Metformin-stimulated AMPK-α1 promotes microvascular repair in acute lung injury

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Jian M-Y, Alexeyev MF, Wolkowicz PE, Zmijewski JW, Creighton JR. Metformin-stimulated AMPK-α1 promotes microvascular repair in acute lung injury. Am J Physiol Lung Cell Mol Physiol 305: L844–L855, 2013. First published October 4, 2013; doi:10.1152/ajplung.00173.2013.— Acute lung injury secondary to sepsis is a leading cause of mortality in sepsis-related death. Present therapies are not effective in reversing endothelial cell dysfunction, which plays a key role in increased vascular permeability and compromised lung function. AMP-activated protein kinase (AMPK) is a molecular sensor important for detection and mediation of cellular adaptations to vascular disruptive stimuli. In this study, we sought to determine the role of AMPK in resolving increased endothelial permeability in the sepsis-injured lung. AMPK function was determined in vivo using a rat model of endotoxin-induced lung injury, ex vivo using the isolated lung, and in vitro using cultured rat pulmonary microvascular endothelial cells (PMVECs). AMPK stimulation using N1-(o-d-ribofuranosyl)-5-aminimidizole-4-carboxamide or metformin decreased the LPS-induced increase in permeability, as determined by filtration coefficient (Kf) measurements, and resolved edema as indicated by decreased wet-to-dry ratios. The role of AMPK in the endothelial response to LPS was determined by shRNA designed to decrease expression of the AMPK-α1 isoform in capillary endothelial cells. Permeability, wounding, and barrier resistance assays using PMVECs identified AMPK-α1 as the molecule responsible for the beneficial effects of AMPK in the lung. Our findings provide novel evidence for AMPK-α1 as a vascular repair mechanism important in the pulmonary response to sepsis and identify a role for metformin treatment in the management of capillary injury.

MILD ACUTE RESPIRATORY DISTRESS SYNDROME (ARDS), formerly classified as acute lung injury (ALI) (24), develops in response to a direct insult to the lung or indirectly through an acute systemic inflammatory response, such as occurs with sepsis. Development of lung dysfunction is highly variable in patients with sepsis; however, alterations in vascular function are principal contributors to disease progression. Damage to the vascular bed resulting from Gram-negative bacterial infections, like Pseudomonas aeruginosa, occurs in distal vessels (18, 19). Bacterial toxins and host inflammatory mediators injure alveolar capillaries, increasing endothelial permeability, leading to accumulation of edema fluid into the alveolar space, deterioration of gas exchange, and compromised lung function (17, 30).

Although various pharmacological and nonpharmacological strategies are employed to reverse this damage, few have proven beneficial (14, 16, 39). The lack of effective treatment modalities is due, in part, to the nature of the injury. The process by which sepsis causes vascular damage is complex, involving a variety of signaling cascades and effector systems. Thus identifying candidate molecules for therapeutic intervention has been elusive. An ideal candidate molecule would interact with endothelial-damaging pathways at diverse levels of vascular physiology and therefore have the potential for interfering with pathological processes at multiple progression points. We propose that AMP-activated protein kinase (AMPK) is such a molecule. Recent studies have shown that activation of AMPK decreases the inflammatory response of many cell types, including endothelial cells, moderates cardiovascular complications associated with ischemia or diabetes, and diminishes the proinflammatory activation of neutrophils and macrophages (26, 35, 42). Functional AMPK consists of an α/βγ-heterotrimer and, as its name implies, is activated by AMP that competes with ATP for binding to the γ-subunit of the complex. It was originally described as a molecular sensor for maintaining metabolic homeostasis in response to stresses that accelerate ATP consumption or inhibit ATP production. This classic view of AMPK regulation is giving way to a more comprehensive understanding of its function. AMPK response to stress is now associated with a broader range of physiological functions, which occur independently of AMP levels and involve signaling mechanisms known to regulate cellular structure and vascular integrity (2, 5, 9, 41). In this way, AMPK serves as an integrator of multiple regulatory signals important in monitoring energy status and maintaining vascular homeostasis.

Having demonstrated recently that AMPK-α1 contributes to endothelial repair in a pharmacologically injured lung (4), we reasoned that AMPK-α1 is involved in the capillary response to a clinically relevant mediator of sepsis-induced vascular dysfunction, Pseudomonas aeruginosa LPS (36). In this study, we demonstrate that activation of AMPK following LPS injury enhances ability of the lung to resolve capillary damage and identifies AMPK-α1 as a molecular target for treatment of sepsis-induced lung disease. Moreover, we provide evidence that metformin, frequently prescribed as a long-term strategy for managing diabetes is beneficial in the treatment of acute pulmonary microvascular injury.

