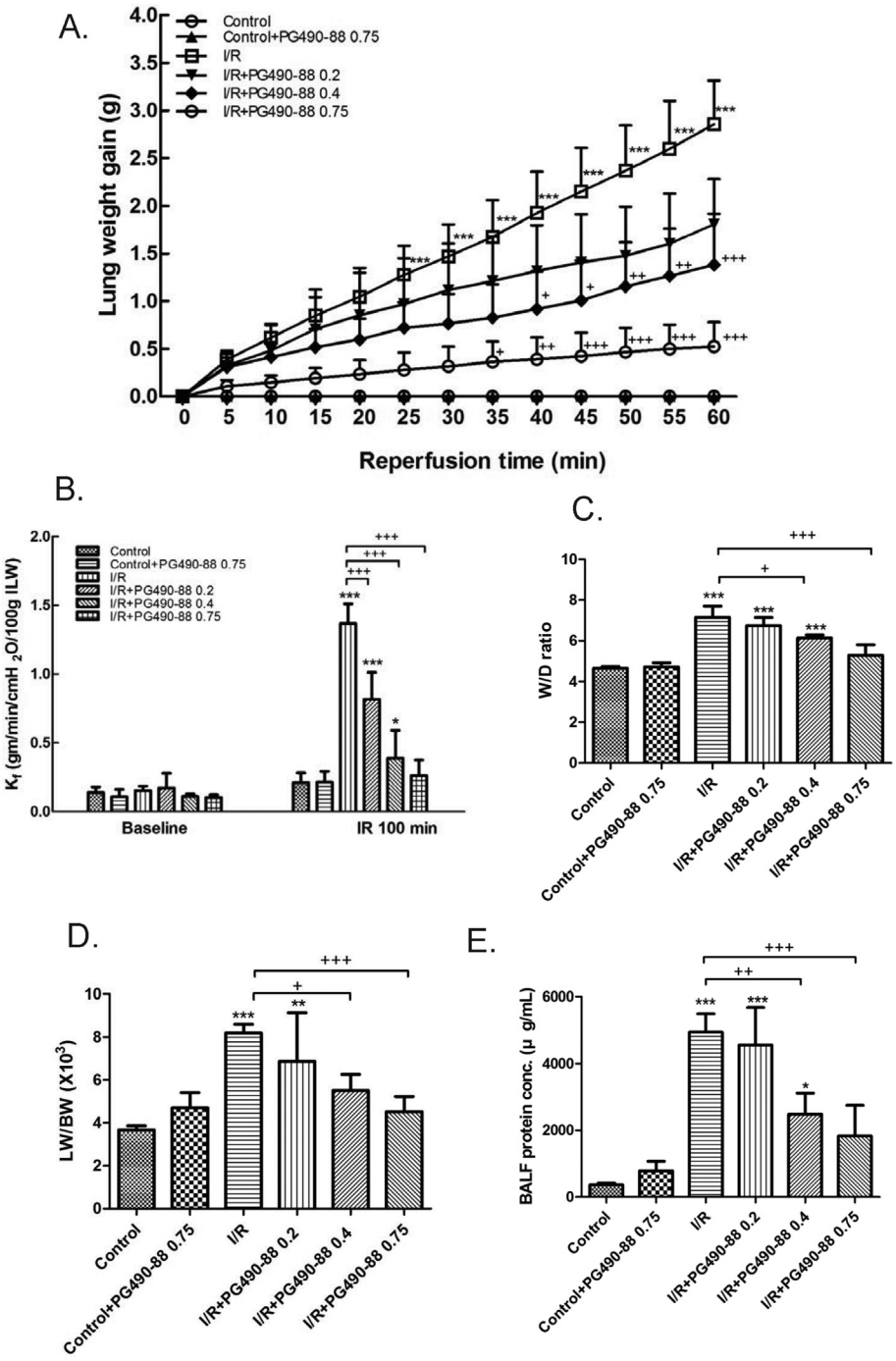

Fig. 1. Effect of PG490-88 on pulmonary edema. Lung weight gain was determined continuously for 60 min of reperfusion (A). K_f (B), lung wet weight/dry weight (W/D) ratio (C), lung weight/body weight (LW/BW) ratio (D), and total protein concentration in bronchoalveolar lavage fluid (BALF) (E) were measured after 60 min of reperfusion. Data are expressed as means ± SDs (6 rats per group); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, compared with the control group; $+p < 0.05$, $++p < 0.01$, $+++p < 0.001$, compared with the I/R group.

or long-term mortality. This may be because multiple complex signaling and cellular interactions mediate the tissue injury associated with ALI/ARDS [7]. Therefore, there is an urgent need to develop effective treatments for this potentially reversible lung damage. Recent research indicated that triptolide had beneficial effects in rat models of ALI induced by bleomycin, ventilator, lipopolysaccharides (LPS), and chlorine [10–13]. The reperfusion of an acutely ischemic lung can elicit an intense inflammatory reaction that injures lung tissue. However, the efficacy of PG490-88 against I/R-induced lung injury is unknown. The aims of the present study were to investigate the effect of PG490-88 on protection from I/R-induced lung injury *in vivo* and *in vitro*, and to identify the underlying mechanisms of these protective effects.

2. Materials and methods

2.1. Isolated perfused rat lung model

The rats in this study were cared for according to the guidelines of National Institutes of Health, and all experiments were performed with the permission of the Animal Review Committee of National Defense

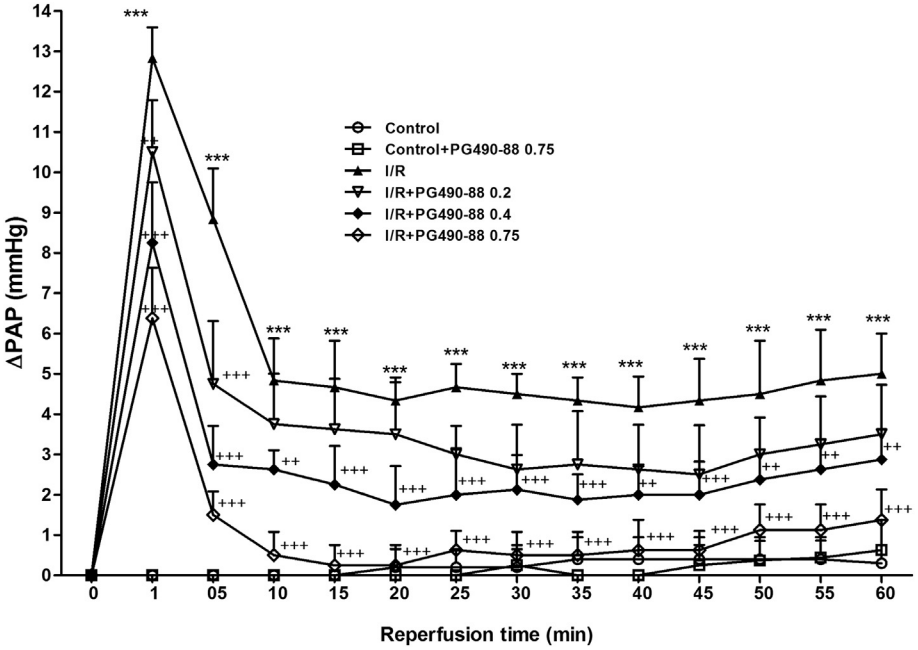

Fig. 2. Effect of PG490-88 on pulmonary artery pressure (ΔPAP). PAP was measured continuously for 60 min of reperfusion. Data are expressed as mean ± SDs (6 rats per group). ***p < 0.001, compared with the control group; + + +p < 0.001, compared with the I/R group.

