

Hypercapnic acidosis confers antioxidant and anti-apoptosis effects against ventilator-induced lung injury

Wan-Chao Yang¹, Chun-Yu Song¹, Nan Wang¹, Li-Li Zhang¹, Zi-Yong Yue^{1,2,3}, Xiao-Guang Cui^{1,2,3} and Hua-Cheng Zhou^{1,2,3}

Hypercapnic acidosis may attenuate ventilator-induced lung oxidative stress injury and alveolar cell apoptosis, but the underlying mechanisms are poorly understood. We examined the effects of hypercapnic acidosis on the role of apoptosis signal-regulating kinase 1 (ASK1), which activates the c-Jun N-terminal kinase (JNK) and p38 cascade in both apoptosis and oxidative reactions, in high-pressure ventilation stimulated rat lungs. Rats were ventilated with a peak inspiratory pressure (PIP) of 30 cmH₂O for 4 h and randomly given FiCO₂ to achieve normocapnia (PaCO₂ at 35–45 mm Hg) or hypercapnia (PaCO₂ at 80–100 mm Hg); normally ventilated rats with PIP of 15 cmH₂O were used as controls. Lung injury was quantified by gas exchange, microvascular leaks, histology, levels of inflammatory cytokines, and pulmonary oxidative reactions. Apoptosis through the ASK1-JNK/p38 mitogen-activated protein kinase (MAPK) cascade in type II alveolar epithelial cells (AECIIs) were evaluated by examination of caspase-3 activation. The results showed that injurious ventilation caused significant lung injury, including deteriorative oxygenation, changes of histology, and the release of inflammatory cytokines. In addition, the high-pressure mechanical stretch also induced apoptosis and caspase-3 activation in the AECIIs. Hypercapnia attenuated these responses, suppressing the ASK1 signal pathways with its downstream kinase phosphorylation of p38 MAPK and JNK, and caspase-3 activation. Thus, hypercapnia can attenuate cell apoptosis and oxidative stress damage in rat lungs during injurious ventilation, at least in part, due to the suppression of the ASK1-JNK/p38 MAPK pathways.

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INTRODUCTION

Mechanical ventilation can result in overdistension of the alveoli, leading to pulmonary edema, the release of cytokines and deterioration in oxygenation, which are described as ventilator-induced lung injury (VILI).^{1,2} The primarily biological mechanisms include the release of inflammatory mediators and oxygen radicals, leading to end-organ epithelial cell apoptosis and organ dysfunction.^{3–5}

In a previous study, a high tidal volume in mouse lung could induce the activation of c-Jun N-terminal kinase (JNK), an increase in macrophage inflammatory protein-2 (MIP-2) expression, and the migration of neutrophil into the lung.⁶ Activated JNK is dependent on the activation of

apoptosis signal-related kinase 1 (ASK1), which is identified as a member of the mitogen-activated protein kinase (MAPK) family, in cultured bronchial epithelium.^{7,8} Activation of ASK1 in turn leads to the activation of other components of MAPKs, such as JNK and p38, and results in an initiation of cell apoptosis.⁹ Another studies also demonstrated that large tidal volume ventilation-induced apoptosis was dependent, at least in part, in the ASK1/JNK/AP-1 pathway¹⁰ and a p38 MAP kinase-xanthine oxidoreductase-dependent pathway.¹¹

There is an evolving body of evidence suggesting that hypercapnic acidosis exerts biologically important beneficial effects in experimental models. Laffey *et al*^{12,13} showed that

¹Department of Anesthesiology, Second Affiliated Hospital of Harbin Medical University, Harbin, China; ²Anesthesiology Key Laboratory, Harbin Medical University, Education Department of Heilongjiang Province, Harbin, China and ³Anesthesiology Key Laboratory, Harbin Medical University, Harbin, China
Correspondence: Dr Z-Y Yue, MD, PhD, Department of Anesthesiology, Second Affiliated Hospital of Harbin Medical University, Harbin 150086, China.
E-mail: yueziyong@126.com

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therapeutic hypercapnia was applied to the animals by the deliberate elevation of CO₂, which also markedly reduced the extent of ischemia-reperfusion injuries. Subsequent studies have demonstrated hypercapnia's protective effects against VILI in both isolated perfused lungs and an animal model.^{14–16} The mechanism by which hypercapnic acidosis affects acute lung injury, includes the attenuation of NF- κ B activation,¹⁷ the inhibition of endogenous xanthine oxidase¹⁸ and oxygen radical formation,¹⁹ and the reducing release of the cytokines.^{12,20,21} However, it is still unclear whether hypercapnic acidosis may affect cell apoptosis and oxidative stress injury of the alveolar by MAPK pathways in VILI.

Therefore, the objectives of this study were to determine whether hypercapnia may attenuate the formation of mechanical stretch-induced oxygen radical, cell apoptosis, and the changes of a MAPK signal transduction of lungs in a rat model of high-pressure ventilation (HPV).

MATERIALS AND METHODS

Adult male Sprague Dawley (s.d.) rats, body weight 250–300 g, were bought from (The Depth of animals experiment, Harbin Medical University, China). The experimental protocols were approved by the Harbin Medical University Animal Care and Use Committee, Harbin, China.

Experimental Protocol

A rat model of VILI was established according the previous study.²² In brief, a tracheostomy was performed with an intraperitoneal injection of 30 mg/kg pentobarbital sodium (Abbott, North Chicago, IL, USA). The internal carotid artery was cannulated to take the blood samples for the blood gas analysis and to monitor the arterial pressure. Body temperature was maintained at 36.5–38.0 °C, anesthesia and supplemental fluids were administered intravenously for the requirement throughout the experiment. Mechanical ventilation was initiated in the pressure-controlled mode (Kent Scientific Ventilator-Dual Mode, USA) with a peak inspiratory pressure (PIP) of 15 cmH₂O, a positive end-expiratory pressure (PEEP) of 2 cm H₂O, a frequency of 30 breaths/min to maintain PaCO₂ at 35–45 mm Hg, an inspiration-to-expiration (I:E) ratio of 1:2, and an inhaled oxygen fraction (FiO₂) of 0.7 for 15 min, after which the baseline data were collected and the animals were randomly allocated to three ventilation strategies, each for 4 h, the groups were described as follows: (1) The control ventilation group (PIP 15 cmH₂O, PEEP 2 cmH₂O, frequency 30 min); *n* = 18, normocapnia (FiO₂ 0.7, FiCO₂ 0, balance N₂). (2) The injurious ventilation group (PIP 30 cmH₂O, PEEP 2 cmH₂O, frequency 30 min); *n* = 18, PaCO₂ was maintained in the normal range through inhaling the gas mixture (FiO₂ 0.7, FiCO₂ 4~5%, balance N₂). (3) The injurious ventilation/hypercapnia group (PIP 30 cmH₂O, PEEP 2 cmH₂O, frequency 30 min); *n* = 18, PaCO₂ was maintained in the target range of 80 mm Hg–100 mm Hg through inhaling the gas mixture (FiO₂ 0.7, FiCO₂ 11~12%, balance N₂). Tidal

