Stretch-Induced Activation of AMP Kinase in the Lung Requires Dystroglycan

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Lung cells are exposed to cyclic stretch during normal respiration and during positive pressure mechanical ventilation administered to support gas exchange. Dystroglycan is a ubiquitously expressed matrix receptor that is required for normal basement membrane formation during embryogenesis and for maintaining the function of skeletal muscle myocytes and neurons where it links cells to matrix. We previously reported that equibiaxial stretch of primary alveolar epithelial cells activated the MAP kinase pathway ERK1/2 through a mechanism that required an interaction between dystroglycan and matrix. We determined whether this mechanism of mechanotransduction activates other signaling cascades in lung epithelium. Exposure of rat epithelial alveolar type II cells (AEC) to cyclic mechanical stretch resulted in activation of 5' AMP-activated protein kinase (AMPK). This response was not affected by pretreatment of AEC with the ERK inhibitor PD98059 but was inhibited by knockdown in dystroglycan expression. Moreover, production of reactive oxygen species was enhanced in mechanically stimulated AEC in which dystroglycan was knocked down. This enhancement was reversed by treatment of AEC with an AMPK activator. Activation of AMPK was also observed in lung homogenates from mice after 15 minutes of noninjurious mechanical ventilation. Furthermore, knockdown of dystroglycan in the lungs of mice using an adenovirus encoding a dystroglycan shRNA prevented the stretch-induced activation of AMPK. These results suggest that exposure to cyclic stretch activates the metabolic sensing pathway AMPK in the lung epithelium and supports a novel role for dystroglycan in this mechanotransduction.

Keywords: stretch; lung injury; mechanical ventilation; acute respiratory distress syndrome

Mechanical stimulation of the cells in the lung is an important regulator of lung development and function (1, 2). In the normal adult lung, cells are under a mechanical tension that is created by the lung architecture (1, 3). In many pulmonary diseases, including acute lung injury, asthma, chronic obstructive lung diseases, and pulmonary fibrosis, the mechanical forces exerted on the cells are altered, which results in the activation of a number of distinct signaling pathways. Some of these pathways may be protective, whereas others may exacerbate the underlying pathology (4–6).

Recently, there has been a considerable recent effort toward defining the molecular pathways activated by mechanical stimulation in a variety of cell and tissue types (1, 3). Stretch-activated ion channels, junction proteins, matrix receptors, and

CLINICAL RELEVANCE

Ventilator-induced lung injury involves excessive stretch on the alveolar epithelium. Stretch triggers signaling pathways that may be protective or deleterious. Defining these pathways is a necessary prerequisite to the design of new pulmonary therapies.

cytoskeleton-associated elements have been implicated as components of mechanical sensitive signaling cascades (1, 3). In alveolar epithelial cells (AEC), mechanical stimulation in the form of stretch is known to activate extracellular signal-regulated kinase 1/2 (ERK1/2) (7, 8). Previously, we implicated the interaction of the matrix receptor dystroglycan with a novel laminin (laminin-311) secreted by AEC in mediating the transduction of signals elicited by mechanotransduction (7). Specifically, we observed that inhibiting dystroglycan with blocking antibodies or knocking down the expression of dystroglycan with shRNA prevented the stretch-induced activation of ERK1/2 in cultured AEC (7).

Here, we report that there is a dystroglycan dependent activation of 5' AMP-activated protein kinase (AMPK) in primary rat AEC exposed to low levels of equibiaxial stretch. The AMPK pathway serves as an evolutionarily conserved metabolic sensor (9–11). In response to metabolic stresses that alter the ratio of AMP to ATP in the cell, the AMPK pathway becomes active and phosphorylates a number of target proteins that tend to enhance ATP production, (e.g., proteins involved in oxidative phosphorylation, fatty acid oxidation, and glucose uptake) while inhibiting proteins involved in energy consumption (e.g., proteins involved in protein, cholesterol, and fatty acid synthesis). The net effect of activation of the AMPK pathway is to restore the energy store of the cell. In this study, we demonstrate that the loss of dystroglycan-dependent AMPK activation induces the production of reactive oxygen species (ROS) in mechanically stimulated AEC. In addition, we report that the transition of mice from spontaneous ventilation to positive pressure ventilation with noninjurious tidal volumes is also associated with a robust activation of AMPK. Knockdown of dystroglycan in the intact mouse lung prevented the mechanical ventilation-induced activation of AMPK. These results are the first to detail a link between stretch and the activation of AMPK and support the importance of dystroglycan in mediating mechanotransduction in the lung.