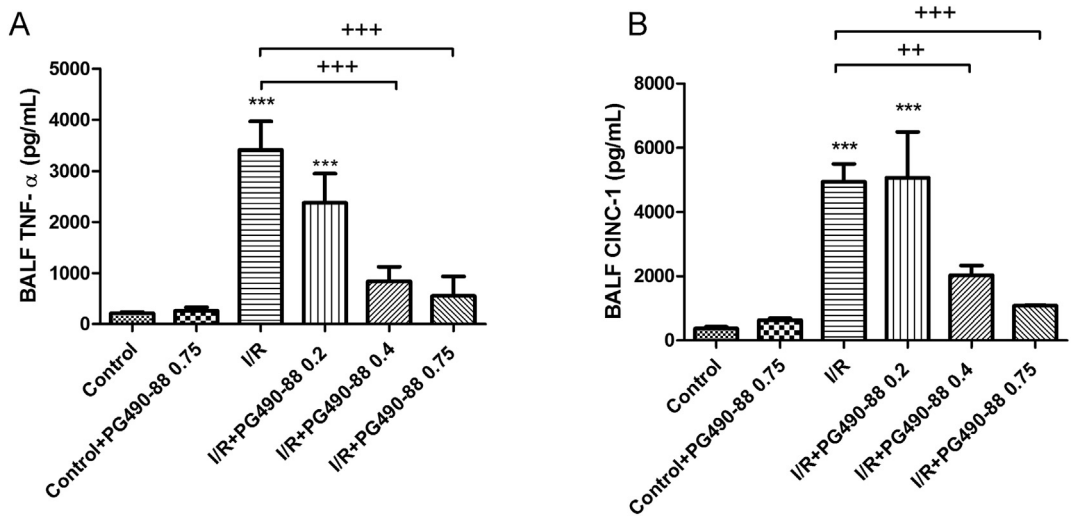

Fig. 3. Effect of PG490-88 on CINC-1 and TNF-α levels in bronchoalveolar lavage fluid (BALF). TNF-α (A) and CINC-1 (B) were measured after 60 min of reperfusion. Data are expressed as mean ± SDs (6 rats per group). ***p < 0.001, compared with the control group; + + +p < 0.001, compared with the I/R group.

Medical Center. A previously described isolated and perfused rat lung model was used [14–16]. Briefly, Sprague-Dawley male rats (body weight [BW]: 350 ± 20 g) received tracheotomies and were ventilated with humidified air containing 5% CO_2 at a tidal volume of 3 mL, a positive end-expiratory pressure of 1 cmH_2O, and a rate of 60 breaths/min. After a sternotomy, heparin was injected into the right ventricle, and 10 mL of intracardiac blood was withdrawn. The pulmonary artery and the left ventricle were cannulated while perfusate was flowing, and were perfused with a physiological salt solution (119 mM NaCl, 4.7 mM KCl, 1.17 mM $MggSO_4$, 22.6 mM $NaHCO_3$, 1.18 mM KH_2PO_4, 1.6 mM $CaCl_2$, 5.5 mM glucose, and 50 mM sucrose) containing 4% bovine serum albumin. The 10 mL of collected blood was added to the perfusate and subsequently mixed with the physiological salt solution to be used as a perfusing fluid for the isolated lungs. The flow rate of the roller pump (Minipuls 2; Gilson Medical Electronic, Middleton, WI, USA) was kept at a constant 8–10 mL/min. The *in situ* isolated rat lung was put on an electronic balance to record real-time changes in lung weight (LW). The left atrial pressure, representing the pulmonary venous pressure (PVP), and the pulmonary arterial pressure (PAP) were continuously monitored from the side arm of the cannula using Gould pressure transducers (Gould Instruments, Cleveland, OH). All isolated lung preparations used for experiments were in the isogravimetric state, had no leakage at the sites of cannula insertion, and had no evidence of bleeding or edema.

2.2. Assessment of microvascular permeability

An index of microvascular permeability to water (K_f) was calculated from the change in lung weight caused by elevation of venous pressure, as described previously [14–16]. K_f was termed as the initial weight gain rate ($g\,min^{-1}$) divided by the PVP ($10\,cmH_2O$) and lung weight, and was presented in units of $g{\cdot}min^{-1}{\cdot}cmH_2O^{-1} \times 100$ g.

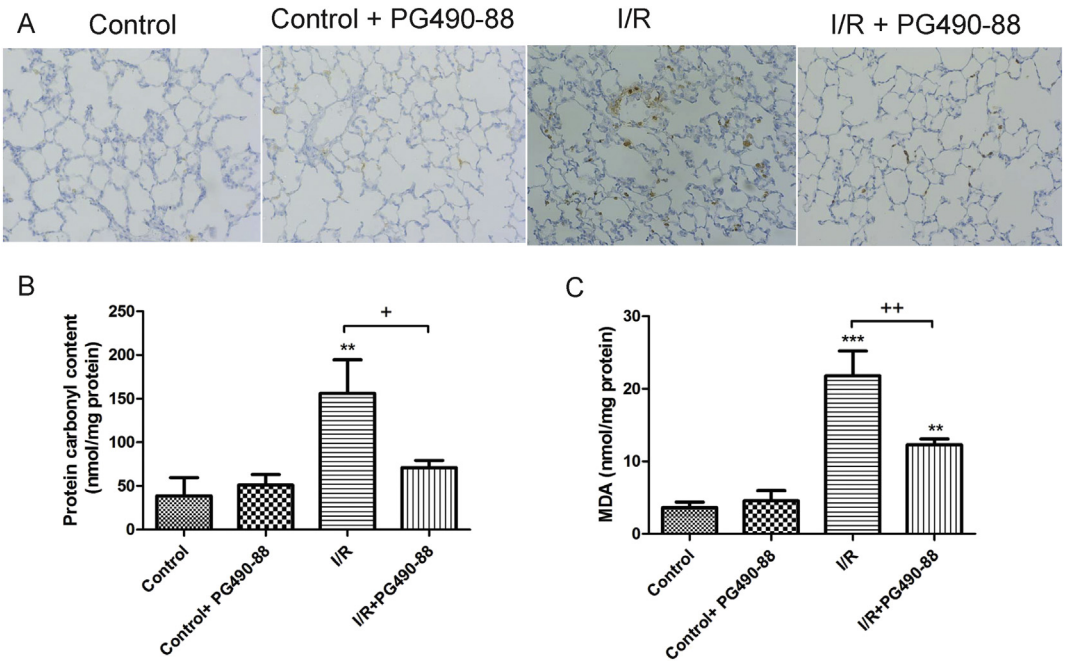

Fig. 4. Effect of PG490-88 on protein carbonyl content, MDA level, and MPO-positive cells in lung tissue. Immunohistochemical analysis (200×) of MPO (A), and measurement of carbonyl content (B) and MDA level (C) were performed after 60 min of reperfusion. Data are expressed as mean ± SDs (6 rats per group). $^{**}p < 0.01$, $^{***}p < 0.001$, compared with the control group; $+p < 0.05$, $++p < 0.01$, compared with the I/R group.

2.3. Ratios of lung weight (LW) to BW and wet lung to dry lung weight

After the experiments, the right lung was removed from the hilar region and LW was measured for determination of the LW/BW ratio. A part of the right upper lung lobe was weighed and incubated at 60 °C for 48 h in an oven. The dry weight was then measured, and the wet to dry (W/D) lung weight ratio was determined.

2.4. Measurement of protein, cytokine-induced neutrophil chemoattractant-1(CINC-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) in bronchoalveolar lavage fluid (BALF)

BALF was recovered by lavaging the left lung twice with 2.5 mL of saline at the end of the experiment. The lavage fluid was centrifuged at 200 × *g* for 10 min. The protein concentration in the supernatant was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The levels of proinflammatory cytokines (TNF-α, IL-6 and CINC-1) in the BALF were measured using a commercial ELISA kit (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

2.5. Assessment of protein carbonyl content and malondialdehyde (MDA) in lung tissue

The levels of protein carbonyl contents and MDA in right upper lung lobe were determined using a Protein Carbonyl Content Assay Kit (Abcam, Cambridge, MA, USA) and a Lipid Peroxidation (MDA) Assay Kit (Abcam, Cambridge, MA, USA) according to each manufacturer's instructions.

2.6. Western blotting analysis

The right middle lung lobe samples and cell protein lysates were separated by 10% SDS polyacrylamide gel electrophoresis and immunoblots were performed as previously described [15]. The membranes were probed with anti-β-actin as a loading control (1:10000, Sigma Chemical Company, St. Louis, MO, USA) and with one of the following antibodies: anti-B-cell lymphoma (Bcl)-2 (1:200, Santa Cruz Biotechnology, Dallas, Texas, USA), anti-NF-κB p65, anti-phospho-NF-κB p65, anti-inhibitor of NF-κB (IκB)-α, anti-extracellular signal-related protein kinase 1/2 (ERK1/2), anti-phosho-ERK1/2, anti-c-Jun N-terminal kinase (JNK), anti-phospho-JNK, p38 protein kinase (p38), anti-phospho-p38, and anti-mitogen-activated protein kinase phosphatase-1 (MKP-1) (1:1000, Cell Signaling Technology, Danvers, MA, USA), or anti-TATA (1:1000, Abcam, Cambridge, MA, USA). All data are presented as the ratio of the target protein to the reference protein (β-actin).

