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Original article

Increased cardiac microvascular permeability and activation of cardiac endothelial nitric oxide synthase in high tidal volume ventilation-induced lung injury

Ming-Jui Hung[a, b], Ming-Yow Hung[b, c], Wen-Jin Cherng[a], Li-Fu Li[b, c]

[a]Cardiology Section, Department of Medicine, Chang Gung Memorial Hospital at Keelung, Keelung 20401; [b]Graduate Institute of Clinical Medical Sciences, Chang Gung University College of Medicine, Taoyuan 33302; [c]Section of Cardiology, Department of Medicine, Taipei Medical University-Shuang Ho Hospital, Taipei 23561; [c]Division of Pulmonary and Critical Care Medicine, Chang Gung Memorial Hospital at Linkou, Taoyuan 33333, Taiwan, ROC

Background: Positive pressure ventilation with large tidal volumes has been shown to cause lung injury via the serine/threonine kinase-protein kinase B (Akt) and endothelial nitric oxide synthase (eNOS)-pathways. However, the effects of high tidal volume (V_T) ventilation on the heart are unclear.

Objectives: Evaluate the effect of V_T ventilation on the cardiac vascular permeability and intracellular Akt and eNOS signaling pathway.

Methods: C57BL/6 and Akt knock-out (heterozygotes, +/–) mice were exposed to high V_T (30 mL/kg) mechanical ventilation with room air for one and/or five hours.

Results: High V_T ventilation increased cardiac microvascular permeability and eNOS phosphorylation in a time-dependent manner. Serum cardiac troponin I was increased after one hour of high V_T ventilation. Cardiac Akt phosphorylation was accentuated after one hour and attenuated after five hours of high V_T ventilation. Pharmacological inhibition of Akt with LY294002 and high V_T ventilation of Akt+/– mice attenuated cardiac Akt phosphorylation, but not eNOS phosphorylation.

Conclusion: High V_T ventilation increased cardiac myocardial injury, microvascular permeability, and eNOS phosphorylation. Involvement of cardiac Akt in high V_T ventilation was transient.

Keywords: Akt, eNOS, lung injury, vascular permeability, ventilation

Endothelial nitric oxide synthase (eNOS) is activated via the phosphatidylinositol 3-kinase (PI3-kinase)/serine/threonine kinase-protein kinase B (Akt) signaling pathway [1]. Akt is a serine/threonine protein kinase that is recruited to the membrane by its binding to PI3-kinase-produced phosphoinositides. At the membrane, Akt is phosphorylated and activated by phosphoinositide-dependent kinase [1, 2]. Akt subsequently phosphorylates and activates eNOS, leading to the production of nitric oxide (No) [3, 4]. NO has been shown to regulate multiple cellular functions, including smooth muscle cell relaxation, neurotransmission, macrophage-induced cytotoxicity, and apoptosis, as well as direct induction of vascular and epithelial hyperpermeability [5]. In an isolated rabbit lung model of ventilation-induced lung injury (VILI), investigators have reported that the nitrite and nitrate of bronchoalveolar lavage fluid significantly increased after high-pressure ventilation (30 cm H_2O) in comparison with control group (15 cm H_2O) [6]. Recently, the involvement of Akt and eNOS in ventilation-induced neutrophil infiltration has been demonstrated in a mouse model of VILI [7]. However, the effect of high tidal volume (V_T) ventilation on the heart is still unknown. In this study,

Correspondence to: Li-Fu Li, MD, PhD, Division of Pulmonary and Critical Care Medicine, Chang Gung Memorial Hospital at Linkou, Taoyuan 33333, ROC. E-mail: lfp3434@adm. asm.cgmh.org.tw

we hypothesized that high V_T causes an increase in cardiac microvascular permeability, a sign of cardiac injury, which is related to the intracellular Akt and eNOS signaling pathway.

Materials and methods

Experimental animals

Male C57BL/6 mice, either wild-type Akt+/+ or Akt+/– (knock-out) on a C57BL/6 background, weighing between 20 and 25 g were obtained from the National Laboratory Animal Center (Taipei, Taiwan) and Jackson Laboratories (Bar Harbor, USA). Heterozygotes (+/–) were used because homozygotes exhibit lower fertility and female homozygotes do not nurse well. Up to 50% perinatal mortality could occur. Mice that were heterozygous for the targeted mutation were viable and did not display any gross behavior abnormalities. The construct Akt containing disrupted exons 4 through 7 and the 5' end of exon 8 was electroporated into 129P2Ola/Hsd-derived E14 embryonic stem (ES) cells. Chimeras were generated by injecting these ES cells into C57BL/6 (B6) blastocytes. The resulting chimeric male animals were crossed to C57BL/6 mice, and then backcrossed to the same for 10 generations. Heterozygotes (+/–) were intercrossed to generate homozygous mutant mice (–/–). The lower expression levels of the Akt protein in Akt+/– mice were confirmed using Western blot analysis.

