

Activated Akt Protects the Lung from Oxidant-induced Injury and Delays Death of Mice

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Abstract

Oxidant-induced injury to the lung causes extensive damage to lung epithelial cells. Impaired protection and repair of the lung epithelium can result in death. The serine-threonine kinase Akt has been implicated in inhibiting cell death induced by different stimuli including growth factor withdrawal, cell cycle discordance, DNA damage, and loss of cell adhesion in different cell types. However, the *in vivo* relevance of this prosurvival pathway has not been explored. Here we show that a constitutively active form of Akt introduced intratracheally into the lungs of mice by adenovirus gene transfer techniques protects mice from hyperoxic pulmonary damage and delays death of mice. This is the first demonstration of the *in vivo* protective function of Akt in the context of oxidant-induced lung injury.

Key words: hyperoxia • lung • Akt • apoptosis • survival

Introduction

Oxygen therapy is administered to decrease tissue hypoxia and to relieve arterial hypoxemia in patients with acute and chronic cardiovascular and pulmonary diseases and also to premature babies with respiratory distress syndrome. However, hyperoxia can cause acute lung injury which results in increased lung water with protein-rich edema fluid spilling into the interstitial and alveolar spaces. This is thought to occur after injury to epithelial and capillary endothelial cells resulting in epithelial cell death and increased pulmonary capillary permeability (1, 2). Current evidence indicates that the damaging effects of oxygen are caused by reactive oxygen intermediates (ROIs) such as the superoxide anion (O_2^-) that are formed in the presence of excessive oxygen. Severe oxidant-induced stress can result in respiratory failure and even death (3).

Akt/PKB is homologous to the protein kinases PKA and PKC (and hence the name PKB or related to A and C protein kinase [RAC-PK]; reference 4). Akt is activated by phosphatidylinositol (PI) 3-kinase in response to growth and survival factors (5). It has been shown that after stimulation by growth factors, Akt undergoes phosphorylation at

residues Thr308 in the activation loop of the kinase and at Ser473 in the carboxy terminus (6, 7). A downstream pathway that is induced by activation of the PI 3-kinase/Akt pathway is the phosphorylation of the Bcl-2 family member Bad, which releases Bcl-XL thereby promoting cell survival (8, 9). A second downstream prosurvival mechanism involves the transcription factor forkhead, the phosphorylation of which by Akt prevents its nuclear translocation and activation of genes that promote apoptosis (10). Several studies indicate that Akt activation plays an important role in inhibiting cell apoptosis in fibroblasts, epithelial and lymphoid cell lines, and neuronal cells (11–16). However, these studies with Akt have been carried out *in vitro* and the *in vivo* significance of this pathway in protection from cell death induced by different stimuli including oxidative stress remains to be investigated. Here we demonstrate that expression of a constitutively active derivative of Akt in the mouse lung by adenovirus gene transfer methods protects lungs and delays death from oxidant-induced injury.

Materials and Methods

Cells and Adenovirus Constructs. Human primary small airway epithelial (SAE) cells were obtained from Clonetics and grown in supplied medium. The virus packaging cell line 293 was grown in DMEM supplemented with 10% FBS. The gener-

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ation of adenovirus constructs expressing myr-Akt has been described previously (17).

Akt Kinase Assay. Akt kinase assay was performed using an "Akt Kinase Assay" kit (Cell Signaling Technology) following the manufacturer's instructions. In brief, SAE cells grown in 100-mm plates were infected by Adeno-myr-Akt virus or control Adeno-green fluorescent protein (GFP) virus at a multiplicity of infection (MOI) of 10. Cells were lysed in immunoprecipitation (IP) buffer and used for immunoprecipitation of Akt with anti-Akt antibody conjugated to agarose beads. The immunoprecipitated Akt was incubated with glycogen synthase kinase (GSK)-3 protein in kinase assay buffer containing ATP. After centrifugation, an aliquot of the supernatant was removed and tested for the presence of phospho-GSK-3 by Western blotting using anti-phospho-GSK-3 α/β (Ser 21/9) antibody. The Akt bound to the agarose beads was released by boiling in SDS-sample buffer and subjected to Western blot analysis using anti-Akt antibody.