MATERIALS AND METHODS

Materials. Texas Red-labeled dextran was obtained from Life Technologies (Grand Island, NY). Transwell 0.4-μm inserts came...
from Costar (Cambridge, MA). N1-(α, β-ribofuranosyl)-5-aminomidazole-4-carboxamide (AICAR) was purchased from Tocris Biosciences (Ellisville, MO). Compound C (6-[4-[2-(piperidin-1-yl ethoxy)phenyl]-3-pyridin-4-yl pyrazolo[1,5-a]pyrimidinum) was obtained from EMD Biosciences (San Diego, CA). AMPK assay was assayed using an AMPK-α [pT172] immunoassay kit from Invitrogen (Carrollton, CA). Pseudomonas aeruginosa LPS was obtained from Sigma-Aldrich (St. Louis, MO). Unless otherwise noted, all other materials and reagents were purchased from Sigma-Aldrich (St. Louis).

**Cell culture.** Rat pulmonary microvascular endothelial cells (PMVECs) were isolated, characterized, and cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin as described previously (4, 13).

**Retroviral constructs and stable transfection of AMPK-α1 shRNA.** PMVECs selectively express the AMPK-α1 catalytic subunit. Expression of AMPK-α1 was reduced in these cells using an shRNA-mediated retroviral approach and selection by antibiotic resistance as described previously (4).

**PCR.** Wild-type PMVECs and cells expressing the shRNA to AMPK-α1 (Δα1) were seeded onto 60-mm cell culture dishes and used at confluence 3–4 days later. For LPS time course studies, single doses of LPS in 250 μl DMEM were added to the cell culture media at a final concentration of 250 μg/ml. Experiments were stopped at the appropriate times using Trizol. Total RNA was isolated from cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s recommendations. RNA was quantitated by absorbance at 260 nm, and RT-PCR reactions were set up using a AccessQuick RT-PCR System (Promega, Madison, WI). Each reaction contained 1 μg of template RNA and 1 μM concentrations of forward and reverse primers: AMPK-α1 forward, (RT-PCR) ACCATCTCTTGTTGCCGAAGACCC; and reverse, (RT-PCR) GGTGCCTTCCTCGGACAACCGAAT (expected PCR product size, 224 bp). GAPDH was used as loading control.

**AMPK activity.** Cells were seeded onto 24-well culture plates (Corning, Corning, NY) and used at confluence 3–4 days later. Medium was aspirated and replaced with fresh medium containing the vehicle control DMEM or the AMPK activator AICAR (1 mM). Incubation was stopped at 2.5 h. AMPK activity was determined using antibody specific to the phosphorylated T-172 (active) form of AMPK-α. Detection was obtained by ELISA following the manufacturer’s protocol. Experiments were conducted in triplicate and repeated at least three times.

**Permeability assays.** Endothelial permeability was analyzed in vitro by diffusing TRITC-labeled dextran through a confluent endothelial monolayer. Immediately preceding experiments, media in the upper chamber was replaced with fresh media containing 125 μg/ml tracer alone or tracer with drug: LPS (250 μg/ml), AICAR (1 mM), or metformin (250 μg/ml). For the LPS + AICAR and the LPS + metformin groups, AMPK activator was added 1 h after the LPS. Unlabeled dextran (125 μg/ml) was added to the media in the lower wells to equilibrate dextran concentrations between the upper and lower chambers. Experiments were conducted in triplicate and repeated at least three times. At each time point, 50 μl media was removed from the lower chamber of each well. The amount of dextran that diffused through the endothelial monolayer was measured using a Spectra Max M3 microplate reader (Molecular Devices, Sunnyvale, CA).

**Transendothelial electric resistance.** PMVEC barrier integrity was measured using an Electric Cell-substrate Impedance Sensing system (Applied Biophysics, Troy, NY) as described in detail (4). Briefly, PMVEC (40 × 10^3 cells/mm²) were plated onto 8W10E arrays in normal culture medium and used when resistances reached >900 Ohm, usually 2–3 days after seeding. Resistance was taken every 15 min for the duration of the experiments. Baseline resistances were measured for 2 h before addition of LPS (250 μg/ml) in 50 μl media. For the LPS + AICAR and the LPS + metformin groups, AICAR (1 mM) or metformin (250 μg/ml) in 50 μl media was added 3 h after the LPS.

**PMVEC wound healing.** Endothelial response to wounding was evaluated by determining the rate (velocity) of gap closure in scratch-wounded PMVEC monolayers. Cells were plated onto sterile 25-mm glass coverslips and grown to confluence. Monolayers were scratched with a sterile 200-μl pipette tip, and coverslips were fitted into an Attofluor cell chamber (Life Technologies). Experiments were performed in cell culture medium under environmentally controlled conditions (5% CO2, 37°C). Baseline velocities were established before the addition of LPS (250 μg/ml), AICAR (1 mM), or metformin (250 μg/ml). Images were taken with a ×10 objective at 10-min intervals for 24 h using a Nikon Eclipse Ti microscope. The rate of velocity for gap closure was determined using the object-tracking module of Nikon Elements software to track the movement of individual cells located along the leading edge of gaps. At least 10 cells, chosen at random, were tracked for each experiment.