MATERIALS AND METHODS

Isolation of AECs

AECs were isolated from pathogen-free male Sprague-Dawley rats (200–225 g) as previously described (8, 12). Briefly, the lungs were perfused via the pulmonary artery, lavaged, and digested with 3 U/ml elastase (Worthington Biochemical, Freehold, NJ). AECs were purified by differential adherence to dishes pretreated with immunoglob-

(Received in original form November 26, 2007 and in final form April 23, 2008)

This work was supported by National Institutes of Health grants PO1 HL071643 (G.R.S.B., J.C.R.J.), RO1 ES015024 (G.M.M.), and RO1 ES013995 (G.R.S.B.).

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Am J Respir Cell Mol Biol Vol 39, pp 666–672, 2008

Originally Published in Press as DOI: 10.1165/rcmb.2007-0432OC on June 12, 2008

Internet address: www.atsjournals.org

ulin G, and cell viability was assessed by trypan-blue exclusion (>95%). Cells were resuspended in Dulbecco's modified Eagle's medium (Cellgro; Mediatech Inc., Herndon, CA) containing 10% FBS (Hyclone, Logan, UT) with 2 mM glutamine, 100 U/ml penicillin, 0.25 µg/ml amphotericin B, and 100 µg/ml streptomycin. Cells were seeded in 6-well, 35-mm, elastomer-bottomed Bioflex plates (Flexcell International, Meckesport, PA) or peptide-coated glass coverslips at a density of 0.5×10^6 cells/cm². Cells were incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. The day of isolation and plating is designated culture Day 0. A similar procedure was used to isolate AEC from mice, except that the cells were purified by negative immunoselection using magnetic beads, followed by differential adherence to CD90 pretreated dishes. The isolated mouse cells were processed immediately after isolation for immunoblotting as detailed below.

Antibodies and Other Reagents

Polyclonal rabbit antisera against ERK1/2, activated (phosphorylated) ERK1/2, acetyl-CoA carboxylase (ACC), and activated (phosphorylated) ACC were obtained from Cell Signaling Technology (Danvers, MA). Mouse IgM IIH6 against α-dystroglycan has been described elsewhere (13). Mouse mAb 8D5 against β-dystroglycan was purchased from Novocastra Labs (Newcastle, UK). Mouse IgM Ab1 against actin was purchased from Roche. The pan-keratin rabbit antiserum was kindly provided by Dr. Robert Goldman (Northwestern University). Control immunoglobulins G and M and fluorescein, rhodamine, and horseradish-peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). The AMPK activator 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) was purchased from Cell Signaling Technology (14).

Short Hairpin RNA Protocol

A set of single-stranded oligonucleotides encoding the dystroglycan target short hairpin RNA (shRNA) and its complement were synthesized by Invitrogen (Carlsbad, CA) and have been previously described (7): 2719Forward, CACCGCTCATTGCTGGAATCATTGCCGAAGCAATGATTCCAGCAATGAGC; 2719Reverse, AAAAGCTCATTGCTGGAATCATTGCTTCGGCAATGATTCCAGCAATGAGC.

