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Activation of AMPK Enhances Neutrophil Chemotaxis and Bacterial Killing

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An inability of neutrophils to eliminate invading microorganisms is frequently associated with severe infection and may contribute to the high mortality rates associated with sepsis. In the present studies, we examined whether metformin and other 5′ adenosine monophosphate-activated protein kinase (AMPK) activators affect neutrophil motility, phagocytosis and bacterial killing. We found that activation of AMPK enhanced neutrophil chemotaxis *in vitro* and *in vivo*, and also counteracted the inhibition of chemotaxis induced by exposure of neutrophils to lipopolysaccharide (LPS). In contrast, small interfering RNA (siRNA)-mediated knockdown of AMPKα1 or blockade of AMPK activation through treatment of neutrophils with the AMPK inhibitor compound C diminished neutrophil chemotaxis. In addition to their effects on chemotaxis, treatment of neutrophils with metformin or aminoimidazole carboxamide ribonucleotide (AICAR) improved phagocytosis and bacterial killing, including more efficient eradication of bacteria in a mouse model of peritonitis-induced sepsis. Immunocytochemistry showed that, in contrast to LPS, metformin or AICAR induced robust actin polymerization and distinct formation of neutrophil leading edges. Although LPS diminished AMPK phosphorylation, metformin or AICAR was able to partially decrease the effects of LPS/toll-like receptor 4 (TLR4) engagement on downstream signaling events, particularly LPS-induced IκBα degradation. The IκB kinase (IKK) inhibitor PS-1145 diminished IκBα degradation and also prevented LPS-induced inhibition of chemotaxis. These results suggest that AMPK activation with clinically approved agents, such as metformin, may facilitate bacterial eradication in sepsis and other inflammatory conditions associated with inhibition of neutrophil activation and chemotaxis.

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INTRODUCTION

Neutrophils are an essential component of the innate immune system, with a primary role in the clearance of extracellular pathogens (1). Both localization and neutralization of microorganisms are important neutrophil functions that are orchestrated by specific inflammatory mediators released from the site of infection (2,3). In particular, chemoattractants and chemokine gradients are major neutrophil guidance signals, whereas the migration of neutrophils from the vasculature to inflammatory sites is mediated by adhesion proteins, including P- and E-selectins and integrin ligands such as vascular cell adhesion molecule 1 (VCAM-1), intracellular adhesion molecule 1 (ICAM-1) and ICAM-2 on endothelium (2,4). Several mechanisms are involved in bacterial killing by neutrophils. For example, release of antimicrobial peptides, generation of reactive nitrogen and oxygen species (ROS/RNS), as well as production of hypochlorous acid by myeloperoxidase, are utilized by neutrophils to kill invading microorganisms (5,6). In addition to phagocytosis and killing internalized microorganisms, neutrophils also can release DNA and DNA-associated proteins to form extracellular traps to prevent bacterial dissemination (7,8).

Although killing of microorganisms is apparently a beneficial function of neutrophil activation, exaggerated activation of neutrophils can result in collateral damage to tissues and also contribute to the development of organ failure in sepsis (9,10). However, impairment of neutrophil activation also can lead to serious complications in infected patients. In particular, diminished neutrophil activation or decreased neutrophil numbers are associated with a high mortality rate in sepsis (11). Neutrophil dysfunction also

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commonly occurs with bacterial pneumonia in critically ill patients after trauma and hemorrhage (12–15).

Beside the central roles that NADPH-oxidase and ROS occupy in killing microorganisms, enhanced propagation and dissemination of bacteria are associated with loss of neutrophil ability to reach the site of infection (16,17). In sepsis, such alterations in neutrophil function are a result of disrupted chemokine signaling and expression of adhesion molecules (18–21). For example, the appearance of detectable levels of the bacterial products lipopolysaccharide (LPS) or lipoteichoic acid in the circulation of severely infected patients is associated with diminished neutrophil chemotaxis, including neutrophil response to interleukin 8 (IL-8), macrophage inflammatory protein 2 (MIP-2), or keratinocyte-derived chemokine (KC). In spite of progress in understanding mechanisms responsible for the inhibition of neutrophil chemotaxis, pharmacologic approaches to prevent or restore neutrophil chemotaxis and bacterial eradication are not available.

Metformin is commonly used in patients with non-insulin-dependent diabetes mellitus to lower blood glucose concentrations and to improve insulin sensitivity (22). Metformin has been shown to prevent cardiovascular complications associated with diabetes and obesity through mechanisms that presumably involve inhibition of adipose tissue lipolysis, reduction of circulating levels of free fatty acids, and inhibition of low density lipoprotein production (23,24). Although metformin has a broad spectrum of effects, inhibition of mitochondrial complex I and activation of 5′ adenosine monophosphate-activated protein kinase (AMPK) appear to be major mechanisms of its action (25,26). Recent studies have shown that besides their ability to regulate cellular metabolism (27), metformin and other AMPK activators can decrease the severity of organ injury in acute inflammatory states (28), including LPS-induced liver injury or acute lung injury (29,30). Metformin, berberine or aminoimidazole carboxamide ribonucleotide (AICAR) were all shown to diminish activation of the toll-like receptor 4 (TLR4)/NF-κB signaling cascade, as well as to release of proinflammatory mediators of neutrophils and macrophages, and also to enhance endothelial integrity *in vitro* and in models for sepsis (31–36). In addition, metformin enhances host defense mechanisms by facilitating the chemotaxis and maturation of T cells (37,38). Besides its antiinflammatory actions, activated AMPK was recently shown to increase the phagocytic ability of macrophages (39). Although these studies revealed a beneficial effect of metformin in acute inflammatory conditions, there is little information concerning the ability of metformin or other AMPK activators to alter primary innate immune responses, and particularly bacterial eradication. In this study, we examined the hypothesis that AMPK activation may affect fundamental neutrophil functions, including chemotaxis and bacterial killing.

