# Valproic Acid Attenuates Acute Lung Injury Induced by Ischemia–Reperfusion in Rats

Shu-Yu Wu, Ph.D., Shih-En Tang, M.D., Ph.D., Fu-Chang Ko, M.D., Geng-Chin Wu, M.D., Kun-Lun Huang, M.D., Ph.D., Shi-Jye Chu, M.D.

## ABSTRACT

**Background:** Evidence reveals that histone deacetylase (HDAC) inhibition has potential for the treatment of inflammatory diseases. The protective effect of HDAC inhibition involves multiple mechanisms. Heme oxygenase-1 (HO-1) is protective in lung injury as a key regulator of antioxidant response. The authors examined whether HDAC inhibition provided protection against ischemia–reperfusion (I/R) lung injury in rats by up-regulating HO-1 activity.

**Methods:** Acute lung injury was induced by producing 40 min of ischemia followed by 60 min of reperfusion in isolated perfused rat lungs. The rats were randomly allotted to control group, I/R group, or I/R + valproic acid (VPA) group with or without an HO-1 activity inhibitor (zinc protoporphyrin IX) (n = 6 per group).

**Results:** I/R caused significant increases in the lung edema, pulmonary arterial pressure, lung injury scores, tumor necrosis factor-α, and cytokine-induced neutrophil chemoattractant-1 concentrations in bronchoalveolar lavage fluid. Malondialdehyde levels, carbonyl contents, and myeloperoxidase-positive cells in lung tissue were also significantly increased. I/R stimulated the degradation of inhibitor of nuclear factor-κB-α, nuclear translocation of nuclear factor-κB, and up-regulation of HO-1 activity. Furthermore, I/R decreased B-cell lymphoma-2, heat shock protein 70, acetylated histone H3 protein expression, and increased the caspase-3 activity in the rat lungs. In contrast, VPA treatment significantly attenuated all the parameters of lung injury, oxidative stress, apoptosis, and inflammation. In addition, VPA treatment also enhanced HO-1 activity. Treatment with zinc protoporphyrin IX blocked the protective effect of VPA.

**Conclusions:** VPA protected against I/R-induced lung injury. The protective mechanism may be partly due to enhanced HO-1 activity following HDAC inhibition. **(ANESTHESIOLOGY 2015; 122:1327–37)**

THE state of histone acetylation/deacetylation balance influences epigenetic modification, which regulates gene expression and has a key role in cell regulation, migration, proliferation, and survival.[1] Reversible histone acetylation is controlled dynamically by histone acetyltransferases and histone deacetylases (HDAC). Histone acetyltransferases relax the chromatin structure by acetylating lysine residues.[1] They increase transcription factor binding to nucleosomal DNA and thus enhance transcription. In contrast, HDAC removes acetyl groups from histones (and other nuclear proteins), tightens the chromatin structure, and suppresses transcription. Thus, HDACs have become therapeutic targets potentially reversing aberrant acetylation during cellular events.[1]

HDAC inhibitors (HDACIs) have shown positive therapeutic potential in animal models of inflammatory and autoimmune diseases including hepatitis, systemic lupus erythematosus, and many neurodegenerative conditions.[2] In most of these models, HDAC inhibition is associated with decreased production of inflammatory mediators. Further research has shown that HDACIs improved survival and attenuated inflammation in animal models of hemorrhagic shock, polytrauma, and septic shock through modulation of cellular signaling pathways including the mitogen-activated protein kinase cascade.[3] Nonetheless, the knowledge of epigenetic modifications due to histone acetylation in lung

### What We Already Know about This Topic

- Lung injury occurs when perfusion is stopped (40 min) to isolated lungs and then restarted (60 min). Some of these manifestations of lung injury include edema, increased inflammatory cytokines in the airspaces, and up-regulation of heme oxygenase-1 activity.

### What This Article Tells Us That Is New

- The administration of valproic acid decreased all the parameters of lung injury, oxidative stress, apoptosis, and inflammation and some of its protection appeared to occur by increasing heme oxygenase-1 activity.

Drs. Huang and Chu contributed equally to this work.

Submitted for publication July 10, 2014. Accepted for publication January 10, 2015. From Institute of Aerospace and Undersea Medicine, National Defense Medical Center, Taipei, Taiwan, Republic of China (S.-Y.W., K.-L.H.); Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, Tri-Service General Hospital, Taipei, Taiwan, Republic of China (S.-E.T., K.-L.H.); Department of Internal Medicine, Taoyuan Armed Forces General Hospital, Taoyuan, Taiwan, Republic of China (F.-C.K., G.-C.W.); and Department of Internal Medicine, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan, Republic of China (S.-J.C.).

 Anesthesiology 2015; 122:1327-37

injury is limited. Fukudome *et al.*[4] reported that valproic acid (VPA) attenuated hemorrhage-induced acute lung injury with decreased production of cytokine-induced neutrophil chemoattractant-1 (CINC-1). Moreover, Ni *et al.*[5] showed that HDACIs attenuated lipopolysaccharide-induced lung injury by inhibiting inflammatory cytokine production and nuclear factor (NF)-κB activation. Recently, Kim *et al.*[6] demonstrated that VPA protected against acute lung injury induced by intestinal ischemia–reperfusion (I/R) in rats. Currently, the role of HDACIs in I/R-induced lung injury is not well understood. In addition, oxidative stress induces the antioxidant enzyme heme oxygenase-1 (HO-1) to maintain oxidative/antioxidant homeostasis. Induction of HO-1 activity exerts a significant protective effect in animal models of lung injury induced by I/R.[7] Therefore, we hypothesized that VPA, a broad-spectrum HDACI, could mitigate I/R-induced lung injury and HO-1 contributed to the beneficial effect of VPA.

## Materials and Methods

### Preparation of Isolated Perfused Lung

Care of the rats used in this study met the guidelines set forth by the National Institutes of Health (National Academy Press 1996). The National Science Council (Taiwan, Republic of China), and Animal Review Committee of the National Defense Medical Center approved the study protocol. The isolated rat lungs were prepared using previously described methods.[8,9] In brief, Sprague–Dawley male rats weighing 350 ± 20 g were anesthetized using intraperitoneal sodium pentobarbital (50 mg/kg). After a cannula had been inserted into the rat's trachea, the lungs were ventilated with humidified air containing 5% $CO_2$. The ventilator settings included a tidal volume of 3 ml, an end-expiratory pressure of 1 cm $H_2O$, and a frequency of 60 cycles per minute. After a median sternotomy, the right ventricle was injected with heparin (1 U/g of body weight [BW]), and 10 ml of intracardiac blood were withdrawn. The pulmonary artery was cannulated and perfused with a physiological salt solution consisting of 119 mM NaCl, 4.7 mM KCl, 1.17 mM $MgSO_4$, 22.6 mM $NaHCO_3$, 1.18 mM $KH_2PO_4$, 1.6 mM $CaCl_2$, 5.5 mM glucose, and 50 mM sucrose. Bovine albumin was added at a concentration of 4 g/dl to maintain the osmolarity of the perfusate. A cannula was inserted into the left ventricle and passed into the left atrium to collect the effluent perfusate for recirculation. The 10 ml of collected blood was added to the perfusate before initiating recirculation with the "half-blood" solution. The perfusion rate of the roller pump was set at 8 to 10 ml/min. The recirculating perfusate–blood solution with the isolated lungs *in situ* was placed on an electronic balance. The left atrial pressure, representing the pulmonary venous pressure (PVP), was monitored from a side arm of the outflow cannula, whereas the pulmonary arterial pressure (PAP) was monitored in the side arm of the inflow cannula.

### Microvascular Permeability

A microvascular permeability index to water ($K_f$) was calculated from the change in lung weight (LW) caused by the increase in venous pressure. The PVP quickly increased by 10 cm $H_2O$ and remained increased for at least 7 min. The slow, steady phase of the weight gain was plotted on semilogarithmic paper as a function of time ($\Delta W/\Delta T$). The slow component of the function was then extrapolated to time 0 to obtain the initial rate of transcapillary filtration. $K_f$ was defined as the *y*-intercept of the plot (in g/min) divided by the PVP (10 cm $H_2O$) and LW, and it was expressed in whole units of $g \cdot min^{-1} \cdot cm\ H_2O^{-1} \times 100\ g$.[10]

### LW/BW and Wet/Dry Weight Ratios

The right lung was removed after the experiments at the hilar region. The wet LW was then determined, and the LW/BW ratio was calculated. For the dry weight, a part of the right upper lung lobe was dried for 48 h at 60°C in an oven, and the wet/dry (W/D) weight ratio was calculated.