The sequence was selected using an algorithm provided by Invitrogen and is identical for rat and mouse dystroglycan. The oligonucleotide pair was annealed, ligated into the pENTR/U6 vector, and used to transform competent *Escherichia coli* cells following an established protocol (Invitrogen). Plasmid DNA was isolated from the kanamycin-resistant colonies and sequenced. The pENTRY/U6 construct was used in a recombination reaction with the adenoviral vector pAD/BLOCK-iT-DEST (Invitrogen). The resulting shRNA adenoviral vector was linearized with *PacI* and transfected into 293A cells using Lipofectamine following Invitrogen protocols. An adenoviral vector containing no DNA was used as a negative control. Approximately 12 days after transfection, the adenovirus-containing 293A cells were harvested and lysed to prepare a crude viral stock. The resultant viral stock was amplified, and the viral concentration was determined. Two days after plating, AECs were infected with the virus at a multiplicity of infection of 1:10. The following day, the medium was replaced, and assays were performed 48 hours later. For experiments in mice, the dystroglycan and null viruses were commercially amplified to high titer ($>10^{11}$ pfu/ml) (Viraquest, North Liberty, IA).

Cyclic Stretching

AECs were cultured for 3 days on YIGSR-coated BioFlex culture plates (Flexcell International, Hillsborough, NC). After serum starvation for 24 hours, the cells were subjected to equibiaxial stretching on Day 4 using the Cyclic Strain Unit FX-4000 (Flexcell International, Hillsborough, NC). This unit consists of a controlled vacuum unit and a base plate to hold the culture plates. A vacuum was cyclically applied to elastomer-bottomed, 6-well culture dishes using the Flexercell device with loading stations in place to impose equibiaxial stretching at 30 cycles per minute and a stretching/relaxation ratio of 1:1, resulting in a 10% linear elongation of the membrane as measured microscopically. This is the same regimen that we have used in previous reports (7, 8).

SDS-PAGE and Immunoblotting

AECs were solubilized in sample buffer consisting of 8 M urea, 1% sodium dodecyl sulfate in 10 mM Tris-HCl (pH 6.8), and 15% β-mercaptoethanol (15). Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and processed for immunoblotting as previously described (15, 16). Immunoblots from a minimum of three separate assays were scanned and quantified using Molecular Analyst (Bio-Rad, Richmond, CA). Dystroglycan levels in AEC were assessed relative to actin protein levels in the same samples. To evaluate the relative levels of phosphorylation of a target protein in a test sample, protein preparations were subjected to SDS-PAGE on two identical gels, and the proteins were transferred to two separate pieces of nitrocellulose. One of the pieces of nitrocellulose was probed with antibody against the phosphorylated form of the protein, and the other was processed with antibody that recognizes the same protein regardless of its phosphorylation state. The level of phosphorylation of an assayed protein was calculated relative to the total amount of the same protein in each sample. We then calculated the difference in phosphorylation of the test protein in each sample relative to that observed in samples of stretched cells; nontreated cells; or control infected, ventilated lungs in the same study set, with the results being expressed as percentages. Results from a minimum of three study sets were combined and are presented as means ±SE. Significant differences between experimental conditions were explored with two-tailed, paired Student's *t* tests. A significant difference was prospectively identified as *P* < 0.05.

Measurement of ROS

To measure the generation of ROS, we infected AEC with an adenovirus encoding an oxidant-sensitive green fluorescent protein (GFP) probe containing a mitochondrial matrix localization sequence (mito-Ro-GFP), as previously detailed (17). This probe was originally described by Remington and colleagues, who validated its responsiveness to superoxide anion and H₂O₂ *ex vivo* and in living cells (18, 19). Oxidation of the Ro-GTP probe was assessed using flow cytometry. In brief, after treatment, AEC were removed from their substrate using TrypLE Express (Invitrogen), and equal aliquots of the resulting suspension were transferred to tubes containing media alone or media containing 1 mM dithiothreitol (DTT) or 1 mM t-butyl hydroperoxide. After 10 minutes, the ratio of fluorescence (emission of 535 nm) at excitations of 400 and 490 nm was measured in 5,000 cells per condition using a DakoCytomation CyAn high-speed multilaser droplet sorter. The oxidation state of the cells was calculated as the completely reduced ration (DTT) less the untreated value divided by the difference in the ration observed with DTT and t-butyl hydroperoxide (17).