**Animals.** All animal experiments were performed using male Sprague-Dawley rats (200–250 g; Charles River, Wilmington, MA) following a protocol approved by the Animal Care and Use Committee of the University of Alabama at Birmingham and in accordance with the National Institutes of Health Guidelines on the Care and Use of Laboratory Animals.

**Isolated perfused lungs.** Animals were anesthetized, and, following sternotomy, the heart and lungs were removed en bloc. Lungs were ventilated and perfused at constant flow with Earl’s balanced salt solution containing 4% BSA (pH 7.4, 37°C). Filtration coefficients (Kt) were taken at baseline, 30, 60, 90, and 120 min. Kt measurements were calculated as the rate of weight gain after a 10-cmH2O increase in pulmonary venous pressure and were normalized to lung dry weight (g). Hemodynamic parameters (pulmonary artery, capillary, venous, and airway pressures) and lung weight gain were determined using ADInstruments PowerLab 8/30 and LabChart pro software. pH, PO2, and PCO2 were monitored during experiments using an OPTI Medical Systems Blood Gas Analyzer (Roswell, GA). For ex vivo treated lungs, LPS was used at 250 μg/ml, AICAR at 1 mM, metformin at 250 μg/ml, and compound C at 50 μM. LPS or drug was diluted in 1 ml perfusate buffer, added to the 20 ml of buffer in the reservoir at the appropriate time point, and carefully triturated (n = 5 lungs per group).

**In vivo model of LPS-induced lung injury.** Animals were randomly assigned to one of six experimental groups: 1) sham control, 2) LPS, 3) AICAR, 4) LPS + AICAR, 5) metformin, or 6) LPS + metformin. For the sham control, AICAR (20 mg/kg), and metformin (15 mg/kg) groups, drug in 250 μl sterile saline was given by i.p. injection 24 h before lung isolation. The LPS group was injected i.p. with 6 mg/kg AICAR, 3 mg/kg AICAR, and metformin (15 mg/kg) groups, drug in 250 μl sterile saline and subdivided into two groups. At 6 h after LPS treatment, lungs were isolated from the first LPS subgroup for use in Kt studies, whereas animals in the second group were given sterile saline only, AICAR (20 mg/kg), or metformin (15 mg/kg) i.p. in 250-μl volumes and monitored for an additional 18 h. Lungs from the second LPS subgroup, the LPS + AICAR group, and the LPS + metformin group were isolated at 24 h after LPS treatment, and Kt was determined as described above (n = 5 animals per group).

**Histology.** Following Kt studies, two to three lungs from each group were chosen at random and perfusion fixed with 10% formalin at 25 cmH2O airway pressure and 15 cmH2O vascular pressure before being embedded in paraffin. Sections were cut into 5-μm slices and stained with hematoxylin and eosin to evaluate changes in morphology.

**Graphing and statistical analysis.** For in vivo and ex vivo studies, at least five animals/lungs were used for each group. Endothelial permeability and resistance studies were conducted in triplicate with a minimum of three independent experiments. Time-lapse studies were repeated at least five times. Data are reported as means ± SE. Statistical analysis for the difference among groups was performed using a one-way or two-way ANOVA with Bonferroni post hoc
Fig. 1. shRNA-mediated knockdown of AMP-activated protein kinase (AMPK)-α1 blunts AMPK stimulation in pulmonary microvascular endothelial cells (PMVECs). A: PCR of wild-type (Wt) PMVECs and cells expressing the shRNA to AMPK-α1 (Δα1) demonstrates reduced AMPK-α1 mRNA content in Δα1 cells. Lung (L) used as positive control. B: ELISA studies confirmed that Δα1 PMVECs exhibit reduced AMPK activity and response to N1-(α-ribofuranosyl)-5-aminimidazole-4-carboxamide (AICAR) and metformin stimulation (n = 3; *P < 0.05; ***P < 0.001).

RESULTS

shRNA to AMPK-α1 reduces AMPK activity in PMVECs. Because AMPK is a master regulator of cellular metabolism, complete loss of AMPK function is not compatible with survival (32). We used a retroviral approach to stably knockdown AMPK-α1 mRNA (Fig. 1A) in PMVECs. The shRNA reduced basal AMPK activity by 60% (1.01 ± 0.034 vs. 0.418 ± 0.043 U/mg protein; P < 0.001, n = 3) (Fig. 1B). This reduction in basal AMPK activity also resulted in a significant decrease in stimulatable AMPK activity in the AMPK-α1 knockdown (Δα1) cells. AICAR- or metformin-stimulated AMPK in Δα1 cells reached levels comparable to the basal activity of wild-type cells (0.835 ± 0.12 and 0.875 ± 0.124 vs. 1.01 ± 0.034 U/mg protein, respectively; P > 0.05, n = 3). Thus Δα1 cells possess sufficient residual AMPK activity, when stimulated with AICAR or metformin, to approximate the unstimulated AMPK activity of wild-type cells.

