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## Pulmonary-derived phosphoinositide 3-kinase gamma (PI3K $\gamma$ ) contributes to ventilator-induced lung injury and edema

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**Abstract** *Background:* Ventilator-induced lung injury (VILI) occurs in part by increased vascular permeability and impaired alveolar fluid clearance. Phosphoinositide 3-kinase gamma (PI3K $\gamma$ ) is activated by mechanical stress, induces nitric oxide (NO) production, and participates in cyclic adenosine monophosphate (cAMP) hydrolysis, each of which contributes to alveolar edema. We hypothesized that lungs lacking PI3K $\gamma$  or treated with PI3K $\gamma$  inhibitors would be protected from ventilation-induced alveolar edema and lung injury. *Methods:* Using an isolated and perfused lung model, wild-type (WT) and PI3K $\gamma$ -knockout (KO) mice underwent negative-pressure cycled ventilation at either  $-25$  cmH $_2$ O and  $0$  cmH $_2$ O positive end-expiratory pressure (PEEP) (HIGH STRESS) or  $-10$  cmH $_2$ O and  $-3$  cmH $_2$ O PEEP (LOW STRESS). *Results:* Compared with WT, PI3K $\gamma$ -knockout mice lungs were partially protected from VILI-induced derangement of respiratory mechanics (lung elastance) and edema formation [bronchoalveolar lavage (BAL) protein concentration, wet/dry ratio, and lung histology]. In PI3K $\gamma$ -

knockout mice, VILI induced significantly less phosphorylation of protein kinase B (Akt), endothelial nitric oxide synthase (eNOS), production of nitrate and nitrotyrosine, as well as hydrolysis of cAMP, compared with wild-type animals. PI3K $\gamma$  wild-type lungs treated with AS605240, an inhibitor of PI3K $\gamma$  kinase activity, in combination with enoximone, an inhibitor of phosphodiesterase-3 (PDE3)-induced cAMP hydrolysis, were protected from VILI at levels comparable to knockout lungs. *Conclusions:* Phosphoinositide 3-kinase gamma in resident lung cells mediates part of the alveolar edema induced by high-stress ventilation. This injury is mediated via altered Akt, eNOS, NO, and/or cAMP signaling. Anti-PI3K $\gamma$  therapy aimed at resident lung cells represents a potential pharmacologic target to mitigate VILI.

**Keywords** Ventilator-induced lung injury · Nitric oxide · Permeability · Lung edema · cAMP · ARDS

### Introduction

Mechanical ventilation is a life-saving therapy but can initiate or exacerbate lung injury [1–4]. This ventilation-

induced lung injury (VILI) results, in part, in increased vascular permeability and impaired alveolar fluid clearance, resulting in the clinical manifestation of alveolar edema [5–7].

Pulmonary mechanical stretch enhances nitric oxide (NO) production [8, 9], which can contribute to the pathogenesis of VILI by increasing alveolar and vascular permeability [10–12]. This mechanism is thought to involve peroxynitrite, which is a potent oxidant produced by the interaction of NO and oxygen radicals [13]. Nitrotyrosine, a marker of peroxynitrite damage, is increased in patients with acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) [13–15]. In addition to permeability alterations, reactive nitrogen species can impair cyclic adenosine monophosphate (cAMP)-dependent alveolar fluid clearance (AFC) [11]. The intracellular level of cAMP is a critical modulator of endothelial permeability, and strategies that increase cAMP can reverse alterations in lung permeability [16, 17].

The phosphoinositide 3-kinase (PI3K) family is found largely in neutrophils, but is also expressed in structural cells of various organs including the lung [18], where they contribute to cyclic, stretch-activated nitric oxide production in endothelial cells [19]. Since the PI3K $\gamma$  isoform can act independently of its kinase activity to modulate cAMP concentrations [20, 21], it may play an essential role in the formation of edema during VILI. Recently, our group demonstrated a role for PI3K $\gamma$  in VILI [22]; however, that study did not examine the effect on pulmonary edema formation, nor identify whether the cells mediating VILI were the circulating neutrophils or the residential cells of the lung, which could be specifically targeted by anti-PI3K $\gamma$  therapies. Therefore, use of inhibitors towards the kinase-dependent and/or kinase-independent functions of PI3K $\gamma$  may each induce protection against the detrimental effects of VILI.

The aim of this study is to investigate the role of pulmonary-derived PI3K $\gamma$  in the development of ventilator-induced lung injury and edema. We hypothesized that isolated and perfused lungs lacking PI3K $\gamma$  would be protected from VILI and edema formation, through the alteration of NO signaling and cAMP lung content. Moreover, we hypothesized that this protection would result from a combination of the blockade of both the kinase-dependent and kinase-independent effects.

## Materials and methods

### Animals, surgery, and ventilation protocol

Experiments were conducted on 129/Sv adult male PI3K $\gamma$  wild-type (PI3K $\gamma^{+/+}$ ) and knockout (PI3K $\gamma^{-/-}$ ) mice (25–30 g). PI3K $\gamma$  wild-type and knockout lungs were isolated, perfused, and ventilated as previously described [23]. After ascertaining pulmonary perfusion, the mode of ventilation was changed to negative pressure to minimize hydrostatic edema formation [23]. After stabilization (60 min), lungs were randomized to two ventilation strategies: LOW STRESS, with end-inspiratory pressure

(EIP) =  $-10$  cmH<sub>2</sub>O and end-expiratory pressure (EEP) =  $-3$  cmH<sub>2</sub>O; and HIGH STRESS, with EIP =  $-25$  cmH<sub>2</sub>O and EEP =  $0$  cmH<sub>2</sub>O. Ventilation was stopped after either 60 or 180 min. Animals used as negative controls ( $n = 8$ , NO STRESS group) were anesthetized and exsanguinated, lung vasculature was flushed, and the hearts and lungs were dissected en bloc without mechanical ventilation or perfusion.