### Cell Counts, Protein Concentration, CINC-1, and Tumor Necrosis Factor-α Levels in Bronchoalveolar Lavage Fluid

At the end of the experiment, bronchoalveolar lavage fluid (BALF) was obtained by rinsing the left lung with 2.5 ml of saline twice. The BALF was then centrifuged at 200*g* for 10 min. The protein level in the supernatant was measured using a bicinchoninic acid test (Pierce, USA). Tumor necrosis factor (TNF)-α and CINC-1 levels in the BALF were quantified using an enzyme-linked immunosorbent assay kit (R&D Systems Inc., USA). Differential and total cell counts in the BALF were assessed as described previously.[11]

### Malondialdehyde Level and Protein Carbonyl Content in Lung Tissue

The lung tissue was homogenized in a 1.15% KCl aqueous solution. A 100 μl aliquot of the homogenized lung tissue was mixed into a solution of 200 μl of 8.1% thiobarbituric acid and 700 μl of distilled water. The mixture was then boiled for 30 min at 100°C and centrifuged at 3,000*g* for 10 min. The malondialdehyde content of the supernatant was measured by absorbance at 532 nm and was expressed as nmol/mg protein. The oxidative damage to the proteins in the lung tissue was assessed by determining the carbonyl group content based on a reaction with dinitrophenylhydrazine as previously described.[9] The carbonyl content was determined from the absorbance at 370 nm assuming a molar absorption coefficient of 220,000 $M^{-1}$ and was expressed as the concentration of carbonyl derivatives in the protein (nmol carbonyl/mg protein).[9]

### Western Blot Analysis

Cytoplasmic and nuclear proteins were extracted according to the manufacturer's instructions (Nuclear/Cytosol Extraction kit; BioVision, Inc., USA) from frozen lung tissue samples and used for Western blot analysis. Protein levels in the extracts were quantified using a bicinchoninic acid protein assay (Pierce). Equal amounts of lung tissue homogenates (30 μg/lane) were electrophoresed on 10 to 12% sodium dodecyl

sulfate–polyacrylamide gels and moved to polyvinylidene fluoride membranes (Hybond; Amersham Biosciences, USA). The membranes were then incubated in phosphate-buffered saline (PBS) with 0.1% Tween 20 (Sigma-Aldrich, USA) and 5% nonfat milk for 1 h at ambient temperature to prevent nonspecific staining. Primary antibodies against acetylated histone H3 diluted 1:250 (Upstate Biotechnology, USA), heat shock protein (Hsp) 70 diluted 1:500 (Santa Cruz Biotechnology, USA), B-cell lymphoma (Bcl)-2 diluted 1:200 (Santa Cruz Biotechnology), and NF-κB p65 and inhibitor of NF-κB (IκB)-α diluted 1:1,000 (Cell Signaling Technology, USA) were incubated at 4°C overnight. The blots were then washed in PBS with Tween 20 (0.1%) for 10 min and rewashed two more times. The blots were then incubated with horseradish peroxidase–linked anti-rabbit immunoglobulin G (diluted 1:40,000) or anti-mouse immunoglobulin G (diluted 1:50,000) at room temperature for 1 h. The blots were washed three times in PBS with Tween 20 (0.1%) for 10 min. The bands were developed using enhanced chemiluminescence and exposure to x-ray film. Next, the blots were stripped of antibodies and incubated again with an anti-TATA antibody (diluted 1:1,000) for detection of nuclear protein (Abcam, USA). Similarly, anti-β-actin antibody (diluted 1:10,000) was used for detection of cytoplasmic protein (Sigma-Aldrich, USA). The antibody dilutions ensured equivalent loading. The band intensity ratios were calculated.

### Immunohistochemical Studies

Formalin-fixed, 4-μm paraffin sections were deparaffinized before antigen retrieval. The slides were incubated for 15 min with a solution of 3% $H_2O_2$ in methanol to block endogenous peroxidase. The slides were then treated with primary rabbit polyclonal antibody to myeloperoxidase (1:100 dilution; Cell Signaling Technology) and the large activated fragment (17/19 kD) of caspase-3 (1:200 dilution; Cell Signaling Technology). The slides were washed and then incubated for 30 min with rat-specific horseradish peroxidase polymer anti-rabbit antibody (Nichirei Corporation, Japan). Horseradish peroxidase substrate was then added and allowed to react for 3 min, and the sections were then counterstained with hematoxylin.

### Measurement of HO-1 Activity

Heme oxygenase-1 activity was measured spectrophotometrically in lung tissue by the determination of the rate of appearance of bilirubin as previously described.[7] HO-1 activity is reported in units of picomoles of bilirubin per milligram of protein per hour.

### Histological Assessment

The lung tissue was histologically prepared and stained with hematoxylin and eosin. The numbers of polymorphonuclear neutrophils in the interstitium were counted in 10 high-power fields (×400) and averaged. Two pathologists examined a minimum of 10 randomly selected fields in a masked manner. Lung injury was scored using a four-point scale indicating none (0), mild (1), moderate (2), or severe (3) injury. The slides were examined for neutrophil infiltration or aggregation in the airspace or vessel wall, and thickening of the alveolar wall. The resulting two scores were added together for the lung injury score.[12]

### Experimental Protocols

For the VPA series, the rat lungs were randomly allocated to receive PBS (control, n = 6), 300 mg/kg VPA (drug control, n = 6), only I/R, or I/R with different doses of VPA (75 mg, 150 mg, or 300 mg/kg; n = 6 per group). VPA (sodium valproate; Sanofi Aventis Company, USA) was added to the reservoir (containing 20 ml of perfusate). The doses of VPA in this study were chosen according to a previous study.[13]

For the HO-1 activity inhibition series, 36 rats were randomized to receive the vehicle (n = 18, dimethyl sulfoxide; Sigma-Aldrich) or zinc protoporphyrin IX (ZnPP), a specific HO-1 inhibitor (n = 18; dose, 20 mg/kg; Sigma-Aldrich) by intraperitoneal injection 24 h before the experiment. The ZnPP dosage used was chosen based on our previous work showing that ZnPP-suppressed HO-1 activity at 20 mg/kg of BW.[7] The rat lungs were randomly assigned to the control (n = 6), I/R (n = 6), or VPA (300 mg/kg) + I/R (n = 6) groups for the HO-1 inhibitor experiment.

The isolated lungs were allowed to equilibrate for 20 min before starting. The baseline PAP, PVP, weight change, and the initial $K_f$ for 7 min were then measured. All parameters were equilibrated to baseline for 10 min after the measurements.

In I/R group, the lung preparations were kept at 25°C. After all parameters had returned to the baseline state, the lungs were deflated by stopping ventilation and perfusion to cause ischemia. They were maintained in the deflated state for 40 min. Perfusion and ventilation were resumed, and the $K_f$ was measured 60 min later.

### Statistical Analysis

The data are expressed as means ± SD. The nature of hypothesis testing was two tailed. For comparisons of LW gain and PAP between groups during 60 min of observation, a two-way ANOVA for repeated measurements was used followed by the *post hoc* Bonferroni test. The comparisons among the groups were performed by using one-way ANOVA followed by a *post hoc* Bonferroni test. Comparisons for $K_f$ within each group were performed using paired Student *t* tests. There were no missing data points in this experiment. The sample sizes in the current study were justified based on our previous works. The six animals in each group were appropriate to avoid intravariation in the experimental group. Significance was set at the *P* value less than 0.05. GraphPad Prism 6 (GraphPad Software, USA) was used to perform the statistical calculations.