Exposure of Mice to Hyperoxic Stress and Intratracheal Administration of Adenovirus. To address the role of Akt in protection from hyperoxic injury in vivo, a constitutively active form of Akt (myr-Akt; reference 17) was instilled into the trachea of C57BL/6 mice (7–9 wk old). Mice were anesthetized by intraperitoneal injection of a 0.1-ml solution of a ketamine/xylazine mixture (10 mg/ml/1 mg/ml) in pyrogen-free saline. The skin of the mice on the ventral neck was cleaned with a cotton swab soaked in isopropyl alcohol. The mice were next placed on a Deltaphase Isothermal pad (Braintree Scientific Inc.) warmed up to 37°C to reduce hypothermia and quicken recovery time. Trachea were exposed by a small incision in the neck skin. Next, 50 μ l of PBS containing adenovirus (Ad-myr-Akt or Ad-EGFP, 10⁹ PFU) was injected into the trachea using a 50- μ l Hamilton syringe fitted

with a 22 S needle. The incision was closed with wound clips and mice were kept warm and monitored until they recovered from anesthesia (usually 30 min). Then they were transferred to their cages with food and water and the cages were transferred to a hyperoxic chamber and exposed to 100% oxygen in a plexiglass hyperoxia exposure chamber. Oxygen from a liquid source was passed through a bubble humidifier and introduced into the sealed chamber at 10 liters/min. The chamber was fitted with a water manometer to maintain normobaric pressure. CO₂ production by the animals was neutralized in the chamber by absorbing with soda lime filter. The FIO₂ was measured continuously with an oxygen analyzer (VTI O₂ gas analyzer; Vacu Med). The mice were fed food and water ad lib and maintained on a 12-h dark-light cycle at sea level and at room temperature. Mice were followed after exposure to O₂ for different lengths of time and assessed for hyperoxic injury.

Lung Histology. Lungs were prepared for histology by perfusing the animal through the right ventricle with PBS to remove all blood as described by us previously (18, 19). Lungs were inflated to 20 cm water pressure with Streck Tissue Fixative (STF; Streck Laboratories Inc.) instilled through a tracheostomy tube. 5- μ M sections were mounted on slides and stained with hematoxylin and eosin according to established procedures.

Terminal Deoxynucleotidyl Transferase-mediated dUTP-biotin Nick-end Labeling Assay. DNA fragmentation was assessed using the DeadEnd Colorimetric Apoptosis Detection System (Promega). Tissue sections were deparaffinized by immersing slides in fresh xylene, washed successively in graded ethanol, and subjected to terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) assay following instructions of the manufacturer. Negative controls were incubated in buffer containing all components except TdT enzyme. Nucleotide incorporation was detected by treatment with horseradish peroxidase-conjugated streptavidin and enzyme substrate.

Statistical Analysis. Where appropriate, data are expressed as means \pm SEM. The significance of difference between two groups was analyzed using the Student's unpaired *t* test. Differences in means were considered significant if *P* < 0.05. Differences in survival between mice infected with control virus and Akt-expressing virus were assessed by χ^2 analysis.

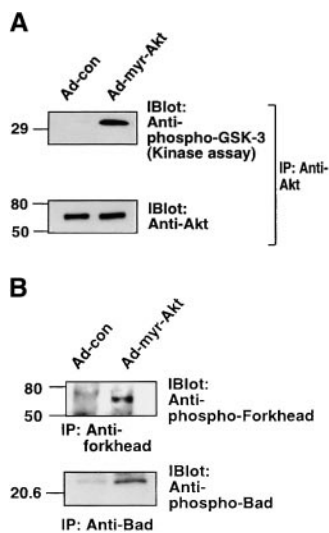


Figure 1. Constitutively active Akt induces phosphorylation of downstream substrates forkhead and Bad in lung epithelial cells. SAE cells were infected with HA-tagged Ad-myr-Akt virus or Ad-con virus. After 24 h of infection, cells were washed and lysed as described in Materials and Methods. (A) Cell extracts were used to determine Akt kinase activity. Endogenous Akt and Akt expressed from the transgene were immunoprecipitated with the same amount of anti-Akt antibody and the kinase activity in the immunoprecipitate was determined using GSK-3 as substrate. The reaction mixtures were centrifuged and the phosphorylation of GSK-3 in the supernatant was