MATERIALS AND METHODS

Mice

Male C57BL/6 mice were purchased from the National Cancer Institute–Frederick (Frederick, MD, USA). Male mice, 8 to 10 wks of age, were used for experiments. The mice were kept on a 12-h light:dark cycle with free access to food and water. All experiments were conducted in accordance with protocols approved by the University of Alabama at Birmingham Animal Care and Use Committee.

Reagents and Antibodies

W-peptide was purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA). Metformin, berberine and IκB kinase (IKK) inhibitor PS-1145 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Compound C and rapamycin were purchased from Calbiochem (La Jolla, CA, USA). Antibodies for phospho-Thr172-AMPK, total AMPK, phospho-Ser240/244-rpS6, total rpS6, 4E-BP1, and IκBα were purchased from Cell Signaling Technology (Danvers, MA, USA). β-actin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Custom antibody mixtures and negative selection columns for neutrophil isolation were obtained from Stem Cell Technologies (Vancouver, BC, Canada). Fluorescein isothiocyanate (FITC)-labeled *E. coli*, *S. aureus* and Alexa Fluor 594–conjugated phalloidin were from Invitrogen/Life Technologies (Carlsbad, CA, USA). Mounting oil solution containing DAPI was from Vector laboratories (Burlingame, CA, USA). The μ-slide for chemotaxis assay was obtained from Ibidi (Mt. Prospect, IL, USA); and transmigration chambers that we used are available from BD Biosciences (San Jose, CA, USA).

Neutrophil Isolation and Culture

Bone marrow neutrophils were isolated as described previously (31,40). Neutrophil purity was consistently greater than 97%, as determined by Wright-Giemsa–stained cytospin preparations. Neutrophils were cultured in RPMI 1640 medium containing 0.5% or 5% fetal bovine serum (FBS) and treated as indicated in the figure legends. Neutrophil viability under experimental conditions was determined using trypan blue staining and was consistently greater than 95%.

HL-60 Cell Culture and Differentiation

The HL-60 cell line was obtained from American Type Culture Collection and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL), streptomycin (25 μg/mL) and L-glutamine (1 mmol/L). To obtain differentiated cells, HL-60 cells were cultured in medium containing dimethyl sulfoxide (DMSO) (1.3%) for four consecutive days (41). Differentiated cells were then used for small interfering RNA (siRNA) treatment and IL-8 mediated chemotaxis.

siRNA Knockdown of AMPKα1

Differentiated HL-60 cells were incubated with specific siRNA (1 μmol/L) to AMPKα1, as described previously (41).

Briefly, cells (3.5×10^6/well) in 12-well plates were incubated in Accell medium (serum free) containing siRNA (1 μmol/L) to AMPKα1 for 72 h. During incubation with siRNA, the cell culture medium was supplemented with 1.3% DMSO to maintain differentiation of the HL-60 cells. The cells were then subjected to Western blot analysis of AMPK or transwell migration assay.

Transwell Migration Assay

Transwell migration assay was performed using 24-well cell plate BD Falcon cell culture inserts (Translucent PET Membrane, BD Biosciences). Briefly, bone marrow neutrophils (10^6 cells/well) or differentiated HL-60 cells in 300 μL of RPMI 1640 medium (5% FBS) were added to the upper reservoir, whereas W-peptide or IL-8 in culture medium (800 μL) was placed in the lower reservoir of transmigration chamber. In all experiments, chemotaxis was determined after neutrophils or HL-60 cells were allowed to migrate for 60 min at 37°C followed by imaging the cells in the lower reservoir (41). Each condition was tested three or more times.

Measurement of Cell Velocity

Bone marrow neutrophils pretreated as described in figure legends and then loaded into the μ-slide for chemotaxis assay (Ibidi). Cell migration was initiated by inclusion of W-peptide (50 nmol/L) to create a concentration gradient. Migration was recorded by imaging cells with 1 min intervals for a total of 60 min and then distance and movement direction of individual cells were plotted to calculate speed and velocity.

Measurement Neutrophil Chemotaxis *In Vivo*

Mice were subjected to application of metformin (125 mg/kg of body weight; intraperitoneal [IP]) for 12 h and 2 h before IP injections of W-peptide (0.43 mg/kg). In additional experiments, mice were treated with compound C (3 mg/kg, IP) or vehicle (saline) for 2 h prior to application of W-peptide. After 6 h, mice were euthanized and peritoneal lavages obtained using 10 mL of RPMI 1640 medium (without serum).

Phagocytosis Assay

Phagocytosis of fluorescent labeled *E. coli* or *S. aureus* by neutrophils was performed as described previously (39). In brief, phagocytosis of fluorescently labeled bacteria by neutrophils pretreated with or without metformin (500 μmol/L, 2.5 h) was determined by adding tenfold excess of *E. coli* or *S. aureus* to the cells. To measure internalization of bacteria, fluorescent *E. coli* or *S. aureus* were incubated for 15 min at 37°C, and cells were then washed three times in ice-cold PBS. Next, cells were incubated with or without trypan blue solution (0.2% trypan blue, 20 mmol/L citrate, and 150 mmol/L NaCl, pH 4.5) for 1 min, then centrifuged, and the cell pellet was resuspended in PBS, and the amount of fluorescence was measured using flow cytometry.