## Results

### Effect of VPA on Acetylated Histone H3 Level

The acetylation of lys9 in histone H3, an index of HDAC inhibition, was measured, and VPA significantly increased the level of acetylated histone H3 in lung tissue when compared

with the I/R group (fig. 1). The HDAC inhibition was dose dependent. This result indicates that the dose of VPA in this study was sufficient to increase acetylation of histone protein.

### Effect of VPA on Lung Edema

Ischemia–reperfusion significantly increased LW gain (fig. 2A). This increase in the LW gain was attenuated by the VPA treatment in a dose-dependent manner. Moreover, the addition of ZnPP blocked the protective effect of VPA. I/R significantly increased $K_P$ LW/BW and W/D weight ratios, and protein levels in the BALF ($P < 0.05$; fig. 2, B–E); VPA treatment significantly reduced these increases in a dose-dependent manner. However, the addition of ZnPP blocked the protective effect of VPA.

### Effect of VPA on PAP

In the control group, the PAP had almost no change during the 100-min observation interval. In the I/R group, the PAP increased and then decreased to its lowest point 20 min after reperfusion. At 60 min after reperfusion, the PAP in the I/R group remained significantly higher than at baseline and that of the control group. Treatment with VPA significantly diminished the increase in this late stage of PAP increase in a dose-dependent manner. The protective effect of VPA was significantly blocked when ZnPP was added ($P < 0.05$; fig. 3).

### Effect of VPA on HO-1 Activity in Lung Tissue

Ischemia–reperfusion significantly increased HO-1 activity when compared with the control group ($P < 0.05$; fig. 4). Furthermore, VPA (300 mg/kg) treatment significantly enhanced lung HO-1 activity in comparison with the I/R group. In addition, ZnPP treatment reduced HO-1 activity significantly in the I/R plus VPA group ($P < 0.001$; fig. 4).

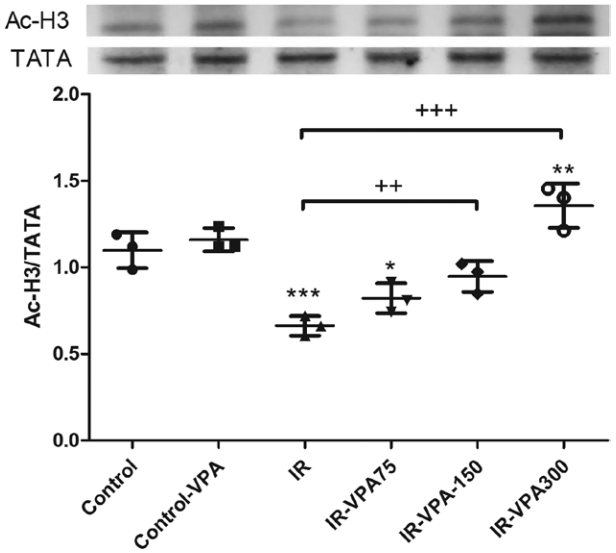


**Fig. 1.** Effect of valproic acid (VPA) on acetylated histone H3 (Ac-H3) expression in lung tissue. VPA up-regulated Ac-H3 expression in a dose-dependent manner in ischemia–reperfusion (I/R) lung injury. TATA served as loading controls for nuclear proteins. A representative blot is shown. Data are expressed as mean ± SD (n = 3 per group). ***$P < 0.001$ compared with the control group; +++$P < 0.001$ compared with the I/R–vehicle group. *$P < 0.05$; **$P < 0.01$; ++$P < 0.01$.

### Effect of VPA on TNF-α and CINC-1 Levels in BALF

The TNF-α and CINC-1 levels were significantly increased in the BALF of the I/R group in comparison with those in the control group (fig. 5). VPA (300 mg/kg) significantly inhibited the production of TNF-α and CINC-1 in the I/R group. ZnPP treatment abolished the protective effects of VPA.

### Effect of VPA on Malondialdehyde Level, Carbonyl Content, and Myeloperoxidase-positive Cells in Lung Tissue

In comparison to the control group, the I/R group had significantly increased malondialdehyde levels, carbonyl contents, and numbers of myeloperoxidase-positive cells in the lung tissue ($P < 0.001$; fig. 6, A–C). Treatment with VPA (300 mg/kg) significantly attenuated these increases ($P < 0.001$). However, the protective effects of VPA were abolished by treatment with ZnPP.

### Effect of VPA on Histopathology and Lung Injury Score

The lung histology disclosed obvious interalveolar septum thickening and increased inflammatory cell infiltration in the I/R group compared with the control group (fig. 7A). The VPA-treated group showed decreased neutrophil infiltration (fig. 7B), lung injury scores (fig. 7C), and neutrophil migration into the alveolar spaces (fig. 7D). In contrast, the protective effect of VPA was abolished by pretreatment with ZnPP.

### Effects of VPA on Hsp70, Bcl-2, and Caspase-3 Protein Expression

Hsp70 and Bcl-2 protein contents in lung tissue were substantially decreased in the I/R groups than in the control groups. However, both proteins were significantly increased upon VPA treatment. The protective effect of VPA was abolished by treatment with ZnPP (fig. 8, A and B). The intensity of activated caspase-3-immunolabeled cells was significantly greater in the I/R group than in the control group. VPA treatment significantly decreased immunolabeled cells, but the protective effect was reduced by the addition of ZnPP (fig. 8C).

### Effect of VPA on NF-κB Signaling Pathway

The nuclear level of NF-κB p65 was increased after I/R injury (fig. 9A), whereas the cytoplasmic level of IκB-α was decreased significantly in the I/R group compared with the control group (fig. 9B). VPA treatment restored suppressed IκB-α levels and reduced nuclear NF-κB p65 levels. Treatment with ZnPP counteracted the protective effect of VPA (fig. 9).

## Discussion

The current study demonstrated that acute lung injury induced by I/R significantly increased lung edema, PAP, neutrophil infiltration, inflammatory cytokine production, oxidative stress, apoptosis, IκB-α degradation, nuclear