Mice were randomized to receive one of the following treatments: (1) vehicle (0.9% NaCl) or (2) LY294002 (PI3-K inhibitor). Confocal immunofluorescence microscopy was used to analyze the level of Akt expression in Akt+/– mice.

This study was performed in accordance with animal experimental guidelines of the National Institute of Health (Bethesda, USA) and with approval of the Research Committee of Chang Gung Memorial Hospital at Keelung.

Ventilator protocol

A 20-gauge angiocatheter was introduced into the trachea of the animal under general anesthesia with intraperitoneal ketamine (90 mg/kg body weight) and xylazine (10 mg/kg body weight) while breathing room air. The C57BL/6 mice then received 0.9% saline containing maintenance ketamine (0.1 mg/g/hour) and xylazine (0.01 mg/g/hour) at a rate of 0.09 mL/10g/hour by a continuous intraperitoneal fluid pump and were ventilated for one and five hours at 30 ml/kg V_T (V_T 30) at a rate of 65 breaths per minute in room air. The V_T delivered by the ventilator was checked by measuring the amount of fluid displaced from an inverted calibration cylinder. End tidal CO_2 was continuously monitored by a microcapnograph (Columbus Instruments, Columbus, USA) and respiratory frequency was adjusted to maintain end tidal CO_2 at 30-40 mm Hg. V_T was increased by 5 mL/min to correct the air loss from sample flow adaptor during monitoring of end tidal CO_2. Animals were placed in a supine position on a heating blanket and then attached to a ventilator, model 55-7058 (Holliston, USA). Peak inspiratory airway pressure was measured with a pressure transducer amplifier (Gould Instrument Systems, Valley View, USA) connected to the tubing at the proximal end of the tracheotomy tube. Mean arterial pressure was monitored every hour during mechanical ventilation using a pressure transducer amplifier connected to a 0.61 mm OD (0.28 mm ID) polyethylene catheter ending in the common carotid artery. Control, non-ventilated mice were anesthetized and sacrificed immediately. Two mice were placed on separate ventilators side-by-side so that pairs of mice could be studied simultaneously. One hour and five hours of V_T 30 were used to collect samples of 1) myocardium for Evans blue dye (EBD) leak analysis, 2) protein for Western blot analysis of eNOS and Akt, and 3) myocardium for confocal immunofluorescence analysis. At the end of the study period, heparinized blood was taken from the arterial line for analysis of arterial blood gas and cardiac troponin I, and the mice were sacrificed. The hearts were then lavaged three times with 0.6 mL of 0.9% normal saline. For histopathology, the hearts were removed en bloc, and fixed with 10% neutral buffered formalin (pH 6.8 to 7.2) at a pressure of 30 cmH_2O and stored in formalin. For measurement of kinase activation, the hearts were homogenized in 3 mL of lysis buffer (20 mM HEPES [pH 7.4], 1% Triton X-100, 10% glycerol, 2 mM ethylene glycol-bis [β-aminoethyl ether]-*N*,*N*,*N*′,*N*′-tetraacetic acid. 50 μM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiotreitol, 400 μM aprotinin, and 400 μM phenylmethylsulfonyl fluoride), transferred to Eppendorf tubes, and placed on ice for 15 minutes. Tubes were centrifuged at 14,000 rpm for 10 minutes at 4°C, and the supernatant was flash-frozen.

EBD analysis

Extravasation of EBD (Sigma Chemical, St. Louis, USA) into the myocardium was used as a quantitative measure of changes of microvascular permeability in acute cardiac injury. Thirty minutes before the end of mechanical ventilation, 30 mg/kg of EBD was injected through the internal jugular vein. At the time of sacrifice, the hearts were perfused free of blood with 1 mL of 0.9% normal saline via the right ventricle and removed en bloc. Evans blue was extracted from heart tissue after homogenization for two minutes in 5 mL of formamide (Sigma Chemical, St. Louis, USA) and incubated at 37°C overnight. The supernatant was separated by centrifugation at 5,000*g* for 30 minutes, and the amount was recorded. Evans blue in the plasma and heart tissue was quantitated by dual-wave length spectrophotometric analysis at 620 nm and 740 nm. The method corrected the specimen's absorbance of contaminating heme pigments at 620 nm absorbance, using the following formula: corrected absorbance at 620 = actual absorbance at 620 nm – [1.426 (absorbance at 740) + 0.03]. We calculated the amount of EBD amount extracted from heart tissue and divided the amount by the weight of heart tissue.

