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RESEARCH PAPER

Inhibition of the phosphatase PTEN protects mice against oleic acid-induced acute lung injury

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Background and purpose: Injury to the lung parenchyma is a constitutional feature shared by many lung diseases. The protein, phosphatase and tensin homologue deleted on chromosome Ten (PTEN) is a major suppressor of phosphoinositide-3 kinase/Akt signalling, a vital survival pathway in lung parenchymal cells. Based on this, we hypothesized that PTEN inhibition *in vivo* would enhance cell tolerance to stress thereby preventing acute lung injury.
Experimental approach: We evaluated the ability of a PTEN inhibitor, potassium bisperoxo (1,10-phenanthroline) oxovanadate [bpV(phen)], to prevent acute lung injury induced by oleic acid (OA) in adult C57BL/6 mice. Lung assessments included bronchoalveolar lavage, tissue morphology, immunostaining for markers of cell death, cell identity, phospho-Akt and phospho-ERK levels and oximetry.
Key results: OA induced acute lung injury in a dose- and time-dependent manner. No injury was observed in the vehicle control or bpV(phen) treatment groups. PTEN inhibition by bpV(phen) increased lung tissue levels of phospho-Akt and ERK and but not focal adhesion kinase. This occurred in conjunction with a statistically significant reduction in protein content, lactate dehydrogenase, as well as tumour necrosis factor-α and chemokines in bronchoalveolar lavage fluid when compared with OA treatment alone. The incidence of alveolar lesions, consistent with acute lung injury, and terminal uridine deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)-positive cells was also significantly reduced. Importantly, PTEN suppression maintained pulmonary function.
Conclusions and implications: Treatment with bpV(phen) significantly reduced the severity of acute lung injury in mice indicating that additional investigation is warranted to understand the important role that this phosphatase may play in the lung.
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Keywords: PTEN; lung; epithelium; phosphatase; bpV(phen); apoptosis; cell death

Abbreviations: PTEN, phosphatase and tensin homologue deleted on chromosome Ten; OA, oleic acid; bpV(phen), potassium bisperoxo (1,10-phenanthroline) oxovanadate; TUNEL, Terminal uridine deoxynucleotidyl transferase dUTP nick end labeling; PI3K, phosphoinositide-3 kinase; ARDS, acute respiratory distress syndrome; OA, oleic acid; LDH, lactate dehydrogenase; ALI, acute lung injury; IT, intratracheal instillation; IACUC, Institutional Animal Care and Use Committee; H&E, haematoxylin and eosin; PCNA, proliferating cell nuclear antigen; RCA, type I epithelial marker *ricinus communis* agglutinin I; SpO_2, Oxygen saturation in peripheral arterial blood; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; FAK, focal adhesion kinase; KGF, keratinocyte growth factor; PI(3,4,5)P_3, phosphatidyl-inositol 3,4,5-trisphosphate; MAPK, mitogen activated protein kinase; PBS, phosphate buffered solution; GSK3, glycogen synthase kinase-3; pAkt, phosphorylated Akt

Introduction

The lung epithelium in collaboration with the endothelium forms an essential barrier that maintains tissue homeostasis, optimal gas exchange, and restricts fluid accumulation in the airway (Mutlu and Sznajder, 2005; Jain and Eaton, 2006). Acute lung injury (ALI) (Bernard *et al.*, 1994) can progress rapidly during the host systemic inflammatory response as a consequence of epithelial and endothelial cell injury and flooding of the alveolar space thereby substantially reducing ventilation. Severe ALI is associated with high mortality (Hudson and Steinberg, 1999), and no therapeutic agents are currently available to protect the lung parenchyma or enhance tissue repair. Therefore, the identification of novel molecular targets that influence protection of the lung parenchyma or wound repair following injury are warranted.

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The phosphoinositide-3 kinase (PI3K)/Akt signal transduction pathway is a potent inducer of cell survival (Ray *et al.*, 2003). We previously reported that activation of this pathway protects the lung epithelium from apoptosis (Bao *et al.*, 2005). The protein, phosphatase and tensin homologue deleted on chromosome Ten (PTEN) is the primary counter-regulator of the PI3K/Akt pathway by virtue of its lipid phosphatase activity (Chan *et al.*, 1999; Maehama *et al.*, 2001). Our group also recently reported that pharmacological suppression of PTEN with bisperoxovanadium compounds, relatively potent PTEN inhibitors, activated Akt-mediated signalling events culminating in enhanced lung epithelial wound repair. In particular, Akt phosphorylation as well as activation of the downstream signalling kinase, glycogen synthase kinase-3, were elevated following PTEN inhibition and most importantly, significantly accelerated wound closure following mechanical injury (Lai *et al.*, 2007). Based on this, we hypothesized that PTEN may also play a pivotal role in the lung in response to noxious stimuli. The purpose of this investigation was to evaluate the role of PTEN in the lung *in vivo* in a model of ALI.

Different preclinical models of ALI have been developed by utilizing different reagents including, but not limited to, bleomycin, bacterial lipopolysaccharide (LPS) and oleic acid (OA) (Julien *et al.*, 1986; Beilman, 1995; Hayashi *et al.*, 2002; Kim *et al.*, 2005; Ulrich *et al.*, 2005; Zhou *et al.*, 2005; Li *et al.*, 2006). OA has been reported to induce ALI in a manner characteristic of acute respiratory distress syndrome (ARDS) but without substantial inflammation. In particular, structural and functional alterations consistent with ARDS, including severe alveolar attentuation and oedema, can be reproducibly attained in mice exposed to OA (Zhou *et al.*, 2005). Based on this, we chose OA-induced ALI (OA-ALI) as our model to evaluate the role of PTEN as a molecular target in parenchymal injury.

We observed that the PTEN inhibitor, potassium bisperoxo (1,10-phenanthroline) oxovanadate [bpV(phen)] augmented activity within the PI3K and ERK signalling pathways in lung tissue and significantly reduced the extent of lung injury when administered before OA. Overall, PTEN inhibition significantly reduced the extent of epithelial and endothelial injury and helped to maintain respiration and reduce mortality. Based on this, we conclude that PTEN is a negative regulator of the parenchymal response to insult in the normal adult mouse lung. Furthermore, the ability to pharmacologically manipulate PTEN function in the lung with relative precision may provide an innovative opportunity to understand and treat lung disease.

Methods

Animal experiments

All animal procedures and studies were approved by the Institutional Animal Care and Use Committee (IACUC) at The Ohio State University. In general, four treatment groups of 5–10 mice were evaluated unless otherwise indicated. The four treatment groups included OA alone, bpV(phen) pretreated prior to OA, bpV(phen) alone and vehicle control for OA.

Induction of ALI

OA was given by intravenous injection, as described previously (Ulrich *et al.*, 2005; Zhou *et al.*, 2005). Female C57BL/6 mice, 8–10 weeks old, were studied. The formulation of OA was optimized as follows: 5% OA, 5% ethanol and 90% ethylene glycol. The mixture of 5% ethanol and 95% ethylene glycol was used as vehicle control.