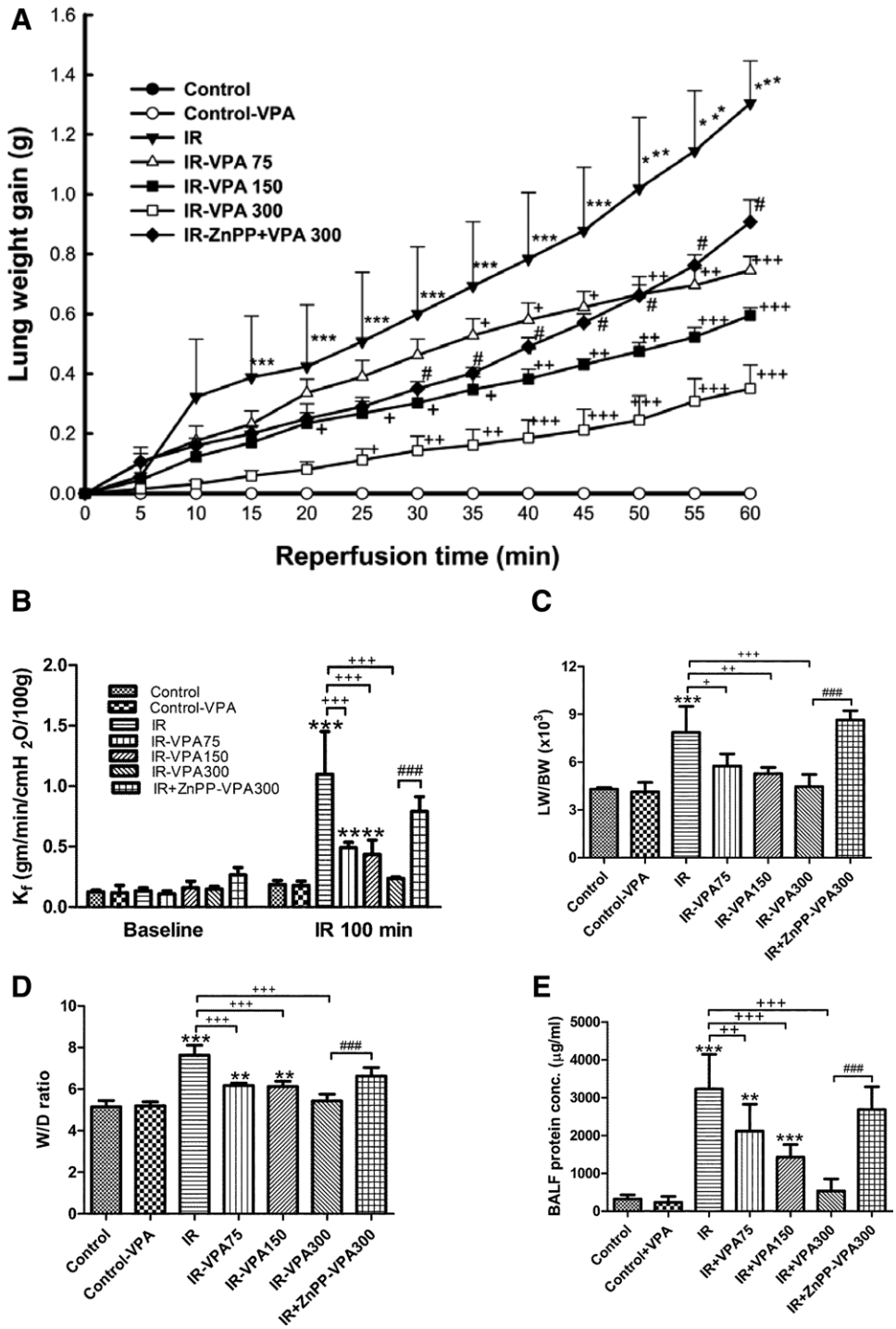


**Fig. 2.** Effect of valproic acid (VPA) and zinc protoporphyrin IX (ZnPP) on pulmonary edema. Lung weight gain (*A*), $K_f$ (*B*), lung weight/body weight (LW/BW) (*C*), and wet/dry (W/D) weight ratios (*D*), and protein concentration in bronchoalveolar lavage fluid (BALF) (*E*) increased significantly in the ischemia–reperfusion (I/R) group. The increase in these parameters was significantly attenuated by treatment with VPA. The protective effect of VPA was abrogated by ZnPP treatment. Data are expressed as mean ± SD (n = 6 per group). $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control group; $+P < 0.05$, $++P < 0.01$, $+++P < 0.001$ compared with the I/R–vehicle group; $\#P < 0.05$, $\#\#\#P < 0.001$ compared with the I/R + VPA 300 group.

translocation of NF-κB, and tissue injury. These effects were significantly attenuated by pretreatment with VPA. However, the protective effect of VPA was reduced by the HO-1 activity inhibitor, ZnPP. This indicates that VPA exerts its antiinflammatory effects partly through enhancing HO-1 activity. Our results suggest an important role for HDAC in I/R-induced lung injury and also that HO-1 could contribute to the protection provided by VPA.

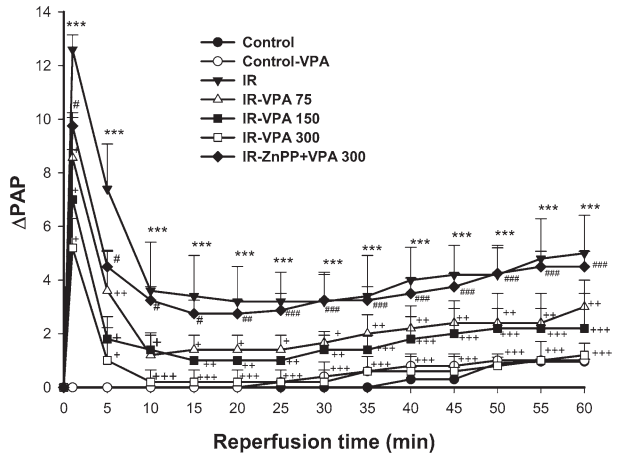


**Fig. 3.** Effect of valproic acid (VPA) and zinc protoporphyrin IX (ZnPP) on pulmonary artery pressure (ΔPAP). PAP increased significantly in the ischemia–reperfusion (I/R) group. The increase in PAP was attenuated significantly by treatment with VPA, but not when ZnPP was added. Data are expressed as mean ± SD (n = 6 per group). ***$P$ < 0.001 compared with the control group; +$P$ < 0.05, ++$P$ < 0.01, +++$P$ < 0.001 compared with the I/R–vehicle group; #$P$ < 0.05, ##$P$ < 0.01, ###$P$ < 0.001 compared with the I/R + VPA 300 group.

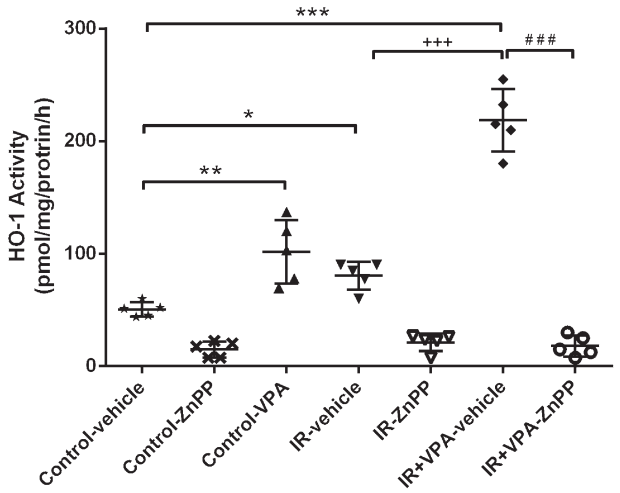


**Fig. 4.** Effect of valproic acid (VPA) and zinc protoporphyrin IX (ZnPP) on heme oxygenase-1 (HO-1) activity in lung tissue. Ischemia–reperfusion (I/R) significantly increased HO-1 activity. VPA further enhanced HO-1 activity. Treatment with ZnPP completely abolished HO-1 activity in both the absence and presence of VPA. Data are expressed as mean ± SD (n = 6 per group). *$P$ < 0.05, ***$P$ < 0.001 compared with the control group; +++$P$ < 0.001 compared with the I/R–vehicle group; ###$P$ < 0.001 compared with the I/R + VPA group. **$P$ < 0.01.

HDAC inhibitors have emerged as potent antiinflammatory therapeutic agents in a variety of diseases based on their ability to increase histone acetylation, alter the transcription of associated genes, and provide tissue protection.[1] After I/R injury, there was a significant decrease in the histone H3 acetylation in the lung tissue, similar to results from a previous study using a rat model of permanent ischemic stroke.[14]