volumes (V_T) were determined every 60 min with a VT Plus HF Gas Flow Analyzer (Fluke Corporation, USA). Inhaled and exhaled CO₂ and O₂ were tested using a gas monitor (DATEX Instrumentarium, Helsinki, Finland).

Tissue Sampling and Assays

At the end of the experiment, the abdomen and chest of each animal was opened under anesthetic. The left lung was isolated with surgical silk tied around the left main bronchus. The right lobe bronchus was lavaged with 4 ml of saline (0.9%, 4 °C) and 2–3 ml of bronchoalveolar lavage fluid (BALF) was collected. A 1.0-ml aliquot was used for differential cell counts. The remainder of the fluid was immediately centrifuged (3000 × *g* for 10 min) at 4 °C. The supernatant was stored at –20 °C for measurement of the inflammatory mediators and total protein concentration (BCA; Pierce, Rockford, IL, USA). The upper sections of the left lungs were excised, weighted, and desiccated at 70 °C for 1 week to measure the wet-to-dry weight ratio (W/D) for quantifying pulmonary edema formation. The one-third of left side lung tissue was homogenized and then centrifuged (4 °C), and the supernatant was stored frozen (–80 °C) to measure reactive oxygen species. The right side of rat lung tissue was snap frozen and used for protein extraction. Subsequently, the two-third of left side rat lung tissues were used for histology. Slices were embedded in paraffin, and sections (5 μm) were used for hematoxylin and eosin (H&E) and immunohistochemical staining.

Isolation of Rat Type II Alveolar Epithelial Cells

Type II alveolar epithelial cells (AECIIs) were isolated from four rats' lung tissues in each group according to established procedures.^{23,24} Briefly, the lungs were lavaged three times via the trachea with 5 ml of Dulbecco's PBS, and then were digested by instilling them with elastase (Worthington Biochem, Freehold, NJ, USA) via the trachea at a specific activity of 4.3 units/ml in JMEM mixed with 100 mg/ml DNase I (Sigma Chemical St Louis, MO, USA) and incubated at 37.8 °C for 35 min. The lungs were minced and the suspension was agitated on ice and filtered sequentially through nylon mesh with pore sizes of 150 μm and 70 μm. The AECIIs were separated from the macrophages by panning on rat IgG-coated Petri dishes and were identified using histochemical staining with alkaline phosphatase,^{25,26} and >90% of the cells were blue positive on day 2. Experiments were performed on the second day after the isolation.

Apoptosis Assay by FACS

The isolated cells were stained with FITC-conjugated Annexin V and propidium iodide (PI) using the Apoptosis Detection Kit (BD Biosciences Pharmingen, USA). Duplicate in each sample were run according to the manufacturer's instructions and analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA).

Measurement of Malondialdehyde Content, Activity of Superoxide Dismutase, Lactate Dehydrogenase, and Myeloperoxidase in the Supernatant of Lung Tissues

The malondialdehyde (MDA) content in the lung tissue was measured by the previous method²⁷ and expressed as nanomoles per milligram of protein. The superoxide dismutase (SOD) activity in the lung tissue was determined according to the previous²⁷ and expressed as units per milligram of protein. Serum lactate dehydrogenase (LDH) activity was measured by the pyruvic acid method²⁸ according to the manufacturer's instructions (Jiancheng Bio-Technology, Nanjing, China). The myeloperoxidase (MPO) activity of the lung tissue used a reagent kit (Jiancheng Bio-Technology, Nanjing, China) and the results were expressed as units per gram of wet lung tissue (U/g). Each measurement was performed in triplicate.

Histology and Immunohistochemistry

The lung sections were stained with H&E and then examined by a pathologist who was blinded to the protocol. Samples were assigned an injury score in each of four categories (interstitial edema, alveolar edema, neutrophil infiltration, and hyaline membrane formation) based on severity (0 = not present, 4 = severe, and present throughout). The overall histologic injury score (HIS) was evaluated according to a previous study.¹⁴

Immunostaining with an antibody against activated caspase-3 (Cell Signaling Technology, USA) was used to quantify the apoptosis in the lung sections.²⁹ The number of cleaved-caspase-3-positive cells was counted at the surface of the airway epithelial cells in 10 fields using the $\times 40$ objective lens.

Enzyme-Linked Immunoassays for Inflammatory Mediators and ASK1

To examine the levels of inflammation in the rat lung tissues, the levels of tumor necrosis factor (TNF)- α and MIP-2 were measured in the BALF by ELISA assay (R&D Systems and BioSource, USA).

The ASK1 phosphorylation expression of rat lung tissues was measured with a Phospho-ASK1 (S966) Sandwich ELISA Kit (ImmunoWay Biotechnology Company, USA). Duplicate in each sample were run according to the manufacturer's instructions.

Western Blot Analysis of JNK, p38 MAPK, and Cleaved Caspase-3

The AECIIs were harvested. The total cellular proteins were extracted by RIPA buffer (Radio-Immunoprecipitation Assay Buffer) containing 0.2% Triton X-100, 5 mmol/l EDTA, 1 mmol/l PMSF, 10 μ g/ml leupeptin, and 10 μ g/ml aprotinin, supplemented with 100 mmol/l NaF, 2 mmol/l Na_3VO_4 , and lysed for 30 min on ice. The total numbers of protein were quantified by using the Bradford method. The proteins in each sample were separated with 12% sodium dodecyl sulfate

polyacrylamide gel electrophoresis (SDS-PAGE) and then were transferred onto polyvinylidene fluoride (PVDF) transfer membranes. The transfer membranes were semidried for 20 min at 15 V. The membranes were washed with PBS three times. After blocking, the membranes were placed into $1 \times$ PBS with an anti-phosphorylated JNK, or an anti-phosphorylated p38 MAPK antibody (rabbit anti-rat) (1:1000), or an anti-cleaved caspase-3 antibody (Cell Signaling Technology) and incubated overnight at 4 °C in a slow shaker. The membranes were washed three times with PBS, and then, incubated the secondary antibody (goat anti-rabbit) (1: 2500) for 2 h. After incubation, the membranes were washed three times with 0.1% Tween in PBS. The membranes were incubated with the substrate for 5 min. The specific brand was quantitatively analyzed by using the Quantity one ChemiDocXRS Imagine Collect System and analysis software (Bio-Rad, USA).