2.7. Immunohistochemical detection of lung myeloperoxidase

Immunohistochemical staining to identify myeloperoxidase (MPO) was performed as described previously [15]. Briefly, paraffin-embedded right lower lung lobe sections were deparaffinized in xylene before antigen retrieval. Then, endogenous peroxidase was quenched with 3% H_2O_2 and 100% methanol for 15 min, and immunostaining of lung sections was performed using a rabbit polyclonal antibody (anti-MPO, 1:100, Cell Signaling Technology). The slides were washed and then incubated with the secondary rat-specific horseradish peroxidase polymer anti-rabbit antibody (Nichirei Corporation, Tokyo, Japan) for 30 min. Then, the horseradish peroxidase substrate was added and incubated for 3 min, and hematoxylin was used for counterstaining.

2.8. Histological analyses

Paraffin sections of right lower lung lobe were stained with hematoxylin-eosin (H&E) to evaluate the extent of lung injury. The number of polymorphonuclear neutrophils in the interstitium were calculated in 10 different high-power fields (×400) and averaged by two pathologists who were blinded and independently performed this procedure. Semiquantitative grading of lung injury based on lung section histology was performed as previously described [17].

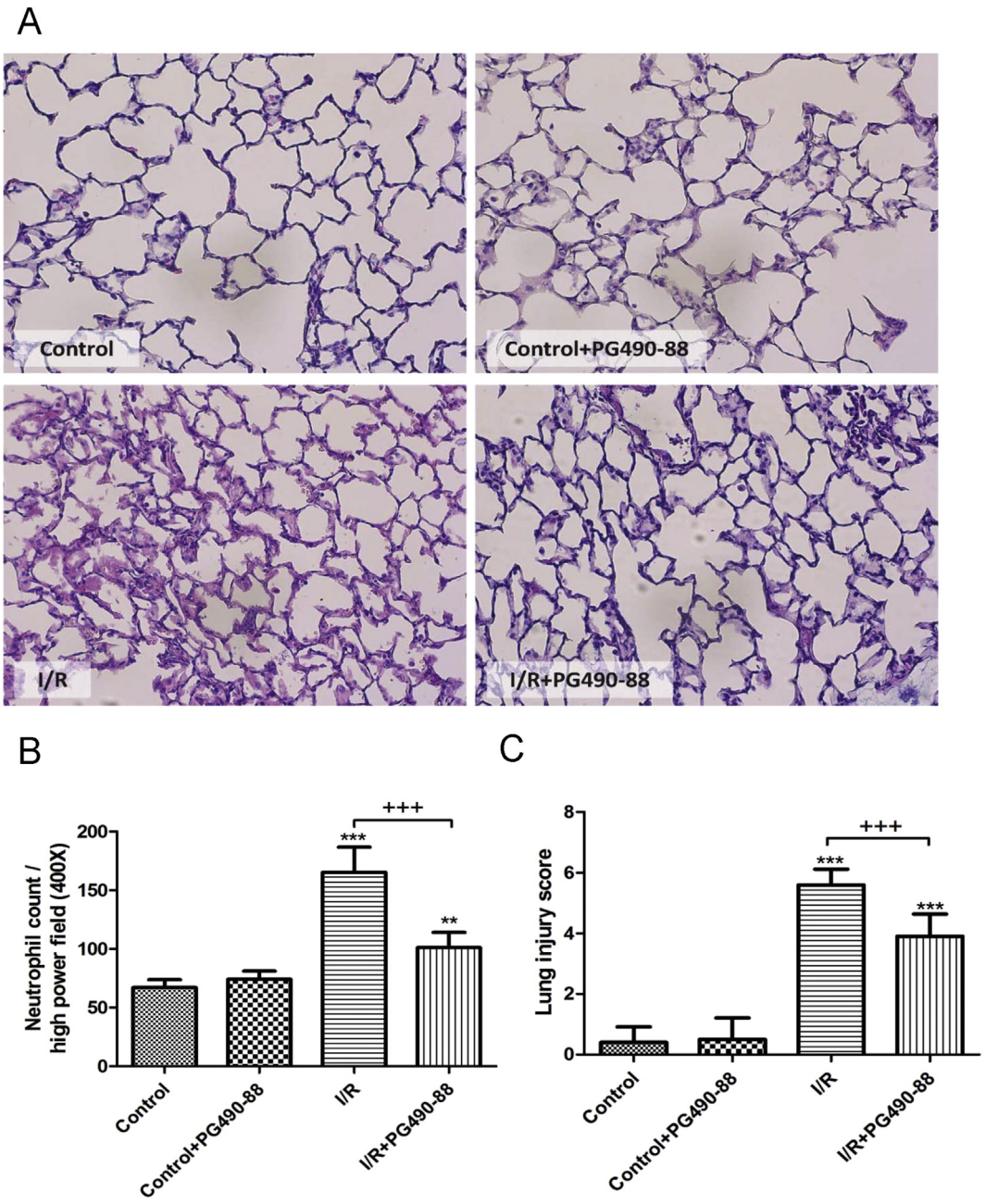

Fig. 5. Effect of PG490–88 on lung histopathology. Microscopy (representative results, 200×, hematoxylin and eosin staining) (A), counting of neutrophils per high power field (400× magnification) (B), and determination of lung injury scores (C) were performed after 60 min of reperfusion. Data are expressed as mean ± SDs (6 rats per group). $^{**}p < 0.01$, $^{***}p < 0.001$, compared with the control group; $+++p < 0.001$, compared with the I/R group.

2.9. Immunofluorescence staining for claudin-4, occludin, and zonula occludens-1 (ZO-1)

Immunofluorescence staining was performed as previously described [18]. Claudin-4, occludin and ZO-1 were identified by immunofluorescence staining using polyclonal rabbit polyclonal antibodies (diluted 1:200, Invitrogen, Carlsbad, CA). Fluorescein isothiocyanate-conjugated chicken anti-rabbit IgG (1:200; Santa Cruz Biotechnology) was used as the secondary antibody for counterstaining. Image assessment was performed using a fluorescence microscope (Leica DM 2500; Leica Microsystems GmbH, Wentzler, Germany) with MetaMorph digital analysis software (Universal Imaging, Downingtown, PA, USA).

2.10. Phosphorylation of claudin-4, occludin, and ZO-1

Claudin-4, occludin, and ZO-1 proteins were immunoprecipitated from the lung homogenates using their respective antibodies as described previously [19]. Bound proteins were electrophoresed on 10% polyacrylamide gels and transferred to Hybond-PVDF membranes. Membranes were blocked and incubated with their respective antibodies or anti-phosphoserine/threonine/tyrosine antibody (Enzo Life Sciences, Farmingdale, NY, USA), followed by horseradish peroxidase-linked goat anti-rabbit antibodies. The protein bands were developed by chemiluminescence.