Immunofluorescence Microscopy

Before tissue harvesting, we inserted a tracheostomy tube in the animal and inflated the lungs with optimal cutting temperature embedding medium (Miles Inc., Elkhart, IN) through the tube. The heart and lungs were removed *en bloc* and snap frozen in methanol on dry ice. Frozen sections (8–12 µm thick) were prepared and processed for indirect immunofluorescence as described previously (20). A mix of primary antibodies was overlaid on the sections on glass slides, and the preparations were incubated at 37°C for 1 hour. The slides were washed in three changes of PBS and then overlaid with secondary antibodies, placed at 37°C for 1 hour, washed extensively, and covered with mounting medium and a coverslip. All preparations were viewed on a Nikon TE2000U microscope (Nikon Instruments Inc., Melville, NY). Microscope images were exported as TIF files, and figures were generated using Adobe Photoshop software.

Mechanical Ventilation of Mice

All animal procedures were approved by the Animal Care and Use Committee of Northwestern University. Male wild-type c57BL/6 mice (20–25 g) were purchased from Charles River Laboratories (Wilmington, MA). The animals were sedated with intraperitoneal pentobarbital (60–80 mg/kg), and a 20-gauge angiocath cut to a length appropriate for the mouse trachea was sutured into the trachea using sterile technique. Animals were allowed to breathe spontaneously though the tracheostomy tube for 15 minutes (spontaneous breathing) or were placed on

a mechanical ventilator and ventilated with a tidal volume of 12 ml/kg, rate of 150, PEEP of +2 cm H₂O, and FiO₂ of 0.21 for 15 minutes. At the end of that time, the animals were killed, and the lungs were homogenized in 1 ml of mild RIPA buffer containing Roche PhosphoSTOP tablets (1 tablet/7 ml buffer) and Na₃VO₄ (1 μ M) on ice for 2 minutes, sonicated for 2 minutes (duty cycle 10) and centrifuged (4°C, 10 minutes, 200 $\times$ g) $\times$ 3 with collection of the supernatant after each centrifugation. The supernatant was aliquoted (100 μ l) and stored at -80°C for subsequent analysis.

RESULTS

Cyclic Stretch of Rat AEC Is Associated with Activation of AMPK

One stimulus known to activate the AMPK pathway is exposure to oxidant stress (9). Because exposure to stretch has been associated with increased oxidant generation by AEC, we reasoned that cyclic stretch might activate the AMPK pathway (21). To test this hypothesis, AEC were isolated from rat lungs and grown on elastomer membranes (Flexcell International) for 3 days. The cells were serum starved for 24 hours and then exposed to equibiaxial cyclic stretch (10%, 10 minutes). Compared with unstretched cells, extracts of AEC exposed to cyclic stretch demonstrated a significant increase in levels of phosphorylated ACC, a target of AMPK (Figure 1).

The Activation of AMPK in Response to Mechanical Stretch Requires Dystroglycan

We previously observed that activation of the ERK1/2 pathway in response to mechanical stretch was blocked by functional inhibitory antibodies to dystroglycan or by knockdown of dystroglycan (7). To determine whether the mechanical stretch-induced activation of AMPK was mediated by the same matrix receptor interaction, AEC were infected with the adenovirus encoding our previously characterized shRNA to dystroglycan or a null adenovirus 48 hours before exposure to cyclic stretch. Infection of AEC with the shRNA against dystroglycan resulted in a significant reduction in the level of dystroglycan ($\sim$ 90%) in the cells 48 hours later, as we reported previously (7, 22). The cells were exposed to equibiaxial cyclic stretch as above and were immediately lysed and immunoblotted using antibodies against total and phosphorylated ACC. Compared with AEC infected with the null virus, cells infected with the shRNA against dystroglycan showed a 50% lower level of phosphorylated ACC after 10 minutes of cyclic stretch (Figure 1).