Statistical Analysis

All data were expressed as mean + s.d. for normally distributed data and as the median (interquartile range) if they were non-normally distributed. The statistical analyses were analyzed with SPSS statistics software (version 13.01S; Beijing Stats Data Mining, Beijing, China). Group comparisons were evaluated by a one-way ANOVA, followed by the Student–Newman–Keuls test, or the Kruskal–Wallis test, followed by the Mann–Whitney *U*-test with the Bonferroni correction for multiple comparisons, as appropriate. The significance was set at $P < 0.05$.

RESULTS

Gas Exchange and Hemodynamic Parameters

There was no significant difference in the pH, PaO₂, PaCO₂, mean arterial pressure, and V_T at the baseline (Table 1). Our data showed that the PaO₂/FiO₂ and MAP in the high-pressure ventilation group decreased progressively after 2 h, and they were significantly lower than those values in the other two groups at the end of the protocol.

Mechanical Ventilation Induces Lung Injury *In Vivo* and is Attenuated by HCA

The rats were ventilated using high-pressure ventilation (PIP: 30 cmH₂O, 4 h) with low PEEP (2 cmH₂O) models, which impaired oxygenation and induced an acute lung inflammatory response, indicating that the levels of TNF- α and MIP-2 in the BALF were significantly higher in the HPV group compared with the control group (Table 1, $P < 0.05$). As shown in Figures 1a and b, BALF protein concentrations and the WW: DW in the injurious group were significantly reduced compared with the control group ($P < 0.05$). We further found that HPV resulted in an increase in the total cell count in the BALF (Figure 1c), and in significant neutrophil recruitment to the alveoli (Figure 1d). In addition, HPV resulted in histological changes marked by alveolar-septal thickening, hyaline membrane formation, and neutrophils

Table 1 Physiological conditions during ventilation

	Normocapnia control ventilation	Normocapnia injurious ventilation	Hypercapnia injurious ventilation
<i>PaCO₂</i> (mm Hg)			
Start	38.7 ± 2.2	38.3 ± 3.6	38.4 ± 4.2
2 h	37.4 ± 4.6	40.6 ± 4.8	95.1 ± 9.9*†,‡
End	35.3 ± 3.4	44.5 ± 4.6	90.7 ± 10.8*†,‡
<i>pH</i>			
Start	7.44 ± 0.03	7.43 ± 0.04	7.43 ± 0.07
2 h	7.42 ± 0.02	7.37 ± 0.03	7.11 ± 0.04*†,‡
End	7.44 ± 0.04	7.33 ± 0.07	7.09 ± 0.03*†,‡
<i>PaO₂/FiO₂</i> (mm Hg)			
Start	355.6 ± 30.9	343.6 ± 33.3	345.1 ± 28.5
2 h	335.8 ± 31.4	319.5 ± 29.4	335.5 ± 32.6
End	346.6 ± 27.4	229.9 ± 50.9*#	275.2 ± 62.6*†,‡
<i>V_T</i> (ml)			
Start	2.32 ± 0.15	2.38 ± 0.25	2.41 ± 0.18
2 h	2.33 ± 0.18	9.22 ± 0.74	9.85 ± 1.06
End	2.39 ± 0.18	5.88 ± 1.27*#	8.78 ± 1.32*†,‡
<i>MAP</i> (mm Hg)			
Start	129.6 ± 18.4	128.9 ± 14.6	133.1 ± 14.1
2 h	124.5 ± 14.6	118.5 ± 12.5	122.5 ± 13.6
End	128.9 ± 12.5	82.1 ± 14.9*#	96.4 ± 19.7*†,‡
BALFTNF-α (pg/ml)	75.12 ± 14.28	181.34 ± 45.3*	141.27 ± 34.5*†
BALFMIP-2 (pg/ml)	82.50 ± 13.70	681.5 ± 113.7*	322.9 ± 66.9*†

Abbreviations: BALF, bronchoalveolar lavage fluid; TNF-α, tumor necrosis factor-α; MIP-2, macrophage inflammatory protein-2; PIP, peak inspiratory pressure; *V_T*, tidal volume.

Values are the mean ± the s.d. *n* = 13 injurious ventilation and 10 control ventilation; **P* < 0.05 vs control ventilated rat with PIP of 15 cmH₂O; †*P* < 0.05 vs injurious ventilation with high pressure (PIP) of 30 cmH₂O; ‡*P* < 0.05 vs injurious ventilation with high pressure (PIP) of 30 cmH₂O; #*P* < 0.05 vs baseline.

infiltration (Figure 2). However, our data demonstrated that the damage changes were much lower in rat lungs from the hypercapnic acidosis group than the injurious group. The lung injury score was lower in the hypercapnia group (7.3 ± 1.6) than that in the injurious group (14.8 ± 2.7, *P* < 0.05), and higher than the control group (4.4 ± 1.3, *P* < 0.05). As described above, hypercapnic acidosis exhibited significant advantages in maintaining stable hemodynamic changes and systemic oxygenation, a decreasing

lung microvascular permeability (Figures 1a and b), attenuated lung-scores (Figure 2), and the decreasing inflammatory injury induced by HPV (Table 1).

Effect of HCA on Oxidative Stress Injury in the Rat Lung Tissues Induced by HPV

The levels of MDA was significantly increased and the level of SOD was significantly decreased in the HPV group when compared with the control group (*P* < 0.05). HCA caused a marked high activities of SOD and a low level of MDA (Figures 3a and b). The LDH activity was also much high in the control group, whereas HCA reversed this change (Figure 3c). The neutrophil migration into the lung lavage fluid and MPO levels was significantly elevated after the HPV for 4 h compared with the control group. HCA significantly decreased the neutrophil influx and MPO levels (Figure 3d) compared with the HPV group.