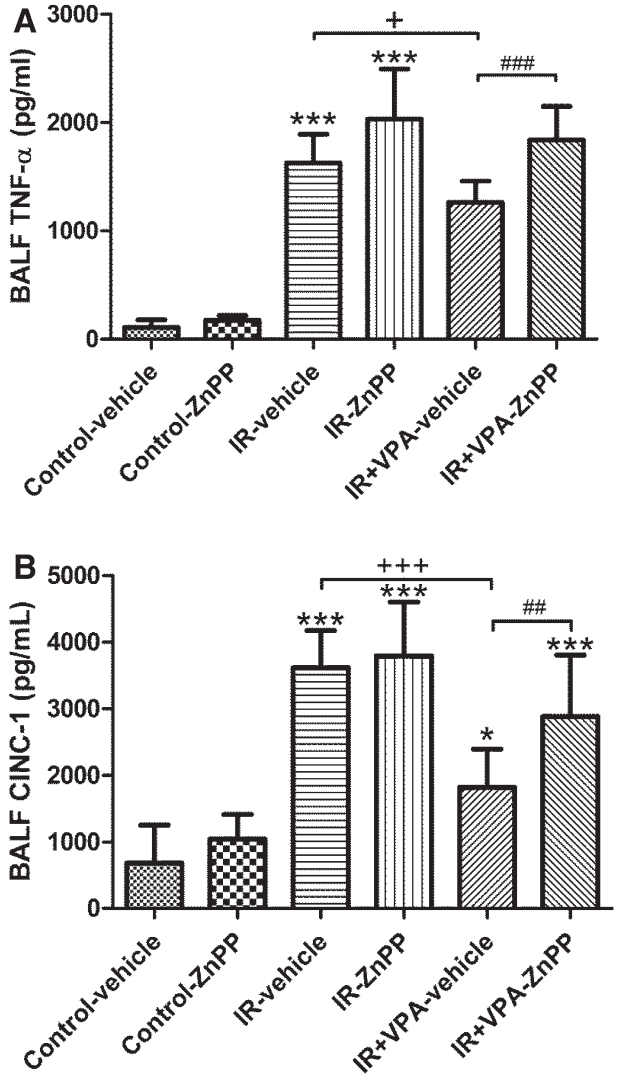


**Fig. 5.** Effect of valproic acid (VPA) and zinc protoporphyrin IX (ZnPP) on tumor necrosis factor (TNF)-α and cytokine-induced neutrophil chemoattractant (CINC)-1 levels in bronchoalveolar lavage fluid (BALF). TNF-α (*A*) and CINC-1(*B*) levels in the BALF increased significantly in the ischemia–reperfusion (I/R) group. The increases in the BALF were significantly attenuated by treatment with VPA. The protective effect of VPA was abrogated by ZnPP treatment. Data are expressed as mean ± SD (n = 6 per group). *$P$ < 0.05, ***$P$ < 0.001 compared with the control group; +$P$ < 0.05, +++$P$ < 0.001 compared with the I/R–vehicle group; ##$P$ < 0.01, ###$P$ < 0.001 compared with the I/R + VPA group.

This reduction in acetylated histone could be related to the suppression of histone acetyltransferase and/or activation of HDAC after an I/R insult. I/R-induced loss of histone acetylation was prevented by VPA treatment. These results indicate that HDAC activity was inhibited in the rat lungs by VPA treatment under the experimental conditions.

Neutrophil sequestration and transmigration in the lung interstitium and alveolar space are the primary characteristics of acute respiratory distress syndrome.[15] The number of infiltrating neutrophils was significantly associated with the

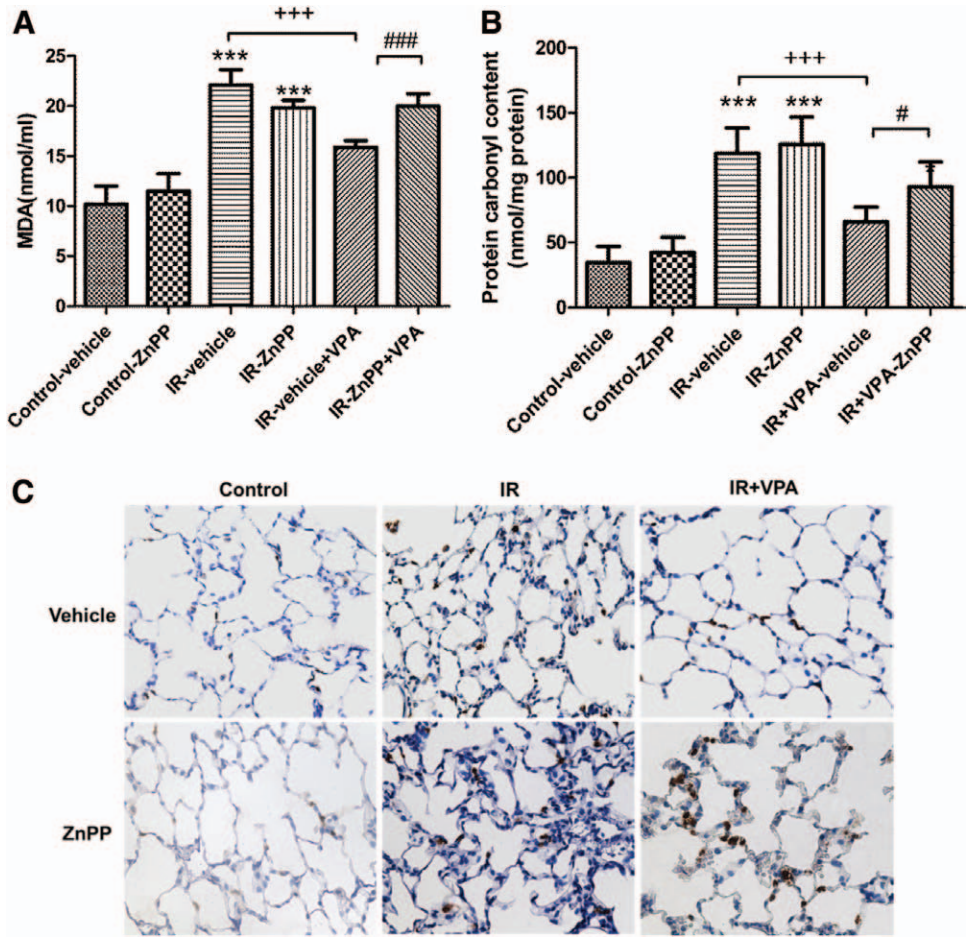


**Fig. 6.** Effect of valproic acid (VPA) and zinc protoporphyrin IX (ZnPP) on malondialdehyde (MDA) levels, carbonyl contents, and myeloperoxidase-positive cells in lung tissue. MDA level (*A*), carbonyl content (*B*), and myeloperoxidase-positive cells (*C*) in lung tissue significantly increased in the ischemia–reperfusion (I/R) group. VPA treatment significantly attenuated these increases. The protective effect of VPA was abrogated by ZnPP treatment. (*C*) Immunohistochemistry for myeloperoxidase in the lung (×200 magnification). Data are expressed as mean ± SD (n = 6 per group). ***$P < 0.001$ compared with the control group; +++$P < 0.001$ compared with the I/R–vehicle group; #$P < 0.05$, ###$P < 0.001$ compared with the I/R + VPA group.

severity of tissue damage after I/R injury.[15] Several studies also showed that suppression of neutrophil infiltration improved reperfusion injury and revealed that I/R lung injury was dependent on neutrophil recruitment.[15,16] The results demonstrated that I/R increased neutrophil infiltration in lung tissue, as evidenced by both increasing myeloperoxidase-positive cells and the number of neutrophils, both of which were attenuated by VPA treatment. This decreased the interaction between neutrophils and the endothelium and reduced the release of proinflammatory cytokines, generation of reactive oxygen species, and free radicals by activated neutrophils. Moreover, this VPA attenuation decreased pulmonary edema as indicated by the reduced $K_F$, lower W/D and LW/BW ratios, and decreasing the protein concentration in the BALF. These results agree with those of other investigations showing that HDACIs have the ability to attenuate vascular permeability and neutrophil infiltration in various models of acute lung injury.[4–6,17]

Investigators of clinical and experimental studies have suggested that the oxidative stress induced by reactive oxygen species plays an important role in I/R-induced lung injury.[9,18] In addition, neutrophil-derived oxygen radicals injure vascular endothelial cells both *in vitro* and *in vivo*, disrupt the functional integrity of the microvasculature, and increase fluid and protein flow from the vascular space into the interstitium.[18] Our results demonstrated that VPA prevented the protein carbonylation and peroxidation of membrane lipids during lung I/R. Similarly, other studies showed that VPA treatment also improved pulmonary oxidative damage and exerted antioxidative effects on the retina after I/R injury.[6,19]

The significance of cytokines in the pathogenesis of acute lung injury has been validated by the widespread use of anticytokine monoclonal antibodies.[20] Our experiment showed that the expression of inflammatory mediators such as TNF-α and CINC-1 in BALF were up-regulated after I/R-induced lung injury. In parallel with this result, activated caspase-3-immunolabeled cells also increased accompanying the decreased expression of Hsp70 and Bcl-2 proteins after