2.11. Study protocol

A total of 36 rat lungs were randomly assigned to one of four

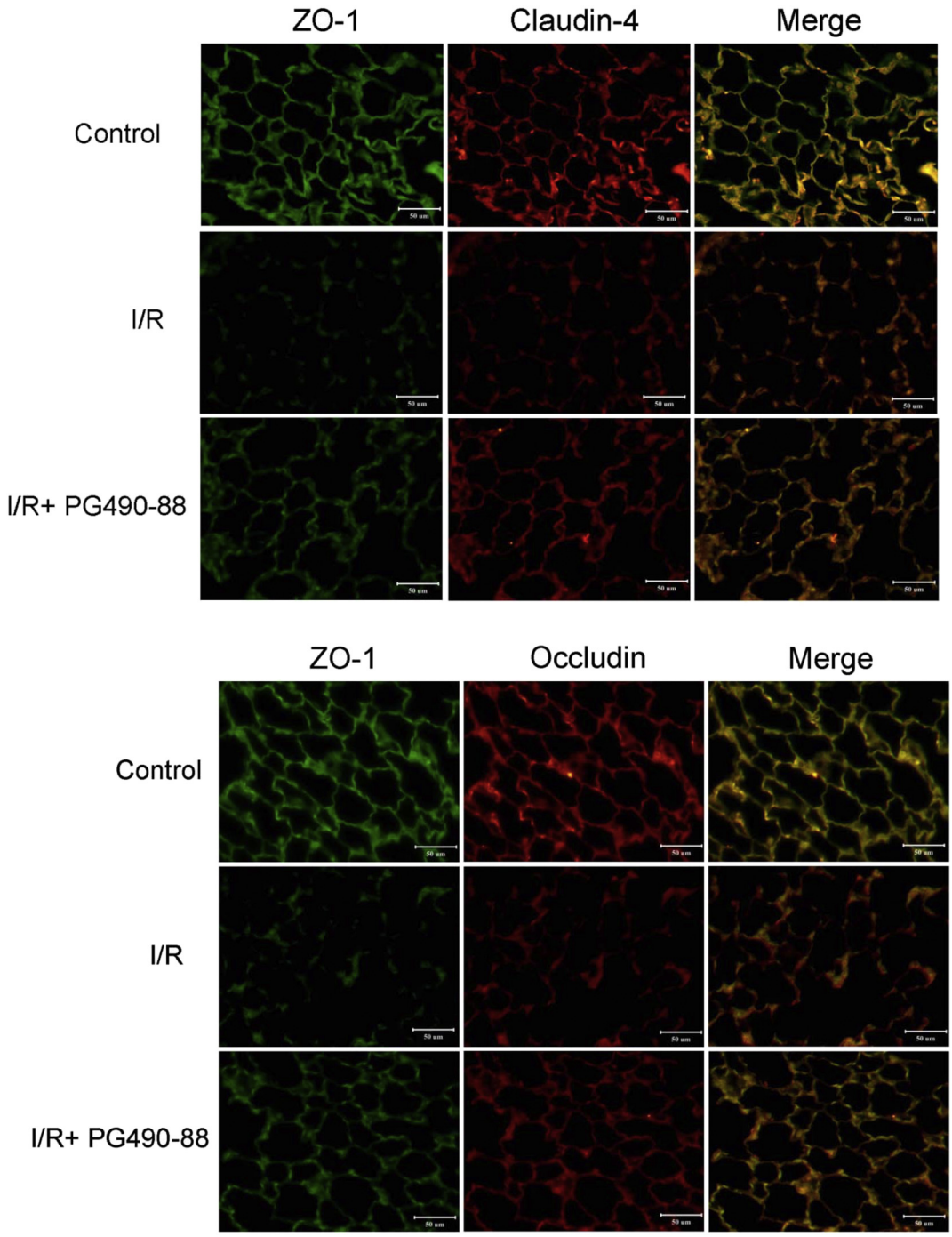

Fig. 6. Effect of PG490-88 on expression of claudin-4, occludin, and ZO-1 in lung tissue. Immunostaining (representative results, 400×) was performed after 60 min of reperfusion.

groups: control (0.9% NaCl, $n = 6$), PG490-88 alone (0.75 mg/kg BW, n = 6), I/R alone (n = 6), or I/R with PG490-88 (0.2, 0.4, or 0.75 mg/kg BW, n = 6). In control group, lungs were perfused with perfusate alone for 100 min. PG490-88 was added to the reservoir (containing 20 mL of perfusate) [11]. I/R was induced in the deflated lungs by stopping ventilation and perfusion to cause ischemia for 40 min. After ischemia, perfusion and ventilation were resumed for 60 min. PG490-88 was generously provided by MyeloRx LLC (Vallejo, CA).

2.12. Cell culture and hypoxia/reoxygenation (H/R) injury

Mouse alveolar type II epithelial (MLE-12) cell lines were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM/F-12 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (Hyclone), penicillin (100 U/mL), and streptomycin (10 μg/mL) in a humidified atmosphere (5% CO_2, 95% air). These cells were exposed to hypoxia (1% O_2, 5% CO_2, and 94% N_2) for 24 h followed by reoxygenation (37 °C with 5% CO_2 and 95% air) for 4 h.

The cells were then pretreated with vehicle or 50 nM PG490-88 [20]. The control group was maintained in the reoxygenated state without the hypoxic stimulus. The supernatant was collected and assayed for macrophage inflammatory protein 2 (MIP-2) using a mouse CXCL2/MIP-2 ELISA kit (R&D, Inc., Minneapolis, MN, USA).

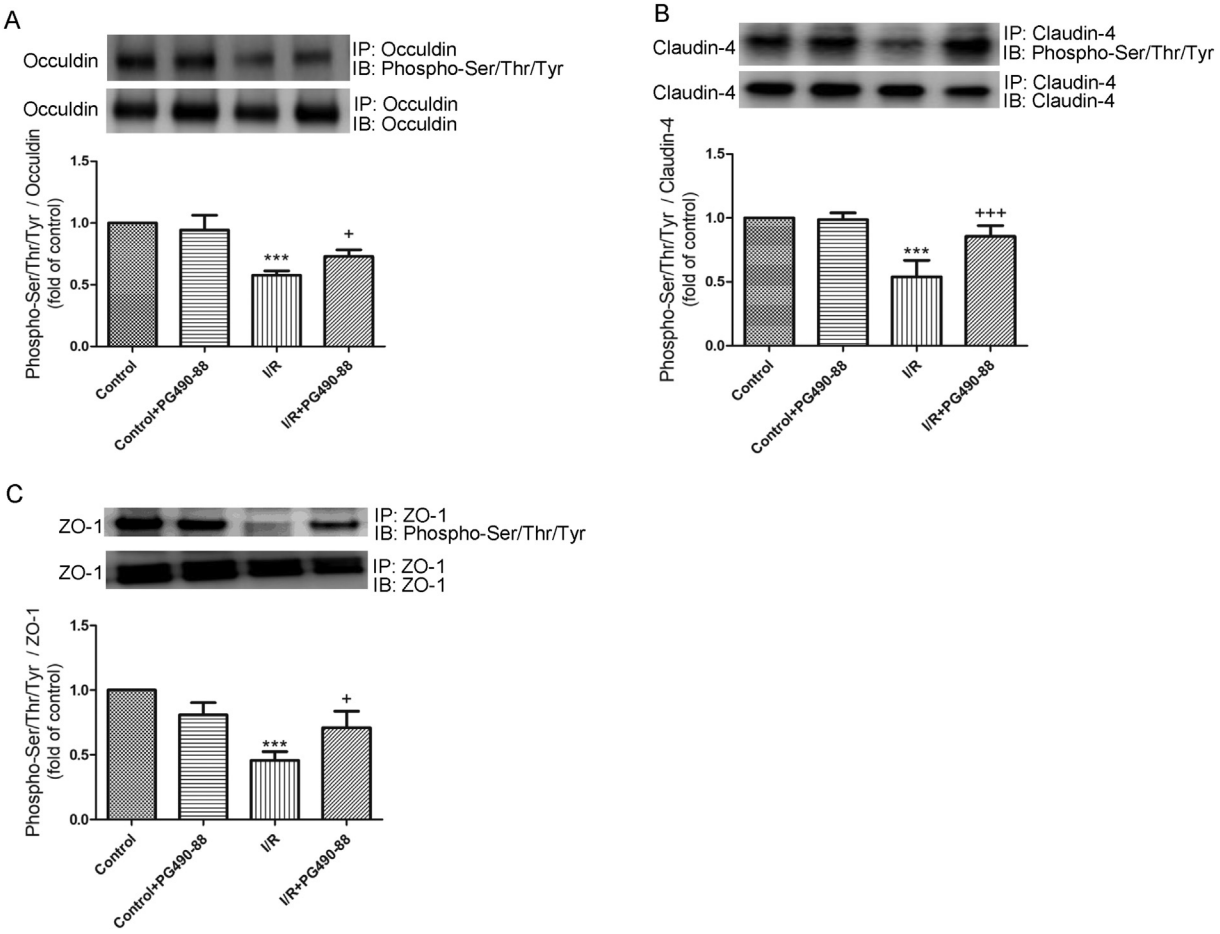

Fig. 7. Effect of PG490-88 on phosphorylation of tight junction proteins in lung tissue. Immunoprecipitation with anti–claudin-4, occludin, and ZO-1 antibodies, followed by immunoblotting with anti-phosphoserine/threonine/tyrosine antibody.
Western blotting was performed after 60 min of reperfusion. Data are expressed as mean ± SDs (6 rats per group). ***$p < 0.001$, compared with the control group; +$p < 0.05$, +++p < 0.001, compared with the I/R group.