Drug administration

Intraperitoneal (i.p.) injection and intratracheal (i.t.) instillation of bpV(phen) was examined to compare systemic and topical administration. For i.p. administration 0.4 μmol of the compound was administered in a total volume of 500 μL. For i.t. administration, mice were anesthetized by using isofluorane with ventilatory support. Some 0.4 μmol of bpV(phen) in 100 μL of vehicle was injected into the trachea.

Bronchoalveolar lavage fluid (BALF)

Bronchoalveolar lavage (BAL) was performed as previously described (Ulrich *et al.*, 2005; Zhou *et al.*, 2005). BALF was analysed for protein content (Bio-Rad, Hercules, CA), lactate dehydrogenase (LDH) release and multiple cytokines release by using a Bio-Plex Kit (Bio-Rad, Hercules, CA).

Histopathological and immunopathological evaluation

Lungs were inflated at 25 cm H_2O, fixed in 10% phosphate buffered solution (PBS)-buffered formaldehyde for 24 h and embedded in paraffin. Lung tissue sections were prepared and stained with haematoxylin and eosin (H&E), AE1/3 (1:200, Boehringer Mannheim, Indianapolis, IN), terminal uridine deoxynucleotidyl transferase dUTP nick end labelling (TUNEL), Masson's trichrome, proliferating cell nuclear antigen and anti-active caspase-3 followed by microscopic evaluation. Immunofluorescence was conducted by utilizing the vascular endothelial and type I epithelial marker *ricinus communis* agglutinin I (RCA) (Dobbs *et al.*, 1985; Alroy *et al.*, 1987), TUNEL, DAPI and evaluated by confocal microscopy. Lung perfusion was conducted as follows: immediately after killing the mice, the thoracic cavity was opened. The left ventricle was cut open prior to perfusion through the right ventricle with 30 mL of PBS. Perfused lungs were collected, snap frozen and stored at –80°C until further evaluation.

Measurement of collagen in formalin fixed tissue

The weight of the right upper lobe was measured from formalin-fixed lung tissue before processing. Tissue was washed with PBS for three times, then cut into small pieces and washed with PBS three times again, followed by homogenization by using a Mini-Beadbeater. Tissue pellets were incubated with 0.1% Sirius red dye in saturated picric acid (Sigma) for 30 min at room temperature. Unbound dye was discarded. Pellets were washed with saturated picric acid and distilled water three times. The bound dye was eluted by 0.1 N NaOH in methanol and shaken for 30 min. The supernatant was measured spectrophotometrically at 490 nm. Rat tail collagen was used as standard control.

Peripheral blood arterial oxygen saturation
Oxygen saturation in peripheral arterial blood (SpO2) was measured as described previously (Davis *et al.*, 2007).

Western blot analysis
The perfused lung was homogenized, and protein was extracted for Western blot analysis. Western blotting was performed as previously described (Lai *et al.*, 2007). The antibodies utilized in this study were anti-Akt (1:2000; Cell signaling, Beverly, MA), anti-p-Akt-Ser^{473} (1:1000; Cell signaling, Beverly, MA), anti-PTEN (1:1000; Cell signaling, Beverly, MA), anti-β-actin (1:5000; MP Biomedicals, OH), anti-ERK, anti-p-ERK, anti-FAK (1:1000; Cell signaling, Beverly, MA), anti-p-FAK (1:1000; Cell signaling, Beverly, MA), goat anti-mouse IgG-HRP (1:3000; Cell signaling, Beverly, MA) and goat anti-rabbit IgG-HRP (1:3000; Zymed, San Francisco, CA).

Statistical analysis
The Student's two sample *t*-test was used to compare the treatment groups against relevant controls. Analysis of variance (ANOVA) was used to evaluate dose response and time course studies as well as other experiments involving more than two study groups. *P* value criteria were set at $\alpha < 0.05$.

Materials
OA was purchased from Sigma-Aldrich (St. Louis, MO). bpV(phen) was purchased from Alexis Biochemicals (San Diego, CA). The cell toxicity assay (LDH kit Roche Applied Sciences Indianapolis, IN) was used according to the manufacturer's guidelines. Bio-Plex analysis (Bio-Rad, Hercules, CA) was used according to the manufacturer's guidelines.

Results

Development of the OA-ALI model
To begin our investigation we first established a more stable dosage suspension for OA by using ethylene glycol as the suspension vehicle (not shown). Using the improved formulation (see *Methods*) we then gave OA intravenously and determined the extent of ALI at different doses (0.1–0.4 μL g^{-1} body weight) and time points (1–4 h at 0.1 μL g^{-1} body weight) in adult mice to optimize the OA-ALI model. The extent of lung injury, as determined by the protein content of BAL, was both dose- and time- dependent. In contrast, no evidence of lung injury was observed with the vehicle control injected mice (Fig. 1A). Based on the initial optimization studies, a dose of 0.1 μL g^{-1} body weight followed by observation at 2 h was used for further analysis of lung injury unless otherwise stated.

Histopathological and immunopathological characterization of parenchymal damage
Lung tissue morphology was first evaluated by H&E staining in inflated, fixed lung sections following OA exposure. Overall, subtle differences in lung architecture were observed in the OA-treated mice when compared with vehicle controls (Fig. 1B). Consistent with a previous report, gross morphological differences were not observed, although attenuated areas of lung parenchyma were dispersed throughout the lung and confined largely within alveolar regions. Within each of these areas we observed alterations consistent with alveolar oedema and haemorrhage. There was no significant evidence of an inflammatory cell infiltrate. Next, we conducted TUNEL staining to directly visualize the distribution and extent of parenchymal injury. A consistently significant increase in consolidated regions of TUNEL-positive cells was observed within the alveolar tissue throughout the entire lung following OA administration (Fig. 1C) but not vehicle control-treated lungs. Fixed lung tissue specimens were then histochemically stained with the epithelial-specific marker AE1/3 and revealed a very similar pattern of localized, intense, staining within consolidated regions (Fig. 1D). The pattern of cellular changes observed by H&E, TUNEL and AE1/3 staining was essentially identical. We then determined if focal lesions were associated with type I lung epithelial and endothelial cell damage by immunofluorescent staining using both TUNEL and RCA, a specific marker for endothelial and type I lung epithelial cells (Dobbs *et al.*, 1985; Alroy *et al.*, 1987). Confocal analysis of Z-stack sections revealed that a majority of TUNEL-positive cells were also RCA-positive indicating that a significant extent of injury was associated with endothelial as well as type I epithelial cells (Fig. 1E).