The Activation of ERK1/2 Is Not Required for the Activation of AMPK in Response to Mechanical Stretch

The pathways leading to the activation of ERK1/2 and AMPK are distinct from one another (10, 21, 23); however, it is possible that the activation of AMPK might occur downstream from the activation of ERK1/2. To test this hypothesis, we exposed AEC to cyclic stretch in the presence or absence of the mitogen-activated protein extracellular signal-regulated kinase inhibitor PD 98059 (50 μ M). AEC were treated with PD 98059 for 60 minutes before treatment with cyclic stretch. The cells were immediately lysed after stretch and immunoblotted using antibodies to phosphorylated and total ERK1/2 and to phosphorylated and total ACC (Figure 2). Treatment with PD 98059 abolished the stretch-induced activation of ERK1/2 but failed to prevent the activation of AMPK (Figure 2). These results suggest that dystroglycan transduces the mechanical signal that activates ERK1/2 and AMPK through distinct mechanisms.

The Loss of Dystroglycan-Dependent AMPK Activation Increases the Stretch-Induced Generation of ROS in Alveolar Epithelial Cells

The application of prolonged cyclic stretch has been shown to increase oxidant generation in AEC and endothelial cells and has been hypothesized to contribute to injury after mechanical ventilation (21). To determine whether the loss of dystroglycan-mediated mechanosignaling altered the generation of ROS in AEC, we infected cells with an adenovirus encoding a ratiometric oxidant-sensitive GFP probe that localizes to the mitochondrial matrix (mito-Ro-GFP) (2 pfu/cell) and no virus, a control virus, or a virus encoding the shRNA against dystroglycan. Forty-eight hours after infection, the cells were exposed to cyclic stretch (10 minutes, 10%) and the oxidation state of the probe was measured using flow cytometry. As previously reported, the brief application of cyclic stretch to uninfected cells or cells treated with a null virus resulted in little if any increase in the generation of ROS (21). By contrast, cells infected with virus encoding the shRNA against dystroglycan showed a substantial increase in ROS generation in response to cyclic stretch. To determine whether inhibition of the AMPK pathway contributed to this increase, AEC were exposed to the same experimental conditions in the presence of the adenosine analog AICAR at a concentration of 0.5 mM for 1 hour. Comparable AICAR treatment activated the AMPK pathway in nonviral treated AEC maintained in serum-free medium (not shown) (14). Treatment with AICAR signifi-

Figure 1. Mechanical stretch-induced activation of the 5' AMP-activated protein kinase (AMPK) pathway in primary rat alveolar epithelial cells (AEC) requires dystroglycan. (A) Day 2 AEC were left untreated (lanes 1 and 2), treated with a control adenoviral vector (lanes 3 and 4), or treated with an adenoviral vector encoding an shRNA to dystroglycan (lanes 5 and 6). On Day 3 the cells were serum starved,

and on Day 4 the cells were exposed to 10 minutes of equibiaxial cyclic stretch in a Flexercell device (10% stretch). The cells were immediately lysed, and the resulting lysates were immunoblotted for phosphorylated (top) and total (bottom) acetyl-CoA carboxylase (ACC), a target of AMPK. (B) Comparison of the percentage phosphorylation of ACC relative to stretched, nontreated cells using densitometry in a minimum of three separate experiments as shown in A. Asterisk indicates a significant difference ($P < 0.05$ compared with stretched, untreated cells and stretched cells treated with control shRNA ($n > 3$)).

Figure 2. Mechanical stretch-induced activation of the AMPK pathway in primary rat AEC does not require the activation of ERK1/2. (A) Serum-starved (24 hours) Day 4 AEC were untreated, treated with vehicle (DMSO), or treated with PD 98059 (50 μ M) for 60 minutes before and during 10 minutes of exposure to stretch. Cell lysates obtained immediately after the stretch were immunoblotted in A for phosphorylated (top panel) and total (bottom panel) ACC or in C using antibodies against phosphorylated (top panel) and total (bottom panel) ERK1/2. Quantification of three independent trials is shown in B and D. In A, there is no significant difference between the relative levels of phosphorylated ACC in the stretched, untreated cells compared with stretched cells treated with DMSO or PD 98059. In B, there is a significant difference ($*P < 0.05$) between relative levels of phosphorylated ERK1/2 in extracts of stretched cells treated with PD 98059 compared with those observed in extracts of stretched, untreated cells and stretched cells treated with DMSO alone ($n > 3$).

cantly reduced the stretch-induced generation of ROS in AEC in which dystroglycan is knocked down (Figure 3).