Cardiac troponin-I measurement

Serum level of cardiac troponin-I was determined on serum from individual animals using mouse cardiac troponin I enzyme-linked immunosorbant assay kit (Life Diagnostics, West Chester, USA) according to the directions of the manufacturer.

Immunoblot analysis

Hearts from control mice without ventilation, ventilated mice exposed to high-V_T ventilation for one hour and five hours were removed and immediately frozen in liquid nitrogen. Crude cell lysates were matched for protein concentration, resolved on a 10% bis-acrylamide gel, and electrotransferred to Immobilon-P membranes (Millipore, Billerica, USA). For measurement of eNOS activation, tissues were immunoprecipitated with eNOS antibodies, which recognize eNOS, for two hours while rotating at 4°C. Immune complexes were collected with protein G-Sepharose beads for one hour while rotating at 4°C. Samples were resolved by sodium dodecyl sulfate-polycarylamide gel electrophoresis, stained with Coomassie blue, exhaustively destained, dried, and analyzed with phosphoimaging or autoradiography. For Akt and eNOS phosphorylation, Western blot analysis was preformed with antibodies to phospho-Akt and phospho-eNOS (serine 1177) (New England BioLabs, Beverly, USA). For determination of Akt and eNOS protein expression, Western blot analysis was performed with the respective antibodies (Santa Cruz Biotechnology, Santa Cruz, USA).

Inhibition of Akt

LY294002 was used to assess the effect of Akt. It was given intraperitoneally at a dose of 5μg/g one hour before starting mechanical ventilation [7].

Confocal immunofluorescent analysis

The heart tissues from control mice (wild-type Akt+/+ and Akt+/–) without ventilation, ventilated mice (wild-type Akt+/+ and Akt+/–) exposed to high-V_T ventilation for one hour while breathing room air were paraffin-embedded, sliced at 4 μm, deparaffinized, antigen-unmasked in 10 mM sodium citrate (pH 6.0), and incubated with rabbit anti-eNOS (1:200; Transduction Laboratories, Inc.), rabbit anti-phospho-eNOS (Thr 495; 1:100; Cell Signaling Technology, Inc.), rabbit anti-Akt (1:100; BioVision Inc.), or rabbit anti-phospho-Akt-1 (1:100; Millipore, Billerica, USA) primary antibody and Cy3-conjugated donkey anti-rabbit secondary antibody (1:800, Millipore, Billerica, USA) according to the instructions of an immunohistochemical kit (Santa Cruz Biotechnology, Santa Cruz, USA). Nuclei were counterstained with bisBenzimide H 33258 (1:10000; Sigma-Aldrich, St. Louis (place), USA). Slides were examined using a fluorescence microscope equipped with a digital camera (Eclipse E800, Nikon, Tokyo, Japan). The signal intensities recorded from the samples were quantified by an independent investigator using Image-Pro Plus (Version 4.1.0.0, Media Cybernetics, Silver Spring, USA).

Statistical evaluation

The Western blots were quantified using a National Institutes of Health image analyzer Image J 1.38x software (National Institute of Health, Bethesda, USA), and presented as the ratio of phospho-Akt to Akt or phospho-eNOS to eNOS (relative phosphorylation) in arbitrary units. Values were expressed as means±SEM for at least three experiments. All Western blot results of were normalized to control, non-ventilated mice breathing room air. Analysis of variance was used to assess

the statistical significance of the differences. Nonparametric testing was employed when data were not normally distributed. The Tukey's post hoc test was employed after analysis of variance on rank. A value of $p < 0.05$ was considered statistically significant. Statistical analyses were performed with SPSS 13.0 for Windows (SPSS, Chicago, USA).

Results

Physiologic data and EBD analysis

There was no statistical difference in pH, PaO_2, $PaCO_2$, mean arterial pressure at the beginning versus the end of mechanical ventilation. EBD analysis was used to measure changes of microvascular permeability in VILI. EBD was significantly higher in mice exposed to V_T 30 for five hours than in mice exposed to V_T 30 for one hour or in control non-ventilated mice (**Table 1**). In addition, the serum cardiac troponin-I was increased after V_T 30 for one hour. These suggest that high-V_T (V_T 30) mechanical ventilation increases cardiac microvascular permeability, a sign of cardiac injury, and myocardial injury.

Lung stretch increased cardiac eNOS phosphorylation

To determine whether cardiac EBD leakage in mice exposed to V_T 30 was associated with eNOS upregulation, we measured phosphorylation and expression of eNOS (**Fig. 1A**). There were time-dependent increases in phosphorylation of eNOS but no change in the expression of total non-phosphorylated protein of eNOS. This suggests that high V_T ventilation-induced cardiac injury accentuates eNOS phosphorylation, indicating that the eNOS pathway may regulate VILI-induced cardiac injury.