Effect of PTEN inhibition on ALI
Having established a reproducible model of ALI, we next determined if pre-treatment with a relatively potent PTEN inhibitor, bpV(phen), could protect the lung parenchyma from OA-induced injury. We first observed that i.p. administration of bpV(phen) within 2–6 h before OA administration resulted in a significant reduction of BALF protein content (Fig. 2A) whereas bpV(phen) alone had no detrimental effect on the lung (inset graph). Next, we evaluated different doses of bpV(phen) and observed that a dose-responsive reduction in BALF protein content occurred within a relatively narrow dose range from 0.1 to 0.4 μmol. The lowest and highest doses evaluated did not significantly reduce BALF protein content (Fig. 2B). In addition, we observed that administration of bpV(phen) alone over the same dose range did not result in any change in BALF protein content, as also observed with the vehicle treatment control group (inset graph). Consistent with these findings, bpV(phen) treatment resulted in a statistically significant reduction in TUNEL positive cells when compared with OA treatment alone (Fig. 2C). The extent of cell injury was determined by measuring total pixel counts of the TUNEL positive cells and then taking the average value of at least 10 random fields per lung section for each individual animal studied. To further verify that bpV(phen) pre-treatment decreased lung injury, we measured LDH in the BALF. Again, bpV(phen) administration resulted in a significant reduction in LDH release when compared with OA treatment alone, indicating that less cell damage had been induced by OA, as a result of PTEN inhibition. LDH was not detected in the BALF from normal controls as well as the vehicle control treatment group (Fig. 3A). Similar results were

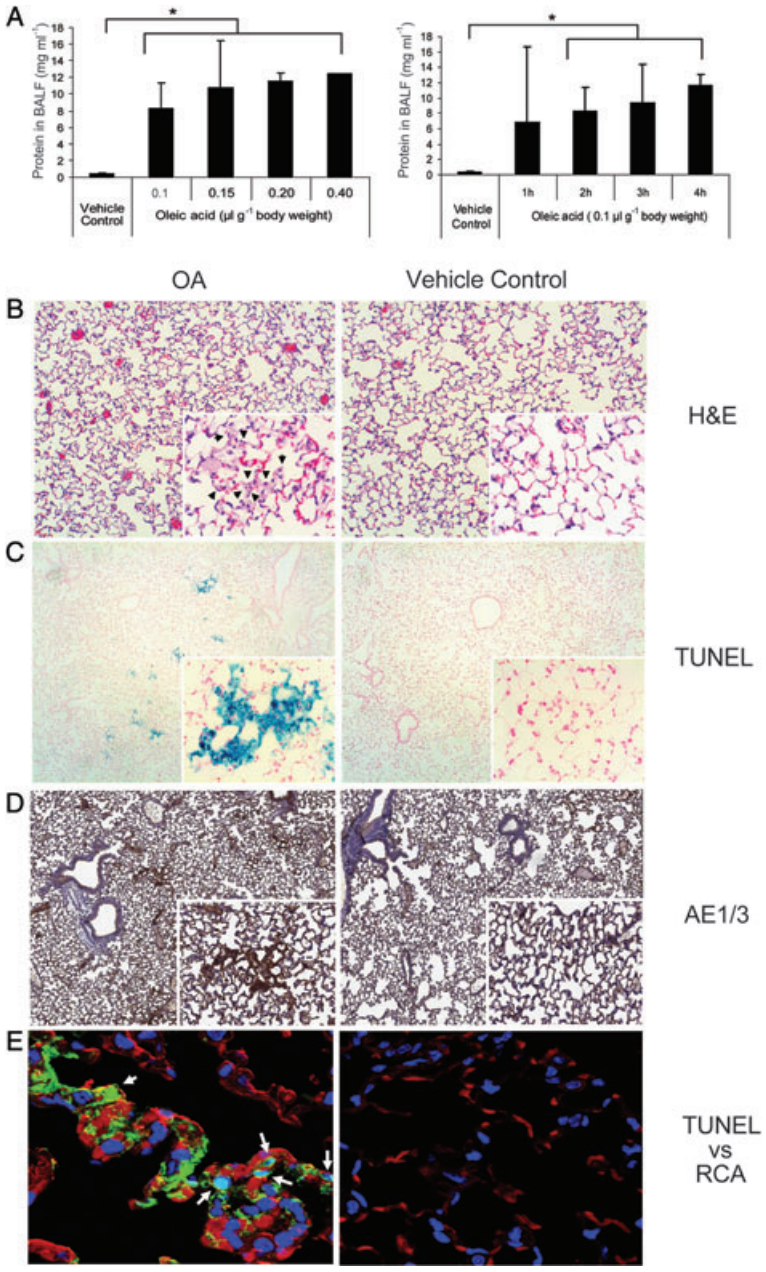

Figure 1 Characterization of acute lung injury (ALI) following oleic acid (OA) administration. (A) A dose-dependent increase in BALF protein content was observed following intravenous OA administration. We first evaluated acute lung injury at 2 h after injection with a dose range from 0.1 to 0.4 µL g^{-1} body weight (left panel). We then observed a time-dependent increase in bronchoalveolar lavage fluid (BALF) protein over 1–4 h following administration of a 0.1 µL g^{-1} body weight OA dose (right panel). No evidence of lung injury was observed following intravenous administration of the vehicle control under all conditions (*$P < 0.05$) ($n = 5$ animals per treatment condition). (B) Representative examples of haematoxylin and eosin (H&E) stained light micrographs of lung from OA-treated and vehicle control groups. The micrographs of the OA group show consolidated areas within the alveolar tissue of mild inflammatory infiltrate, alveolar haemorrhage and proteinaceous alveolar oedema whereas the vehicle control group appeared normal (Original magnification ×10 and ×40). (C) Terminal uridine deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining demonstrated a significant increase in TUNEL-positive cells in a pattern similar to that observed after H&E staining (×4). The lower right inset shows a representative higher magnification image of a consolidated region of TUNEL-positive cells (×40). (D) A very similar pattern was observed with the epithelial-specific marker AE1/3 demonstrating that a majority of the cells in the injured area are epithelia (×4 and ×20). (E) Next, fluorescent confocal microscopy and Z-axis analysis was conducted on the samples from the same animals by using TUNEL staining (green) and the type I lung epithelial marker *ricinus communis* agglutinin I (RCA) (red) (×40). TUNEL and RCA staining colocalized within a majority of the cells that comprised the consolidated areas (arrows). DAPI (blue) was utilized as a nuclear stain.