Effect of HCA on Apoptosis in Rat Lung Tissues Induced by HPV

The number of caspase-3-positive cells at the surface of the airway epithelial cells and alveoli were taken as a marker of apoptosis. As shown in Figure 4, cleaved-caspase-3-positive cells were markedly increased in the HPV group compared with the control group (Figure 4a). The number of positive cells was reduced in the HCA group in comparison with the control group (Figure 4b). The level of cleaved-caspase-3 in the HPV (4 h) stimulated lung tissues was significantly higher than that in the normal ventilation group. However, the level of cleaved-caspase-3 in the lung tissues was decreased in HCA (Figure 4c).

Although caspase-3 activation is typical of apoptotic cell death, it is not synonymous with apoptosis and must be interpreted in conjunction with other data demonstrating apoptotic changes. The apoptosis of pulmonary AECIIs has been proven to have an important role in high tidal volume mechanical ventilation-induced lung injury.¹¹ Therefore, we planned to isolate enriched populations of the AECIIs with sufficient viability and purity for functional studies. As shown in Figure 5b, there was a significant difference in the rate of early apoptosis or late apoptosis/necrosis between the control and the HPV groups. HCA attenuated apoptosis in the AECIIs compared with the HPV-only rat lung.

Effect of HCA on Lung Mechanical Stretch-Induced ASK1 and JNK/ p38 MAPK Activation

We performed an ELISA to observe the potential changes in phosphorylated ASK1 levels, which were significantly increased following the HPV (30 cmH₂O, 4 h) but attenuated by HCA (*P* < 0.05) (Figure 6a).

The levels of phosphorylated JNK and p38 MAPK were also increased following mechanical ventilation in the HPV-only group when compared with the control group (Figures 6b and c). Accordingly, HCA also significantly decreased the

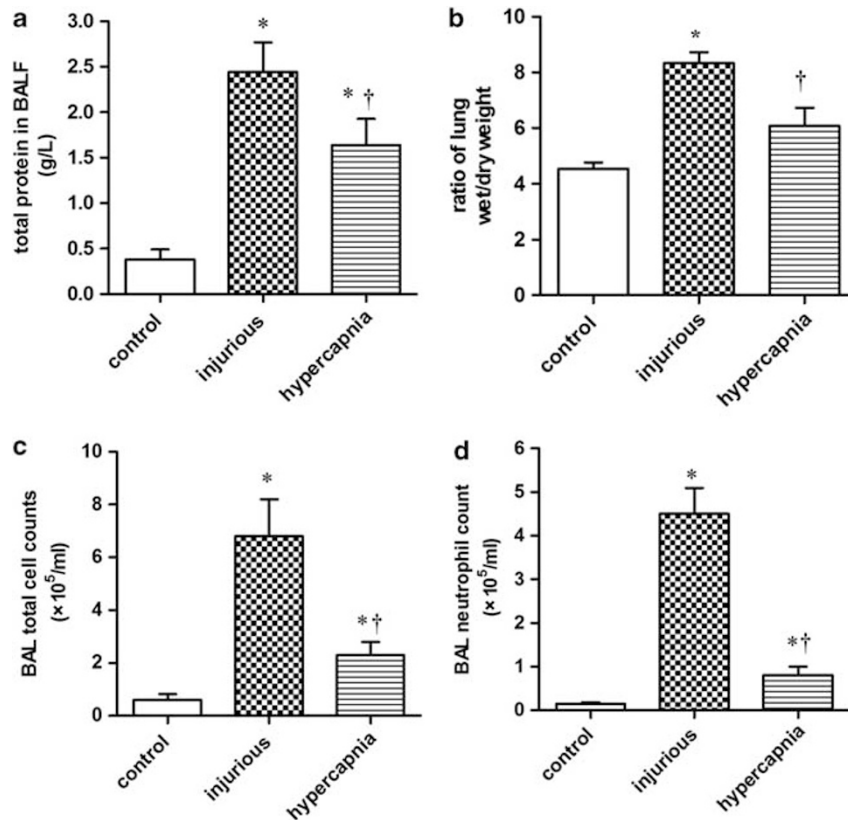


Figure 1 Effect of hypercapnic acidosis on major markers of injury in rats' lungs induced by high-pressure ventilation (HPV) for 4 h. **(a)** Bronchoalveolar lavage fluid (BALF) total protein levels (microgram/ml) were augmented by high-pressure ventilation but diminished by hypercapnia acidosis. **(b)** Pulmonary edema formation was quantified by measuring the wet and desiccated dry weights of the lung tissue. **(c, d)** The total and neutrophil cell count in the lavage group increased following HPV but were attenuated in the hypercapnia group. The data are representative of eight rats in each group. * $P < 0.05$ vs control group (normal ventilation with a peak inspiratory pressure (PIP) of 15 cmH₂O), † $P < 0.05$ vs injurious group (ventilation with a PIP of 30 cmH₂O).

ASK1/JNK and p38 MAPK activities in comparison with the control group ($P < 0.05$).

DISCUSSION

Intracellular signaling pathways such as MAPK pathways have been reported to be involved in the development of VILI.^{30–32} However, it is still unclear that HCA attenuated the oxidative stress and inflammatory injury induced by the lung mechanical stretch, inhibited the activation of ASK1/JNK and p38 MAP kinase, which have the ability to mediate apoptosis, and contributed to increase capillary permeability. This study demonstrates that high-pressure mechanical ventilation in the rat for 4 h caused lung oxidative stress and inflammatory injury, lung edema formation, and apoptosis, which were attenuated by HCA (PaCO₂ at 80–100 mm Hg) and accompanied by a decrease in the activity of ASK1/JNK and p38 MAP kinase.