Adenoviral Infection of Rats and Mice with an shRNA against Dystroglycan Decreases Protein Expression

To determine whether cyclic stretch activated AMPK through a similar mechanism in the intact lung, we sought to decrease the expression of dystroglycan in the lungs of mice using adenoviral delivered shRNA against dystroglycan. To confirm that our adenoviral technique is capable of delivering RNA to the lung

Figure 3. Loss of dystroglycan-dependent AMPK activation increases the generation of mitochondrial reactive oxygen species in alveolar epithelial cells exposed to cyclic stretch. Primary alveolar epithelial cells were infected with an adenovirus-encoding mito-Ro-GFP (2 pfu/cell) and no adenovirus, a control adenovirus encoding human lamin A shRNA, or an adenovirus encoding the shRNA against dystroglycan. After 48 hours the cells were exposed to cyclic mechanical stretch in the presence or absence of a pharmacologic activator of the AMPK pathway 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR). The oxidation state of the probe was assessed using flow cytometry in three trials. Asterisk indicates a significant difference ($P < 0.05$) when compared with control treated cells ($n = 3$). Open bars, control; solid bars, AICAR. DG = dystroglycan.

epithelium, we first infected mouse lungs with adenovirus encoding β -galactosidase and analyzed the lungs using X-gal staining 7 days later. Infection of mouse lungs with adenovirus led to high levels of β galactosidase activity, primarily in the airway epithelium (Figures 4A and 4B). Next we examined the efficacy of adenoviral-delivered dystroglycan shRNA in knocking down dystroglycan expression *in vivo* in mouse lungs. To do so, mouse lungs were infected with adenovirus encoding the shRNA to dystroglycan or a control virus. After 7 days, there was a significant reduction in immunostaining for dystroglycan in sections of the lungs of mice infected with the shRNA to dystroglycan compared with sections of lungs of mice infected with control virus (Figure 4C). Sections of lungs from both sets of mice stain equally well with antibodies against keratin (Figure 4C). We also sought to confirm that the abundance of dystroglycan was reduced in the AEC of the lungs after adenoviral infection. Mice were infected with an adenovirus encoding an shRNA to dystroglycan or a null virus, and 7 days later AEC were isolated. Immediately after isolation, the cells were lysed, and the resulting lysate was separated by SDS-PAGE and processed for immunoblotting. The level of dystroglycan in the AEC from animals infected with the shRNA against dystroglycan was reduced by approximately 60% when compared with AEC from mice infected with the null adenovirus (Figures 4D and 4E). Collectively, these results suggest that infection with an adenovirus encoding a shRNA against dystroglycan can effectively reduce the levels of dystroglycan in the airway epithelium of the intact lung.

Mechanical Ventilation Results in the Activation of AMPK in Mouse Lungs

To determine whether stretch results in the activation of AMPK *in vivo*, we subjected mice to 15 minutes of mechanical ventilation with a tidal volume of 12 ml/kg, rate of 150, PEEP of 0, and FI_{O_2} of 0.21. Ventilation with these tidal volumes was not injurious as assessed by examination of hematoxylin and eosin stained sections and measurements of wet-to-dry weight and quasi-static elastance of the lung and chest wall in animals before and after mechanical ventilation (not shown). Lung homogenates from spontaneously breathing mice and from