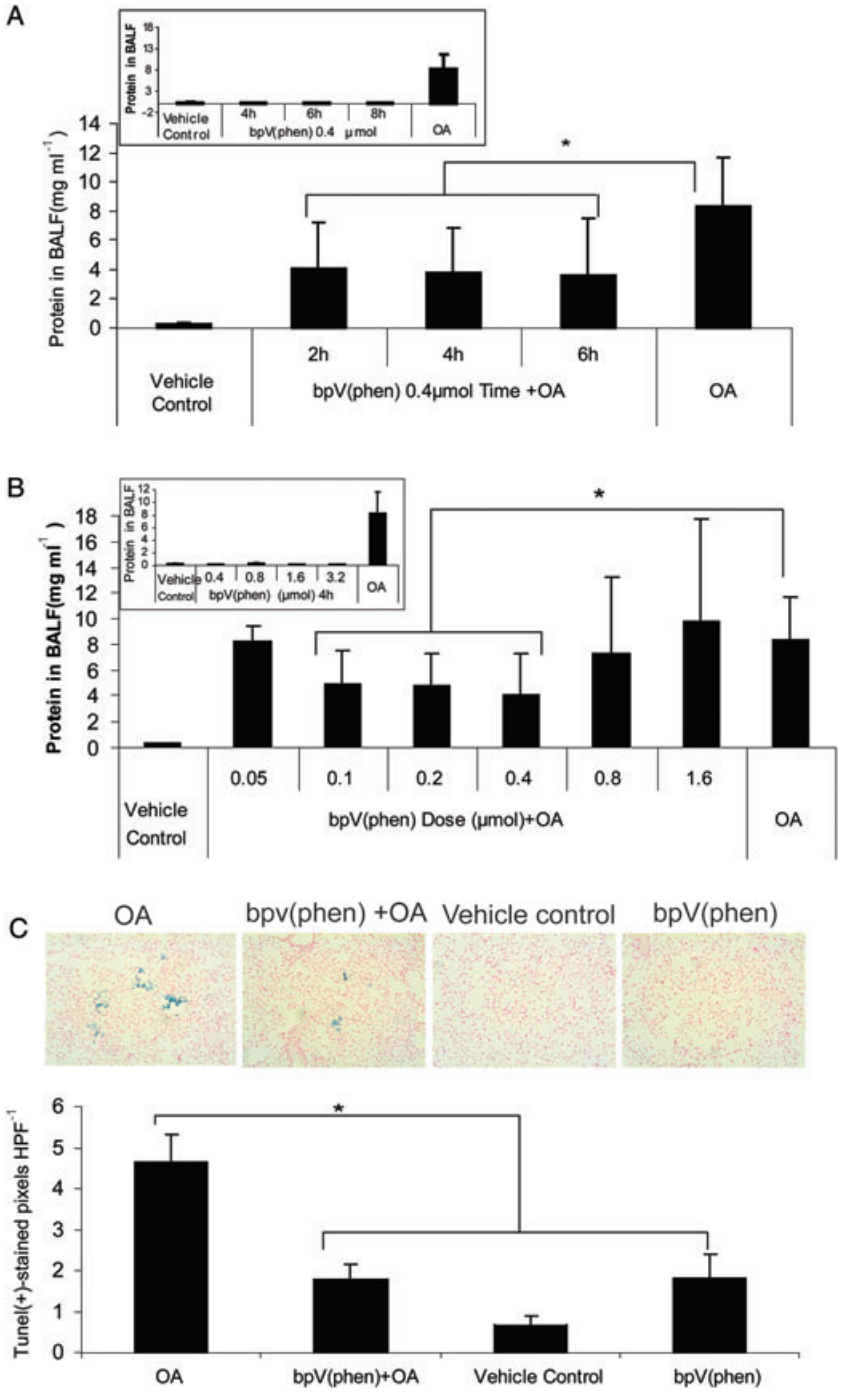

Figure 2 bpV(phen) prevents ALI. Initially, bpV(phen) was administered intraperitoneally (i.p.) at different times and doses before OA administration, and BALF protein was monitored as an index of acute lung injury (A) A significant decrease in BALF protein content was observed when bpV(phen) (0.4 µmol) was administered between 2 and 6 h before OA administration. No evidence of lung injury was observed following administration of bpV(phen) alone at the same times (upper left inset). (B) A biphasic dose response effect was observed over the dosage range studied (0.05–1.6 µmol). The most significant reduction in BALF protein content was observed between 0.1 and 0.4 µmol whereas at the lower and higher doses the benefit was diminished. BpV(phen) treatment alone over the same dose range showed no evidence of lung injury (upper left inset). (C) The magnitude of TUNEL staining (representative image for each treatment group, upper panel) was quantified by measuring the total pixel number as shown in the bottom panel. The average values from 10 fields for each tissue and from three animals for each treatment condition are shown. A statistically significant reduction in TUNEL-positive events was observed in the bpV(phen)-pre-treated lungs when compared with the OA treatment group (*$P < 0.001$). ALI, acute lung injury; BALF, bronchoalveolar lavage fluid; bpV(phen), potassium bisperoxo (1,10-phenanthroline) oxovanadate; OA, oleic acid; TUNEL, terminal uridine deoxynucleotidyl transferase dUTP nick end labelling.