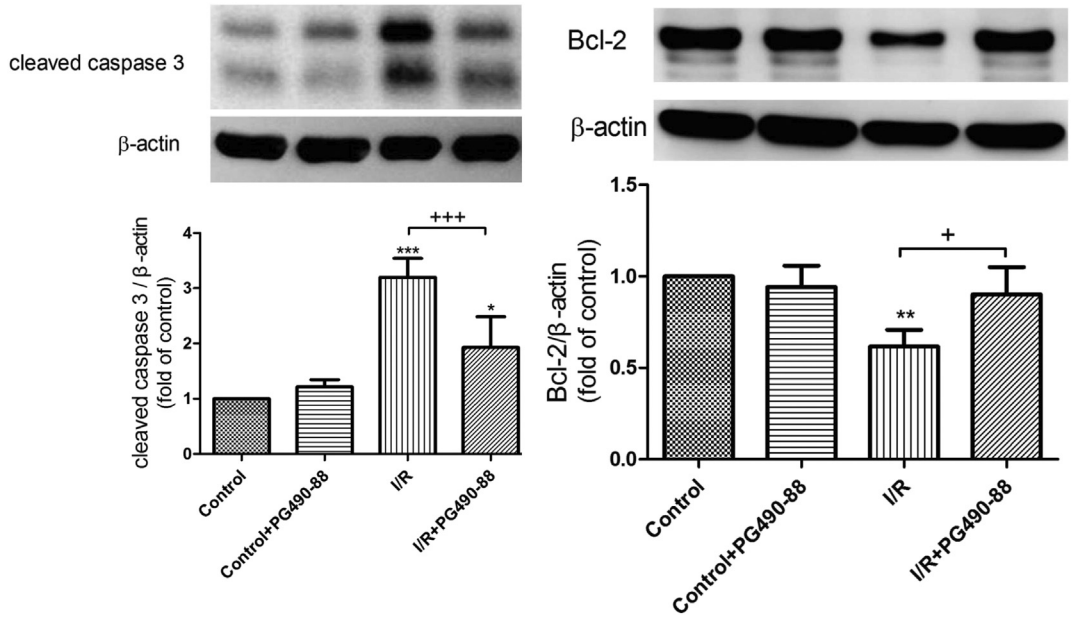

Fig. 8. Effect of PG490-88 on expression of caspase-3 and Bcl-2 in lung tissue.
Western blotting (top: representative results; bottom: quantitation of data) was performed after 60 min of reperfusion. Equal loading was confirmed with β-actin. Representative blots are shown. Data are expressed as mean ± SDs (6 rats per group). *p < 0.05, **$p < 0.01$, ***p < 0.001, compared with the control group; +p < 0.05, +++p < 0.001, compared with the I/R group.

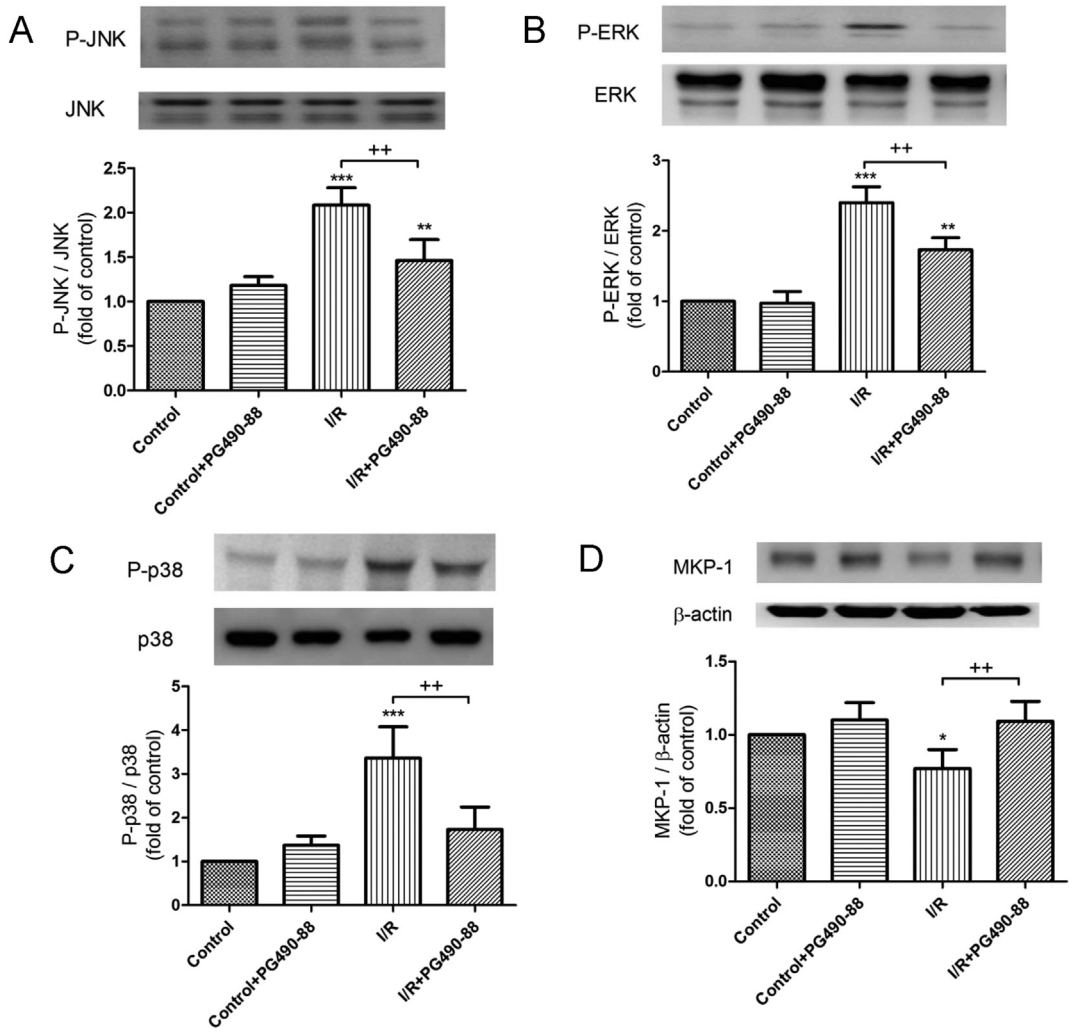

Fig. 9. Effect of PG490–88 on MAPK and MKP-1 expression in lung tissue. Western blotting (top: representative results; bottom: quantitation of data) was used to measure the phosphorylation of JNK (A), ERK (B), and p38 (C) and expression of MKP-1 (D) after 60 min of reperfusion. Equal loading was confirmed with β-actin. Representative blots are shown. Data are expressed as mean ± SDs (6 rats per group).
*p < 0.05, **p < 0.01, ***p < 0.001, compared with the control group; ++p < 0.01, compared with the I/R group.

2.13. Data analysis

GraphPad Prism 5 statistical software (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. The results of each group are presented as means ± SD, and groups were compared using one-way ANOVA followed by a *post-hoc* Bonferroni test. The comparisons of LWG and PAP between groups was utilized two-way ANOVA for repeated measurements followed by the *post-hoc* Bonferroni test. $p < 0.05$ was considered significant.

3. Results

3.1. PG490-88 reduced I/R-induced lung edema

I/R significantly increased lung weight gain over time, and PG490-88 treatment suppressed this effect (Fig. 1A). I/R also significantly increased the K_f, LW/BW and lung W/D weight ratios, and protein concentrations in the BALF after 60 min of reperfusion ($p < 0.05$, Fig. 1B–E); PG490-88 treatment significantly reduced these increases in a dose-dependent manner.