assessed by immunoblotting using anti-phospho-GSK-3 α/β (Ser 21/9) antibody. (B) The phosphorylation of downstream substrates of Akt, forkhead, and Bad was determined by sequential immunoprecipitation and Western blot analysis. Forkhead and Bad were immunoprecipitated with anti-forkhead antibody (Cell Signaling Technology) and anti-Bad antibody (Santa Cruz Biotechnology, Inc.), respectively. Immunoprecipitates were collected by centrifugation, washed four times with IP buffer, and subjected to Western blot analysis with anti-phospho-Bad antibody (Ser 136; Cell Signaling Technology) or anti-phospho-forkhead antibody (Ser 256; Cell Signaling Technology).

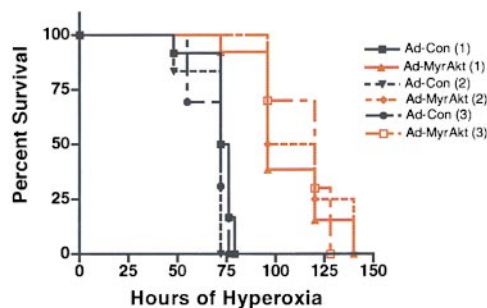


Figure 2. Constitutively active Akt delays death of hyperoxic mice. Mice were infected intratracheally with Ad-con or Ad-myr-Akt and then exposed to 100% oxygen. The survival of the mice was followed after initiation of hyperoxic exposure. In each experiment, each group consisted of 10 animals. Mice expressing Ad-myr-Akt showed a significant increase in survival time in all three experiments (*P* < 0.0001, *P* < 0.0003, and *P* < 0.0001 in experiments 1, 2, and 3, respectively). The data of three independent experiments are shown.

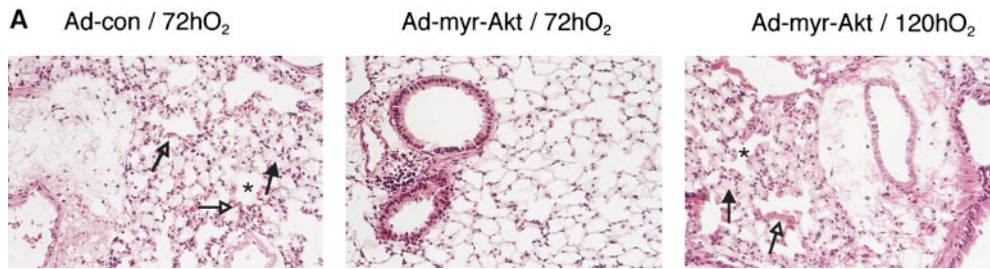


Figure 3. Constitutively active Akt inhibits hyperoxic lung injury in mice. (A) C57BL/6 mice were infected intratracheally with 10^9 PFU of either control virus (Ad-EGFP) or Ad-myr-Akt (HA tagged) and then exposed to 100% O_2 . Lungs were prepared for histology by perfusing the animal through the right ventricle with PBS to remove all blood. Lungs were then inflated to constant pressure (20 cm water) with Streck Tissue fixative (Streck Labs.) instilled through a tracheostomy tube. 5- μ M lung sections were mounted on slides and stained with hematoxylin and eosin according to established procedures. All of the control mice died by 72 h of O_2 exposure and displayed hyaline membrane formation (white arrows), edema (black arrow), widening of alveolar septae (asterisk), and widespread inflammation and hemorrhage (left), whereas none of the mice expressing myr-Akt died at 72 h of

initiation of O_2 exposure and also had no signs of lung injury at this time point (middle). Mice expressing myr-Akt displayed hyaline membrane formation, patchy edema, and hemorrhage at later time points (\sim 120 h after hyperoxia; right). (B) Expression of constitutively active, HA epitope-tagged Akt in mouse lungs at different times of hyperoxic exposure. Lung homogenates were prepared and immunoprecipitations were carried out with anti-HA antibody coupled to agarose followed by Western blot analysis using antibody against phospho-Akt. As myristoylation of Akt causes constitutive phosphorylation of Akt, the increased level of phospho-Akt with time was a reflection of increased level of protein expression from the transgene as confirmed by stripping the blot and reprobing with anti-Akt antibody. This experiment was repeated twice with similar results.