Lung stretch-accentuated cardiac Akt phosphorylation transiently

To further define the effects of high V_T ventilation on Akt, we measured the phosphorylation and expression of the Akt in mice exposed to V_T 30 mechanical ventilation for one and five hours while breathing room air. V_T 30 mechanical ventilation for one hour but not for five hours increased phosphorylation of Akt, but did not change the expression of total non-phosphorylated Akt proteins (**Fig. 1B**). This suggests that high V_T ventilation-induced cardiac injury increases Akt phosphorylation earlier than eNOS phosphorylation, indicating the involvement of the Akt/eNOS pathway in this mouse model.

Table 1. Experimental conditions at the beginning and end of ventilation.

	Non-ventilated C57BL/6	V_T 30ml/kg, one hour	V_T 30ml/kg, five hours
pH	7.40 ± 0.01	7.37 ± 0.01	7.33 ± 0.04
PaO_2 (mm Hg)	98.8 ± 1.4	94.6 ± 2.4	86.1 ± 0.8
$PaCO_2$ (mm Hg)	40.2 ± 0.3	35.6 ± 1.8	34.4 ± 1.5
MAP (mmHg)			
Start	86.0 ± 1.9	85.3 ± 2.1	84.6 ± 2.6
End		74.3 ± 4.3	73.5 ± 5.6
PIP (mm Hg)			
Start		11.4 ± 1.3	22.9 ± 2.7
End		12.1 ± 1.6	28.2 ± 3.4
EBD (ng/mg heart weight)	43.7 ± 0.7	68.2 ± 3.3*	131.9 ± 5.2†
EBD (ng/mg lung weight)	16.4 ± 3.1	25.8 ± 4.9	95.4 ± 6.3*
Troponin I (ng/mL)	10.0 ±2.5	73.8 ± 24.3*	NA

EBD = Evans blue dye, MAP = mean arterial pressure, NA = not available, PIP = peak inspiratory pressure. Arterial blood gas, MAP, PIP, and EBD analysis (ng/mg heart weight) of normal non-ventilated mice and mice were placed on a high V_T mechanical ventilation for one or five hours (n = 5/group). *$p < 0.01$ *vs.* control non-ventilated mice. †$p < 0.001$ *vs.* control non-ventilated mice.

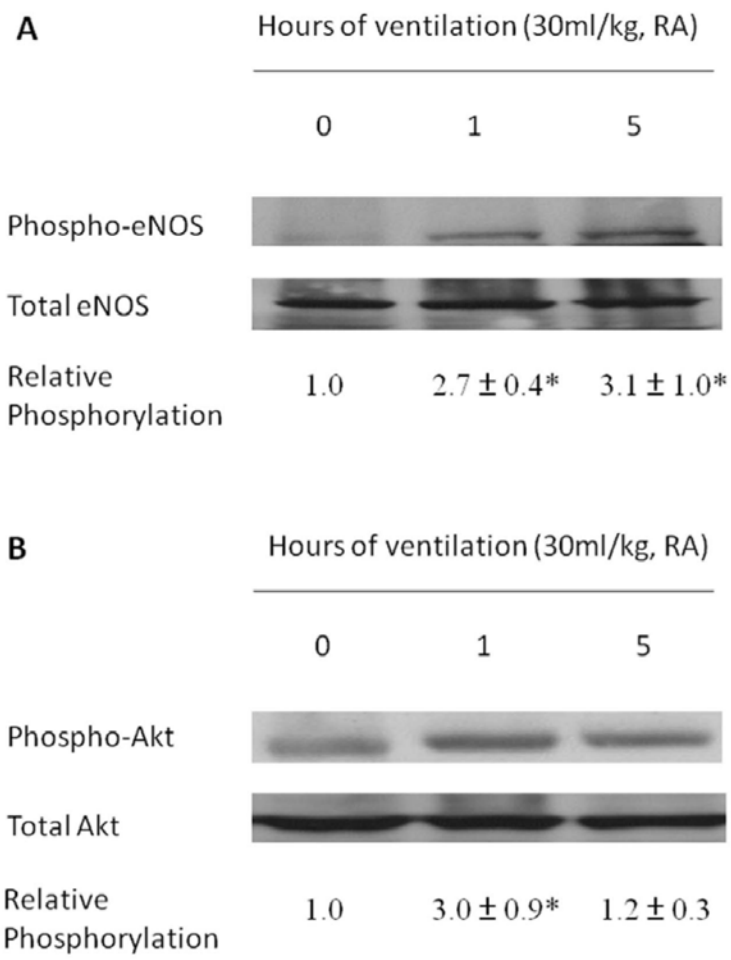

Fig. 1 High V_T ventilation caused a time-dependent accentuation of eNOS and Akt phosphorylation. Western blot was performed using an antibody that recognizes the phosphorylated eNOS and Akt expression (**A** and **B**, Top Panel) and an antibody that recognizes total eNOS and Akt protein expression in cardiac tissue (**A** and **B**, Middle Panel) from control non-ventilated mice, and mice ventilated with V_T 30 ml/kg breathing room air at indicated time periods. RA: mice with room air. Arbitrary units were expressed as relative eNOS and Akt phosphorylation (**A** and **B**, Bottom Panel) (n=5/group). *p <0.01 *versus* control, non-ventilated mice.