3.2. PG490-88 suppressed I/R-induced increase of PAP

Induction of I/R led to an initial increase of the PAP, and then a decline after reperfusion (Fig. 2). After 60 min of reperfusion, the PAP was still significantly greater than at baseline and greater than in the controls, which had a constant PAP during the 100 min observation period. Treatment with PG490-88 significantly suppressed the effect of I/R in a dose-dependent manner ($p < 0.05$; Fig. 2).

3.3. PG490-88 suppressed I/R-induced increase of TNF-α and CINC-1 in BALF

Induction of I/R led to significantly increased levels of TNF-α and CINC-1 in the BALF after 60 min of reperfusion ($p < 0.05$; Fig. 3). PG490-88 significantly suppressed these effects in a dose-dependent manner, although the lowest tested dose of PG490-88 had no significant effect on the level of either cytokine.

3.4. PG490-88 attenuated I/R-induced increase of protein carbonyl content, MDA level, and MPO-positive cells in lung tissue

Induction of I/R led to significantly increased number of MPO-positive cells (based on immunohistochemistry) and increased levels of

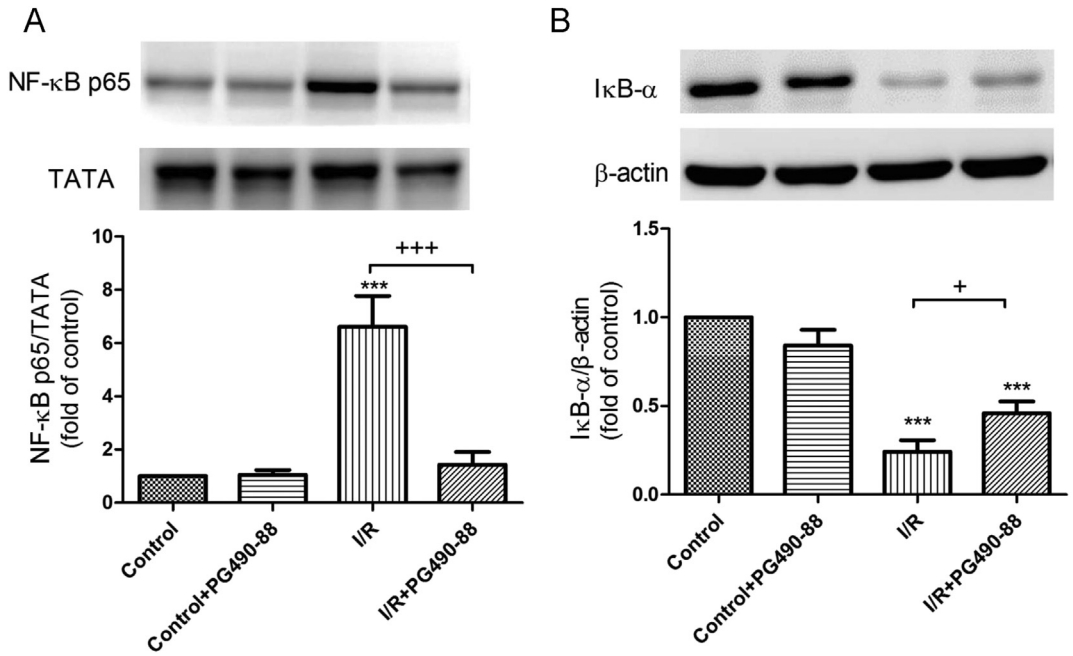

Fig. 10. Effect of PG490–88 on NF-κB activation in lung tissue. Western blotting (top: representative results; bottom: quantitation of data) was used to measure nuclear NF-κB p65 (A) and cytoplasmic IκB-α (B) after 60 min of reperfusion. TATA and β-actin served as loading controls for nuclear and cytoplasmic proteins, respectively. Representative blots are shown. Data are expressed as mean ± SDs (6 rats per group). ***$p < 0.001$, compared with the control group; +$p < 0.05$, + + +$p < 0.001$, compared with the I/R group.

MDA and protein carbonyl content in lung tissue after 60 min of reperfusion ($p < 0.05$, Fig. 4A–C). Treatment with PG490-88 (0.75 mg/kg) significantly suppressed these effects.

3.5. PG490-88 improved I/R-induced histopathology of lung tissue

Induction of I/R led to histologic abnormalities in lung tissues, including infiltration of leukocytes and marked thickening of interalveolar walls. These alterations were attenuated in I/R-treated rats that received PG490-88 after 60 min of reperfusion (Fig. 5A). Treatment with PG490-88 (0.75 mg/kg) significantly reduced neutrophil infiltration (Fig. 5B) and lung injury scores (Fig. 5C).

3.6. PG490-88 restored the I/R-induced disruption of lung tissue tight junctions

Immunostaining of lung sections from control rats indicated smooth and linear distributions of claudin-4, occludin, and ZO-1 along the alveolar epithelium, consistent with the presence of intact tight junctions (Fig. 6). Induction of I/R led to decreased expression and irregular and interrupted distribution of all three proteins after 60 min of reperfusion. Treatment with PG490-88 (0.75 mg/kg) strongly reversed the effect of I/R, indicating restoration of intact tight junctions.

3.7. Effect of PG490-88 on phosphorylation of tight junction protein

Immunoprecipitation with anti-claudin-4, occludin, and ZO-1, followed by immunoblotting with anti-phosphoserine/threonine/tyrosine antibody antibodies, showed reductions in phosphorylation of claudin-4, occludin, and ZO-1 in I/R (Fig. 7). Treatment with PG490-88 (0.75 mg/kg) attenuated the effect of I/R.

3.8. PG490-88 suppressed I/R-induced down-regulation of caspase-3 and up-regulation of Bcl-2 in lung tissue

Western blot analysis of lung tissue indicated that I/R significantly increased the level of caspase-3 and decreased the level of Bcl-2 after 60 min of reperfusion ($p < 0.05$, Fig. 8A-B), consistent with the presence of increased apoptosis. Treatment with PG490-88 (0.75 mg/kg) significantly reversed these effects.

3.9. PG490-88 suppressed I/R-induced activation of mitogen-activated protein kinase (MAPK) pathway and inhibition of MKP-1 expression in lung tissue

Western blot analysis of lung tissue indicated that I/R significantly reduced the expression of MPK-1 and increased the phosphorylation of JNK, ERK, and p38, indicating activation of the MAPK pathway after 60 min of reperfusion (Fig. 9).

Treatment with PG490-88 (0.75 mg/kg) significantly suppressed all of these effects.

3.10. PG490-88 inhibited I/R-induced activation of NF-κB pathway in lung tissue

Western blot analysis of lung tissue indicated that I/R led to a significantly increased nuclear level of NF-κB p65 and a significantly decreased level of cytoplasmic IκB-α after 60 min of reperfusion (Fig. 10), indicating activation of the NF-κB pathway. Treatment with PG490-88 (0.75 mg/kg) significantly suppressed these effects.

3.11. PG490-88 attenuated H/R injury in MLE-12 cells

We also tested the protective effect of PG490-88 on H/R injury in MLE-12 cells. Induction of H/R led to increased phosphorylation of p65, and decreased expression of IκB-α, and increased level of MIP-2 at 2 h and 4 h (Fig. 11). In addition, H/R increased the phosphorylation of JNK, ERK, and p38 at 2 h and 4 h (Fig.12). PG490-88 (50 nM) significantly suppressed all of these effects.