Figure 4. A shRNA against dystroglycan effectively reduces dystroglycan abundance in the mouse lung epithelium. (A) Mice were infected with an adenovirus encoding β -galactosidase, and 7 days later the lungs were harvested and stained using X-gal. Two different views of the same lung are shown. (B) Lung sections from β -galactosidase-infected mice were stained with X-gal (bar in left panel, 500 μ m; bar in right panel, 250 μ m). (C) Mice were infected with a control adenovirus (left panels) or an adenovirus encoding a shRNA to dystroglycan (right panels). Seven days later, the lungs were harvested, and lung sections were immunostained using primary antibodies against dystroglycan (DG) (top panels) or keratin (bottom panels) as indicated (bar, 100 μ m). (D) Primary alveolar type II cells were isolated from mice 7 days after infection with the control or DG adenovirus (DG shRNA) and immediately lysed. The resulting lysates were immunoblotted for DG and actin (loading control). (E) Analysis of relative DG levels in repeated experiments using densitometry (DG/actin).

ventilated mice were resolved by SDS-PAGE followed by immunoblotting using antibodies against phosphorylated and total ACC. Mechanical ventilation resulted in a significant increase in the levels of phosphorylated ACC (Figures 5A and 5B). To determine whether dystroglycan was required for this response, mice were infected with the adenovirus encoding the shRNA against dystroglycan or control adenovirus. Seven days later, spontaneously breathing and mechanically ventilated mice were killed, and the lungs were harvested for measurement of phosphorylated ACC. Infection with the adenovirus encoding the shRNA against dystroglycan, but not the control adenovirus, significantly inhibited the mechanical ventilation-induced activation of the AMPK substrate ACC (Figures 5C and 5D).

DISCUSSION

Cells within the lung are continuously exposed to cyclic mechanical forces associated with normal respiration (2, 24). The initiation of positive pressure ventilation to support the failing lung has been associated with the activation of signaling pathways within the lung and, at large tidal volumes, with the development of lung injury (4–6). The mechanisms by which cells within the lung sense and respond to stretch, however, are incompletely understood (1–3). In lung epithelial cells, Correa-Meyer reported that G-proteins and activation of the EGF receptor were required for the stretch activation of ERK1/2 in AEC (8). Moreover, Tshumperlin and colleagues have reported that clustering of the EGF receptor in response to lateral deformation of the cell may be sufficient for its

Figure 5. Brief treatment with mechanical ventilation at low tidal volume activates the AMPK pathway through a dystroglycan-dependent mechanism. Untreated mice were sedated and allowed to breathe spontaneously or were treated with mechanical ventilation for 15 minutes, 12 ml/kg, PEEP 0, $F_{I_{O_2}}$ 0.21. (A) Immediately after treatment, the lungs were harvested and homogenized, and the resulting lysate was immunoblotted for phosphorylated and total ACC. Each lane represents one animal. (B) Comparison of the percentage phosphorylation of ACC in the lungs of untreated

animals relative to the lungs of animals on ventilation (results are from five or more animals). (C) Mice treated 7 days earlier with a control adenovirus or an adenovirus encoding a shRNA to DG shRNA were treated as described in A, and the lung homogenates were immunoblotted for phosphorylated and total ACC. Each lane represents one animal. (D) Comparison of the percentage phosphorylation of ACC in nonventilated animals treated with control virus, nonventilated animals treated with adenovirus encoding dystroglycan shRNA, and ventilated animals treated with DG shRNA relative to ventilated animals treated with control virus (results are from five or more animals). Asterisk indicates a significant difference ($P < 0.05$) between the relative phosphorylation of ACC in the lungs of ventilated animals treated with virus encoding dystroglycan shRNA relative to the lungs of ventilated animals treated with control virus. Open bars, control; solid bars, ventilation.

activation (25). Our previous study has implicated the matrix receptor dystroglycan as an important regulator of the response of AEC to mechanical stimulation *in vitro* (7). We have extended these findings here by demonstrating that a stretch-induced activation of the AMPK pathway in AEC *in vitro* is inhibited after knockdown of dystroglycan. Furthermore, our *in vivo* data support the importance of dystroglycan in mediating mechanical signaling in the intact lung. Specifically, we have demonstrated a dystroglycan-dependent activation of the AMPK pathway in extracts of intact lungs of mice subjected to mechanical ventilation. Although we cannot rule out a contribution of the nonepithelial cells in the intact lung in the latter phenomenon, we suggest that airway epithelial cells likely account for the ventilator-induced AMPK activation we have observed *in vivo*. We base this conclusion on the finding that AMPK activation in mechanically stimulated lungs is inhibited by adenoviral-mediated knockdown of dystroglycan because infection of the virus is primarily restricted to the airway epithelium.