JNK and p38 MAPK in pulmonary vascular endothelial cells (PVE) are activated in response to various stimuli including proinflammatory cytokines and oxidative stress.³³ JNK and p38 MAPK are activated by each upstream MAPK

kinase, ASK1 is one MAPKKK and has an important role in cytokines and apoptosis.³⁴ In the present study, we examined the effect of HCA on high-pressure stretching induced apoptosis and cytokine production, and oxidative stress in the lung, with the activity of ASK1/JNK and p38 MAP kinase in the isolated type II cells of lung. To examine the potential effects of HCA on the oxidation, we explored whether high-pressure stretching induced the content of MDA and SOD activity and MPO production in the rat lung tissue. Our data show that HCA can acutely and effectively inhibit the MPO pathway with its related oxidant reactions in the inflamed lung. These results were consistent with the previous study demonstrating that acidosis can inhibit this myeloperoxidase-dependent oxidant pathway both *in vitro*^{35,36} and *in vivo*.¹⁹ The results suggest that HCA could attenuate oxidative injury and that the HCA interference might be a protective strategy for AECIIs undergoing HPV.

The signaling pathway by which hypercapnic acidosis acts as an anti-inflammatory agent is not fully understood. In this study, to determine whether the attenuation of the MAPK

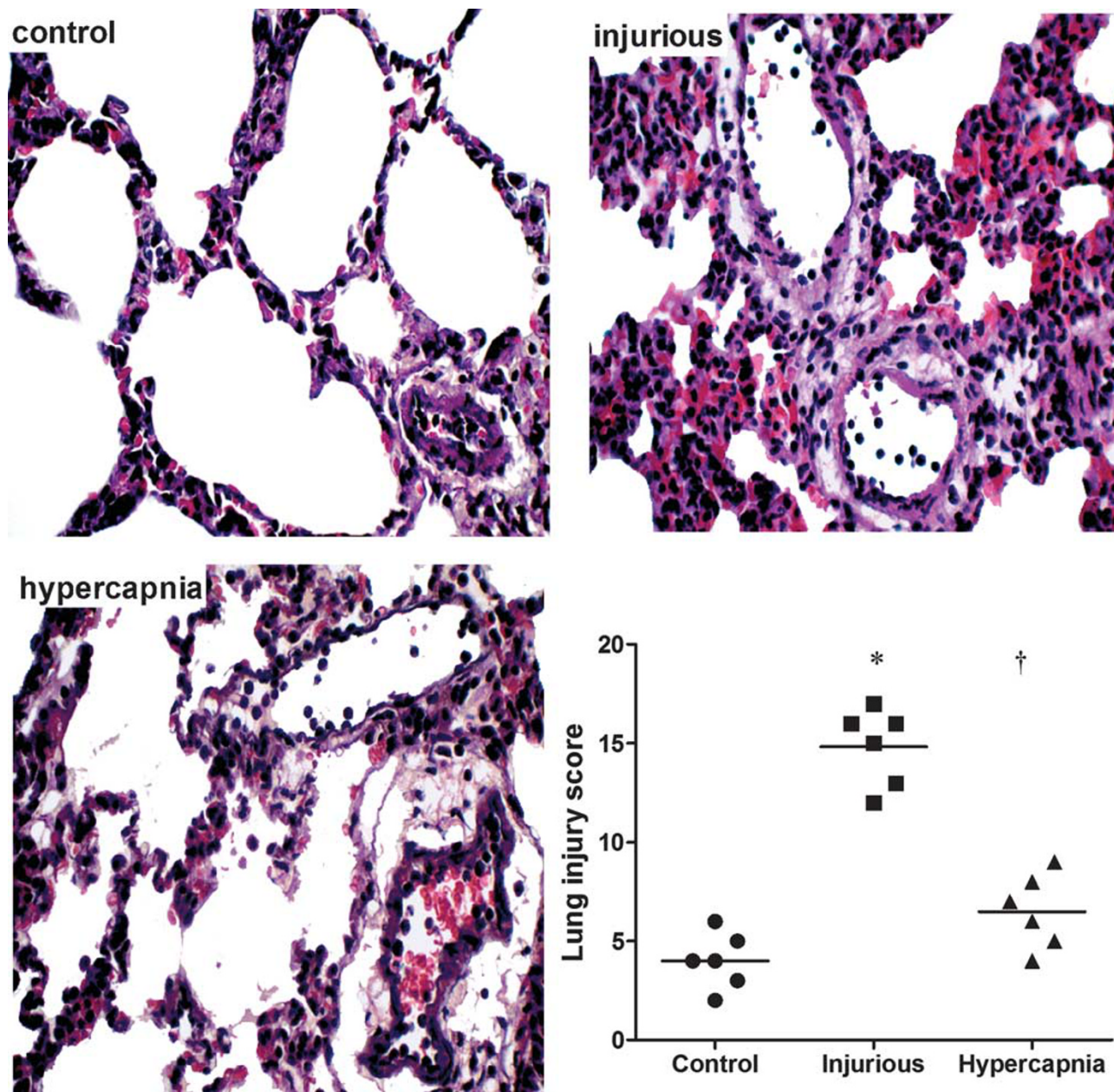


Figure 2 Histological analysis of lungs including the lung injury score in the three groups. Formalin-fixed lung sections were stained with hematoxylin and eosin (H&E). All panels represent $\times 40$ original magnification. The data are representative of six rats in each group. * $P < 0.05$ vs control group; † $P < 0.001$ in the hypercapnia group vs the injurious group.

activation by hypercapnic acidosis actually exerts an important effect on reducing HPV-induced inflammation and apoptosis, we examined the infiltration of cytokines and inflammatory cells into pulmonary tissues. Our findings demonstrated that ventilating rats with hypercapnic gas decreased the MIP-2 and TNF- α levels in BALF, which was in accord with the study of Laffey *et al*³⁷ To further characterize the potential effect of HCA on the ASK1-JNK/p38 MAPK cascade in HPV-induced injury, we isolated specific type II cells after high-pressure ventilation for 4 h with hypercapnic

gas. We found that the effects of mechanical stretch could induce cell apoptosis and phosphorylation of ASK1-JNK and p38 MAPK protein in the type II cells of lung. Furthermore, we determined the activation of caspase-3 because caspase-3 is one of the key executors of apoptosis.^{38,39} High-pressure ventilation activated caspase-3 and concomitantly promoted apoptosis in alveolar epithelial cells, indicating that caspase-3, at least in part, is involved in apoptosis in mechanical stretch-stimulated alveolar epithelial cells. Although several lines of evidence suggest that both caspase and the JNK/p38 MAPK