4. Discussion

Our results showed that PG490-88 treatment had a dose-dependent therapeutic effect on rats subjected to I/R injury. In particular, PG490-

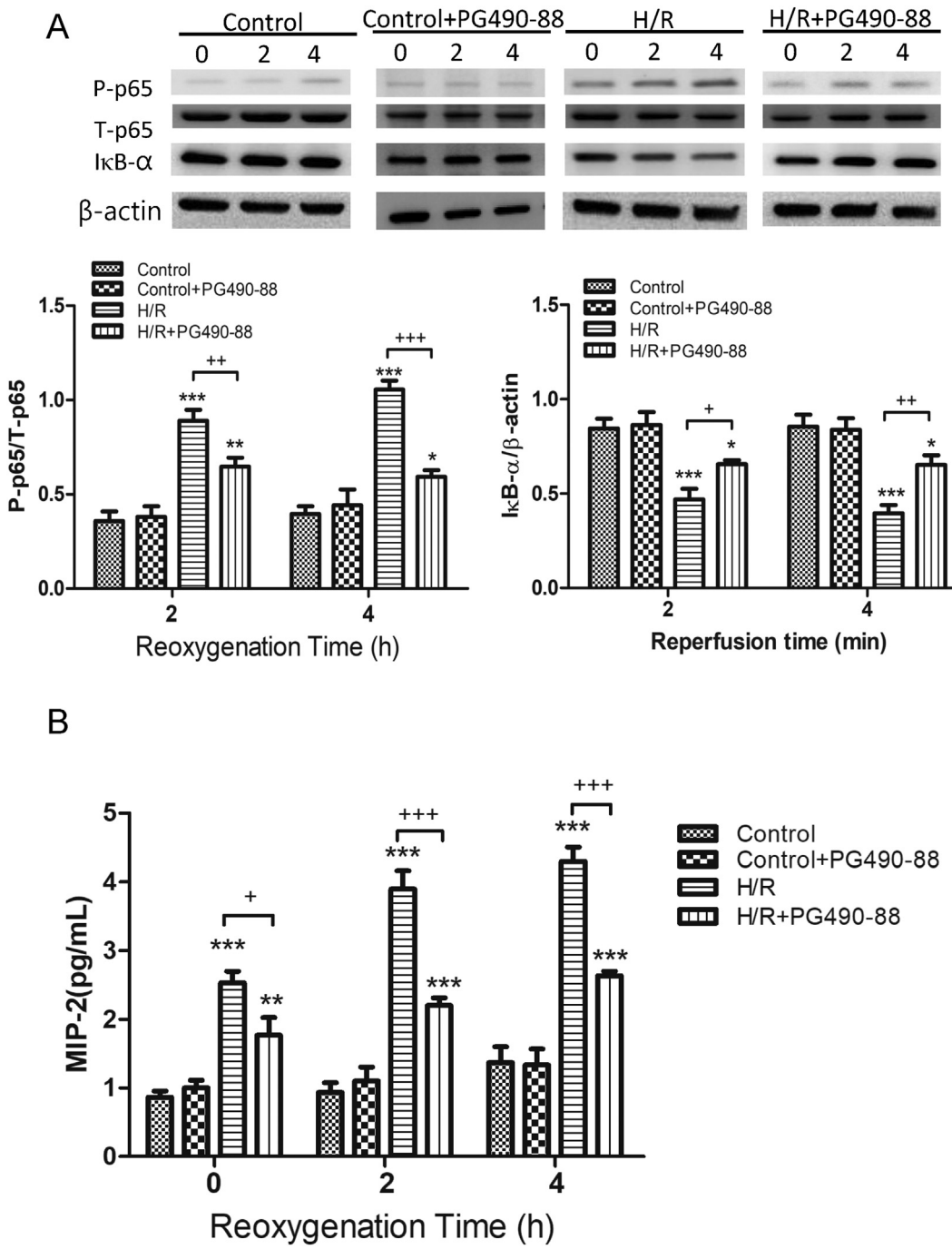

Fig. 11. Effect of PG490–88 on MLE-12 cells subjected to hypoxia/reoxygenation (H/R). The phosphorylation of p65, degradation of IκB-α, and MIP-2 level were measured after 2 h and 4 h of H/R. β-actin served as the loading control. A representative blot is shown. Data are expressed as mean ± SDs. *p < 0.05, **p < 0.01, ***p < 0.001, compared with the control group. +p < 0.05, ++p < 0.01, +++p < 0.001, compared with the H/R group.

88 reduced lung edema due to its reduction of vascular permeability, PAP, LW/BW and W/D ratios, LWG, and protein levels in BALF. PG490-88 treatment also suppressed the I/R-induced disruption of tight junction proteins, production of pro-inflammatory cytokines and free radicals, influx of pulmonary neutrophils, and reduced cell death and tissue damage. At the molecular level, PG490-88 inhibited I/R-induced activation of the MAPK and NF-κB signaling pathways. The results of our *in vitro* experiments with PG490-88 and MLE-12 cells exposed to H/R confirmed the *in vivo* experiments. These results imply that PG490-88 may have potential as an adjunct treatment for I/R lung injury, and should be considered for future clinical studies.

Recent studies indicate that tight junctions have a key role in the pathogenesis of ALI/ARDS [21,22]. These structures are complexes consisting of transmembrane and cytosolic proteins that create a primary barrier which restricts the diffusion of solutes through adjacent epithelial cells. Disruption of the epithelial barrier alters pulmonary permeability. Disruption of the epithelial barrier lead to altered pulmonary permeability [22]. Indeed, considerable evidence suggests that maintaining tight junction complexes can prevent lung injury [21,22]. Occludin, claudin, and ZO-1 proteins are major components of the intact intercellular barrier at tight junctions [22]. Downregulation of these proteins during hyperoxia and LPS-induced lung injury disrupts the tight junction barrier [23,24]. In our study, we observed that induction of I/R impaired the expression of all three of these proteins and

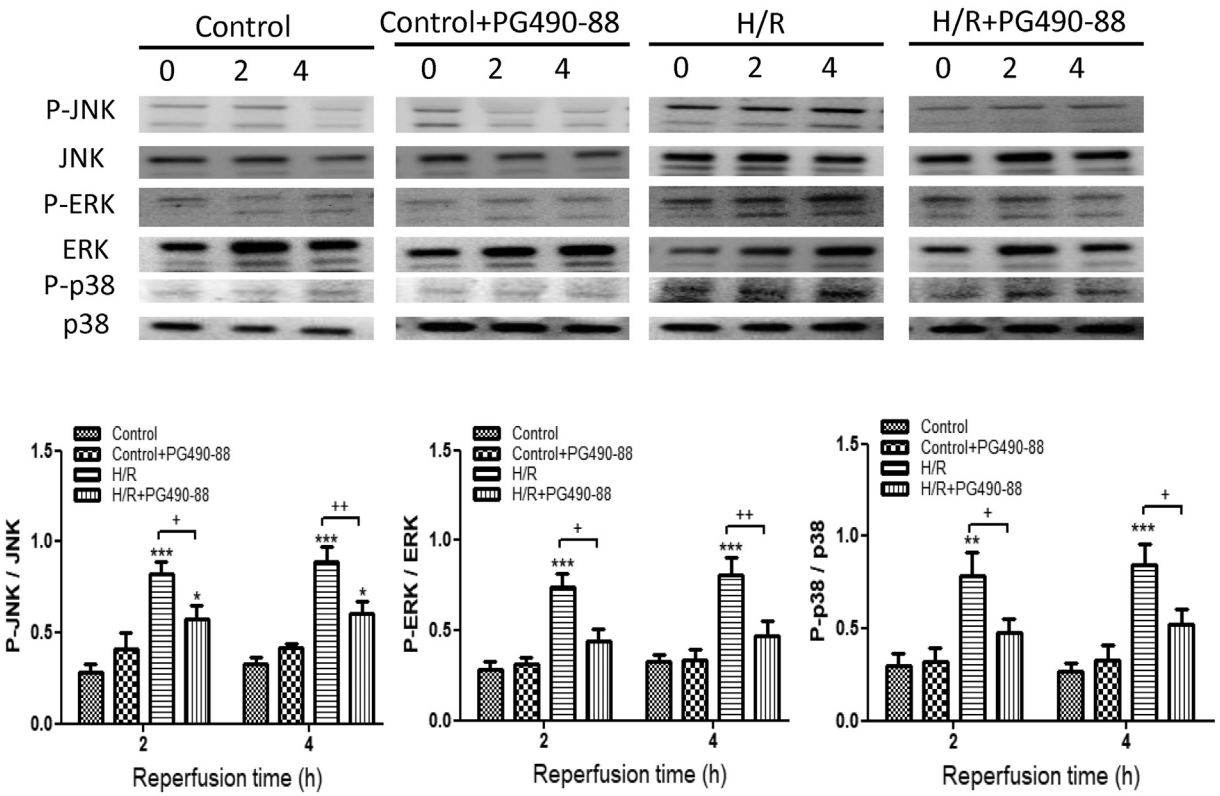

Fig. 12. Effect of PG490–88 on MAPK expression in MLE-12 cells subjected to hypoxia/reoxygenation (H/R). Western blotting was used to measure the phosphorylation of JNK, ERK, and p38. Data are expressed as mean ± SDs. *p < 0.05, **p < 0.01, ***p < 0.001, compared with the control group. +p < 0.05, ++p < 0.01, compared with the H/R group.

thereby increased the permeability of tight junctions, and that PG-490-88 treatment suppressed this effect. Thus, the beneficial effects of PG490-88 on the epithelial barrier could be mediated by its upregulation of tight junction proteins, which reduces the severity of lung edema.