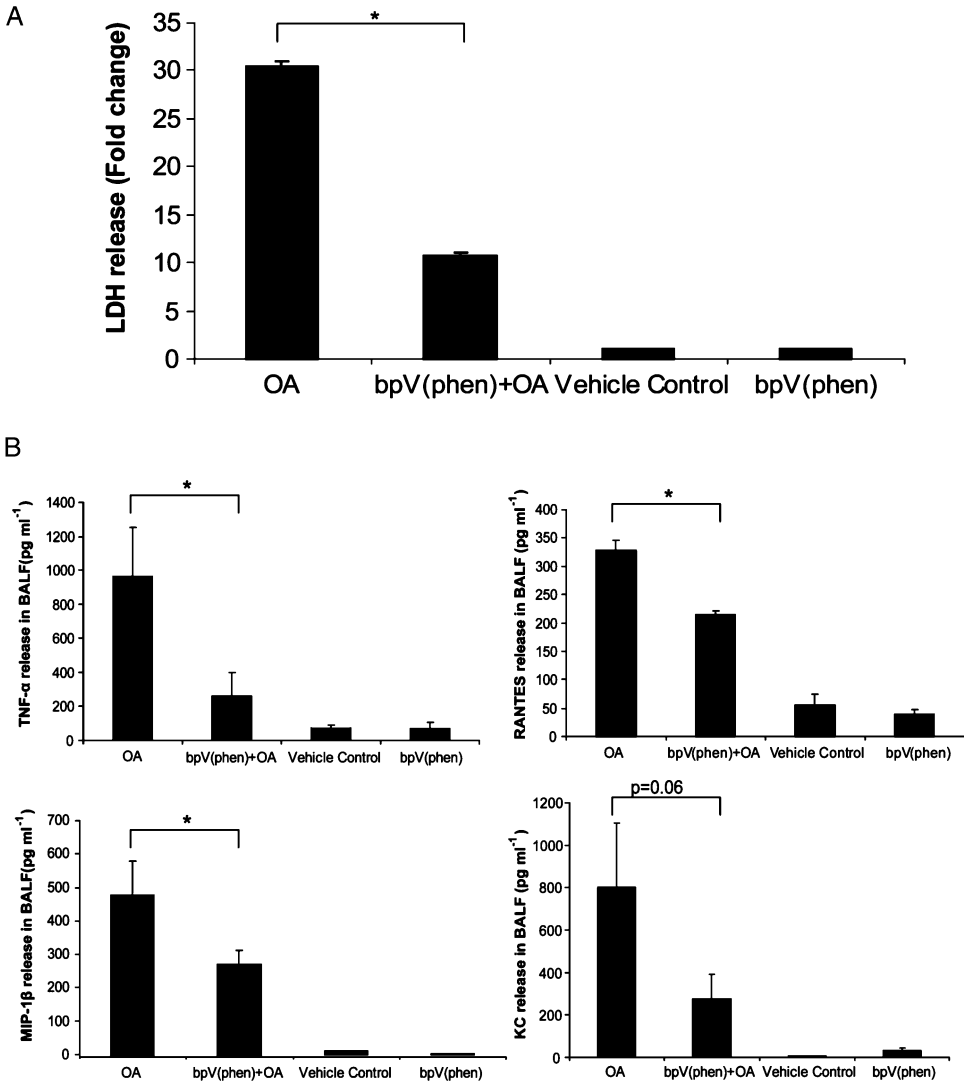

Figure 3 bpV(phen) pre-treatment preserves the lung environment. To verify the reduction in lung injury by bpV(phen) previously observed, BALF was assayed for LDH, TNFα and chemokine content. (A) Lactate dehydrogenase release was first evaluated as an index of cell damage in the airway environment. Consistent with previous observations, OA treatment alone caused a significant increase in LDH in BALF whereas pre-treatment with bpV(phen) (0.4 μmol at 2 h before OA) consistently resulted in a significant reduction in LDH content. Vehicle or bpV(phen) treatment alone did not cause LDH release. (B) Similar results were observed when we evaluated TNFα, RANTES and MIP-1β concentration in BALF. Pre-treatment with bpV(phen) resulted in a significant reduction in TNFα and RANTES. We also observed a similar reduction in KC, but this did not reach statistical significance (*$P = 0.06$). ($n = 12$ animals per treatment condition). BALF, bronchoalveolar lavage fluid; bpV(phen), potassium bisperoxo (1,10-phenanthroline) oxovanadate; LDH, lactate dehydrogenase; OA, oleic acid; TNFα, tumour necrosis factor-α.

observed when we analysed BALF for tumour necrosis factor-α (TNFα) as well as the chemokines RANTES, MIP-1β and KC. In particular, OA treatment increased BALF concentrations of all factors, whereas pre-treatment with bpV(phen) resulted in a significant reduction in most. A similar decrease in KC levels occurred but did not achieve statistical significance ($P = 0.06$) (Fig. 3B).

Effect of PTEN inhibition on cell signalling in the lung

To determine if the protection afforded by bpV(phen) pre-treatment was mediated through PTEN inhibition, we evaluated the phosphorylation status of the kinases Akt, ERK1/2 and focal adhesion kinase (FAK) in lung parenchymal tissue. Each protein is a potential downstream target of the PI3K signalling pathway and associated with mediating cell survival or repair. As a result, we predicted that PTEN inhibition would result in phosphorylation of some or all of these molecules. To determine this we evaluated homogenates of perfused lung at 1, 2 and 4 h after bpV(phen) exposure. We observed that PTEN is constitutively expressed in the lung and that total levels did not change following drug exposure (Fig. 4A). Most striking, bpV(phen) treatment resulted in a highly reproducible increase in Akt and ERK1/2 phosphorylation, but not FAK, suggesting that these two pathways are responsible for the lung protection observed following bpV(phen) treatment. Further, immunostaining of lung tissue revealed that only bpV(pic)-treated specimens exhibited a significant increase in phosphorylated Akt in lung parenchymal cells in the alveolar region (Fig. 4B). In