### Lung function and morphology

Airway opening pressure (Pao), pressure inside the chamber, and tidal volume (Vt) as the integral of the flow, were measured [24]. When ventilation was stopped after 1 h, lung tissue was isolated, snap-frozen, and stored at  $-80^{\circ}\text{C}$ . When ventilation was stopped after 3 h, the right lung was excised, snap-frozen, and stored at  $-80^{\circ}\text{C}$ . The left lung was then inflated and fixed with 4% paraformaldehyde (Sigma-Aldrich, Milwaukee, WI, USA) [25]. Lung sections (3  $\mu\text{m}$ ) were stained with hematoxylin and eosin, and nine random fields of view were analyzed by two pathologists blinded to experimental groups. For histopathological evaluation of lung damage, two separate scores relating to edema and inflammatory cell infiltration were calculated. Interstitial edema was scored as: 0, absent; 1, focally present; 2, diffusely present. Alveolar edema was scored as: 0, absent; 1, mild, focal; 2, mild, diffuse; 3, severe, focal; 4, severe, diffuse. Total lung edema was scored by adding the interstitial and alveolar edema scores. Endoalveolar macrophage quantification (alveolar macrophage score) was scored as: 0, absence; 1, mild infiltration; 2, moderate infiltration; 3, severe infiltration.

### BAL protein concentration

The right lung was lavaged four times with 0.5 mL phosphate-buffered saline (PBS). Total protein concentration was measured by Micro BCA protein assay (Pierce, Rockford, IL), as per the manufacturer's instructions.

### Lung weight-to-dry (W/D) ratio

After 3 h of ventilation, the left lung was tied at the left hilum, weighed (wet weight), desiccated at  $80^{\circ}\text{C}$  for 1 week, then reweighed (dry weight).

### Lung protein expression

Expression of phosphorylated and/or total protein kinase B (Akt), endothelial nitric oxide synthase (eNOS), and inducible nitric oxide synthase (iNOS) was measured by Western blot using monoclonal antibodies (Cell Signaling

Technology; BD Biosciences). Densitometry was analyzed using Quantity One 4.6.1 software (BioRad, CA).

#### Perfusate analysis of nitrite/nitrate and cytokines

Perfusate samples were collected every 30 min throughout ventilation from the left atrium cannula. Nitrite/nitrate generation in lung perfusate samples was measured by Griess reagent (Sigma). Perfusate levels of interleukin-6 (IL6) and macrophage inflammatory protein-2 (MIP2) (R&D Systems, Minneapolis, MN) were assessed by enzyme-linked immunosorbent assay (ELISA).

#### Immunohistochemistry of pAkt, peNOS, and nitrotyrosine

Paraffin-fixed lung sections were incubated with primary antibodies against pAkt (Cell Signaling Technology), peNOS (BD Transduction Laboratories, USA) or nitrotyrosine (clone HM11; Santa Cruz Biotechnology). Immunohistochemical quantitation of nitrotyrosine accumulation in lung tissue was evaluated in the alveolar wall, endoalveolar acellular material, and macrophages and was scored as: 0, absent; 1, 1–5% of the evaluated structure; 2, 5–10% of the evaluated structure; 3, >10% of the evaluated structure.

#### Determination of cAMP content in lungs

Lung tissue cAMP content was determined by radiolabeled competitive protein binding assay (Cyclic AMP 3H Biotrak Assay System; GE Healthcare Life Science, Buckinghamshire, UK), as per the manufacturer's instructions.

#### Determination of myeloperoxidase in lungs

Myeloperoxidase was measured in lung homogenate using an enzyme-linked immunosorbent assay kit, according to the manufacturer's instructions (Hycult Biotechnology, Uden, The Netherlands). Lungs from septic mice [induced by cecal ligation and perforation (CLP)] [26] were used as a positive control.

#### Treatment with the PI3K $\gamma$ inhibitor AS605240 and enoximone

In wild-type animals, AS605240, a PI3K $\gamma$  inhibitor [27], was administered subcutaneously (10 mg/kg) 16 h before ventilation and was added to the perfusate (0.1 mg/kg/min) during ventilation. Enoximone (Perfan; INCA-

Pharm), a phosphodiesterase-3 (PDE3) inhibitor, was used to mimic the increased cAMP observed in PI3K $\gamma$ -knockout mice. In wild-type and knockout animals, enoximone was administered ex vivo into the pulmonary artery (1 mg/kg bolus) and into the perfusate (90  $\mu$ g/kg/min). Additionally, in wild-type animals, both AS605240 and enoximone were administered. Control animals received the respective drug vehicles. During the 3 h of HIGH STRESS or LOW STRESS ventilation, lung perfusate samples were obtained for nitrite/nitrate determination. Following ventilation, dynamic lung compliance was calculated as:  $C_{LUNG} = \text{tidal volume}/(\text{end inspiratory pressure} - \text{end expiratory pressure})$ . The left lung was obtained for cAMP determination and pAkt expression, and BAL was performed on the right lung for total protein determination.

## Statistics

Results are presented as mean  $\pm$  standard error of the mean (SEM). Comparison among groups was performed using the Kruskal–Wallis test, followed by Dunnett multiple-comparison post hoc analysis. A  $p$  value <0.05 was considered statistically significant (SPSS Inc., Chicago, IL).