Protein phosphorylation provides post-translational modification that alters their physical properties and function. Previous study showed that rat glomerular epithelial cells exposed to high glucose caused a reduction in phosphorylation of ZO-1 [19]. Treatment with protamine sulfate increased phosphorylation of ZO-1 in glomeruli of rats [25]. In addition, acute alcohol intoxication combined with burn injury induced a significant decreased phosphorylation of occludin and claudin-1 in small intestine [26]. Our study was consistent with these findings that I/R was associated with reduction in phosphorylation of all three tight junction proteins in the lung. Treatment of animals with PG-490-88 prevented the decrease in phosphorylation. Because the phosphorylation of tight junction proteins is important for tight junction assembly, a decrease in it as noted in this experiment may disturb the tight junction assembly resulting in impaired epithelial barrier function. In contrast, other investigations have shown that phosphorylation of tight junction proteins may increase paracellular permeability in epithelial and endothelial cells [27]. However, the phosphorylation is often induced by different kinases on distinct residues on the same tight junction proteins [27]. This could explain for the difference from previous studies; however, a definitive reason for these differences remains to be investigated.

It is well recognized that oxidative stress can cause significant lung damage. Oxidative stress is accompanied by extensive infiltration of leukocytes, especially neutrophils [28,29]. The damage from oxidative stress also induces epithelial and endothelial damage, which leads to increased vascular permeability and ultimately to the development of ALI/ARDS [28,29]. Previous animal studies reported that limiting oxidative stress can reverse acute lung injury [29]. Our results showed that PG490-88 suppressed oxidative stress, as indicated by its inhibition of protein carbonyl and MDA production in lung tissue subjected to I/R. Furthermore, PG490-88 diminished neutrophil infiltration into lung tissue subjected to I/R, as indicated by the smaller number of neutrophils and MPO-positive cells. This blocked the interaction between neutrophils and the endothelium, and therefore suppressed the production of proinflammatory cytokines and free radicals by activated neutrophils. Thus, the anti-oxidative effects of PG490-88 likely account for its attenuation of lung edema. Our observations agreed with those of Wu et al., who found that PG490-88 alleviated hepatic I/R injury by suppressing oxidative stress [5].

Apoptosis or also recognized as programmed cell death is important for the tissues remodeling during repair processes, and in the pathogenesis of some diseases [30]. Accumulating evidence demonstrates that upregulation of apoptosis following I/R has a crucial role in the development of ALI/ARDS [30]. We and other investigators have shown previously that apoptosis has a role is implicated in the process of lung damage after I/R [18,30–32]. In mammals, the anti-apoptotic protein Bcl-2 had shown to suppress apoptosis by hampering the activity of the pro-apoptotic protein caspase-3 [30]. I/R is accompanied by with reduced pulmonary perfusion, and the decreased level of oxygen can lead to increased apoptosis of lung epithelial and vascular endothelial cells, which may contribute to the pulmonary dysfunction. Further, other research has shown that I/R lung injury is reduced by suppression of apoptosis cell death pathways [30,32]. In our study, we found that PG490-88 significantly attenuated apoptosis in lung tissue by decreasing the level of the pro-apoptotic cleaved-caspase-3 and increasing the level of the anti-apoptotic Bcl-2. Our results concur with a previous report which found that PG490-88 had a neuroprotective effect due to its elicitation of anti-apoptotic responses in rats with cerebral I/R injury [2]. TNF-α is a pro-inflammatory cytokine with a major role in ALI/ARDS, and it can initiate the apoptotic cascade through the death receptor/caspase pathway [32]. Because PG490-88 inhibits TNF-

α production, it is possible that PG490-88 suppresses apoptosis in I/R-evoked lung injury, at least partly, *via* an indirect pathway. This may explain why PG490-88 has different effects on apoptosis in tumor cells and inflammatory cells.

An increase in apoptosis may be another reason for tight junction barrier dysfunction in I/R lung injury. In addition, several other inflammatory mediators (*i.e.*, TNF-α, and IL-6 *etc.*) are also implicated in altered tight junction function and enhanced apoptosis of endothelial and epithelial cells [33]. Many studies have demonstrated an increase in the levels of many of these cytokines following I/R injury [15,17,18]. Recently, one study showed that IL-18 can modulate intestine tight junction protein phosphorylation [26]. Further, I/R caused an increase in neutrophil infiltration into the lung. Neutrophils produce reactive oxygen species that may contribute to altered tight junction proteins and apoptosis in the lung following I/R [17,18,34]. Therefore, multiple mechanisms may modulate tight junction proteins and its phosphorylation in the lung.

NF-κB is an important transcription factor that regulates the expression of many pro-inflammatory cytokines and chemokines [35]. When cytoplasmic NF-κB is activated, the IκB family of inhibitory proteins of NF-κB are phosphorylated, and active NF-κB moves into the nucleus, where it augments the production of pro-inflammatory cytokines, such as TNF-α and CINC-1, and exacerbates lung injury by triggering the production of additional pro-inflammatory cytokines and enhancing the accumulation and activation of leukocytes [35]. Previous studies have also suggested that NF-κB activation has a role in the pathogenesis of I/R lung injury [15,34], and several previous investigations shows that triptolide (PG490) is a strong inhibitor of NF-κB [2–4,13]. Our results clearly showed that PG490-88 significantly suppressed IκB degradation and NF-κB activation, and led to decreased production of proinflammatory cytokines, such as TNF-α and CINC-1, and reduced infiltration of leukocytes. Furthermore, our *in vitro* studies using MLE-12 epithelial cells subjected to H/R, which investigated the direct effects of PG490-88 on alveolar epithelial cells, indicated that PG490-88 significantly inhibited IκBα degradation, NF-κB p65 phosphorylation, and the production of MIP-2 (functional homologs of human IL-8). Thus, the anti-inflammatory action of PG490-88 could be partly explained by its inhibition of NF-κB signaling and the consequent production of pro-inflammatory cytokines.

The MAPKs are serine/threonine protein kinases, whose most studied family members are p38, ERK, and JNK. These proteins participate in inflammatory signaling and other pathological processes associated with I/R injury [36]. Previous research reported that suppression of p38, ERK, or JNK, can reduce peritonitis and LPS-induced lung injury [37–39]. MKP-1 is regarded as an important regulator of inflammation, in that it down-regulates the MAPK and NF-κB pathways [40,41]. In support of this, MKP-$1^{-/-}$ mice had worse outcome following LPS- and ventilator-induced lung injury than MKP-$1^{+/+}$ mice [11]. Previous studies reported that I/R increased p38, ERK, and JNK phosphorylation, and decreased MKP-1 expression in lung tissue [17,18,42]. We found that PG490-88 treatment suppressed these effects and therefore limited the extensive inflammation associated with I/R-induced lung injury. This finding was also consistent with a study by Park et al., who demonstrated that PG490-88 induced MKP-1 expression and protected against ventilator-induced lung injury [11]. In contrast, triptolide (PG490) suppressed peptidoglycan-induced expression of MKP-1 in RAW264.7 murine macrophage cells and primary alveolar macrophages, and led to prolonged activation of JNK and p38 [43]. Further, PG490-88 did not affect MKP-1 activity in cisplatin-induced acute kidney injury [6]. Therefore, the effects of PG490-88 on MKP-1 expression may be context -specific. Further studies are necessary to determine the reasons for these disparate results.

The results of our study indicate that PG490-88 provides many protective effects in rats subjected to I/R injury. In particular, PG490-88 appears to increase the integrity of tight junction barriers, reduce the production of reactive oxygen species and apoptosis, and suppresses the activation of NF-κB and MAPK signal pathways, and thereby inhibit the production of proinflammatory mediators and cytokines. Therefore, PG490-88 appears to be an effective adjunct for treatment of I/R-induced lung injury. A better understanding of the physiological action of PG490-88 is needed before it can be recommended for future use.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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