Inhibition of Akt with LY294002 reduced cardiac Akt phosphorylation but not cardiac eNOS phosphorylation

To determine the role of Akt phosphorylation in ventilator-induced cardiac injury, we pretreated mice for one hour with the specific PI3-kinase inhibitor (LY294002). High V_T ventilation did attenuate cardiac Akt phosphorylation, but still accentuated cardiac eNOS phosphorylation in a time-dependent manner (**Fig. 2**). This suggests that Akt is not the only pathway activated in high V_T ventilation-induced cardiac injury, and the transient accentuation of Akt phosphorylation indicates that Akt lose its protective effect at the later stage of high V_T ventilation.

Immunofluorescent analysis

Using histopathology, we confirmed the eNOS and Akt results. Cardiac Akt phosphorylation (**Fig. 3A**) was accentuated significantly in mice exposed to high V_T mechanical ventilation for one hour, but no significant change in Akt phosphorylation was noted in mice exposed to high-V_T mechanical ventilation for five hours. In contrast, eNOS phosphorylation (**Fig. 3B**) was accentuated in a time-dependent manner in both groups of mice exposed to high V_T mechanical ventilation. High V_T ventilation attenuated the activity of Akt but accentuated the activity of eNOS in Akt+/–mice heart (**Fig. 4**). These findings are consistent with those from the Western blot analysis.

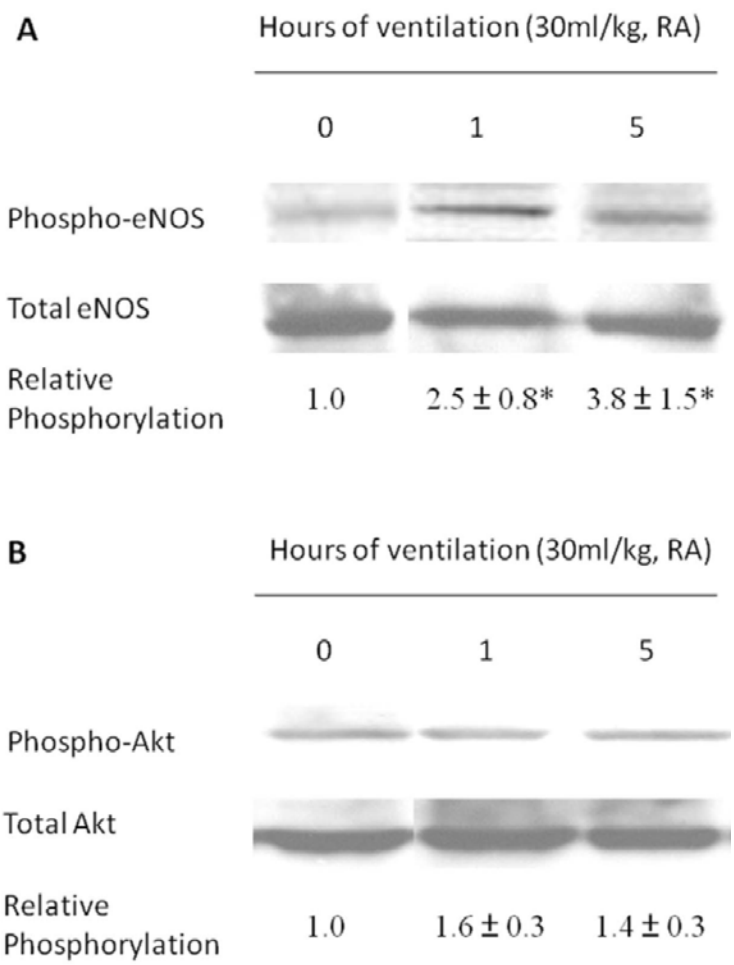

Fig. 2 LY294002 reduced lung stretch-induced cardiac Akt but not eNOS phosphorylation. Mice ventilated at a V_T of 30 mL/kg for one hour were pretreated with 5 μg/g LY294002 intraperitoneally one hour before ventilation. Phosphorylated eNOS or Akt expression (**A** and **B**, Top Panel) and total eNOS or Akt protein expression (A and B, Middle Pane), and quantitation by arbitrary units (**A** and **B**, Bottom Panel) (n=5/group). Arbitrary units were expressed as relative phosphorylation. *p <0.01 *versus* control, non-ventilated mice.