## Results

### Respiratory mechanics and vascular permeability changes

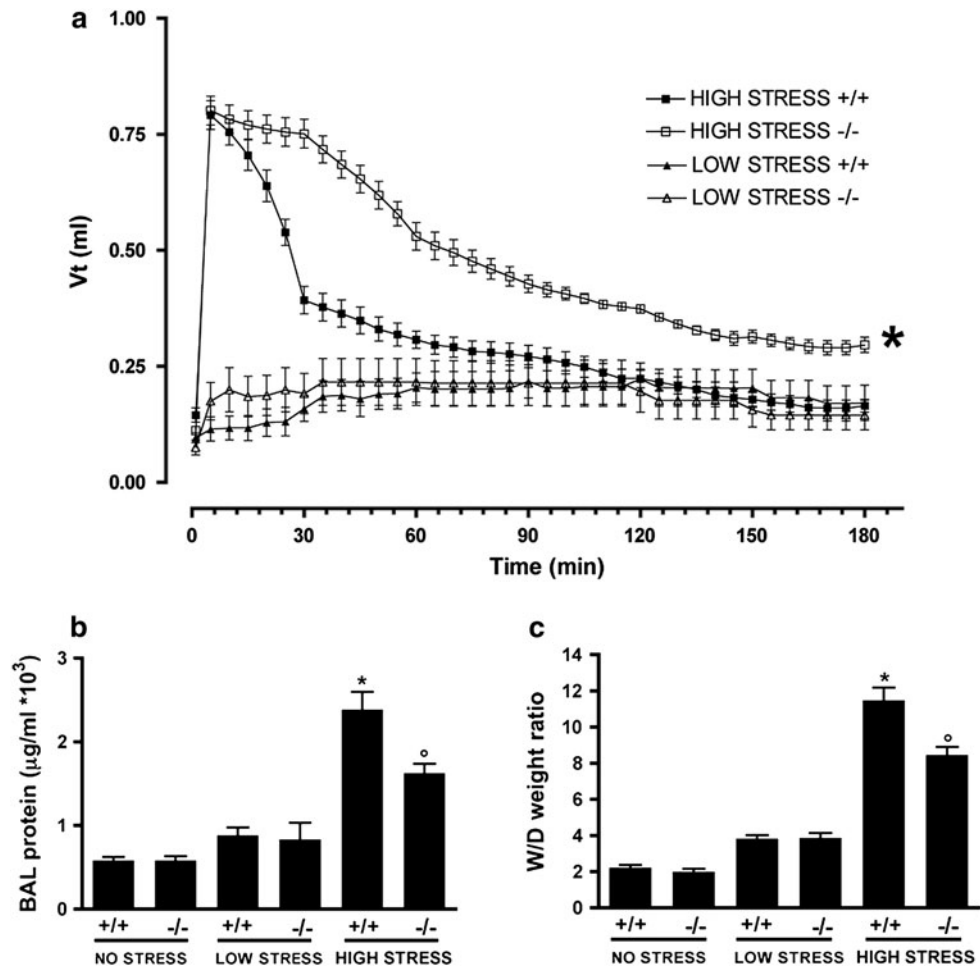
During the initial 60 min stabilization period prior to randomization, tidal volume (Vt) did not differ among groups. Immediately following randomization, there was a significant increase in Vt in both HIGH STRESS groups (Fig. 1a). However, from 20 min until the end of the experimental period, the Vt of the HIGH STRESS<sup>-/-</sup> group remained significantly higher than that of the HIGH STRESS<sup>+/+</sup> group ( $p < 0.05$ ; Fig. 1a).

HIGH STRESS induced significant increases in BAL protein and W/D ratio compared with controls. Lungs from HIGH STRESS<sup>+/+</sup> animals had significantly increased BAL protein concentration and edema ( $p < 0.05$ ) compared with lungs of HIGH STRESS<sup>-/-</sup> animals (Fig. 1b, c).

### Lung edema and alveolar macrophage scores

In NO STRESS animals there were no observable differences in lung morphology or edema score (Fig. 2a, b). Following HIGH STRESS ventilation, wild-type mice

**Fig. 1 a** Changes in tidal volume versus time. Given that we used a fixed transpulmonary pressure throughout the study, decreases in tidal volume over time are a surrogate for decreases in lung compliance. Experimental groups are: LOW STRESS (EEP -10 cmH<sub>2</sub>O, EEP -3 cmH<sub>2</sub>O) wild type (+/+) and PI3K $\gamma$  knockout (-/-), HIGH STRESS (EEP -25 cmH<sub>2</sub>O, EEP 0 cmH<sub>2</sub>O) wild type (+/+) and PI3K $\gamma$  knockout (-/-), and NO STRESS (flushed but no ventilation) wild type (+/+) and PI3K $\gamma$  knockout (-/-), where  $n = 8$  for all groups. \*HIGH STRESS<sup>-/-</sup> was significantly different from all other groups, starting from 20 min and throughout the experimental period. **b** and **c** BAL protein concentrations and W/D weight ratios of ventilated and perfused lungs. \*HIGH STRESS<sup>+/+</sup> was significantly different from all other groups; °HIGH STRESS<sup>-/-</sup> was significantly different from both LOW STRESS and NO STRESS groups



had higher edema scores compared with knockout ( $p < 0.01$ ), LOW STRESS and NO STRESS ( $p < 0.001$ ) animals. By contrast, HIGH STRESS knockout lungs developed higher lung edema scores only compared with NO STRESS ( $p < 0.05$ ).

During HIGH STRESS ventilation there was a statistically significant increase in the alveolar macrophages score compared with LOW STRESS and NO STRESS ( $p < 0.05$ ), but there was no significant difference between genotypes (Fig. 2c).

#### Intracellular signaling activation

Phosphorylated Akt, eNOS (Fig. 3a, b), and iNOS (ESM Fig. 1) were each not significantly different between LOW STRESS<sup>+/+</sup> and LOW STRESS<sup>-/-</sup>. However, HIGH STRESS<sup>+/+</sup> lungs had significantly increased phosphorylated Akt and eNOS compared with HIGH STRESS<sup>-/-</sup> ( $p < 0.05$ ), LOW STRESS, and NO STRESS groups ( $p < 0.05$ ; Fig. 3a, b). The HIGH STRESS<sup>-/-</sup> group exhibited a significant increase in pAkt and peNOS

compared with only NO STRESS ( $p < 0.05$ ; Fig. 3a, b). The concentration of iNOS was not different between groups (ESM Fig. 1).