LPS increases AMPK activity but not AMPK-α1 expression in PMVECs. We have shown previously that LPS does not alter AMPK-α levels in the lung (29, 41). However, LPS may alter AMPK-α1 expression or activity in PMVECs, especially over longer time courses. PMVECs were treated with LPS over 6, 24, 48, and 72 h and AMPK-α1 mRNA levels determined by PCR (Fig. 2A). Although there was a trend toward an increase in AMPK-α1 mRNA over the time course studied, the change was not statistically significant (Fig. 2B). We also determined the phosphorylation status of AMPK-α at Thr172 in PMVECs in response to LPS. Results indicated that LPS increased AMPK activity by 3 h (0.92 ± 0.06 vs. 1.123 ± 0.03 U/mg protein; P < 0.001, n = 3) and remained elevated at 24 h (0.81 ± 0.03 vs. 1.27 ± 0.03 U/mg protein; P < 0.001; n = 3) before returning to baseline levels over the next 48 h.

Activation of AMPK-α1 reduces LPS-induced increase in endothelial permeability. To determine the effect of AMPK stimulation on LPS-induced vascular permeability in vitro, PMVECs grown on Transwell inserts were challenged with LPS over 6, 24, 48, and 72 h and LPS-induced vascular permeability measured (Fig. 2C). Results indicated that LPS increased vascular permeability by 24 h (Con: 0.001 ± 0.000 vs. 6 h: 1.02 ± 0.01 U/mg protein; P < 0.001). This increase was not statistically significant at 24 h (Con: 0.001 ± 0.000 vs. 24 h: 1.02 ± 0.01 U/mg protein; P > 0.05).

Fig. 2. LPS increases AMPK-α activity, but not AMPK-α1 expression in PMVECs. A: PCR of LPS time course in PMVECs. B: exposure of PMVECs to LPS over 72 h does not alter AMPK-α1 expression. C: LPS causes a slight increase in pAMPK-α levels by 3 h that remains elevated at 24 h (n = 3; ns; P > 0.05; ***P < 0.001; AMPK-α1 normalized to GAPDH).
LPS before addition of AICAR or vehicle control (DMEM). AMPK-α1 stimulation blocked the LPS-induced increase in permeability (Fig. 3A). In Δα1 cells, AICAR partially blocked the effect of LPS (Fig. 3B) consistent with decreased AMPK-α1 content in these cells. The next studies were performed in the same manner as the AICAR studies except that metformin was added to the medium after LPS. Results were consistent with the AICAR studies; metformin completely blocked the LPS effect (Fig. 3C) in wild-type cells and partially attenuated the increase in permeability in Δα1 monolayers (Fig. 3D). The difference between the wild-type and Δα1 response to AMPK stimulation in the presence of LPS is highlighted in Fig. 3, E and F, where the final time points for each experimental group are compared. Wild-type and Δα1 cells demonstrate a similar permeability response to LPS [257 ± 12 vs. 290 ± 18 relative fluorescence units (RFU), respectively; P > 0.05, n = 5] (Fig. 3E). The LPS-induced increase in permeability of the wild-type cells returns to control levels in the LPS + AICAR-treated groups (163 ± 8 vs. 137 ± 6 RFU, respectively; P > 0.05, n = 5), whereas, in the Δα1 cells, permeability in the LPS + AICAR groups remains elevated compared with controls (197 ± 10 vs. 115 ± 8 RFU; P < 0.01, n = 5). Similar results were obtained in the metformin studies (Fig. 3F). Permeability in the wild-type cells returned to baseline levels in the LPS + metformin groups (123 ± 13 vs. 156 ± 9 RFU, respectively; P < 0.05, n = 5), whereas permeability remained elevated in the Δα1 LPS + metformin groups (223 ± 10 RFU) compared with control (129 ± 8 RFU; P < 0.001, n = 5).