The role of dystroglycan in signal transduction is somewhat controversial. Although numerous studies have demonstrated that dystroglycan is a structural protein involved in regulating basement membrane formation during embryogenesis and in the maintenance of the structure and function of skeletal muscle myocytes and neurons, the notion that it mediates signaling is less well accepted (26). Langenbach and Rando have reported that interrupting the interaction of dystroglycan with laminin resulted in decreased phosphorylation of the prosurvival kinase Akt, sensitizing myocytes to apoptotic signals (27). Moreover, Winder and colleagues have demonstrated that dystroglycan is a scaffold for the ERK-MAP kinase cascade (28). Our results support this notion and directly implicate dystroglycan in two signal transduction pathways initiated by cyclic stretch of AEC.

We do not know how dystroglycan regulates AMPK activity. It is unlikely that there is a direct interaction between the AMPK and dystroglycan. Although AMPK and its upstream kinase LKB1 are located throughout the cytosol and in the nucleus, two recent reports have identified plectin as an AMPK and a dystroglycan binding partner (29, 30). Plectin is a high-molecular-weight, cytoskeleton-associated protein with a broad tissue and cell expression pattern (31). It has been proposed to be a scaffolding protein for a variety of signaling protein intermediates. Thus, we speculate that plectin serves as a likely candidate molecule linking dystroglycan to ERK1/2 and AMPK signaling. We plan to test this possibility in future studies.

Several groups of investigators have found that mechanical ventilation or exposure of isolated lung epithelial cells, endothelial cells, and vascular smooth muscle cells to cyclic stretch induces the generation of ROS from the mitochondrial electron transport, the xanthine/xanthine oxidase system, and/or the NAD(P)H oxidase complex (21, 32–37). Low-level ROS generation might activate signaling pathways that protect the lung from stretch-induced injury, whereas higher levels of oxidant generation might induce epithelial cell damage. Activation of the AMPK pathway results in decreased protein and lipid synthesis and an increase in the flow of electrons through the mitochondrial electron transport chain (10, 11). This may decrease the rate of mitochondrial oxidant production by enhancing the coupling of electron transport and the generation of the proton gradient (37). We observed that the loss of dystroglycan-dependent signaling increased mitochondrial oxidant generation in AEC exposed to cyclic stretch. This effect appeared to be mediated by activation of the AMPK pathway because the pharmacologic activation of AMPK reduced the stretch-induced generation of ROS in cells lacking dystroglycan. We speculate that activation of the AMPK pathway might protect AEC from the injurious effects of stretch by attenuating the stretch-induced generation of ROS and preserving cellular energy

stores. Thus, together with the activation of ERK1/2, which inhibits cell cycle arrest and protects the cell against apoptosis, mechanosensing through dystroglycan is likely to be an important protective mechanism against stretch-induced injury. However, we cannot rule out that activation of AMPK, although protective at a single cell level, may be injurious to the lung at an organ level. For example, Woolhead and colleagues have reported that the activation of the AMPK pathway decreased vectorial Na⁺ transport in lung epithelial cells, which would impair gas exchange in the injured lung (38).

Conclusions

Cyclic stretch of AEC results in activation of the AMPK pathway through a mechanism that requires dystroglycan. Activation of this pathway attenuates the mitochondrial generation of ROS in AEC exposed to cyclic stretch. The importance of this pathway *in vivo* is supported by the observation that the change from spontaneous breathing to positive pressure ventilation in normal mice is associated with activation of AMPK and that shRNA-mediated knockdown of dystroglycan in the intact lung inhibits the mechanical ventilation-induced activation of the AMPK pathway.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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