In Vitro Killing-Activity Assay

Neutrophils (2×10^6 cells/mL) were incubated with ampicillin-resistant *E. coli* (2×10^7/mL) in RPMI medium without serum for 90 min at 37°C. Next, 20 μL of cell/bacterial suspension was incubated with 480 μL Triton X-100 (0.1%) for 10 min to lyse neutrophils. Serial dilutions were then plated on agar plates with ampicillin and incubated overnight at 37°C. The number of bacterial colonies on agar plates was determined using colony counter software (Bio-Rad, Hercules, CA, USA).

Peritonitis-Induced Sepsis

The efficiency of bacterial eradication *in vivo* was performed as described previously (42,43). Mice were subjected to administration of metformin (125 mg/kg of body weight; IP) for 12 h, compound C (3 mg/kg of body weight; IP) or vehicle (PBS) for 2 h before IP injection of ampicillin-resistant *E. coli* (2×10^8). After 6 h, mice were euthanized and peritoneal lavages obtained using 10 mL RPMI 1640 medium without serum. The number of surviving bacteria was determined by incubation of 95 μL of peritoneal lavages with 5 μL of Triton X-100 (1%) for 10 min to lyse cells, and then serial dilutions were placed on agar plates with ampicillin and incubated overnight at 37°C. Bacterial colonies were counted using colony counter software (Bio-Rad).

Imaging Actin in Neutrophils

Neutrophils were incubated with 4% paraformaldehyde in PBS for 30 min at room temperature then washed with PBS and permeabilized with 0.1% Triton X-100/PBS for 4 min. The cells were then incubated with 3% BSA in PBS for 1 h, followed by the addition of Alexa Fluor 594-conjugated phalloidin (25 μL/mL) for 20 min at room temperature. After the cells were washed with PBS, they were mounted with emulsion oil solution containing DAPI to visualize nuclei. Confocal microscopy was performed as described previously, using a Leica DMIRBE inverted epifluorescence/Nomarski microscope (Leica Microsystems, Wetzlar, Germany) outfitted with Leica TCS NT laser confocal optics (40).

Western Blot Analysis

Western Blot analysis was performed as described previously (44,45). Briefly, cell lysates of murine bone marrow neutrophils (3.5×10^6/well) were prepared using lysis buffer containing Tris pH 7.4 (50 mmol/L), NaCl (150 mmol/L), NP-40 (0.5%, vol/vol), EDTA (1 mmol/L), EGTA (1 mmol/L), okadaic acid (1 nmol/L) and protease inhibitors. Cell lysates were sonicated and then centrifuged at 10,000*g* for 15 min at 4°C to remove insoluble material. The protein concentration in the supernatants was determined using the Bradford reagent (BioRad) with BSA as a standard. Samples were mixed with Laemmli sample buffer and boiled for 15 min. Equal amounts of proteins were resolved by 8% to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred onto polyvinylidene fluoride (PVDF) membranes (Immobilon P; Millipore, Billerica, MA, USA). The membranes were probed

with specific antibodies as described in the figure legends followed by detection with HRP-conjugated goat anti-rabbit IgG. Bands were visualized by enhanced chemiluminescence (Super Signal; Pierce Biotechnology, Rockford, IL, USA) and quantified by AlphaEaseFC software (Alpha Innotech, San Leandro, CA, USA). Each experiment was carried out two or more times using cell populations obtained from separate groups of mice.

Statistical Analysis

Statistical significance was determined by the Wilcoxon rank sum test (independent two-group Mann-Whitney *U* test) as well as Student *t* test for comparisons between two groups. Multigroup comparisons were performed using one-way analysis of variance (ANOVA) with Tukey *post hoc* test. A value of *P* less than 0.05 was considered significant. Analyses were performed on SPSS version 16.0 (IBM, Armonk, NY, USA) for Windows (Microsoft Corporation, Redmond, WA, USA).

RESULTS

Effects of AMPK Inhibition or Activation on Neutrophil Chemotaxis

In initial experiments, mouse neutrophil chemotaxis was determined using W-peptide, a mouse homolog of human IL-8. As shown in Figures 1A–C, W-peptide enhanced, in a dose- and time-dependent manner, neutrophil migration in transmigration chambers. To determine if neutrophil chemotaxis affected AMPK activation, neutrophils were incubated with W-peptide for 0, 20, 40 or 60 min and the amounts of total and phosphorylated AMPK were determined using Western blot analysis. As shown in Figure 1D, transient activation of AMPK was detected in neutrophils 20 min after exposure to W-peptide. Similar to the effects of W-peptide, enhanced chemotaxis and activation of AMPK was found in IL-8-treated HL-60 cells, a differentiated human neutrophil-like cell line (Figures 1E, F) (41).