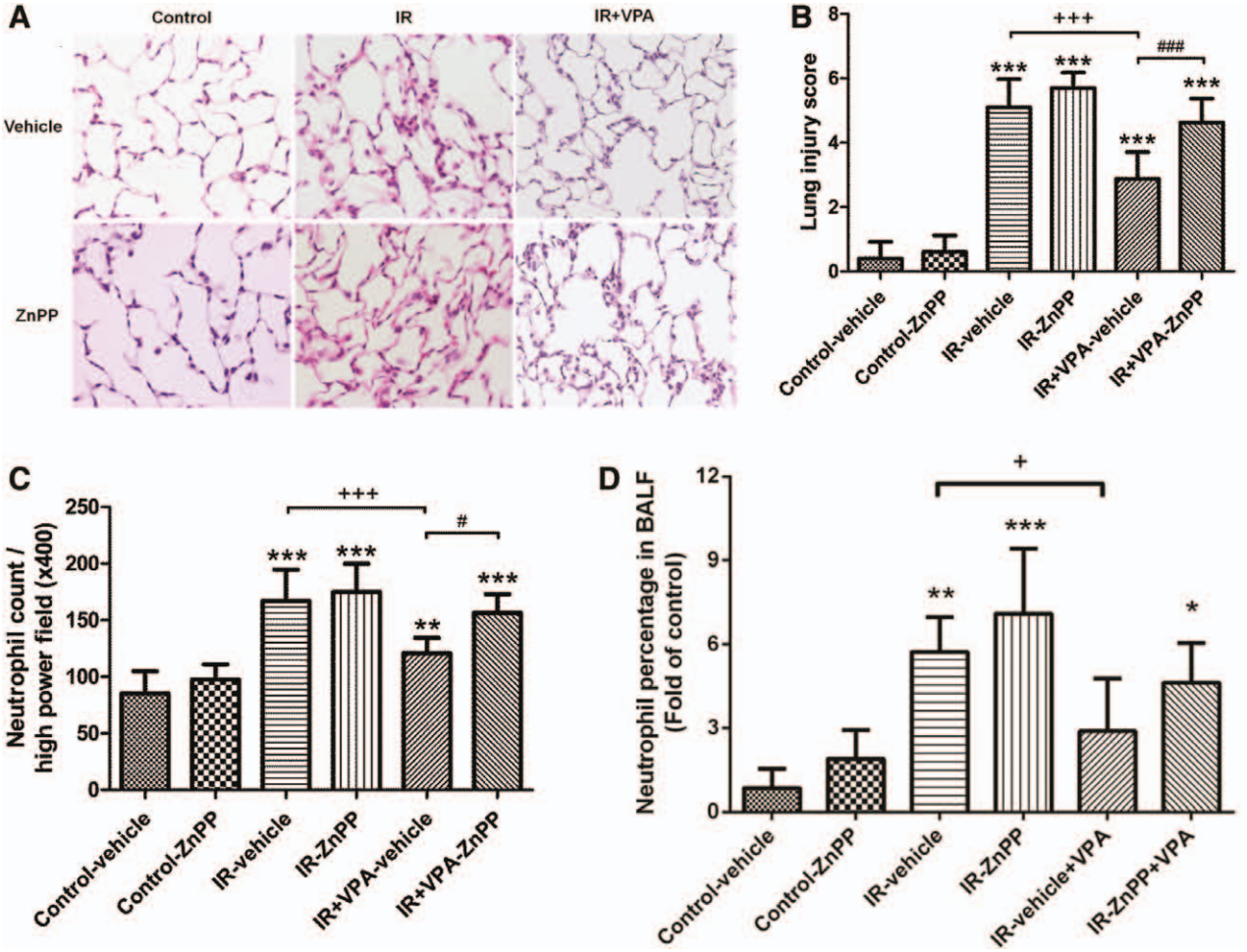


**Fig. 7.** Effect of valproic acid (VPA) and zinc protoporphyrin IX (ZnPP) on inflammatory cell infiltration and the histological appearance of lung tissue. As shown by a representative micrograph of lung tissue (×400 magnification) (*A*), infiltrating neutrophils and sepal widening were increased in the ischemia–reperfusion (I/R) group. VPA treatment improved the histopathological changes, but the improvement was abolished by ZnPP treatment. The lung injury scores (*B*), the numbers of neutrophils per high power field (×400 magnification) (*C*), and the percentage of neutrophils in bronchoalveolar lavage fluid (BALF) (*D*) were significantly higher in the I/R group than in the control group. VPA treatment significantly decreased the rise, but these improvements were abolished by ZnPP treatment. Data are expressed as mean ± SD (n = 6 per group). $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control group; $+++P < 0.001$ compared with the I/R–vehicle group; $\#P < 0.05$, $\#\#\#P < 0.001$ compared with the I/R + VPA group. $^{*}P < 0.05$; $+P < 0.05$.

lung injury. Furthermore, VPA significantly attenuated the increased levels of inflammatory mediators and apoptotic cell death. Indeed, HDACIs reduce proinflammatory cytokine production as well as inhibit cytokine effects.[1,3] Our data were also comparable to that of several investigations showing that the HDACI-induced neuroprotection is due to its antiapoptotic effects. In the rodent model of middle cerebral artery occlusion, HDACIs suppressed ischemia-induced neuronal caspase-3 activation and increased antiapoptotic molecule expression of Hsp70 and Bcl-2, both of which protected against ischemic neuronal death.[14,21] However, further studies are required to examine the VPA-associated epigenetic control mechanisms of proinflammatory cytokines and apoptotic processes.

Nuclear factor-κB is essential for activating the transcription of a proinflammatory cascade of cytokines and chemokines to induce early inflammatory responses. NF-κB-modulated gene expression is also regulated by posttranscriptional modifications, such as phosphorylation and acetylation, which can be altered upon stimulation.[3] NF-κB activation is tightly regulated by its endogenous inhibitor, IκB, which complexes with NF-κB in the cytoplasm. Previous studies showed that I/R lung injury degraded IκB and induced NF-κB activation.[7,12] Our results demonstrated that VPA prevented nuclear translocation of NF-κB and inhibited the degradation of IκB as well as NF-κB-regulated gene expression. Indeed, HDACIs induce hyperacetylation and repress NF-κB signaling *in vitro* and *in vivo*.[3,5,22] The reduction of NF-κB translocation in macrophages within the lamina propria was beneficial in patients with ulcerative colitis treated with HDACI.[23] However, other investigators suggest that HDACIs enhanced NF-κB-dependent gene expression.[22] This indicates that the