Discussion

This study demonstrates that high V_T ventilation may induce cardiac injury, cardiac microvascular hyperpermeability, and cardiac eNOS phosphorylation upregulation. As previous investigators found that eNOS mediates the systemic microvascular leakage [8], the cardiac microvascular hyperpermeability was suggested to be caused by upregulation of cardiac eNOS phosphorylation. We found that LY294002, a selective PI3-kinase inhibitor, significantly reduced the level of Akt phosphorylation. However, the same inhibitor accentuated eNOS phosphorylation indicating other pathways involves eNOS regulation in the setting of high V_T ventilation.

VILI is characterized by non-cardiogenic pulmonary edema, release of cytokines, and influx of neutrophils [8, 9]. The responses include plasma leakage followed by edema formation [10, 11]. Evans blue is a hydrophilic dye, and is often used as a tracer of plasma leakage because of its high affinity for plasma proteins. Plasma leakage causes Evans blue to extravasate from the blood vessels. Measuring the amount of EBD that is extravasated from tissues is useful for evaluating the degree of plasma leakage. Choi et al. [8] exposed rats to a moderately large V_T of 20 mL/kg for two hours; using the EBD technique, they found that VILI results in lung and renal microvascular leakage of protein. In our study, the amounts of EBD of heart and lung were significantly higher in mice exposed to V_T 30 for five hours than in mice exposed to V_T 30 for one hour or control non-ventilated mice. Furthermore, we also found that