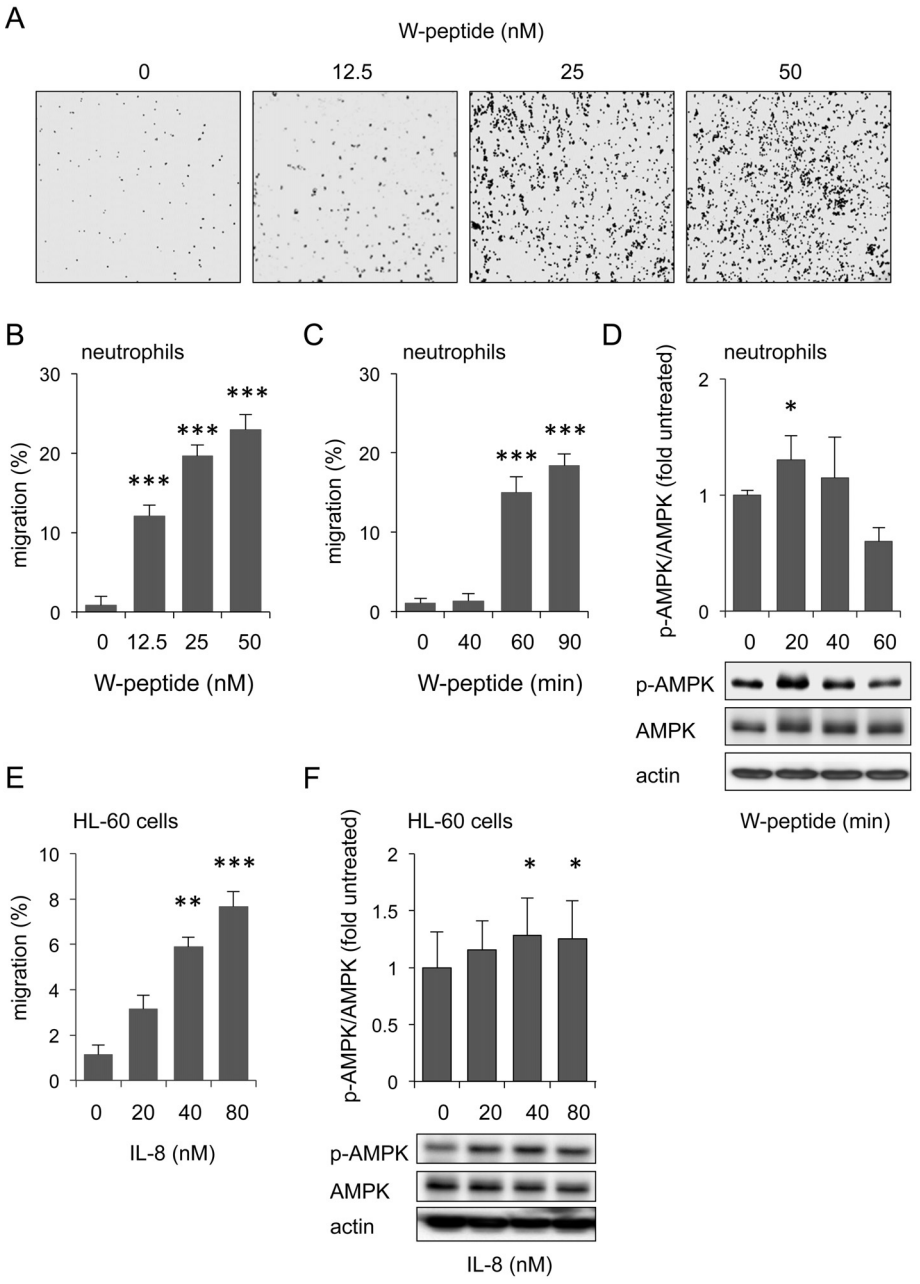

Figure 1. Effects of metformin or AMPK inhibitor compound C on neutrophil chemotaxis. (A, B, C) Representative images (A) and quantitative data (B, C) show neutrophil migration after dose- and time-dependent stimulation with W-peptide. In (B), transmigration assay was performed by inclusion of W-peptide (0, 12.5, 25 or 50 nmol/L) for 60 min, whereas, in (C), migration was determined after inclusion of 50 nmol/L W-peptide for the indicated time period. Means ± SD (n = 3), ***P < 0.001, compared with untreated neutrophils. (D) Representative Western Blots and quantitative data show the amount of phosphorylated and total AMPK in neutrophils treated with W-peptide (50 nmol/L) for 0, 20, 40 or 60 min. Average (mean ± SEM) of optical bend density was obtained from three independent experiments (*P < 0.05, compared with untreated). (E, F) HL-60 cells were treated with IL-8 for 60 min followed by chemotaxis assay (E) and Western Blot analysis (F) for phospho and total AMPK. Means ± SD (n = 3), *P < 0.05, **P < 0.01, ***P < 0.001, compared with untreated cells.

To evaluate the effects of AMPK activation on neutrophil chemotaxis, assays were performed using neutrophils that were left untreated (control) or that were incubated with the AMPK inhibitor compound C. Compound C dose dependently inhibited neutrophil chemotaxis (Figures 2A, B). Additional experiments using the AMPK activators metformin or berberine demonstrated that exposure of neutrophils to such compounds resulted in enhanced chemotaxis (Figure 2C). The confirmatory experiments also were performed using control (scrambled) and specific siRNA to diminish expression of AMPKα1. As shown in Figures 2D and 2E, siRNA mediated knockdown of AMPKα1 resulted in significant decrease in HL-60 chemotaxis. Although metformin and berberine stimulated AMPK activation and neutrophil motility, AICAR did not increase chemotaxis (data not shown) likely due to effects mediated by enzymatically produced ZMP in the AICAR-treated cells (46–48).

AMPK Activation Prevents LPS-Induced Inhibition of Chemotaxis

Previous studies have shown that engagement of TLR4 diminished IL-8–dependent chemotaxis of human neutrophils (49). Similar to the previously reported results, we found that exposure of mouse bone marrow neutrophils to LPS also resulted in inhibition of neutrophil chemotaxis (Figures 3A, B). Western blot analysis showed that inhibition of neutrophil chemotaxis was associated with dose- and time-dependent decrease in LPS-induced AMPK phosphorylation (Figures 3C, D). To further determine the relationship between LPS and AMPK, neutrophils were treated with AICAR before or after exposure to LPS. As shown in Figures 3E and 4F, the addition of AICAR to the cell cultures, at either pre- or postexposure to LPS, was able to increase activity of AMPK.