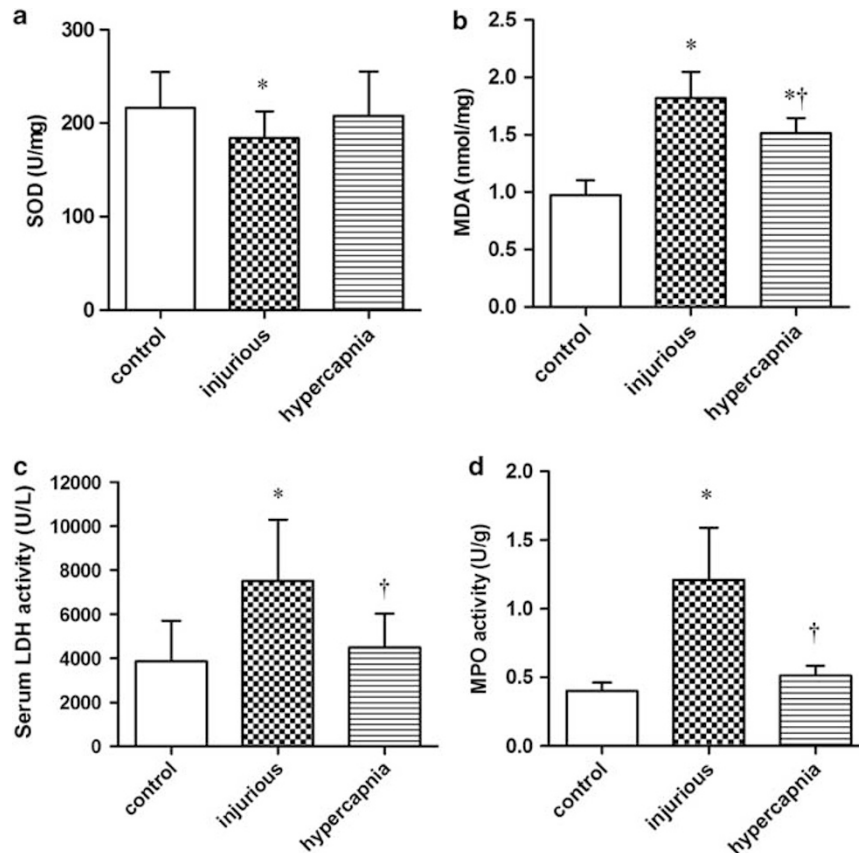


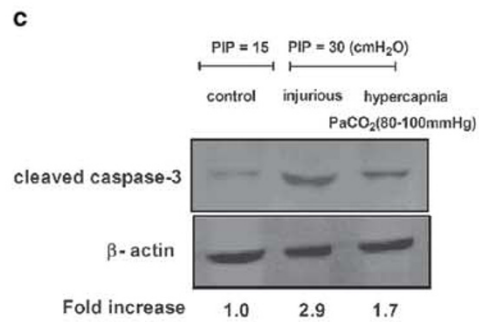
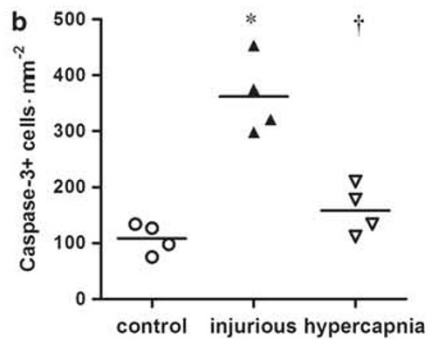
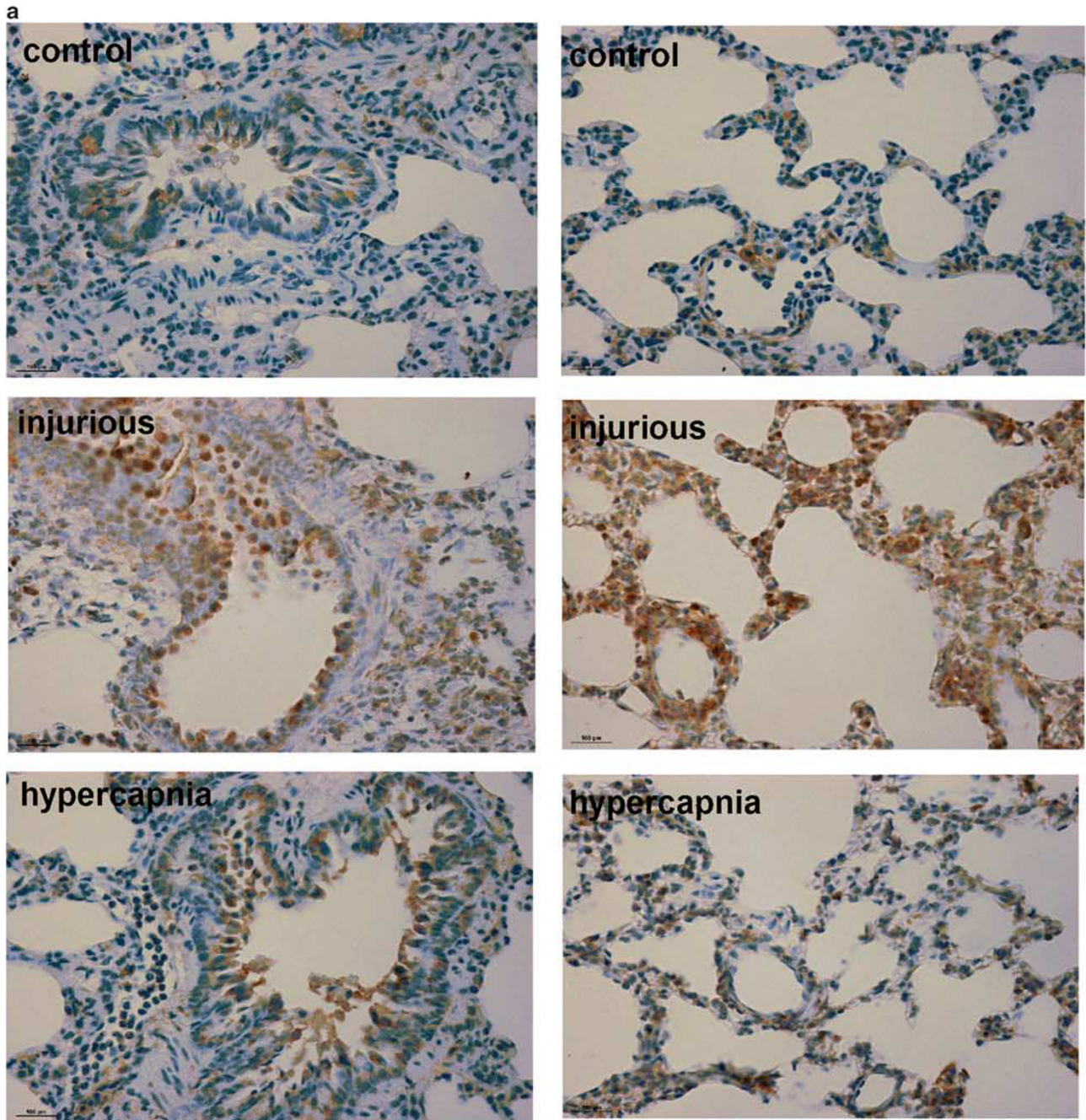
Figure 3 Treatment of hypercapnic acidosis markedly enhanced superoxide dismutase (SOD) activity (a), decreased malondialdehyde (MDA) content (b) lactate dehydrogenase (LDH) activity (c), and myeloperoxidase (MPO) activity (d). Treatment with HCA caused a significant elevation in the SOD activities and a decrease in the MDA levels of the lung tissue. The HCA significantly decreased the LDH and MPO activity compared with the only HPV group. The data are representative of eight rats in each group. * $P < 0.05$ vs control group (normal ventilation with a peak inspiratory pressure (PIP) of 15 cmH₂O), † $P < 0.05$ vs injurious group (ventilation with a PIP of 30 cmH₂O).