Fig. 3. AMPK activation reverses LPS-induced increase in PMVEC permeability. In wild-type cells, LPS increases permeability that is reduced to control levels by AICAR (A) or metformin (Met) (C). In cells expressing the shRNA to AMPK-α1 (Δα1), LPS-induced permeability in AICAR (B) or metformin-treated (D) groups remains elevated compared with control levels yet is reduced compared with LPS only-treated monolayers. Final experimental time points in E and F highlight the difference between the wild-type and Δα1 response to AMPK stimulation. RFU, relative fluorescence units. *P < 0.05; ** and ###P < 0.01; ####P < 0.001; n = 3.
Activation of AMPK reverses LPS-induced decrease in PMVEC barrier resistance. A functional endothelial barrier involves cell-cell interactions, so we examined the effect of AMPK-α1 activation on endothelial monolayer resistance. Stable baseline resistances in confluent PMVEC monolayers indicate tight junctions and mature cell-cell contacts (Fig. 4, A and C). LPS decreased barrier resistance over the course of the experiment (Fig. 4, A and C). Addition of AICAR (Fig. 4A) or metformin (Fig. 4C) rescued barrier integrity, allowing resistances in LPS-treated monolayers to reach control levels. In Δα1 cells, LPS caused a loss of resistance that persisted in the presence of AICAR (Fig. 4B) or metformin (Fig. 4D). Differences between the wild-type and Δα1 response to AMPK stimulation in LPS-treated cells are highlighted in Fig. 4, E and F. Consistent with reduced AMPK activity in Δα1 cells, resistance values of AICAR-stimulated (832 ± 1 Ohm) (Fig. 4E) or metformin-stimulated (753 ± 1 Ohm) (Fig. 4F) Δα1 cells remained reduced compared with control levels (957 ± 1 and 942 ± 1 Ohm, respectively) (P < 0.001 between treatment groups of the same cell type, n = 5) (Fig. 4, E and F) yet demonstrated an increased resistance compared with LPS only-treated monolayers (697 ± 1 and 611 ± 1 Ohm, respectively) (P < 0.001 between treatment groups of the same cell type, n = 5). These data provide evidence that AMPK-α1 serves a role in the restoration of disrupted endothelial cell-cell contacts.

AMPK-α1 stimulation restores LPS-impaired wound repair in endothelium. We next investigated whether AMPK-α1 contributes to endothelial gap resealing in scratch-wounded

Fig. 4. AMPK stimulation repairs LPS-induced disruption of the PMVEC barrier. AICAR (A) or metformin (C) increase resistance in LPS-injured monolayers to control levels. Cells expressing the shRNA to AMPK-α1 (Δα1) exhibit an attenuated ability to respond to AICAR (B) or metformin (D) in the presence of LPS. Differences between wild-type and Δα1 response to AMPK stimulation are highlighted in E and F. Bars were generated by averaging the time points of the last 3 h of the experiment for each group (** and ###P < 0.001, n = 3).
PMVEC monolayers. Images were generated at 10-min intervals to determine the light micrographic appearance of the monolayers at each time point. In wild-type monolayers, gaps resealed within 12 h (Fig. 5, row 1). LPS increased the resealing time in wild-type cells with prominent gaps remaining at 12 h (Fig. 5, row 2). Activation of AMPK-α1 using AICAR or metformin restored resealing times in LPS-treated wild-type cells to control values (Fig. 5, rows 3 and 5). In Δα1 cells, monolayers resealed with similar times as control cells, indicating that the residual AMPK activity remaining in these cells was sufficient to repair the scratch-wounded monolayer (Fig. 5, row 1). These cells also remained sensitive to LPS-induced attenuation of the repair response and exhibited a prolonged resealing time in its presence (Fig. 5, row 1). Addition of AICAR or metformin to LPS-treated Δα1 cells reduced the time required for gaps to close (Fig. 5, rows 3 and 5) compared with LPS alone. However, unlike wild-type cells, AMPK stimulation in Δα1 cells did not return the resealing times to control values. These data indicate that AMPK activity enhances PMVEC repair following barrier disruption.

To quantify the results of the wounding studies, we employed a complementary approach using time-lapse video microscopy to determine the rate (velocity) of PMVEC resealing. In control monolayers, cells along the edges of the wound moved toward the opposing edge at a rate of 0.007 ± 0.0002 μm/s, n = 5 (Fig. 6A). LPS slowed the rate of migration by 42 ± 5% to 0.004 ± 0.0003 μm/s (P < 0.001, n = 8). The addition of AICAR to LPS-treated cells returned the velocity to baseline levels (0.007 ± 0.0003 μm/s, P > 0.05, n = 7). In the presence of LPS, the velocity of metformin-treated wild-type cells was similar to untreated control cells (0.008 ± 0.0007 vs. 0.007 ± 0.0002 μm/s, respectively, P > 0.05, n ≥ 5). In Δα1 cells, LPS slowed the rate of migration by 43 ± 6% to 0.004 ± 0.0002 μm/s (P < 0.001, n = 7). AICAR and metformin partially rescued the rate of resealing in LPS-treated Δα1 cells, returning velocities to 72 ± 6% (P < 0.05, n = 7) and 58 ± 7% (P < 0.05, n = 7) (respectively) of control values (Fig. 6, A and C). Data obtained from typical experiments are given in Fig. 6, B and D. These studies highlight two important aspects of endothelial biology. 1) LPS is involved in multiple aspects of vascular injury. In addition to initiating barrier-disruptive events, LPS subsequently hinders the ability of endothelial cells to reestablish barrier integrity. 2) AMPK-α1 activation enhances restoration of a damaged endothelial monolayer in the presence of an LPS challenge.