Immunohistochemistry for pAkt demonstrated that LOW STRESS<sup>-/-</sup> lungs had less alveolar staining (brown) than LOW STRESS<sup>+/+</sup> (ESM Fig. 2). HIGH STRESS<sup>+/+</sup> had qualitatively more pAkt staining than LOW STRESS controls, which was nearly completely blocked in the HIGH STRESS<sup>-/-</sup> group. Immunohistochemistry for peNOS confirmed that eNOS activation in both LOW STRESS groups was minimal (ESM Fig. 3). In the HIGH STRESS<sup>+/+</sup> group, alveolar cells were strongly positive for peNOS, while the HIGH STRESS<sup>-/-</sup> group displayed observably less staining. NO STRESS groups displayed no observable difference in pAkt or peNOS staining (ESM Figs. 2, 3).

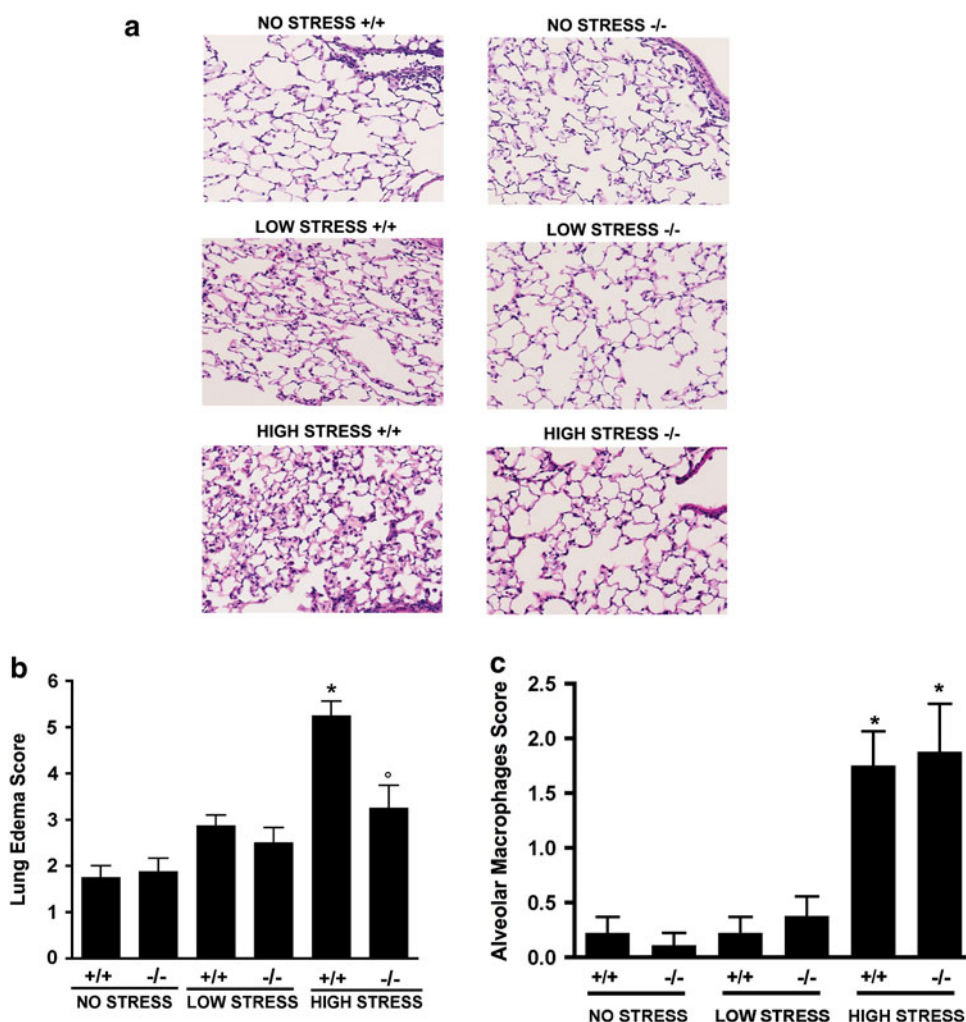
#### Nitrate/nitrite and cytokines release from the lung

The nitrate/nitrite levels in HIGH STRESS<sup>-/-</sup> were equal to those of LOW STRESS lungs (Fig. 3c).



**Fig. 2** Histological assessment of lung edema and macrophage infiltration: **a** representative lung micrographs stained with hematoxylin and eosin (200× magnification); **b** histological lung edema score: combined assessment of alveolar and interstitial edema formation.

\*The HIGH STRESS<sup>+/+</sup> group was significantly different from all the other groups; °The HIGH STRESS<sup>-/-</sup> group was different from both NO STRESS groups. **c** Alveolar macrophage score: an assessment of alveolar macrophages infiltration. \*Both HIGH STRESS groups were different from all other groups. Experimental groups and the number of animals are as in Fig. 1



However, in HIGH STRESS<sup>+/+</sup> there was a progressive increase in nitrate/nitrite throughout the 180 min of ventilation compared with HIGH STRESS<sup>-/-</sup> ( $p < 0.05$ ) and LOW STRESS groups ( $p < 0.05$ ). Perfusate IL6 and MIP2 concentrations increased over time in both LOW STRESS and HIGH STRESS groups, but increased more rapidly in HIGH STRESS groups (ESM Fig. 4). Cytokine concentrations were not significantly different between knockout and wild-type lungs within each ventilation strategy.