Because metformin-induced activation of AMPK was shown to diminish LPS–mediated neutrophil proinflammatory activation (31), we hypothesized that there might be similar effects of AMPK

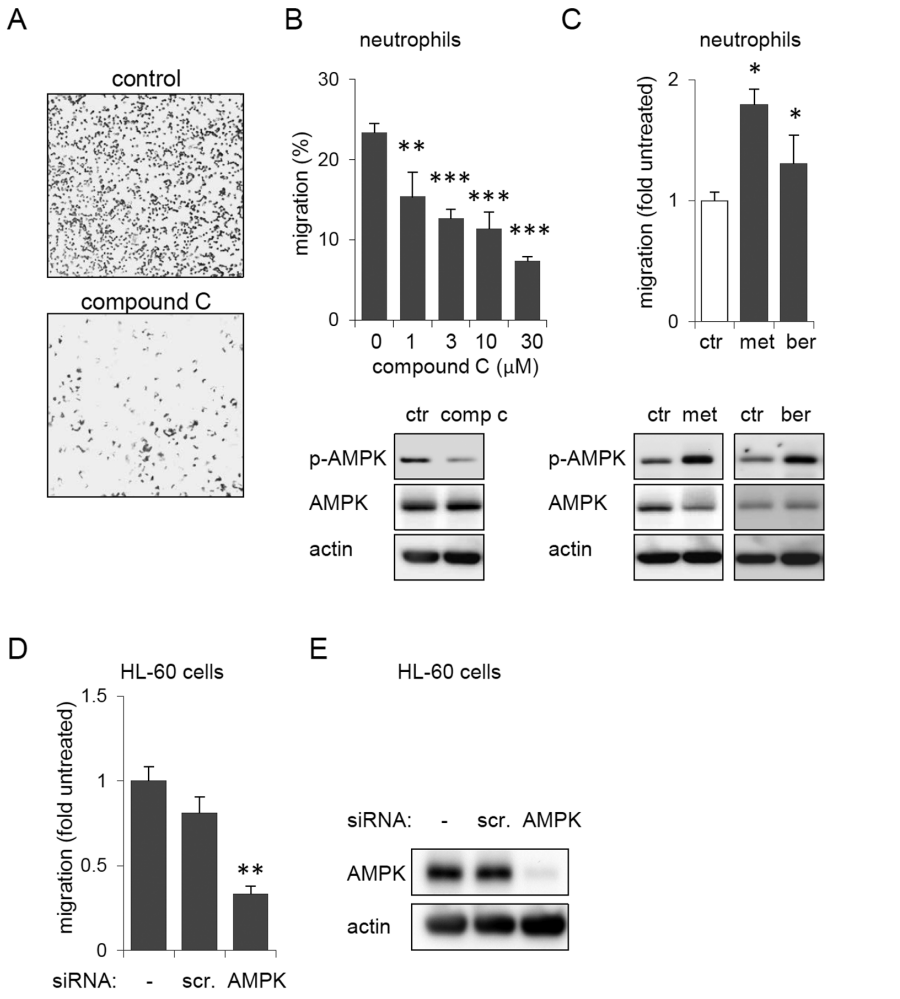

Figure 2. Effects of AMPK activation or inhibition on neutrophil chemotaxis. Neutrophils were pretreated with compound C (0, 1, 2, 3 or 10 μmol/L) for 60 min, metformin (500 μmol/L) or berberine (10 μmol/L) for 2.5 h and then chemotaxis was measured after inclusion of cells in transmigration chambers and exposure to W-peptide for 60 min. (A) Representative images show amount of control (untreated) or compound C–treated (30 μmol/L) neutrophils that migrated into the lower reservoir. Panel (B) shows dose-dependent inhibition by compound C, whereas (C) metformin exposure or berberine exposure enhanced neutrophil chemotaxis. Means ± SD ($n = 3$), $^*P < 0.05$, compared with control. Lower panels B or C show Western blots of phospho AMPK, total AMPK and actin obtained from neutrophils treated with compound C (10 μmol/L) for 60 min, metformin (500 μmol/L) for 2.5 h, or berberine (10 μmol/L) for 2.5 h. (D) Chemotaxis assays were performed in control (untreated) cells and cells treated with control (scrambled siRNA) or specific siRNA to AMPKα1 subunit. (E) Representative Western blots show amounts of AMPKα1 and actin before and after treatment with scrambled or siRNA to AMPKα1. ctr, Control; met, metformin; ber, berberine; scr., scrambled.

activation in preventing the inhibitory effects of LPS on chemotaxis. As shown in Figure 4A, incubation of neutrophils with metformin and LPS significantly improved chemotaxis as compared with that found when neutrophils were treated with LPS alone. Metformin increased neutrophil chemotaxis when included in the cell cultures before or after exposure of the cells to LPS. In additional experiments, the effects of metformin on LPS-associated diminishment