Permeability and edema induced by LPS in the lung ex vivo are resolved by AICAR and metformin. For our next studies, we used the isolated lung technique to discriminate between the systemic inflammatory response and the innate pulmonary response to LPS injury. Pulmonary vascular permeability was determined by Kf increased by twofold 30 min after addition of LPS (0.036 ± 0.006 vs. 0.017 ± 0.0007 ml·min⁻¹·cmH2O⁻¹·g dry weight⁻¹; P < 0.01, n = 5) to the perfusate and reached threefold over control levels (0.079 ± 0.01 vs. 0.25 ± 0.0024 ml·min⁻¹·cmH2O⁻¹·g dry weight⁻¹; P < 0.001, n = 5) by 2 h (Fig. 7A). In LPS + AICAR-treated lungs, AICAR was added 30 min after LPS. By 60 min, Kf in the AICAR-treated lungs had returned to near baselines levels (0.021 ± 0.003 ml·min⁻¹·cmH2O⁻¹·g dry weight⁻¹; P < 0.05, n = 5) and remained at control levels for the duration of the experiment (Fig. 7A). In studies where metformin was added to the perfusate instead of AICAR, the LPS-induced increase in permeability was similarly reduced by AMPK stimulation. By 60 min, Kf values in metformin-treated lungs (0.022 ± 0.003 ml·min⁻¹·cmH2O⁻¹·g dry weight⁻¹) were similar to controls (0.017 ± 0.0007 ml·min⁻¹·cmH2O⁻¹·g dry weight⁻¹) (P > 0.05, n = 5) and were significantly less than the 30-min Kf values (0.022 ± 0.003 vs. 0.032 ± 0.001 ml·min⁻¹·cmH2O⁻¹·g dry weight⁻¹; P < 0.05, n = 5) (Fig. 7A). In parallel with the Kf studies, we calculated the wet-to-dry ratios of isolated lungs to determine the effect of AMPK stimulation on edema in LPS-treated lungs. LPS increased the wet-to-dry ratio by 46 ± 2% over controls (8.9 ± 0.2 vs. 6.1 ± 0.2, respectively; P < 0.001, n = 5) (Fig. 7B). In groups where AICAR or metformin was given following LPS, there was a significant decrease in edema compared with LPS alone (6.9 ± 0.2 and 6.6 ± 0.3 vs. 8.9 ± 0.2, respectively; P < 0.001, n = 5) (Fig. 7B). These studies indicate that the beneficial effects of AMPK stimulation occur independently of systemic inflammatory influences and represent a repair response mechanism inherent to the lung.
Taken together, the lung data are consistent with the idea that because activation of AMPK resolved LPS-injured lungs. However, vascular damage was attenuated in lungs treated with AICAR or metformin. Septal regions in these lungs displayed marked improvement in septal structure with many thin septae present (Fig. 7C, bottom, left middle, arrows) appeared abnormal with congestion, alveolar distortion, thickening, and marked interstitial edema compared with the thin septal networks observed in control lungs. However, vascular damage was attenuated in lungs treated with AICAR or metformin. Septal regions in these lungs displayed marked improvement in septal structure with many thin septae present (Fig. 7C, bottom, left middle, arrows). Taken together, the lung data are consistent with the idea that AMPK serves as an injury-response mechanism involved in vascular repair.

Inhibition of AMPK increases permeability and edema in LPS-injured lungs. Because activation of AMPK resolved LPS-induced vascular damage, we reasoned that inhibition of AMPK would enhance permeability in LPS-treated lungs. The AMPK inhibitor compound C caused a gradual increase in permeability that was twofold greater than control lungs by 2 h (0.027 ± 0.002 vs. 0.013 ± 0.0009 ml·min⁻¹·cmH₂O⁻¹·g dry weight⁻¹; P < 0.001, n = 5) (Fig. 8A). In LPS-treated lungs (solid bars), the addition of compound C (shaded bars) increased permeability that was significant by 90 min (0.029 ± 0.003 vs. 0.046 ± 0.005 ml·min⁻¹·cmH₂O⁻¹·g dry weight⁻¹; P < 0.001, n = 5) (Fig. 8A). At the final Kᵢ measurement, permeability was 50 ± 4% greater in the LPS + compound C group than the LPS only-treated group (0.058 ± 0.01 vs. 0.037 ± 0.002 ml·min⁻¹·cmH₂O⁻¹·g dry weight⁻¹, respectively; P < 0.00, n = 5) (Fig. 8B). Wet-to dry ratios indicate that LPS and compound C increased edema compared with controls (7.3 ± 0.1 and 8.2 ± 0.3 vs. 6.1 ± 0.2, respectively; *P < 0.05, ***p < 0.001, N = 5) (Fig. 8C). Edema in the LPS + compound C-treated lungs was elevated compared with LPS alone (9.0 ± 0.2 vs. 7.3 ± 0.1, respectively; P < 0.001, n = 5) (Fig. 8C). These studies indicate that AMPK activity...
serves a role in pulmonary vascular homeostasis and contributes to vascular repair following endotoxin-induced injury.