#### Nitrotyrosine immunohistochemistry

HIGH STRESS<sup>+/+</sup> demonstrated the greatest nitrotyrosine staining (Fig. 4a), with high accumulation in the alveolar wall compared with HIGH STRESS<sup>-/-</sup> ( $p < 0.05$ ; Fig. 4b). Lung macrophage nitrotyrosine accumulation also tended to increase in HIGH STRESS<sup>+/+</sup> compared

with HIGH STRESS<sup>-/-</sup> (Fig. 4c), but did not reach statistical significance.

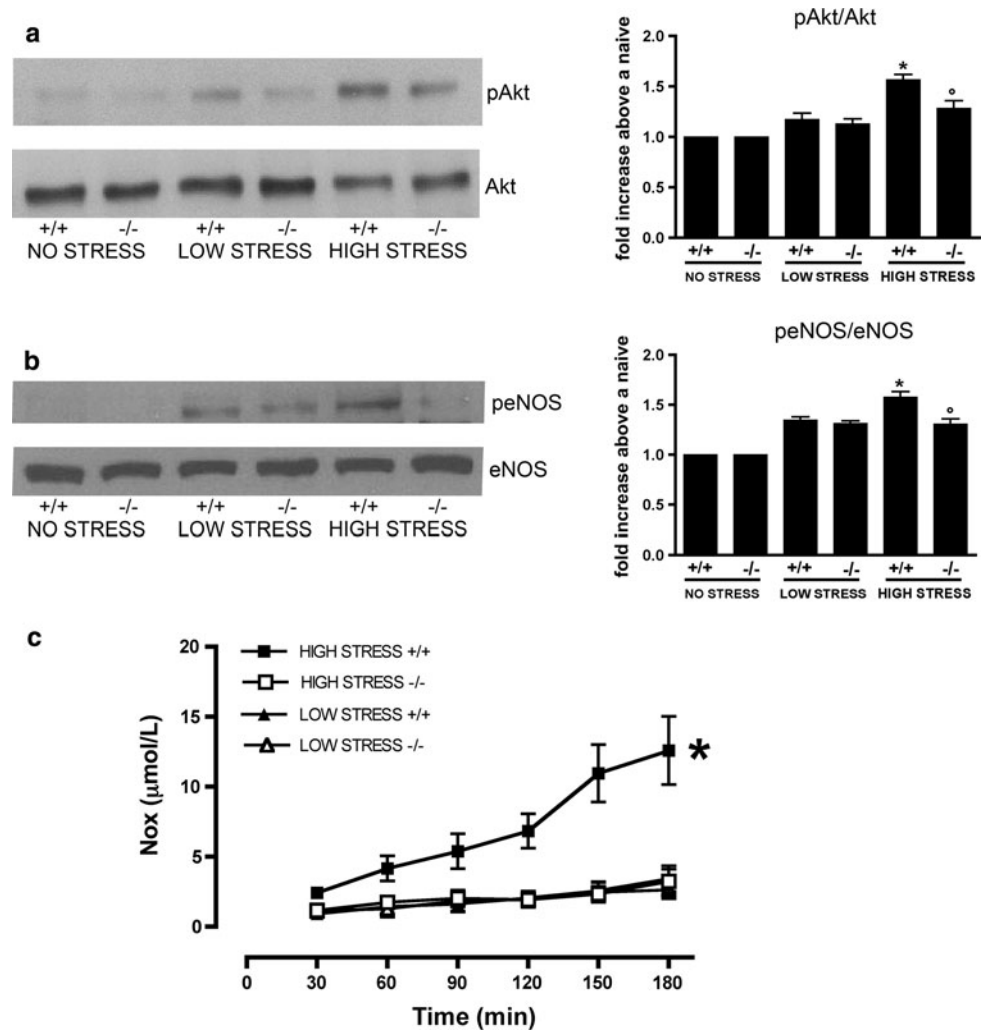
#### cAMP content in lungs

The HIGH STRESS<sup>+/+</sup> lungs had no significant increase in cAMP levels compared with LOW STRESS and NO STRESS controls. However, the HIGH STRESS<sup>-/-</sup> lungs had significantly increased cAMP levels versus HIGH STRESS<sup>+/+</sup>, LOW STRESS, and NO STRESS ( $p < 0.05$ ; Fig. 5).

#### MPO content in lungs

Lung MPO concentration was not different between wild-type and knockout in NO STRESS, LOW STRESS, and HIGH STRESS ventilation, but were each significantly

**Fig. 3** Ventilation-induced activation of: **a** pAkt and **b** phosphorylated endothelial NOS (peNOS), assessed in lung homogenate by Western blot. Densitometric values are displayed as a phosphorylated/unphosphorylated ratio fold increase. Experimental groups are identical to those described in Fig. 1, and  $n = 5$  for all groups. **c** Time course of nitrate/nitrite concentration in lung perfusate, with  $n = 8$  for all groups. \*HIGH STRESS<sup>+/+</sup> was different from all other groups



lower than the CLP (positive control;  $p < 0.05$ ) (ESM Fig. 4).

#### Effect of PI3K $\gamma$ kinase-dependent and kinase-independent inhibition

We used the inhibitors AS605240 to block the PI3K $\gamma$  kinase activity and enoximone, a PDE3 inhibitor, to mimic the increased cAMP resulting from the lack of PI3K $\gamma$  kinase-independent activity [20]. Wild-type lungs treated with both inhibitors showed significantly higher compliance compared with single inhibitor administration or vehicle-treated lungs ( $p < 0.05$ ; Fig. 6a). Moreover, this compliance was similar to PI3K $\gamma$ -knockout lungs (Fig. 6a). Lungs treated with either AS605240 or enoximone had significantly lower BAL protein concentration compared with wild type ( $p < 0.05$ ), and lungs treated with both inhibitors showed BAL protein concentration similar to knockout lungs (Fig. 6b).