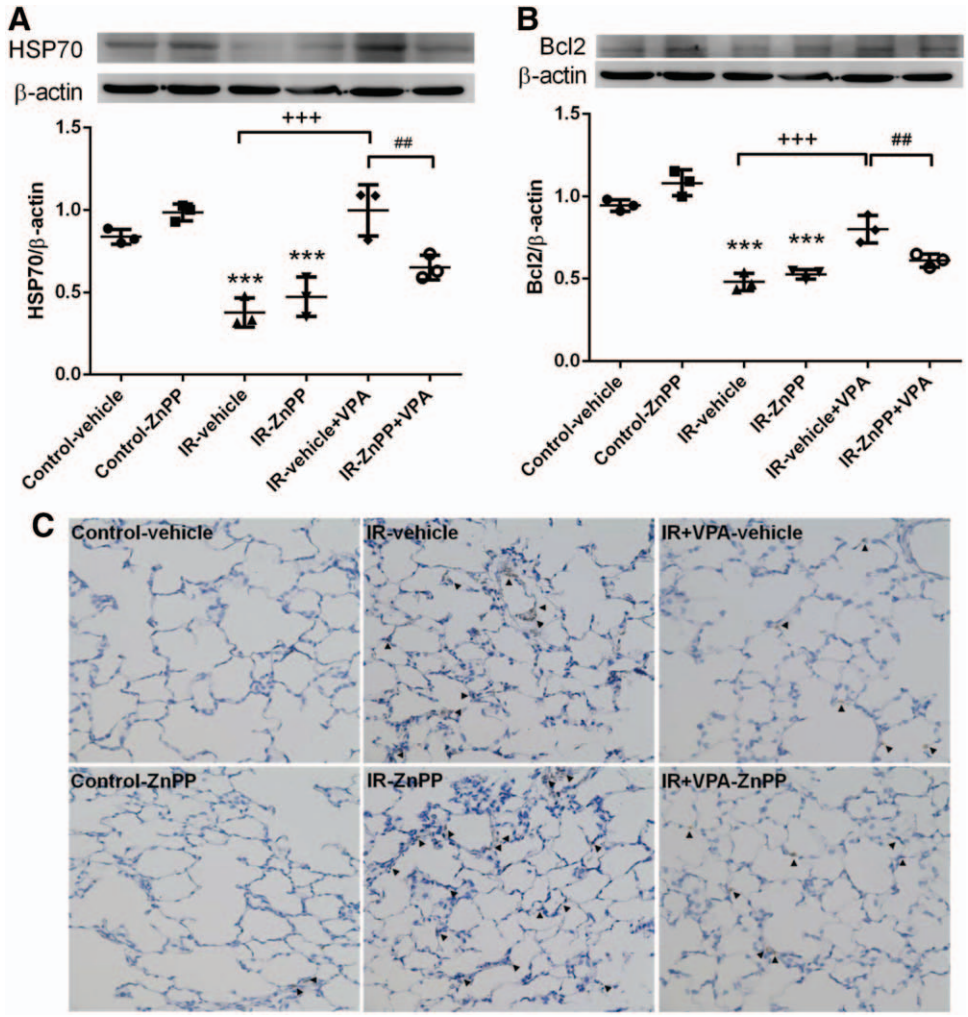


**Fig. 8.** Effect of valproic acid (VPA) and zinc protoporphyrin IX (ZnPP) on the expression of heat shock protein (Hsp) 70, B-cell lymphoma (Bcl)-2, and caspase-3 in the lung tissue. (*A* and *B*) Western blot and densitometry analysis of Hsp70 and Bcl-2 protein in the lung tissue. β-Actin served as loading control for cytoplasmic proteins. (*C*) Immunohistochemistry for active caspase-3 in the lung (indicated with *arrowhead*) (×200 magnification). Ischemia–reperfusion (I/R) significantly decreased Hsp70 and Bcl-2 protein expression and induced caspase-3 activation in the lung tissue. VPA treatment significantly increased Hsp70 and Bcl-2 protein expression and attenuated the signals for active caspase-3. When ZnPP was added, the protective effect was blocked. Representative blots are shown. Data are expressed as mean ± SD (n = 3 per group). ***$P < 0.001$ compared with the control group; +++$P < 0.001$ compared with the I/R–vehicle group; ##$P < 0.01$ compared with the I/R + VPA group.

effects of both histone acetyltransferases and HDAC inhibition depend on acetylation/deacetylation of NF-κB at different lysine residues and the cell type.[22] The precise molecular mechanisms by which HDAC inhibition exerts its effects in these models remain to be elucidated.

The protective effects of VPA involve multiple mechanisms. HDACIs not only directly modulate acetylation of nuclear histones but other nonhistone proteins as well, including regulatory proteins involved in the gene expression, cell death (apoptosis), autophagy, cell cycle progression, redox pathways, DNA repair, cell migration, and angiogenesis.[1,3,24] Furthermore, other mechanisms still need clarification. Our results showed that VPA treatment enhanced HO-1 activity in I/R lung injury. HO-1 is the inducible form of HO and the rate-limiting enzyme in heme degradation. Many studies revealed that HO-1 has a much larger role in tissue damage and protection, including antioxidant, antiapoptotic, and antiinflammatory actions. In the current study and previous experiments, I/R alone resulted in increased HO-1 enzyme activity.[7] VPA further enhanced HO-1 activity and most importantly, it attenuated I/R-induced lung injury. In contrast, HO-1 inhibitor reversed the protective effect of VPA. Our results agree with those of previous investigations that increased HO-1 activity due to ischemic postconditioning, administration of HO-1 adenovirus, and hypercapnic acidosis reduces lung reperfusion injury.[7,25,26] Furthermore, two recent studies demonstrated that HDACIs significantly increased HO-1 expression in the brain, cardiomyocytes, and hearts subjected to ischemic injury.[27,28] In addition, HO-1-deficient mice were unprotected by pharmacologic inhibition of HDAC after cerebral ischemia.[28]

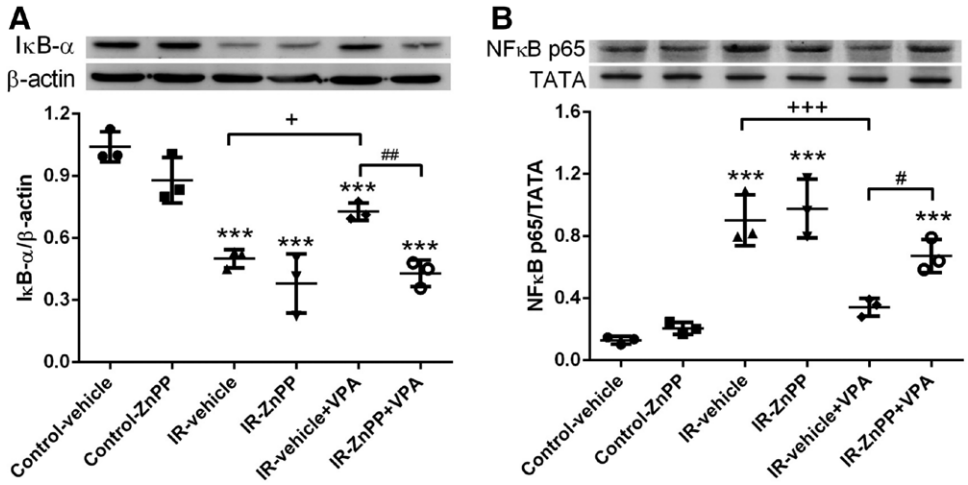


**Fig. 9.** Effect of valproic acid (VPA) and zinc protoporphyrin IX (ZnPP) on the expression of nuclear levels of nuclear factor (NF)-κB p65 and cytoplasmic levels of inhibitor of NF-κB (IκB)-α in the lung tissues. VPA increased IκB-α level (*A*) and reduced nuclear NF-κB p65 level (*B*) in ischemia–reperfusion (I/R)–induced lung injury. ZnPP treatment partially attenuated the protective effect of VPA. TATA and β-actin served as loading controls for nuclear and cytoplasmic proteins, respectively. Representative blots are shown. Data are expressed as mean ± SD (n = 3 per group). ***$P < 0.001$ compared with the control group; +++$P < 0.001$ compared with the I/R–vehicle group; #$P < 0.05$, ##$P < 0.01$ compared with the I/R + VPA group. +$P < 0.05$.

Valproic acid is widely used for epilepsy therapy, bipolar disorder, and some painful neuropathies. It is well known to inhibit HDAC classes I and II, but not class III.[3] In addition, different HDACIs have particular actions on individual acetylation sites, which could provide unique therapeutic effects. Currently, 18 different HDACs have been identified in mammals.[1–3] A recent study showed that HDAC 7 represented a potential inflammatory disease target to enhance the Toll-like receptor-4–dependent inflammatory response.[29] However, the roles of the different HDAC isoforms and HDACIs in I/R-induced lung injury remain poorly understood. Therefore, we believe that further investigations will focus on identifying the functions of the different HDAC enzymes and investigating isoform-specific HDACIs in animal models of acute respiratory distress syndrome.

In summary, we demonstrated that VPA attenuated lung I/R injury by decreasing lung edema, production of inflammatory cytokines, reactive oxygen species, NF-κB signaling, and apoptosis, whereas it enhanced HO-1 activity. The protective effect of VPA was mitigated by the presence of ZnPP, a HO-1 activity inhibitor. These data support the notion that VPA-induced HO-1 expression is responsible, at least in part, for the beneficial effects of VPA. Our results suggest that VPA provides effective treatments for I/R-induced acute lung injury. Further studies should provide greater knowledge for a better understanding of the protective mechanism of individual HDAC isoform inhibitors.

## Acknowledgments

This work was supported, in part, by grants NSC-101-2314-B-016-039 and NSC-102-2314-B-016-034 from the National Science Council, Taipei, Taiwan, Republic of China; TSGH-C103-073 from Tri-Service General Hospital, Taipei, Taiwan, Republic of China; grants 10202 and 10301 from Taoyuan Armed Forces General Hospital, Taoyuan, Taiwan, Republic of China; and MAB-102–34, MAB-102–103, and 103-M051 from the National Defense Medical Center, Taipei, Taiwan, Republic of China.

## Competing Interests

The authors declare no competing interests.