Metformin and AICAR reduce lung damage and increase survival in a rat model of ALI. For our next studies, AICAR or metformin was administered to LPS-treated rats to determine the effects of AMPK stimulation on lung injury in vivo. Six hours after i.p. injection of LPS, animals received a second i.p. injection of saline (sham control), AICAR, or metformin. Permeability studies of lungs obtained from rats 6 h after LPS treatment indicated a 1.5-fold increase in $K_f$ compared with control values ($0.023 \pm 0.001$ vs. $0.015 \pm 0.0007$ ml-min$^{-1}$cmH$_2$O$^{-1}$g dry weight$^{-1}$; $P < 0.00, n = 5$) (Fig. 9A). By 24 h after LPS treatment, $K_f$ values had increased 2.5-fold over control values ($0.038 \pm 0.002$ vs. $0.015 \pm 0.0007$ ml-min$^{-1}$cmH$_2$O$^{-1}$g dry weight$^{-1}$; ***$P < 0.001$, $n = 5$) (Fig. 9A). In contrast to the 24-h LPS-treated lungs, the $K_f$ values of LPS + AICAR- and LPS + metformin-treated lungs at 24 h were similar to the 6-h LPS group, suggesting that AMPK activation blocked further damage to the lung ($0.026 \pm 0.001$ and $0.024 \pm 0.001$ vs. $0.023 \pm 0.001$ ml-min$^{-1}$cmH$_2$O$^{-1}$g dry weight$^{-1}$, respectively; ns, $P > 0.05, n = 5$) (Fig. 9A). To determine whether AMPK stimulation was involved in resolving lung injury in vivo, we calculated the wet-to-dry ratios. Edema in the LPS + AICAR and the LPS + metformin groups was less than the LPS-treated lungs at 6 h, indicating that AMPK stimulation increased clearance and resolved lung damage in the intact animal ($6.3 \pm 0.09$ and $6.6 \pm 0.2$ vs. $7.5 \pm 0.2$, respectively; $\#P < 0.05$; $++P < 0.01$; $***P < 0.001, n = 5$) (Fig. 9B). It is interesting to note that only a single dose of metformin was required for its beneficial effects. Moreover, the dose of metformin (20 mg/kg) we used is equivalent, when normalized by body weight, to the lower range of dose recommendations used in the treatment of diabetes ($\pm 15$–$36$ mg/kg) (21). These data suggest that a single stimulatory event is sufficient to trigger the barrier repair function of AMPK.

Survival rate 24 h after LPS treatment was 62% (Fig. 9C). By contrast, survival rates increased to 100% in groups where AICAR or metformin was given following LPS. AMPK-mediated resolution of edema and lung damage is dramatically illustrated in the images of lungs taken from the in vivo animal model (Fig. 9D). The lung (Fig. 9D, left middle) was removed from the animal 24 h after LPS injection. Extensive hemorrhaging is evident with splotchy red patches covering the entire lung. In the lungs where the animal received AICAR or metformin 6 h after LPS, little to no vascular damage is apparent by 24 h.

**DISCUSSION**

AMPK has been studied extensively in many organ systems, and its role in regulating metabolic processes is well known (8, 26, 34). However, relatively little is known regarding function of this enzyme in the lung. Our present studies sought to understand the role of AMPK in the pulmonary response to vascular damage and determine whether a clinically relevant...
AMPK activator had potential for therapeutic use in the lung. To address the second objective, we assessed the efficacy of metformin treatment in ALI. Metformin is widely used in the treatment of diabetes. In addition to lowering blood glucose levels, metformin may benefit vascular complications of diabetes independently of its conventional hypoglycemic effects (25). The molecular mechanisms underlying metformin action remain a topic of debate; however, it is generally agreed that metformin administration results in activation of AMPK (8, 31, 33, 40). To confirm that metformin was acting through AMPK, we used it in parallel with the AMPK activator AICAR.

We first determined the endothelial response to AMPK at the cellular and molecular level. Increased permeability and edema associated with endotoxin-induced damage originate with the capillary circulation. Thus we used lung microvascular endothelial cells for our in vitro studies. Using cells stably expressing shRNA to AMPK-α1 (Δα1) and complimentary approaches to determine changes in monolayer permeability and barrier integrity, we found that AMPK-α1 activation enhanced the ability of capillary endothelial cells to restore a damaged monolayer in the presence of LPS. Moreover, these data provide evidence that AMPK-α1 localizes to a discrete subcellular compartment in lung capillary cells contributing to the growing body of evidence elucidating the molecular basis of endothelial heterogeneity and, by extension, provides insight into the complex nature of vascular injury in lung disease (15, 27, 28). It will be interesting to determine in future studies whether AMPK activity in caveolar membranes represents a distinct signaling compartment in lung endothelium.