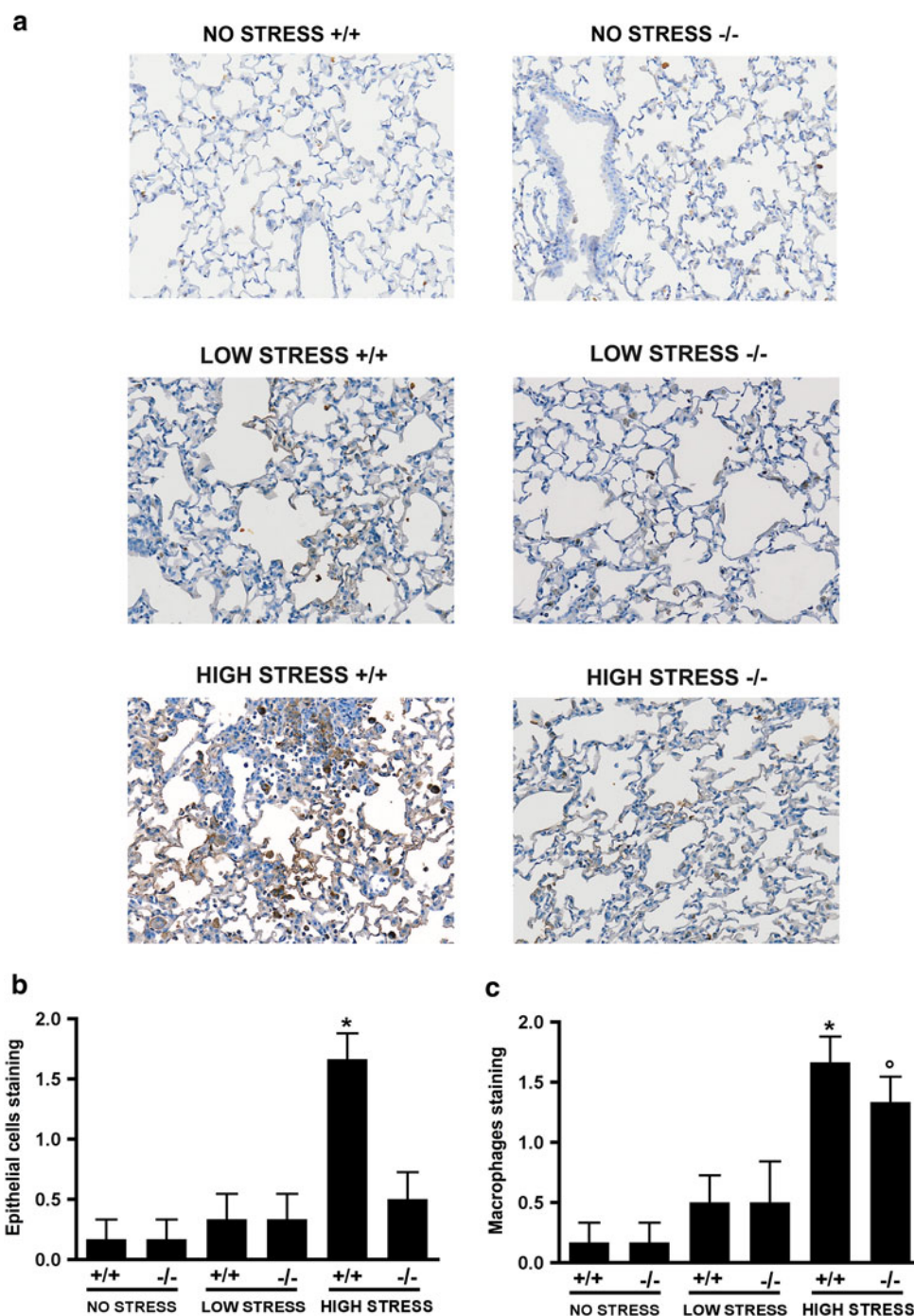
Administration of the inhibitors was also associated with reduced pAkt lung expression, reduced nitrate/nitrite

release, and increased cAMP levels. Lungs treated with AS605240 only or in combination with enoximone showed a significant reduction ( $p < 0.05$ ) of pAkt lung expression (ESM Fig. 8) and nitrate/nitrite release (Fig. 6c) compared with wild-type lungs, with comparable levels to knockout lungs (ESM Figs. 8 and 6c). Moreover, lungs treated with enoximone only or in combination with AS605240 showed increased ( $p < 0.05$ ) cAMP levels compared with wild-type lungs, with similar levels to knockout lungs (Fig. 6d). There were no differences among all inhibitor study groups during LOW STRESS ventilation (ESM Fig. 6). Enoximone had no effect when administered to PI3K $\gamma$ -knockout lungs during HIGH STRESS mechanical ventilation (ESM Fig. 7).

## Discussion

The major finding of this study is that, in the absence of circulating leukocytes, PI3K $\gamma$  of resident lung cells