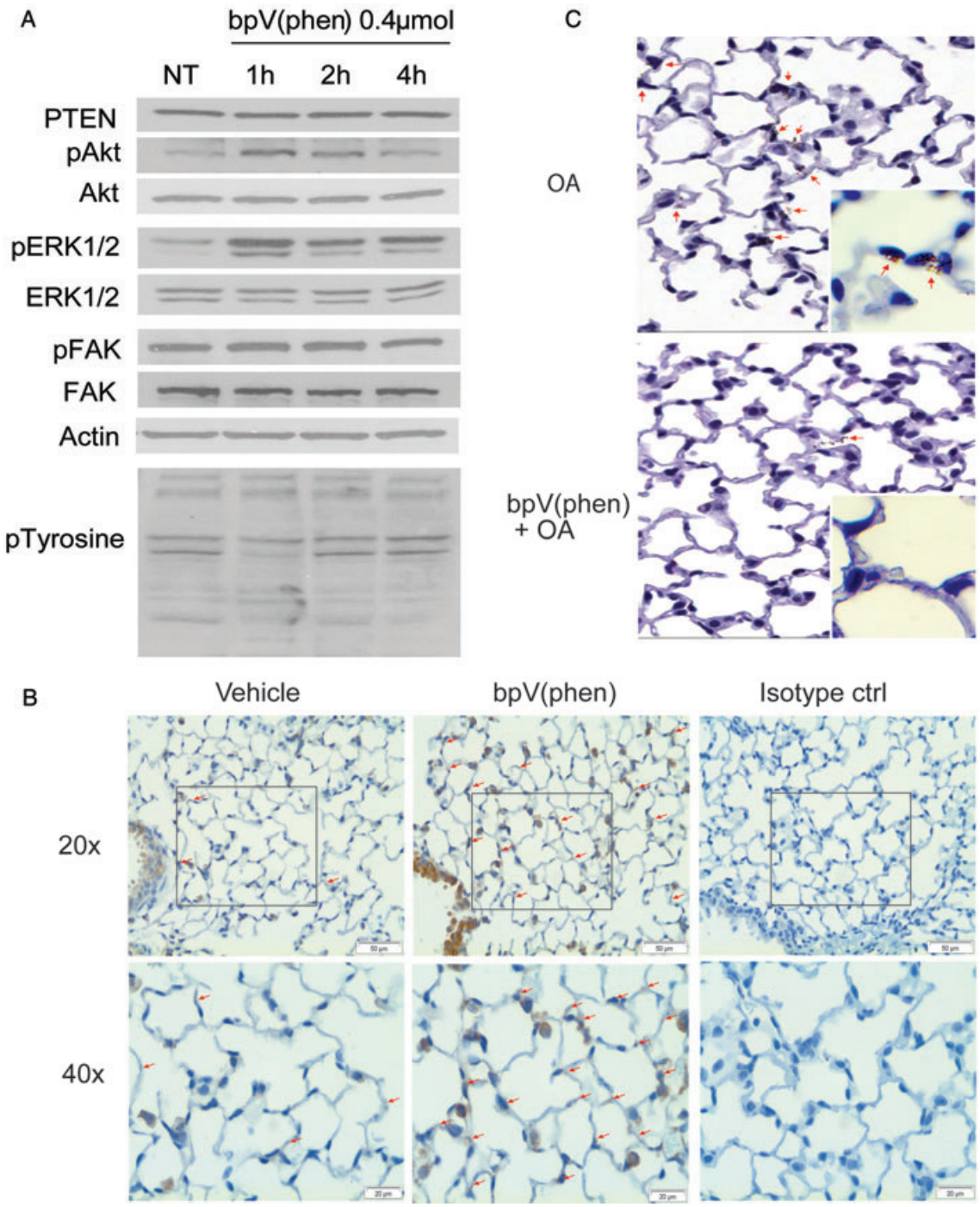

Figure 4 PTEN inhibition increases Akt and ERK signalling in the lung. In order to determine whether the protection provided by bpV(phen) is a consequence of PTEN inhibition, we examined lung homogenates for phosphorylated Akt, ERK and FAK, known factors that function downstream of PTEN, following administration of 0.4 μmol bpV(phen) for up to 4 h. (A) As shown, PTEN is constitutively present in lung tissue and following bpV(phen) administration, the phosphorylation of Akt and ERK consistently increased within 1 h. Phosphorylation of FAK was not observed. Furthermore, inspection of the same lung homogenates for phosphorylated tyrosine residues revealed that there were no differences between bpV(phen)-treated or untreated lungs suggesting that the molecular events and protection observed following bpV(phen) treatment were not a consequence of non-specific effects. The results are representative of three separate experiments. (B) The same lung tissue was also examined by immunostaining using an antibody that detects phosphorylated Akt. As shown, vehicle control-treated animals exhibited a predominant pattern of staining in alveolar macrophages and the bronchiolar epithelium with low levels of staining in alveolar parenchymal cells. In sharp contrast, treatment with bpV(phen) resulted in an increase in the presence of phosphorylated Akt throughout the lung and particularly in parenchymal cells including the lung epithelium in the alveolar region (arrows highlight parenchymal cells that stain positive for phosphorylated Akt). (C) Further, knowing that activation of the Akt and ERK pathways promotes cell survival and prevents apoptosis, we immunostained tissues obtained from the same animals for the presence of active caspase 3. As shown, the incidence of active caspase-3-positive cells was increased following OA treatment (upper panel) whereas the number of caspase-3-positive cells was decreased by bpV(phen) pre-treatment (bottom panel). The results are representative of three experiments. bpV(phen), potassium bisperoxo (1,10-phenanthroline) oxovanadate; FAK, focal adhesion kinase; OA, oleic acid; PTEN, phosphatase and tensin homologue deleted on chromosome Ten.

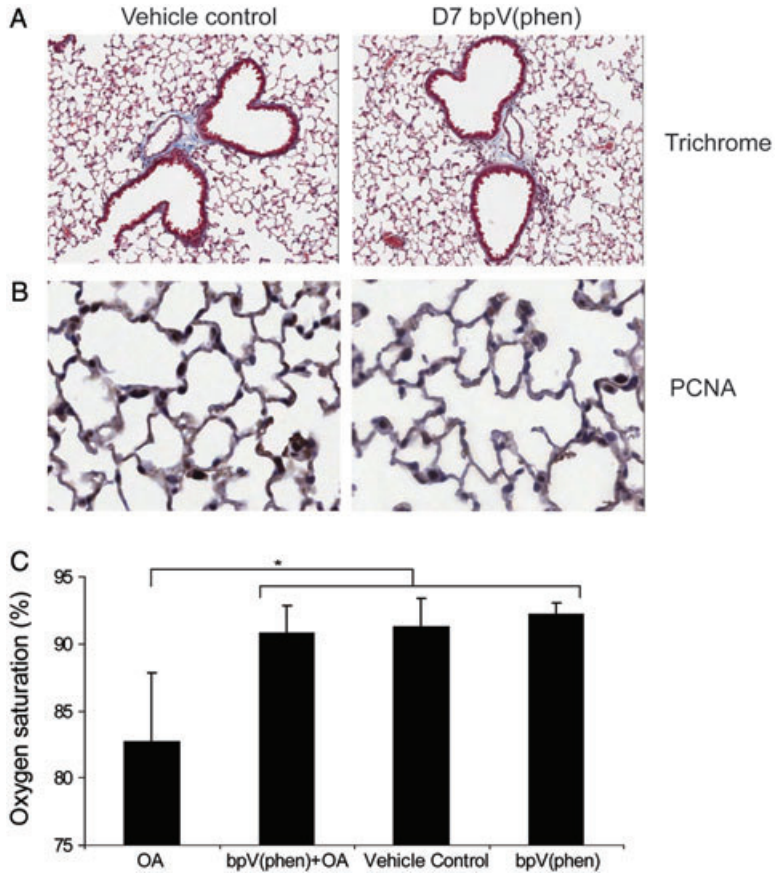

Figure 5 Physiological impact of pharmacological PTEN suppression. (a) Trichrome staining was performed to determine if bpV(phen) and hence, PTEN inhibition increased collagen formation in the lung. No detectable change in trichrome staining was observed in either the vehicle control (left panel) or bpV(phen) treatment groups (right panel), following a single dose, for up to 7 days. Similar results were also observed on day 1 and day 3 (data not shown). (b) The cell proliferation marker PCNA was also used to determine whether drug treatment changed the number of replicating cells. No evidence of changes in cell division was observed for up to 7 days (right panel) suggesting that increased proliferation does not contribute significantly to cytoprotection. (c) Next, whole animal oximetry was performed to determine the extent of oxygen saturation in peripheral arterial blood arterial. As shown, OA caused a statistically significant decrease in oxygen saturation down to 82% when compared with the expected normal values of the vehicle control treatment group (92%). Administration of bpV(phen) prior to OA completely prevented oxygen desaturation and maintained arterial oxygen levels at normal baseline values 92% (*$P < 0.05$) ($n = 7$ animals per treatment condition). bpV(phen), potassium bisperoxo (1,10-phenanthroline) oxovanadate; OA, oleic acid; PCNA, proliferating cell nuclear antigen; PTEN, phosphatase and tensin homologue deleted on chromosome Ten.

addition, we examined the same samples for the presence of phosphorylated tyrosine residues. Presumably, if bpV(phen) functioned through a non-specific mechanism that affected other phosphatases, then we would expect to see an increase in the presence of phosphotyrosine residues. As shown, no significant changes were observed across the different time points evaluated, further suggesting that bpV(phen) mediated its effect in a specific manner by inhibiting PTEN (Fig. 4A). Knowing that activation of the Akt as well as ERK signalling pathways can protect against cell death by preventing caspase-3 activation (Buckley *et al.*, 1999), we then evaluated the lung sections for the presence of active caspase-3. As shown, OA treatment alone resulted in a significant increase in active caspase-3-positive cells located within the lung parenchyma (red arrows), whereas bpV(phen) significantly decreased the number of positive cells suggesting that PTEN inhibition prevented apoptosis of parenchymal cells following OA treatment (Fig. 4C). The location and extent of active caspase-3-positive cells was highly consistent with the staining patterns previously described by using AE1/3 and TUNEL.

Physiological impact of PTEN inhibition

PTEN is expressed by many cell types. Further, inherited defects in PTEN function are associated with detrimental consequences including dysregulation of the normal cell cycle, leading to cancer, as well as increasing invasive migratory behaviour. To begin to address these concerns associated with using a PTEN inhibitor, we determined the effect of bpV(phen) in lung tissue for up to 7 days following a single i.p. dose. Trichrome staining was performed to determine if there was an increase in collagen formation. We observed no detectable changes in the trichrome staining for up to 7 days when compared with untreated controls (Fig. 5A), suggesting that no change in collagen formation had occurred. Identical results were also observed on day 1 and day 3 (data not shown). Lung collagen content was also analysed in tissue

homogenates from all samples and revealed no quantitative difference in collagen content between the different treatment groups and normal control animals (data not shown). Similarly, we could detect no significant change for up to 7 days in cell proliferation within the lung parenchyma, as determined by proliferating cell nuclear antigen staining, following treatment with bpV(phen) in the OA injury model, also indicating that changes in cell proliferation did not appear to contribute to the protective effect of bpV(phen) treatment (Fig. 5B). Again, similar results were also observed on day 1 and day 3 (data not shown).