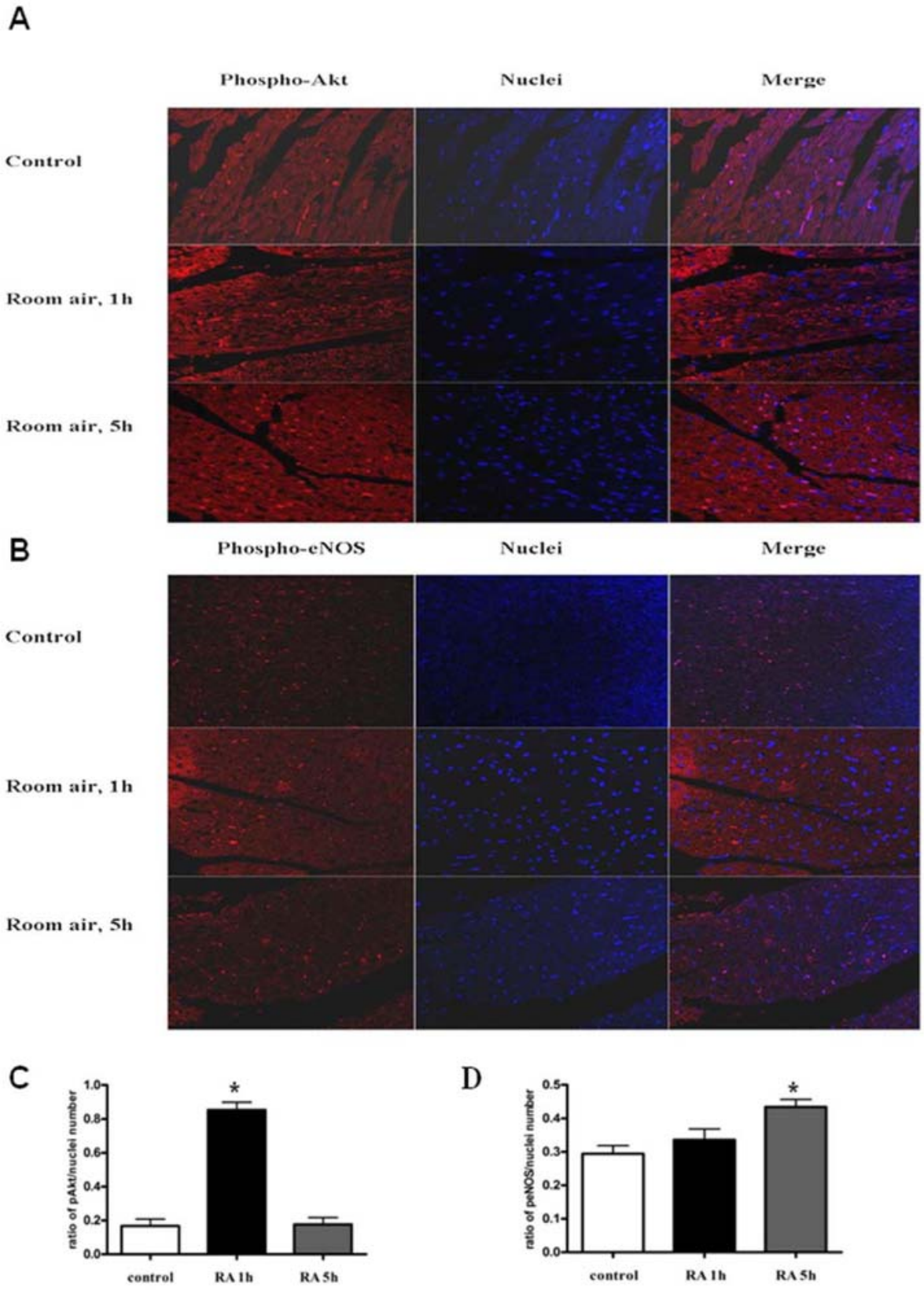

Fig. 3 Effects of high V_T ventilation on Akt and eNOS activation in mice cardiac tissue. Representative photomicrographs (x400) with phospho-Akt (**A**) or phospho-eNOS (**B**) staining of the cardiac sections after one and five hours of high V_T mechanical ventilation with room air (n=3/group). Arbitrary units are expressed as ratios of red immunofluorescence signal in cytoplasm (phospho-Akt or phospho-eNOS)/blue immunofluorescence signal in nuclei. Phospho-Akt expression was accentuated after one hour (**C**) of mechanical ventilation and phospho-eNOS expressions were accentuated in a time-dependent manner (D) after mechanical ventilation. *p <0.05 *versus* control, non-ventilated mice.