Results and Discussion

A Constitutively Active Form of Akt Can Phosphorylate Its Downstream Substrates Bad and Forkhead in Lung Epithelial Cells. In cell culture systems, activation of Akt has been shown to result in phosphorylation of protein substrates that are involved in cytoprotection. To study the in vivo function of activated Akt, we generated replication-defective adenovirus containing a constitutively active form of Akt (myristoylated Akt; Ad-myr-Akt). The functional activity of myr-Akt was assessed by infecting primary SAE cells with either Ad-myr-Akt or control replication-defective

adenovirus (Ad-con) and by performing Akt kinase assay by immunoprecipitating Akt from cell extracts and using GSK-3 as substrate. Fig. 1 A demonstrates that myr-Akt is an active kinase as revealed by phosphorylation of GSK-3. One mechanism by which Akt promotes cell survival involves phosphorylation of the Bcl-family member Bad (8, 9). Phosphorylation of Bad at Ser-136 by Akt results in its dissociation from Bcl- X_L and association with the adapter protein 14-3-3. The free Bcl- X_L released from sequestration by Bad promotes cell survival. Another downstream substrate of activated Akt involved in cell survival is the transcription factor forkhead (10). Phosphorylation of forkhead by Akt

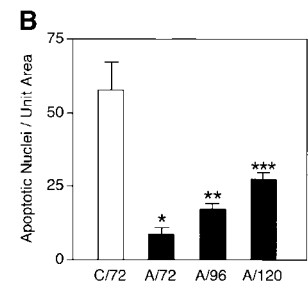
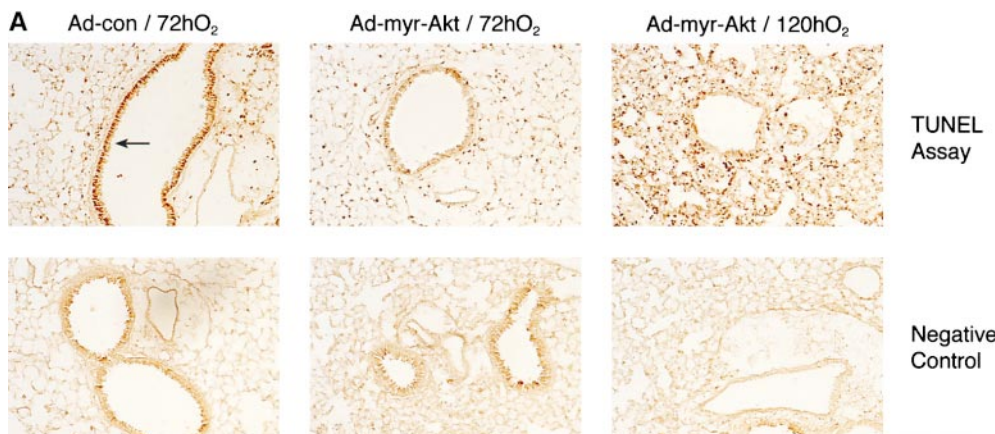


Figure 4. Inhibition of cell death in the airway epithelium by expression of constitutively active Akt (Ad-myr-Akt). (A) Lung sections from mice infected with control adenovirus or Ad-myr-Akt were subjected

to TUNEL assay for detection of DNA fragmentation/cell death in lung tissue. Lung sections were deparaffinized. Sections were then subjected to TUNEL assay (Dead End; Promega) following instructions of the manufacturer. Brown staining of the cells represent apoptotic nuclei. (B) A quantitative estimate of apoptotic nuclei (brown stain) per unit area. At 72, 96, and 120 h after hyperoxic treatment, there was a significant suppression of airway epithelial cell death in mice expressing myr-Akt compared with mice infected with control virus at 72 h after hyperoxia (* $P < 0.001$, ** $P < 0.0134$, and *** $P < 0.035$).

inhibits its apoptotic function by preventing its nuclear translocation. Fig. 1, B and C, show the ability of myr-Akt to cause phosphorylation of Bad and forkhead. Therefore, these data show that myr-Akt is able to phosphorylate its signaling substrates that are involved in cytoprotection.