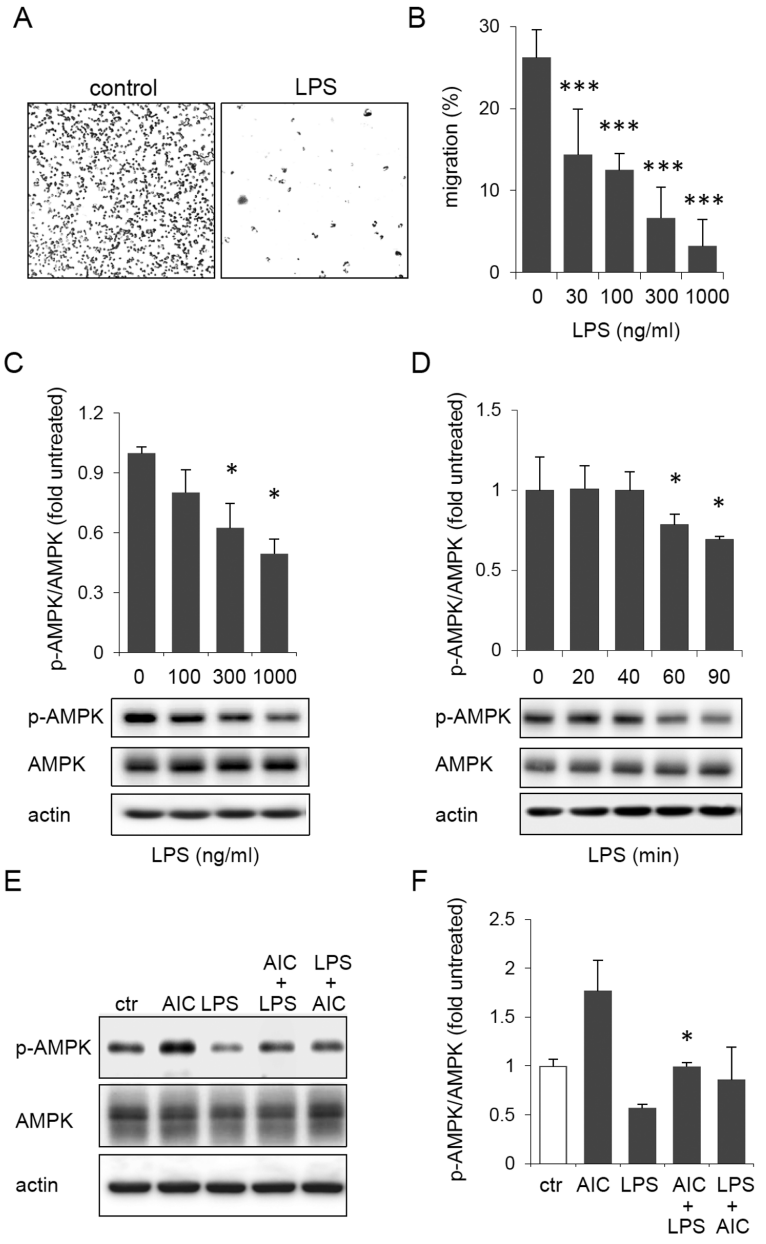

Figure 3. Exposure to LPS diminished neutrophil chemotaxis and AMPK phosphorylation. (A) Representative images show neutrophil transmigration before and after treatment with LPS. Neutrophils were treated with LPS (0 or 1 μg/mL) for 90 min. The cells were then washed and chemotaxis examined over a 60 min period. In (B), neutrophils were incubated with LPS for 90 min followed by measurement of neutrophil chemotaxis. (C, D) Representative Western blots and quantitative data show the amounts of phosphorylated and total AMPK obtained from neutrophils treated with LPS (0–1,000 ng/mL) for 90 min (C) or after exposure to LPS (300 ng/mL) for the indicated time periods (D). Means ± SEM (n = 3), *P < 0.05. (E, F) LPS suppresses activation of AMPK. Representative Western Blots and quantitative data show amounts of phospho and total AMPK, and actin. Neutrophils were treated with AICAR (0 or 250 μmol/L) for 60 min and then cultured with LPS (0 or 300 ng/mL) for an additional 60 min. Cells also were treated with LPS (300 ng/mL) for 60 min followed by inclusion of AICAR (250 μmol/L) in the cultures for an additional 60 min. Means ± SD, n = 3, *P < 0.05, compared with neutrophils treated with LPS alone. ctr, Control; AIC, AICAR.

of neutrophil velocity were determined using μ-slide for chemotaxis assay. As shown in Figures 4B and 4C, metformin increased neutrophil velocity and also prevented neutrophil immobilization after exposure to LPS. In contrast, treatment of LPS-exposed neutrophils with the AMPK inhibitor compound C resulted in significant inhibition of neutrophil chemokinesis.

Given our *in vitro* results showing that AMPK activation modulated neutrophil chemotaxis, we determined if activation or inhibition of AMPK can affect neutrophil chemotaxis *in vivo*. To examine this possibility, control mice or mice treated with metformin or compound C were then given an IP injection of W-peptide and the number of neutrophils migrating into the peritoneum was measured 5 h later (Figure 4D). As shown in Figure 4E, treatment with metformin before application of W-peptide was associated with an increased number of peritoneal neutrophils as compared with treatment with W-peptide alone. In contrast to metformin, administration of compound C diminished W-peptide–induced peritoneal accumulation of neutrophils.