cascade are key pathways resulting in apoptosis,⁴⁰ the exact relationship between the caspase pathways and the MAPK pathways is still unclear. Li Fu *et al*^{6,10} reported that the ASK1 and JNK activity inhibitor was used in an animal of VILI model and their results suggested that the ASK1-JNK pathway had a key role in both stretch-induced apoptosis and cytokine production. However, it difficult to assess the possible mechanisms without the use of specific inhibitors to these pathways. Although a specific inhibitor of ASK1 was not used in this study, our findings showed high-pressure stretch-induced injury triggers by an ASK1-mediated signaling cascade, neutrophil infiltration, MIP-2 expression, and apoptosis initiation in the model of VILI. Our study suggests that the ASK1 activation induced by high-pressure stretch is another way of the cytokine production and apoptosis. Interrogation of the hypercapnia's influence on MAPK pathways as relates to apoptosis is important, but administration of an apoptosis inhibitor (inhaled or IP) would have been interesting to assess both the physiological and inflammatory impact to understand the relevance of apoptosis and hypercapnia.

In addition to the MAPK pathway, we have previously demonstrated the activation of NF- κ B subjected to high-pressure ventilation in the rats (unpublished results). Ventilation leading to the nuclear translocation of NF- κ B has been previously shown in cell culture,⁴¹ in isolated perfused rat lungs,⁴² and in animal lung injury induced by ventilation.⁴³ In this study, the activation of NF- κ B was markedly attenuated by HCA accompanied by the reduction of oxidative stress from neutrophil-mediated injury. It has also been reported that hypercapnic acidosis inhibits the adhesion of endotoxin-induced neutrophils to pulmonary endothelial cells through attenuating I κ B- α degradation, which in turn neutralizes the DNA-binding activity of NF- κ B.²¹ Thus, our study demonstrated that hypercapnic acidosis may also affect the VILI by mediating the pathways of ASK1-JNK and p38 MAPK, apoptosis, and inflammatory cytokines in a rat model.⁴⁴

Clinical Implications

In our study, hypercapnic acidosis inhibited mechanical stretch-induced lung inflammatory injury and cell apoptosis



through an underlying molecular mechanism of attenuating the phosphorylation levels of ASK1-JNK and p38 MAPK protein. Although a lot of evidence suggests that hypercapnic acidosis has a valuable role in experimental

models,^{14,15,18,37,45} we also should pay attention to consider harmful effects of hypercapnia and/or acidosis on its potential clinical application. It is clear that hypercapnic acidosis may exert adverse hemodynamic effects in patients