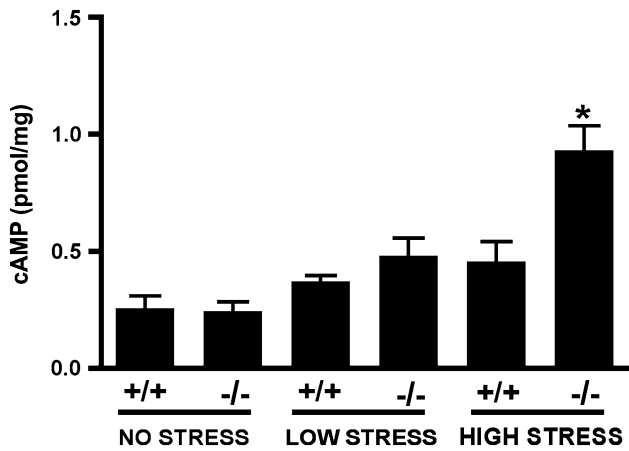
**Fig. 4** Lung nitrotyrosine analysis. **a** Representative immunohistochemical staining of nitrotyrosine; note the highest staining intensities for nitrotyrosine in the alveolar cells in the HIGH STRESS<sup>+/+</sup> group. **b** Lung epithelial cell nitrotyrosine score: an assessment of alveolar epithelial cells that have positive staining. \*HIGH STRESS<sup>+/+</sup> was significantly different from all other groups. **c** Histological lung macrophages nitrotyrosine score: an assessment of macrophage positive staining. \*HIGH STRESS<sup>+/+</sup> was significantly different from LOW STRESS and NO STRESS groups; °HIGH STRESS<sup>-/-</sup> was significantly different from NO STRESS groups. Experimental groups are the same as those described in Fig. 1, and  $n = 6$  for all groups



directly contributes to the development of ventilation-induced lung injury (VILI) and stretch-induced alveolar edema. Furthermore, pharmacological blockade of PI3K $\gamma$  kinase activity alone or in combination with a therapy aimed to increase cAMP levels may be a suitable strategy to attenuate VILI.

The isolated and perfused lung model provided necessary advantages in that it: (1) mimics normal circulation and hemodynamic forces, (2) maintains pulmonary

viability and function, and (3) allows for analysis of lung-derived mediator production [23]. Nonetheless, this model has some weaknesses. First, we assumed that our ventilated, perfused ex vivo lung model has a minimum number of neutrophils, which hence do not play a role in the increase in lung injury observed during HIGH STRESS ventilation. This assumption is based on the fact that the perfusate used is a cell-free solution that effectively “washes out” intravascular cells. However, to ensure that margined and/or



**Fig. 5** Lung cAMP content after 3 h of mechanical ventilation. Experimental groups are identical to those described in Fig. 1, and  $n = 5$  for all groups. \*HIGH STRESS<sup>-/-</sup> was significantly different from all other groups

resident macrophages were not playing a major role, MPO and macrophage infiltration was evaluated, showing no differences between genotypes, strongly suggesting that inflammatory cells did not play a significant role. Finally,

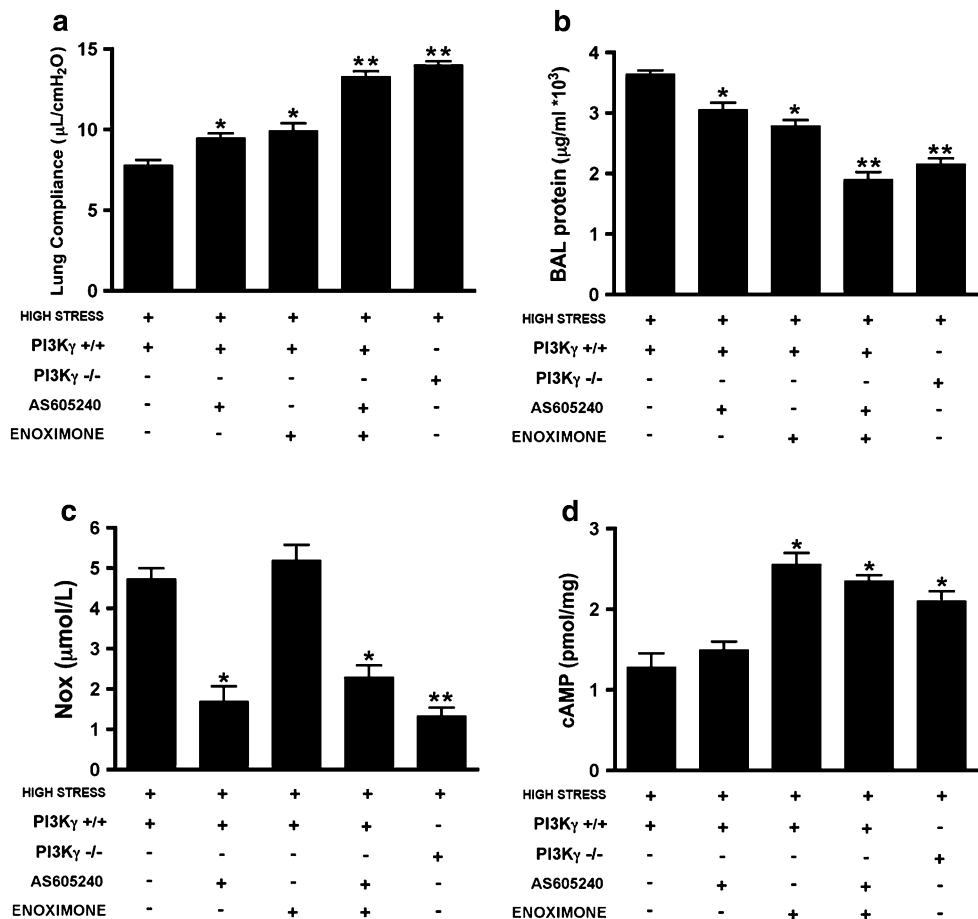
while this model was ideally suited to answer the questions posed in this study, the in vivo response may vary due to circulating leukocytes and inflammatory mediators.

PI3K $\gamma$  and its downstream target Akt influence migration, inflammation, and survival [18]. PI3K $\gamma$  also possesses kinase-independent scaffolding activity, which regulate intracellular cAMP concentrations [20, 21]. We previously demonstrated that PI3K $\gamma$ -knockout lungs were protected from VILI through modulation of the apoptosis/necrosis balance [22]. The novelty of this study specifically lies in the novel role of PI3K $\gamma$  in VILI-induced edema formation related to its kinase-dependent and kinase-independent activities. Additionally, we demonstrate that blocking PI3K $\gamma$  kinase activity and increasing lung cAMP levels may be a useful strategy to mitigate VILI.