AMPK Activation Facilitates Neutrophil-Dependent Bacterial Uptake and Killing

Although our experiments found that treatment with the AMPK activator metformin or the AMPK inhibitor compound C significantly affect neutrophil chemotaxis, the effects of AMPK activation on bacterial killing were not determined. In addition to the generation of ROS, phagocytosis is central in bacterial eradication. To measure phagocytosis, control (untreated) neutrophils or neutrophils treated with metformin or compound C were incubated with fluorescein isothiocyanate (FITC)-tagged *E. coli* or *S. aureus* and then subjected to flow cytometry. As shown in Figures 5A and 5B, pretreatment with metformin increased the ability of neutrophils to phagocyte bacteria. Next, we determined if activation or inhibition of AMPK can affect bacterial killing. To examine this possibility, neu-

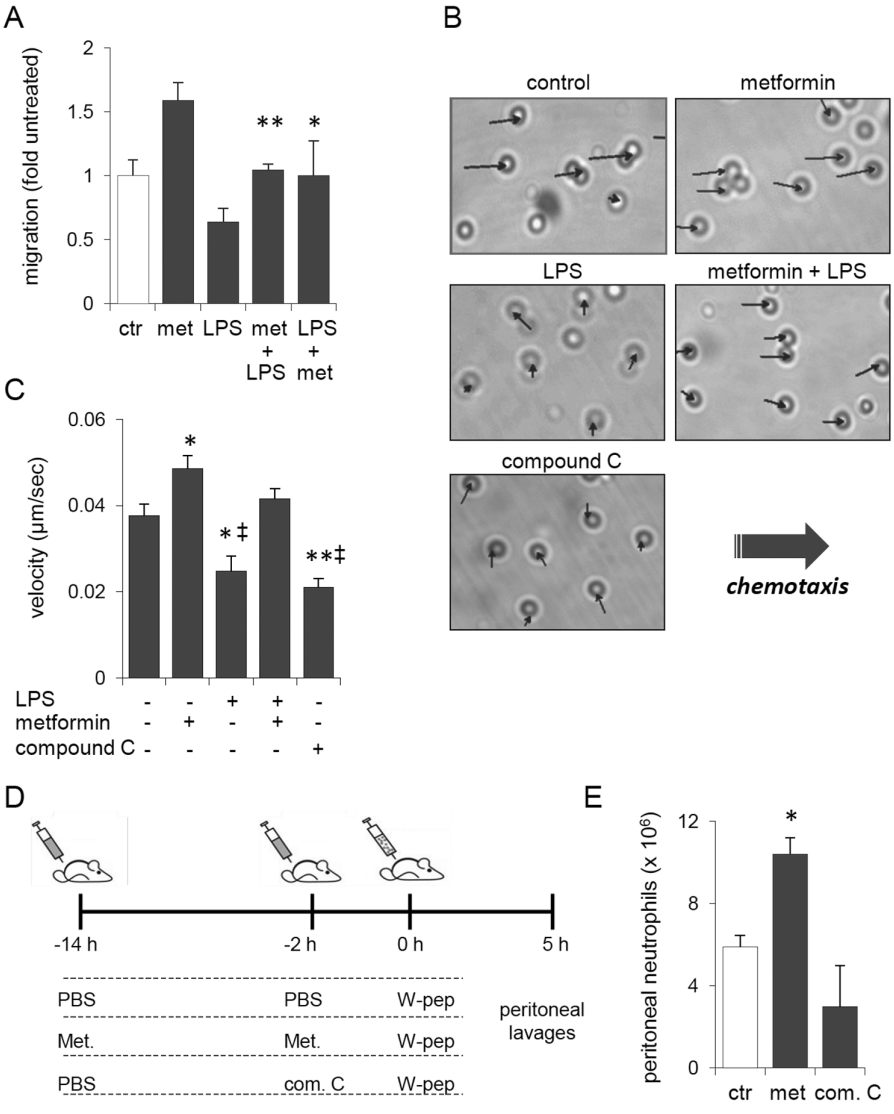

Figure 4. Metformin diminished LPS-induced inhibition of neutrophil chemotaxis. (A) Neutrophils were treated with metformin (met; 0 or 500 μmol/L) for 90 min and then LPS (0 or 300 ng/mL) for 60 min, or cells were first treated with LPS (300 ng/mL) for 60 min followed by inclusion of metformin (500 μmol/L) in the cultures for an additional 90 min. Neutrophil chemotaxis was then examined. Means ± SD, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with neutrophils treated with LPS alone. (B) Representative images show direction and distance (length of arrows) passed by the neutrophils a pretreated with metformin, LPS, compound C or combination of metformin and LPS. Panel (C) shows neutrophil velocity. Mean ± SEM, $n \geq 20$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control, $^{\ddagger}P < 0.001$ compared with metformin and $^{\S}P < 0.001$ compared with LPS alone. Large arrow indicates direction to W-peptide. (D, E). Metformin stimulates, whereas compound C diminishes neutrophil chemotaxis *in vivo*. Panel (D) shows time line administration of metformin (125 mg/kg; IP), compound C (3 mg/kg; IP), PBS (200 μL; IP) or W-peptide (0.43 mg/kg; IP) followed by acquisition of peritoneal neutrophils. Panel (E) shows amount of peritoneal neutrophils obtained from mice treated as indicated in (D). Mean ± SEM ($n \geq 3$). $^{*}P < 0.05$, compared control or mice treated with compound C. ctr, Control; met or Met., metformin; W-pep, W-peptide; com. C, compound C.

trophils were left untreated or were incubated with metformin, AICAR or compound C. As shown in Figures 5C and 5D, both metformin and AICAR increased, whereas compound C diminished, bacterial killing. Results obtained from bacterial killing assays showed that a modest increase in the amount of bacteria was recovered after incubation of neutrophils with LPS (Figures 5C, D).

Previous studies have shown that signaling cascades downstream of AMPK activates regulation of mTORC1. Therefore, in additional experiments, neutrophils were treated with the specific mTOR inhibitor rapamycin. However, rapamycin exposure did not affect the ability of neutrophils to eradicate bacteria (see Figures 5C, D).