## Correspondence

Address correspondence to Dr. Chu: Department of Internal Medicine, Tri-Service General Hospital, National Defense Medical Center, No. 325, Sec. 2, Chenggong Road, Neihu 114, Taipei, Taiwan, Republic of China. d1204812@mail.ndmctsh.edu.tw. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. Anesthesiology's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

## References

1. Dinarello CA, Fossati G, Mascagni P: Histone deacetylase inhibitors for treating a spectrum of diseases not related to cancer. Mol Med 2011; 17:333–52
2. Halili MA, Andrews MR, Sweet MJ, Fairlie DP: Histone deacetylase inhibitors in inflammatory disease. Curr Top Med Chem 2009; 9:309–19
3. Li Y, Alam HB: Modulation of acetylation: Creating a pro-survival and anti-inflammatory phenotype in lethal hemorrhagic and septic shock. J Biomed Biotechnol 2011; 2011:523481
4. Fukudome EY, Li Y, Kochanek AR, Lu J, Smith EJ, Liu B, Kim K, Velmahos GC, deMoya MA, Alam HB: Pharmacologic resuscitation decreases circulating cytokine-induced neutrophil chemoattractant-1 levels and attenuates hemorrhage-induced acute lung injury. Surgery 2012; 152:254–61
5. Ni YF, Wang J, Yan XL, Tian F, Zhao JB, Wang YJ, Jiang T: Histone deacetylase inhibitor, butyrate, attenuates

lipopolysaccharide-induced acute lung injury in mice. Respir Res 2010; 11:33

6. Kim K, Li Y, Jin G, Chong W, Liu B, Lu J, Lee K, Demoya M, Velmahos GC, Alam HB: Effect of valproic acid on acute lung injury in a rodent model of intestinal ischemia reperfusion. Resuscitation 2012; 83:243–8
7. Wu SY, Li MH, Ko FC, Wu GC, Huang KL, Chu SJ: Protective effect of hypercapnic acidosis in ischemia-reperfusion lung injury is attributable to upregulation of heme oxygenase-1. PLoS One 2013; 8:e74742
8. Chu SJ, Chang DM, Wang D, Chen YH, Hsu CW, Hsu K: Fructose-1,6-diphosphate attenuates acute lung injury induced by ischemia-reperfusion in rats. Crit Care Med 2002; 30:1605–9
9. Peng CK, Huang KL, Wu CP, Li MH, Hu YT, Hsu CW, Tsai SH, Chu SJ: Glutamine protects ischemia-reperfusion induced acute lung injury in isolated rat lungs. Pulm Pharmacol Ther 2011; 24:153–61
10. Chang H, Li MH, Chen CW, Yan HC, Huang KL, Chu SJ: Intravascular FC-77 attenuates phorbol myristate acetate-induced acute lung injury in isolated rat lungs. Crit Care Med 2008; 36:1222–9
11. Tang SE, Wu CP, Wu SY, Peng CK, Perng WC, Kang BH, Chu SJ, Huang KL: Stanniocalcin-1 ameliorates lipopolysaccharide-induced pulmonary oxidative stress, inflammation, and apoptosis in mice. Free Radic Biol Med 2014; 71:321–31
12. Wu SY, Wu CP, Kang BH, Li MH, Chu SJ, Huang KL: Hypercapnic acidosis attenuates reperfusion injury in isolated and perfused rat lungs. Crit Care Med 2012; 40:553–9
13. Kochanek AR, Fukudome EY, Li Y, Smith EJ, Liu B, Velmahos GC, deMoya M, King D, Alam HB: Histone deacetylase inhibitor treatment attenuates MAP kinase pathway activation and pulmonary inflammation following hemorrhagic shock in a rodent model. J Surg Res 2012; 176:185–94
14. Kim HJ, Rowe M, Ren M, Hong JS, Chen PS, Chuang DM: Histone deacetylase inhibitors exhibit anti-inflammatory and neuroprotective effects in a rat permanent ischemic model of stroke: Multiple mechanisms of action. J Pharmacol Exp Ther 2007; 321:892–901
15. Williams AE, Chambers RC: The mercurial nature of neutrophils: Still an enigma in ARDS? Am J Physiol Lung Cell Mol Physiol 2014; 306:L217–30
16. Adkins WK, Taylor AE: Role of xanthine oxidase and neutrophils in ischemia-reperfusion injury in rabbit lung. J Appl Physiol (1985) 1990; 69:2012–8
17. Ximenes JC, de Oliveira Gonçalves D, Siqueira RM, Neves KR, Santos Cerqueira G, Correia AO, Félix FH, Leal LK, de Castro Brito GA, da Graça Naffah-Mazzacorati M, Viana GS: Valproic acid: An anticonvulsant drug with potent antinociceptive and anti-inflammatory properties. Naunyn Schmiedebergs Arch Pharmacol 2013; 386:575–87
18. McCord JM: Oxygen-derived free radicals in postischemic tissue injury. N Engl J Med 1985; 312:159–63
19. Zhang Z, Qin X, Zhao X, Tong N, Gong Y, Zhang W, Wu X: Valproic acid regulates antioxidant enzymes and prevents ischemia/reperfusion injury in the rat retina. Curr Eye Res 2012; 37:429–37
20. de Perrot M, Liu M, Waddell TK, Keshavjee S: Ischemia-reperfusion-induced lung injury. Am J Respir Crit Care Med 2003; 167:490–511
21. Faraco G, Pancani T, Formentini L, Mascagni P, Fossati G, Leoni F, Moroni F, Chiarugi A: Pharmacological inhibition of histone deacetylases by suberoylanilide hydroxamic acid specifically alters gene expression and reduces ischemic injury in the mouse brain. Mol Pharmacol 2006; 70:1876–84
22. Ghizzoni M, Haisma HJ, Maarsingh H, Dekker FJ: Histone acetyltransferases are crucial regulators in NF-κB mediated inflammation. Drug Discov Today 2011; 16:504–11
23. Lührs H, Gerke T, Müller JG, Melcher R, Schauber J, Boxberge F, Scheppach W, Menzel T: Butyrate inhibits NF-κB activation in lamina propria macrophages of patients with ulcerative colitis. Scand J Gastroenterol 2002; 37:458–66
24. Xie M, Kong Y, Tan W, May H, Battiprolu PK, Pedrozo Z, Wang ZV, Morales C, Luo X, Cho G, Jiang N, Jessen ME, Warner JJ, Lavandero S, Gillette TG, Turer AT, Hill JA: Histone deacetylase inhibition blunts ischemia/reperfusion injury by inducing cardiomyocyte autophagy. Circulation 2014; 129:1139–51
25. Xu B, Gao X, Xu J, Lei S, Xia ZY, Xu Y, Xia Z: Ischemic postconditioning attenuates lung reperfusion injury and reduces systemic proinflammatory cytokine release *via* heme oxygenase 1. J Surg Res 2011; 166:e157–64
26. Zhang X, Shan P, Jiang D, Noble PW, Abraham NG, Kappas A, Lee PJ: Small interfering RNA targeting heme oxygenase-1 enhances ischemia-reperfusion-induced lung apoptosis. J Biol Chem 2004; 279:10677–84
27. Kessler-Icekson G, Hochhauser E, Sinai T, Kremer A, Dick J, Tarasenko N, Nudelman V, Schlesinger H, Abraham S, Nudelman A, Rephaeli A: A histone deacetylase inhibitory prodrug—butyroyloxymethyl diethyl phosphate—protects the heart and cardiomyocytes against ischemia injury. Eur J Pharm Sci 2012; 45:592–9
28. Wang B, Zhu X, Kim Y, Li J, Huang S, Saleem S, Li RC, Xu Y, Dore S, Cao W: Histone deacetylase inhibition activates transcription factor Nrf2 and protects against cerebral ischemic damage. Free Radic Biol Med 2012; 52:928–36
29. Shakespear MR, Hohenhaus DM, Kelly GM, Kamal NA, Gupta P, Labzin LI, Schroder K, Garceau V, Barbero S, Iyer A, Hume DA, Reid RC, Irvine KM, Fairlie DP, Sweet MJ: Histone deacetylase 7 promotes Toll-like receptor 4-dependent proinflammatory gene expression in macrophages. J Biol Chem 2013; 288:25362–74