We also determined if the decrease in cellular injury translated to a physiologically relevant improvement in lung function. To determine this we monitored the SpO_2. OA resulted in a statistically significant reduction in oxygen saturation (down to 82%) when compared with vehicle control (92%). Pre-treatment with bpV(phen) resulted in complete preservation of SpO_2 at normal levels thereby indicating that temporary PTEN suppression prevented lung injury and maintained pulmonary gas exchange functions (Fig. 5C). In addition, the effect of bpV(phen) pre-treatment on survival, 10 days after OA, was assessed. In comparison with OA treatment alone (0.1 μL g^{-1} body weight), mice that received bpV(phen) prior to OA showed a significant improvement in survival (data not shown; the vehicle and bpV(phen) control treatment groups experienced 100% survival).

Topical administration of bpV(phen)

Having observed that the majority of lung injury caused by OA was associated with damage to the alveolar epithelium, we conducted a head-to-head comparison between bpV(phen), given by i.t. instillation or i.p. The primary objective of this pilot study was to determine if topical administration of bpV(phen) would direct the compound to the intended target and be more effective. Consistent with our previous results, i.p. administration of bpV(phen) 2 h before OA administration resulted in a statistically significant reduction in protein BALF concentration (Fig. 6A). In comparison, IT administration of bpV(phen) resulted in a very consistent but modest reduction in BALF protein content that did not reach statistical significance (Fig. 6B). The most logical explanation to account for these findings is that i.t. administration did not provide sufficient delivery of bpV(phen) across the alveolar tissue. However, these encouraging results provide a basis for future studies that will evaluate aerosolized administration as a more effective method to test the impact of localized pharmacologic PTEN inhibition and its impact on preventing lung injury.

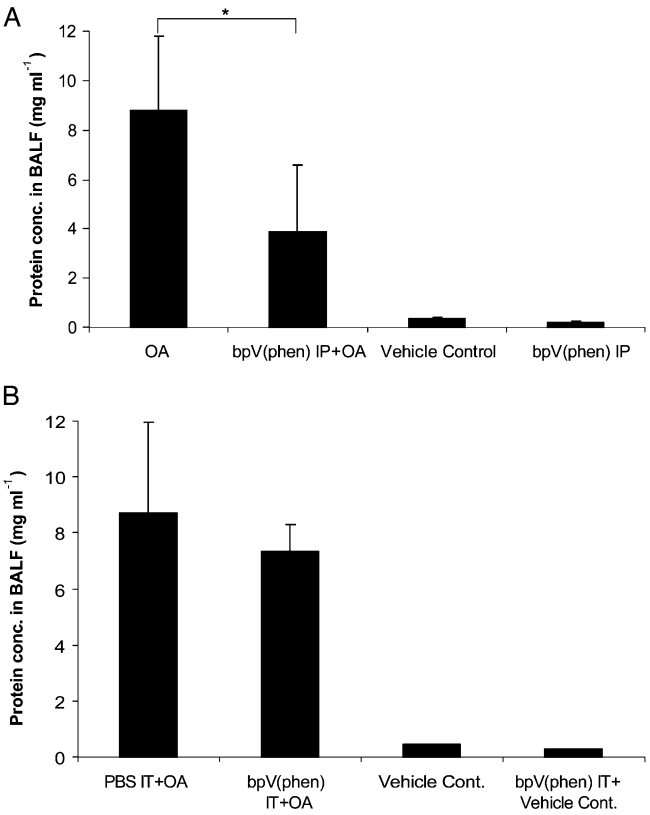

Figure 6 Comparison of topical and systemic PTEN suppression. (a) We directly compared the ability of bpV(phen) to prevent OA-induced ALI following i.p. injection or i.t. As previously shown, i.p. administration resulted in a statistically significant reduction in BALF protein content when compared with OA alone. (*$P < 0.05$) ($n = 12$ animals per treatment condition). (b) In comparison, a highly consistent but mild reduction in BALF protein content was observed following i.t. administration of bpV(phen) that did not reach statistical significance. ($n = 12$ animals per treatment condition). ALI, acute lung injury; BALF, bronchoalveolar lavage fluid; bpV(phen), potassium bisperoxo (1,10-phenanthroline) oxovanadate; i.p., intraperitoneal; i.t., intratracheal; OA, oleic acid; PTEN, phosphatase and tensin homologue deleted on chromosome Ten.

Discussion

Stimulation of membrane receptor tyrosine kinases and G-protein-coupled receptors leads to activation of the class I family of PI3Ks. Upon activation, PI3K phosphorylates the 3′ position of the inositol ring of phosphatidylinositol 4,5 bisphosphate (PIP_2) generating phosphatidylinositol 3,4,5 trisphosphate (PIP_3), which in turn leads to phosphorylation of Akt and other downstream effectors, resulting in enhanced cell proliferation and survival (Engelman *et al.*, 2006). We and others have shown that the PI3K/Akt axis is a vital signalling pathway in the lung epithelium that protects against inflammatory (Bao *et al.*, 2005) and oxidant stress (Lu *et al.*, 2001). PTEN's primary role is to reverse the effects of PI3K action by converting PIP_3 back to PIP_2, through dephosphorylation of PIP_3 at the 3′-position of the inositol ring (Cully *et al.*, 2006). Therefore, the critical balance between PI3K and PTEN activities has a significant influence on cell survival. PTEN was originally named TEP-1 that stands for [TGF]-regulated and epithelial cell-enriched phosphatase (Li and Sun, 1997). Consistent with this, we observed that PTEN is relatively abundant in primary, differentiated human lung epithelia. Based on this, we hypothesized that inhibition of PTEN in lung epithelia would facilitate repair as well as protect this important barrier from injury. With regard to the former, we recently reported that pharmacological inhibition of PTEN enhances repair of lung epithelium following mechanical injury *in vitro* (Lai *et al.*, 2007). A similar important role for PTEN in wound healing of the gastric mucosa has also been reported (Tsugawa *et al.*, 2003). In particular, we have compared two bisperoxovanadium compounds, both relatively

potent irreversible PTEN inhibitors (Schmid *et al*., 2004), and observed that suppression of PTEN phosphatase activity significantly accelerated the time for wound closure and functionally restored the epithelium to an intact monolayer with high electrical resistance. In the current investigation, we have extended our studies to determine if pharmacological suppression of PTEN can protect the lung parenchyma against acute injury *in vivo*, using the OA-ALI model.

Although different small animal models for ALI exist, we chose OA-ALI because it is a well-studied model that reproducibly induces structural histopathology consistent with ARDS but without substantial inflammation (Schuster, 1994; Zhou *et al*., 2005). Our rationale was to evaluate the impact of PTEN inhibition upon the lung parenchyma without having to control for the biological heterogeneity associated with an inflammatory milieu. The extent of OA-induced damage that we observed was highly consistent with previously published work in terms of both the extent and location of injury. The majority of injury was located in alveolar regions with structural alveolar damage and oedema that also lead to a significant decrease in oxygenation and survival. Further inspection of lung tissue revealed that a significant portion of TUNEL-positive cells were type I alveolar epithelia as well as vascular endothelia. Interestingly, we observed that OA-ALI provoked a localized inflammatory response in the airspace, as determined by the presence of cytokines and chemokines in the BALF, despite the lack of evidence of an inflammatory cell infiltrate. There are likely to be many reasons for this. First, we evaluated the lung within 2–6 h of injury, a time frame that is typically too early to encounter significant cellular infiltrates. Second, we suspect that TUNEL positive cells were the result of both apoptosis (Mantell *et al*., 1997) and necrosis as previously described (Ulrich *et al*., 2005). And third, inflammation following acute injury was likely to be due to provocation of parenchymal and mesenchymal cells along with lung macrophages. This is supported by a significant increase in LDH release in BALF, consistent with necrosis, and that active caspase-3 immunostaining was not observed in all TUNEL-positive cells. Based on this, we predict that necrosis invoked a localized inflammatory response by resident parenchymal and immune cells resulting in cytokine and chemokine release. Interestingly, we observed that PTEN inhibition not only significantly decreased the extent of tissue injury but also decreased cytokine and chemokine production. Whether the beneficial effects of PTEN inhibition is entirely due to cell protection or the result of alteration in cellular signalling networks remains to be determined.