During VILI, pulmonary edema occurs through two different mechanisms: tensile failure in the endothelial-epithelial barrier, and inhibition of alveolar fluid clearance [28, 29]. Since PI3K $\gamma$  may be involved in both of these mechanisms, we examined signaling pathways known to contribute to these systems.

The nitric oxide signaling pathway contributes to increased endothelial-epithelial barrier breakdown, vascular permeability, and edema formation during ventilation

**Fig. 6** Protective effects of AS605240 and enoximone on the lungs during HIGH STRESS mechanical ventilation. Experimental groups are HIGH STRESS<sup>+/+</sup>, HIGH STRESS<sup>+/+</sup> plus AS605240, HIGH STRESS<sup>+/+</sup> plus enoximone, HIGH STRESS<sup>+/+</sup> plus AS605240 and enoximone, and HIGH STRESS<sup>-/-</sup>, where  $n = 5$  for all groups. **a** Lung compliance. **b** BAL protein concentrations. **c** Nitrate/nitrite concentration in lung perfusate. **d** Lung cAMP content. \*Significant difference with  $p < 0.05$  compared with HIGH STRESS<sup>+/+</sup>. \*\*Significant difference with  $p < 0.001$  compared with HIGH STRESS<sup>+/+</sup>





[10, 30, 31]. Additionally, eNOS activation is dependent on PI3K/Akt during mechanical ventilation [32]; therefore, we evaluated the effects of pulmonary PI3K $\gamma$  on nitric oxide synthesis alteration and byproduct production. We found that pAkt and peNOS, but not iNOS, were diminished in pulmonary tissue of PI3K $\gamma$ -knockout mice following HIGH STRESS ventilation. However, contrary to these findings, mice overexpressing eNOS showed attenuated VILI, which may result from the in vivo role of eNOS-derived NO in inhibiting leukocyte infiltration [33].

Specific downstream activation of PI3K pathways may alter the effect on vascular permeability, since activation of Src family kinase by PI3K increased permeability while PI3K/Akt/glycogen synthase kinase 3 $\beta$  activation limited lung edema [34]. Our findings provide new insights into the effects of PI3K $\gamma$  on NO and cAMP levels relative to edema formation during high-stress mechanical ventilation.

Nitric oxide has an extremely short half-life; however, the quantity of NO can be approximated by analysis of nitrate/nitrite, which are formed following its reaction with oxygen. NO can also induce production of peroxynitrite, which is a potent oxidant capable of damaging alveolar epithelium [13]. We found increased production of nitrate/nitrite and nitrotyrosine, a marker of peroxynitrite, in response to HIGH STRESS ventilation, which was completely abrogated in lungs lacking PI3K $\gamma$ . Several previous studies have correlated nitrotyrosine with the development of acute lung injury in patients [14, 35]. Therefore, we speculate that, since PI3K $\gamma$ -knockout lungs are protected from pulmonary nitrosylation and VILI, the PI3K $\gamma$  isoform plays a pivotal role in the loss of endothelial–epithelial barrier integrity.

Both animal and human studies have demonstrated that high-stress ventilation leads to cytokine/chemokine release associated with subsequent lung damage known as biotrauma [23, 36–38]. In addition, mediator release can be further altered by nitric oxide signaling [30]. While we confirm that pulmonary cytokine production was increased throughout HIGH STRESS ventilation, it was not be significantly different between genotypes, despite differences in NO activity. This may be due to the fact that iNOS-derived NO, unaltered in our experiment, is more closely linked with biotrauma. Moreover, the rise in chemotactic cytokines and subsequent neutrophil

infiltration significantly lags behind the early induction of increased vascular permeability [22].

In addition to altering barrier permeability, PI3K $\gamma$  may also affect alveolar fluid clearance, further contributing to edema. Nitric oxide can inhibit alveolar fluid clearance during high-stress mechanical ventilation [11, 39]. Additionally, high-tidal-volume ventilation can induce a sustained reduction in cAMP-dependent alveolar fluid clearance [11]. PI3K $\gamma$  is known to modulate cAMP concentrations independent of its kinase activity [20, 21]. Since in our model HIGH STRESS<sup>-/-</sup>, but not HIGH STRESS<sup>+/+</sup>, displayed a significant augmentation of cAMP, this mechanism may contribute to decreased vascular permeability and increased alveolar fluid clearance.

In other disease models, such as sepsis [40] and arthritis [27], pharmacological inhibition of PI3K $\gamma$  kinase activity has been shown to modulate inflammation and improve outcomes. This study is the first showing that blocking PI3K $\gamma$  kinase activity in resident lung cells attenuates VILI through reduction in NO release. Moreover, a combined strategy using enoximone to increase cAMP, thereby mimicking the lack of PI3K $\gamma$  kinase-independent function, further attenuated VILI to levels similar to those in PI3K $\gamma$ -knockout mice.

In conclusion, this study demonstrates that pulmonary-derived PI3K $\gamma$  significantly contributes to the development of ventilation-induced lung injury and pulmonary edema. Furthermore, we correlate the development of VILI and edema with eNOS-derived nitric oxide signaling and cAMP hydrolysis, suggesting that PI3K $\gamma$  contributes to pulmonary edema by increasing endothelial–epithelial permeability and/or reducing alveolar fluid clearance. Ultimately, this study identifies PI3K $\gamma$  as a possible therapeutic target in the treatment and/or prevention of VILI and edema. Moreover, a pharmacological strategy able to block the effects of PI3K $\gamma$  in residential lung cells can mitigate the deleterious effects of mechanical stress, hopefully with minimal effect on the innate immune response.

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