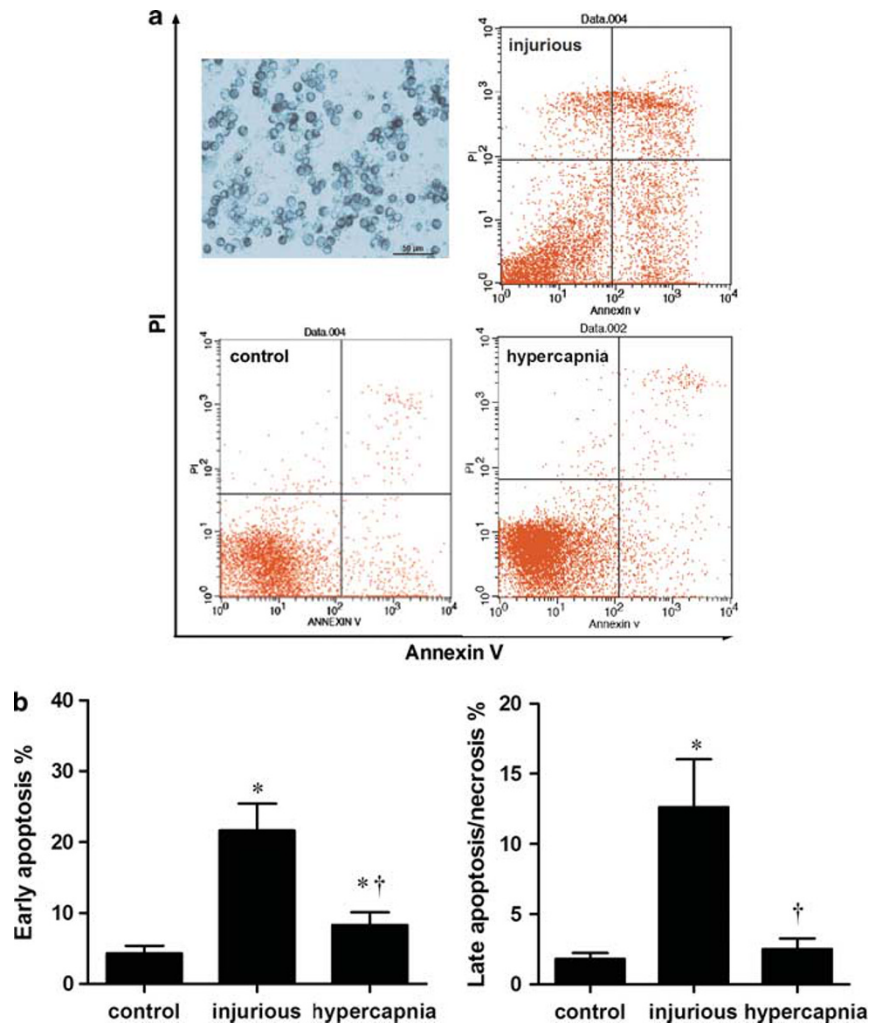


Figure 5 Annexin V-FITC/propidium iodide (PI) staining of type II alveolar epithelial cells (AECII) in the lung tissue of rats. (a) Alkaline phosphatase staining of type II cells. Blue reaction product identifies alkaline phosphatase activity (bar = 50 μm). (b) Cells were stained with annexin V and PI and analyzed by flow cytometry. Annexin V-positive/PI-negative staining (lower right quadrant) represented early apoptosis; Annexin V-positive/PI-positive (upper right quadrant) represented late apoptosis/necrosis. (c) Histogram of early apoptosis and late apoptosis/necrosis in the three groups. There were significant differences in the rate of early apoptosis and late apoptosis/necrosis between the control and injured groups. HCA apparently attenuated apoptosis in AECII compared with the injury-only ventilated rat lung. For the definitions of group abbreviations, see Figure 4. The data are representative of four rats in each group. * $P < 0.05$ vs control group; † $P < 0.001$ in the hypercapnia group vs the injurious group.

Figure 4 Detection of caspase-3 protein in the lung tissues of rats. (a) Immunohistochemical staining of caspase-3 in the airway epithelial cells and the alveoli of lung tissues. The presence of brown granules in the nucleus was defined as a positive cell. (b) Scatter plot of individual caspase-3-positive (+) cells/mm² in the lung tissue of animals ventilated at a peak inspiratory pressure (PIP) 15 cmH₂O/positive end-expiratory pressure (PEEP) 2 cmH₂O (control group) and PIP 30 cmH₂O/PEEP 2 cmH₂O with normocapnia (injurious group) or hypercapnia treatment (hypercapnia group) ($n = 4$ per group). (c) Western blot for cleaved caspase-3 in the lung tissues. The amounts of cleaved caspase-3 proteins were quantified and are presented as the amount of cleaved caspase-3 proteins relative to the control cells (1.0). β-Actin for loading control. The data are representative of four rats in each group. * $P < 0.05$ vs control group; † $P < 0.05$ vs injurious group. Internal scale bar = 50 μm.

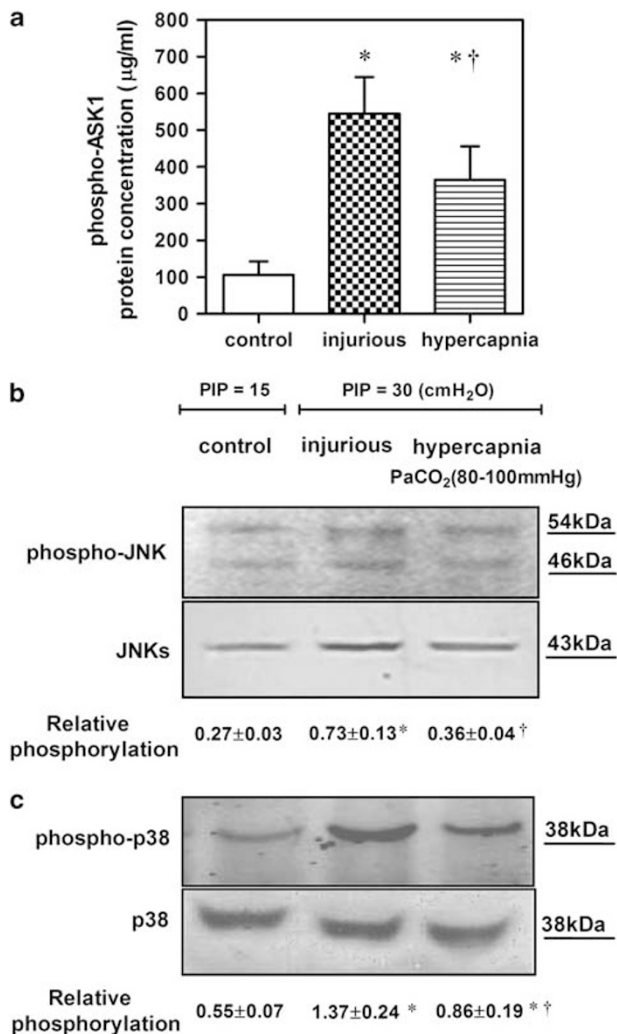


Figure 6 Hypercapnic acidosis attenuated the increase apoptosis signal-regulating kinase 1 (ASK1), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) protein activation of type II alveolar epithelial cells (AECII) in the lung tissue of rats following high-pressure ventilation for 4 h. ELISA was performed using an antibody that recognizes the phosphorylated ASK1 (a); Western blot was performed using an antibody that recognizes the phosphorylated JNK expressions (b) and the antibody that recognizes the phosphorylated p38 expressions (c). Arbitrary units were expressed as relative phosphorylation. For the definitions of the group abbreviations, see Figure 4. The data are representative of four rats. * $P < 0.05$ vs control group, † $P < 0.05$ vs injurious group.

with ARDS.⁴⁶ In addition, our previous study (unpublished data) also demonstrated that moderating hypercapnic acidosis (PaCO₂ of 80–100 mm Hg) could result in the improving oxygenation, histologic characteristics, and a lessened inflammatory response, compared with severe hypercapnic acidosis (PaCO₂ of 130–150 mm Hg). A previous study has also shown that the beneficial effects of moderating hypercapnia may be offset by the high level of adverse effects caused by itself.⁴⁷ Thus, although acidosis induced by acute hypercapnia may be as a protective factor in acute models of

ALI, the effects of hypercapnia during long-term exposure need to be further studied.

In conclusion, the present study proved that the administration of HCA significantly decreases lung vascular leakage and inflammation in a rat model of HPV-mediated VILI. The protection effect of HCA may involve the inhibition of inflammation and apoptosis via the inhibition of ASK1/JNK and p38 MAP kinase phosphorylation signaling pathway.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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