Vanadium was first recognized as a competitive, reversible, broad-spectrum inhibitor of tyrosine phosphatases (Gordon, 1991; Huyer *et al*., 1997). New and improved analogues, including bpV(phen), were subsequently developed through derivatization of the parent compound by addition of polar side chains that resulted in a significant increase in affinity towards PTEN (IC_{50}; 20–40 $nmol \cdot L^{-1}$) (Posner *et al*., 1994; Cerovac *et al*., 1999; Rumora *et al*., 2004; Schmid *et al*., 2004). Synthesis of these compounds took advantage of the fact that PTEN is distinct from all other cysteine-based phosphatases by virtue of its wide catalytic pocket and elliptical opening. The bisperoxovanadium compounds pharmacologically interact with PTEN by irreversibly oxidizing a key cysteine residue within the CX5R motif, the primary reactive centre that provides the lipid phosphatase activity, thereby inhibiting phosphatase activity. Based on the natural half-life of PTEN (48–72 h) (Wu *et al*., 2000) and the relatively short half-life of bpV(phen) (<6 h) (Cerovac *et al*., 1999), we hypothesized that short-term inhibition of PTEN following a single injection of bpV(phen) could protect the lung parenchyma from injury over a desirable, limited time-frame without significant adverse effects. Careful evaluation of this strategy *in vivo* is paramount when considering that germ-line mutations that render PTEN dysfunctional cause cancer in humans and further, that attenuation of PTEN function has been implicated in the pathogenesis of non-malignant disorders including asthma (Kwak *et al*., 2003) and pulmonary fibrosis (White *et al*., 2003; 2006). In particular, White and colleagues reported that PTEN inhibition with the related compound bpV(pic) activated myofibroblasts thereby augmenting bleomycin-induced pulmonary fibrosis in mice. Therefore, PTEN inhibition may confer distinctly different cellular phenotypes between epithelia and fibroblasts in the lung. Using the OA-ALI model, we observed that administration of bpV(phen) intraperitoneally prior to OA resulted in Akt and ERK phosphorylation in lung parenchymal tissue, which occurred within the same time that we observed protection against cell death. Further, we found no evidence of pathological alterations in tissue structure, cell division or collagen deposition for up to 7 days following drug administration. The differences between our investigation and previous reports may be related to different experimental approaches. In this investigation we suppressed PTEN in the normal lung before injury, whereas the previous study intervened days after significant injury had already been established with bleomycin. Collectively, this suggests that modulation of PTEN function relative to the timing and extent of lung injury may be crucial and will require further investigation.

We initially chose the i.p. route in order to prolong systemic drug concentrations in comparison with intravenous administration, following a single dose of bpV(phen). In addition, we evaluated i.t. administration as a first step to determine if PTEN suppression of the lung parenchyma could be more precisely controlled through topical application, thereby avoiding systemic drug exposure. i.t. administration resulted in a consistently mild protective effect, but this did not reach statistical significance. This finding most likely reflects insufficient delivery of the compound over the alveolar surface, thereby necessitating further evaluation with more effective aerosol-based delivery strategies. It may also reflect insufficient biodistribution of the compound into the endothelium.

The exact mechanism(s) to account for the protective effects of bpV(phen) in this model will require further study. PTEN is a dual-specificity lipid and protein phosphatase. Although abundant data demonstrate that PTEN is a negative regulator of the PI3K/Akt pathway, it is becoming clear that PTEN influences other signalling kinases, including mitogen activated protein kinase and FAK. Under the experimental conditions studied here, bpV(phen) administration resulted in a reproducible increase in both Akt and ERK1/2 phosphorylation in the lung parenchyma. Activation of these pathways occurred without an increase in phosphotyrosine staining in

lung tissue homogenates, further suggesting that cytoprotection was specifically the result of PTEN inhibition and not caused by non-specific drug–protein interactions. Based on well-established studies (Davies *et al.*, 1998; Myers *et al.*, 1998) the most logical explanation to account for these findings is that PTEN inhibition enhanced PI3K/Akt and possibly ERK signalling, thereby raising the threshold against cell death in differentiated cells that constitute the parenchyma. No evidence of invasive cell behaviour including collagen deposition or cell division were observed following bpV(phen) administration. Recently, it has been shown that PTEN can interact directly with and dephosphorylate FAK thereby inhibiting integrin-mediated cell spreading and migration (Tamura *et al.*, 1998; 1999). Consistent with the lack of invasive migratory behaviour exhibited, no changes in FAK phosphorylation status were observed under these experimental conditions. Recent evidence suggests that PTEN may also suppress cell proliferation through protein interactions with its C2 domain (Okumura *et al.*, 2005). This raises the potential for PTEN to exert its influence on cell migration and proliferation independent of its lipid phosphatase activity and the PI3K pathway.

Based on this investigation, we contend that PTEN plays an important role in the lung in response to OA-ALI in mice. Specifically, inhibition of PTEN by bpV(phen) prior to ALI protected the lung parenchyma from injury. Protection of the parenchyma from OA-induced injury translated into significant improvements in ventilation and survival when compared with animals that received OA alone. This corroborates recent work demonstrating that the balance between PI3K and PTEN is a vital component in wound healing that is conserved across species (Zhao *et al.*, 2006). Interestingly, it was observed that deletion of PTEN enhances wound repair by augmenting electrotaxis, an innate response that is PI3K dependent and guides the repair programme (for commentary see Huttenlocher and Horwitz, 2007). Whether the beneficial effects of temporary PTEN suppression in the lung apply to other models, as well as to ALI in humans, remains to be determined. Our findings support the concept that transient PTEN inhibition did not induce a major alteration in the phenotype of epithelia, endothelia or fibroblasts. In comparison with studies utilizing mice with genetically modified forms of PTEN, pharmacological inhibitors such as bpV(phen) have an advantage in that the intended target remains endogenously present and retains its scaffolding function and ability to interact with other proteins, but its enzymatic activity can be reduced in a dose-dependent manner. Based on their relatively straightforward structure that is amenable to chemical derivatization (Rosivatz *et al.*, 2006), and known stability in both powder and solution, we propose that the bisperoxovanadium compounds should be further investigated to understand the emerging role of PTEN in the lung and also be considered as potential therapeutic agents to prevent or facilitate epithelial wound repair.

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Conflict of interest

The authors state no conflict of interest.

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