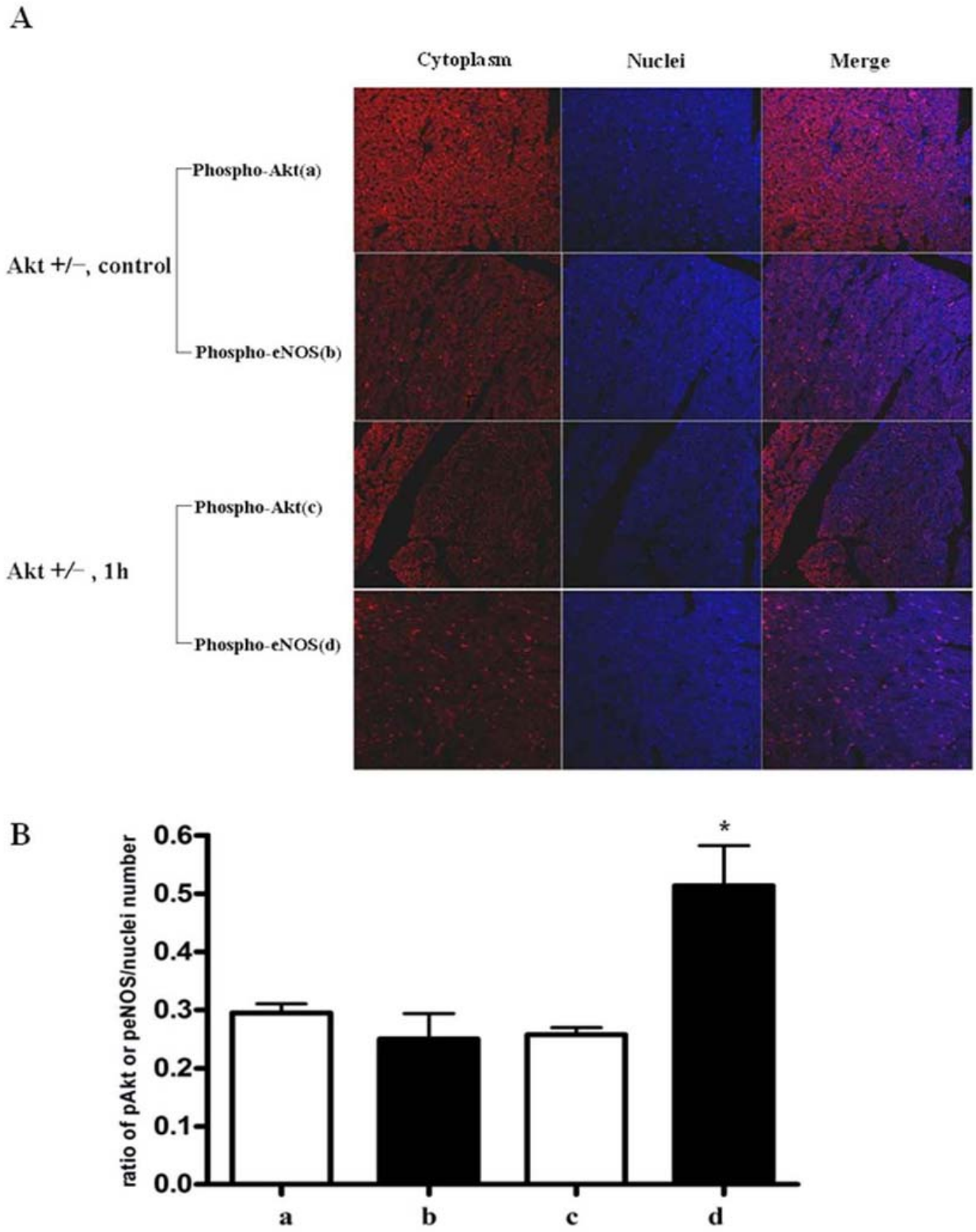

Fig. 4 Effects of lung stretch on Akt and eNOS activation in Akt+/– mice cardiac tissue. (**A**) Representative photomicrographs (×400) with phospho-Akt (**a, c**) and phospho-eNOS (**b, d**) staining of the cardiac sections after one hour of mechanical ventilation with room air (n=3/group). Arbitrary units were expressed as ratios of red immunofluorescence signal in cytoplasm (phospho-Akt or phospho-eNOS)/blue immunofluorescence signal in nuclei. (**B**) Phospho-Akt expression was not accentuated in Akt+/– mice after one hour (**c**) of mechanical ventilation as compared with control (**a**). Phospho-eNOS expression was accentuated in Akt+/– mice after one hour (**b**) of mechanical ventilation as compared with control (**d**). *p <0.05 *versus* control, non-ventilated mice.

the serum cardiac troponin-I was increased after V_T 30 for one hour. These suggest that high V_T ventilation may result in cardiac injury, including plasma leakage and myocardial damage.

The physical forces of mechanical ventilation are sensed and converted into the reactions of intracellular signal transduction via stress failure of plasma membrane, stress failure of epithelial and endothelial barriers, mechanical strain, or shear stress [12]. Petroff et al. [13] demonstrated that PI3-kinase was activated in endothelial cells in response to shear-stress and in cardiac myocytes in response to stretch. PI3-kinase and the downstream serine/threonine kinase Akt/protein kinase play important roles in regulating neutrophil influx and chemotaxis [14, 15]. We found that mechanical ventilation augmented phosphorylation of Akt in the early stage and attenuated phosphorylation in the later stage. This finding might be explained by disappearance of protective role Akt in the high V_T ventilation-induced cardiac injury in a time-dependent manner. The contribution of Akt was further explored by using a highly specific competitive inhibitor of the PI3-kinase, LY294002, which binds to the ATP-binding site [16]. Using immunohistochemistry, we confirmed that ventilation with a high V_T induces Akt phosphorylation in cardiomyocyte.

Nitric oxide synthase can be induced in many cell types, including endothelial cells and cardiomyocytes [13]. It has been shown that eNOS is a target of Akt, and that inhibition of the PI3-kinase and Akt pathways or mutation of the Akt site on the eNOS protein (at serine 1177) attenuates the phosphorylation of serine, thereby preventing the activation of eNOS [3]. We found high V_T ventilation increased eNOS phosphorylation in cardiomyocytes. This effect was not changed after pretreatment with Akt inhibitor, LY294002. Other investigators have found that eNOS, but not iNOS, may mediate the systemic microvascular leakage [8]. Petroff et al. [13] found that cardiomyocyte NO produced by activation of the PI3-kinase/Akt/eNOS axis acts as a second messenger of stretch by enhancing ryanodine-receptor calcium-release channel activity, contributing to myocardial contractile activation. We found that high V_T mechanical ventilation could cause phosphorylation of eNOS and the up-stream regulator of Akt without other stimuli. We also found that the Akt is not the only up-stream regulator of eNOS. Based on our findings and those from previous reports, we suggest that high V_T ventilation causes cardiac injury, in part, by attenuating the phosphorylation of Akt.

During past years, the importance of small G proteins in regulating endothelial barrier function has been established. Rho kinase was reported to interfere with eNOS protein expression [17], and altered eNOS activity results in alterations of endothelial barrier function [18]. Recently, Rho kinase was found to have an intrinsic barrier-protective activity at the cell margins and an induced barrier-disruptive activity at contractile F-actin stress fibers [19]. The role of Rho kinase in the high V_T ventilation-induced cardiac microvascular hyperpermeability requires further investigation.

Conclusion

Using an *in vivo* mouse model, we disclosed that high V_T ventilation increased cardiac myocardial injury, microvascular permeability, and eNOS phosphorylation. Involvement of cardiac Akt in high V_T ventilation was transient.

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M-J H and L-F L collected and analyzed the data. M-Y H and W-J Cherng reviewed and coordinated the study.

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