Constitutively Active Akt Prolongs Survival of Hyperoxic Mice and Inhibits Oxidant-induced Injury to the Lung. Having confirmed the ability of myr-Akt to induce activation of downstream signaling pathways in lung epithelial cells, we investigated whether expression of Ad-myr-Akt could prevent oxidant-induced lung injury and death in animals. In these experiments, control adenovirus or adenovirus expressing myr-Akt (17) was introduced intratracheally into mice and the mice were immediately exposed to 100% O₂. Although all of the control mice died within 72 h of initiation of exposure to 100% O₂, all of the mice infected with Ad-myr-Akt lived longer (Fig. 2). The mice were killed at 120 h at which point they had begun to display respiratory distress. Thus, mice expressing activated Akt showed a significant increase in survival time ($P < 0.0001$). Histological examination of lung sections showed hyaline membrane formation, hemorrhage, inflammation, and gross pulmonary edema in the lungs of the control mice at 72 h (Fig. 3 A). In contrast, the lungs of mice infected with Ad-myr-Akt appeared normal and free of any hyaline membrane, hemorrhage, inflammation, or edema at 72 h after hyperoxia. At 120 h of exposure to 100% O₂, the lungs of the myr-Akt-expressing mice displayed hyaline membrane formation, hemorrhage, inflammation, and edema. To confirm expression and activation of myr-Akt in the lungs of the infected mice, lung extracts were immunoprecipitated with anti-hemagglutinin (HA) antibody and the immunoprecipitates were analyzed by immunoblotting with antibody to phosphorylated Akt. As shown in Fig. 3 B, adenovirus-derived transgenic expression of activated Akt was evident within 24 h of infection in mice infected with Ad-myr-Akt but not in those infected with the control virus, and the expression was detectable at 96 h after infection, the last time point tested in this assay. It is well established that hyperoxic injury involves an initiation phase after exposure to hyperoxia which is followed by an inflammatory phase and a destructive phase. Collectively, our data suggest that activated Akt can significantly delay the initiation phase of acute lung injury.

Constitutively Active Akt Protects Lung Epithelial Cell Death In Vivo. We next examined whether expression of constitutively active Akt is sufficient to suppress cell death induced by hyperoxia. In these experiments, lung sections from mice infected with control adenovirus or adenovirus expressing constitutively active Akt were subjected to TUNEL assay for nuclear DNA fragmentation, an indicator of cell death. As shown in Fig. 4 A, mice infected with control virus and subjected to hyperoxia displayed extensive TUNEL⁺ nuclei in the airway epithelium as well as in alveolar epithelial cells at 72 h after hyperoxia. In contrast, at this time point, cell death in the airway epithelium was almost completely blocked and alveolar epithelial cell death was partially blocked when mice were infected with Ad-myr-Akt. The better protection of the airway epithelium

was probably due to the fact that the airway epithelium is more accessible to adenovirus mediated gene transfer than the alveolar epithelium. At 120 h after exposure to hyperoxia, when hemorrhage, inflammation, and edema were evident in the lungs of the Ad-myr-Akt-infected mice (Fig. 4 A), correspondingly, cell death was apparent in both airway and alveolar epithelial cells. Thus, the increased survival of Ad-myr-Akt-expressing animals at 72 h after hyperoxia correlated with decreased hemorrhage, inflammation, and edema in the lung and a dramatic suppression of airway cell death. These results suggest that a better means of activating Akt in alveolar epithelial cells will provide a better survival advantage to the animals.

In summary, our data demonstrate that Akt activation in vivo promotes resistance to oxidant-induced cell death and significantly delays the onset of acute lung injury resulting in increased survival of animals. Recently, activation of Akt by the 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitor simvastatin was shown to promote angiogenesis in the ischemic limbs of normocholesterolemic animals (20). Similarly, Akt activation may be a useful adjunct to oxygen therapy in hypoxic stress in cardiovascular and pulmonary diseases.

The authors thank R. Homer for advice on lung pathologies and members of the Yale Pulmonary Section for helpful discussions.

This work was supported by grants HL60207 and HL60995 from the National Institutes of Health to P. Ray.

Submitted: 22 November 2000

Revised: 21 December 2000

Accepted: 3 January 2001

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