Because LPS initiates a systemic inflammatory response, we resolved the respective contributions of the systemic response and the innate pulmonary response to injury by exposing isolated lungs to LPS. We found that AICAR and metformin resolved lung edema and reduced permeability. We then evaluated the effect of AMPK stimulation in an animal model of LPS-induced sepsis. The dose of LPS administered to the animals was sufficient to induce overt respiratory symptoms by 6 h and approximated the LD₅₀ by 24 h after exposure. Lung injury was confirmed by Kᵥ measurements at both time points. Intervention with AICAR or metformin, administered 6 h after LPS, resulted in Kᵥ values similar to controls and increased survival to 100% at 24 h. These studies demonstrate that vascular injury is repaired by AMPK activation independent of systemic inflammatory influences and suggest that AMPK is a key component of the innate endothelial response to inflammation.

Fig. 8. Inhibition of AMPK exacerbates LPS-induced lung injury. A: lungs treated ex vivo with the AMPK inhibitor compound C exhibit a gradual increase in permeability (Kᵥ) compared with controls. B: In LPS-treated lungs (solid bars), the addition of compound C (gray bars) increased permeability over LPS alone. C: wet-to-dry ratios indicate LPS (solid bar), and compound C (dark gray bar) increases edema compared with controls (open bar). Edema in LPS + compound C-treated lungs was greater than LPS alone (###black vs. light gray bar). (n = 5 animals per group; Cmpd C, compound C; *P < 0.05; *** and +++ and ###P < 0.001).

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Although our findings contribute to a better understanding of AMPK function, the picture is far from complete. What seem to be contradictory findings in AMPK research only serve to emphasize the gaps in our knowledge. AMPK was found to regulate Na\(^+/\)H\(^+\) handling in the renal system where epithelial sodium channel (ENaC) levels were upregulated in AMPK-/H\(^+\) knockout mice. However, there were only mild effects on renal salt handling, and the finding that blood pressure in these animals was reduced was counterintuitive (1). In the lung, both beneficial and detrimental roles for AMPK have been observed. During hypoxia, Na,K-ATPase is inhibited in a process dependent on reactive oxygen species and activation of AMPK-\(\alpha\)1. Downregulation of this ion channel by AMPK led to alveolar epithelial dysfunction and impaired alveolar fluid reabsorption (7). These data were supported in cultured H441 lung epithelial cells where activation of AMPK-\(\alpha\)1 by AICAR or metformin inhibited ENaC and decreased monolayer resistance (37). In contrast, other groups have found that activation of AMPK using AICAR protected against lung endothelial barrier dysfunction and neutrophil accumulation induced by direct pulmonary injury resulting from intratracheal LPS instillation (38). In this last study, AICAR was administered prophylactically, before administration of LPS. In our model system, treating the lung with AICAR or metformin before LPS had no effect on reducing lung permeability (J. R. Creighton, unpublished results). Clearly, more studies will be required to understand better the links between stimulatory events, AMPK activity, cellular function, and physiological outcomes.

![Diagram of AMPK-\(\alpha\)1 and ALI](image)

**Fig. 9.** Stimulation of AMPK increases survival and enhances lung repair in LPS-treated rats. A: LPS increased permeability by 6 h that was further elevated at 24 h (solid bars). AICAR and metformin decreased permeability compared with LPS alone (***black vs. gray bars). B: AICAR and metformin decreased LPS-induced pulmonary edema. (+ + and #black vs. gray bars). C: survival at 24 h was increased to 100% in animals that received metformin or AICAR after LPS. D: images of lungs used in Kf studies. Left: control lung, no evidence of hemorrhaging. Middle: lung isolated 24 h after animal was given LPS. Hemorrhaging is present over the entire lung surface. Right middle and right: lungs from LPS + metformin- or LPS + AICAR-treated animals, no hemorrhaging is evident. In groups receiving an AMPK activator, animals received drug 6 h after LPS. Unless stated otherwise, studies were performed 24 h after animals were given LPS (\(n = 5\) animals per group; #\(P < 0.05\); ++\(P < 0.01\); ***\(P < 0.001\)).
In conclusion, our studies are supported by recent reports of the beneficial effects of AMPK in other animal models (38) and are consistent with metformin-mediated vascular-protective effects observed in patients with diabetes. Interestingly, several clinical and observational studies have linked metformin use in diabetic patients with a decreased risk of developing ALI (6, 10, 12, 20). In sum, data from our group and others provide evidence that AMPK-mediated mechanisms of vascular repair are conserved across species. Thus the current study wherein we demonstrate a use for metformin in ALI and identify the metabolic sensor AMPK-α1 as a novel therapeutic target for reversing endothelial cell dysfunction and increased permeability has potential for application in human disease.

REFERENCES