AMPK Activation Enhances Bacterial Killing in Peritonitis-Induced Sepsis

Considering our *in vitro* results that AMPK activation with metformin increased neutrophil chemotaxis, as well as enhanced uptake and killing bacteria *in vitro*, we determined if metformin treatment can improve bacterial clearance *in vivo*. This possibility was examined using a murine model for peritonitis-induced sepsis (42,43). As shown in Figures 5E and 5F, there were significant decreases in the numbers of viable bacteria recovered from mice that received metformin as compared with controls. Of note, the number of peritoneal neutrophils was found to be increased, compared with the control group (Figure 5G).

AMPK Activation Stimulates Actin Rearrangement, Neutrophil Leading Edge Formation, and Diminishes LPS/TLR4-Mediated Inhibition of Neutrophil Chemotaxis

Although our experiments demonstrated that AMPK activation enhanced neutrophil chemotaxis and bacterial killing, the mechanism responsible for such effects was not delineated. AMPK activation induced by cellular exposure to metformin or AICAR results in cytoskeletal rearrangement (17). As shown in Figure 6, whereas significant decrease

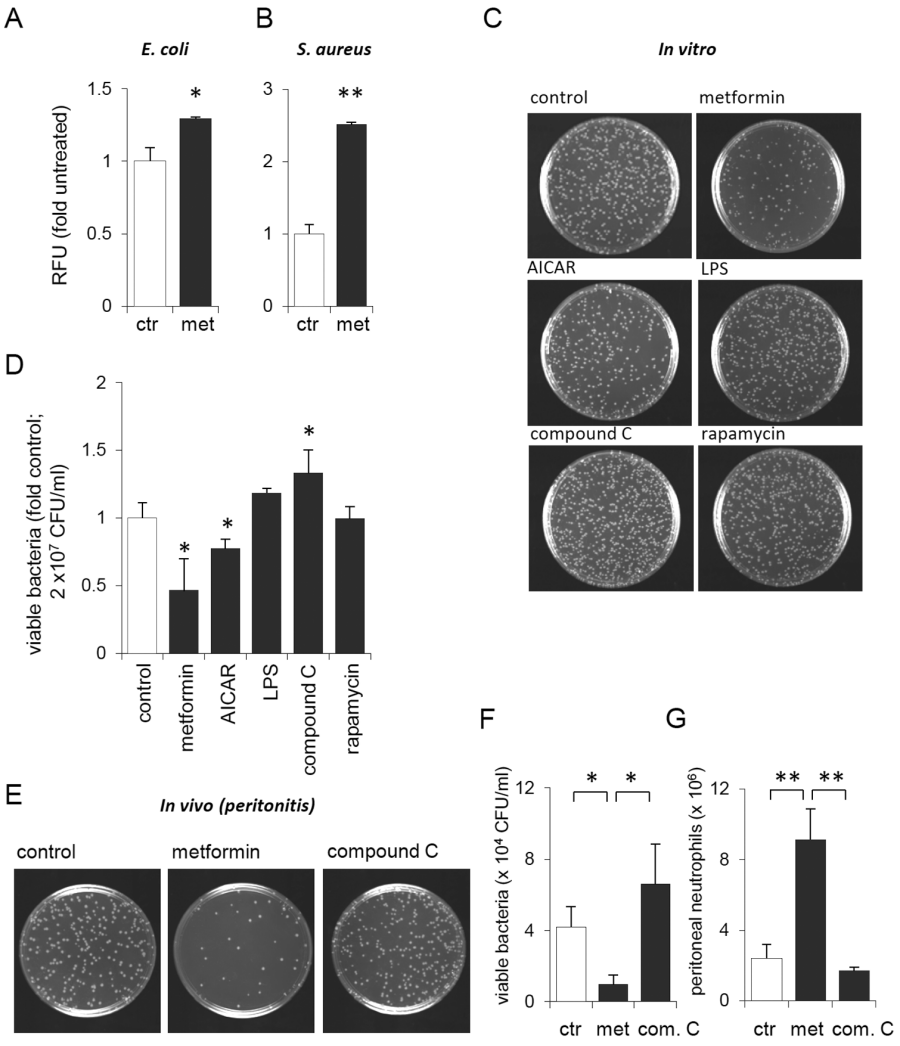

Figure 5. Metformin increases bacteria uptake and killing *in vitro* and *in vivo*. (A, B). Neutrophils (10^6 cells/mL) were pretreated with or without metformin (500 μmol/L) for 2.5 h and then incubated with fluorescently tagged *E. coli* (10^7/mL) (A) or *S. aureus* (10^7/mL) (B) for additional 20 min. Neutrophil-dependent uptake of bacteria was determined using flow cytometry. Means ± SEM (n = 3), *P < 0.05 or **P < 0.01. (C, D) Neutrophils (2 × 10^6 cells/mL) were pretreated with metformin (0 or 500 μmol/L) for 2.5 h, AICAR (0 or 250 μmol/L) for 2 h, LPS (1 μg/mL), compound C (10 μmol/L) or rapamycin (10 nmol/L) for 60 min. Next, neutrophils were incubated with *E. coli* (2 × 10^7/mL) for 90 min. Images (C) show agar plates with colonies formed by viable *E. coli* that were obtained after incubating bacteria with neutrophils. Panel (D) shows the number of viable bacteria recovered from killing assay. Means ± SEM (n = 4), *P < 0.05 compared with control. (E, F, G) Mice were subjected to the administration of metformin (125 mg/kg; IP) for 12 h, compound C (3 mg/kg; IP), or vehicle (0.9% saline) for 2 h followed by IP injection of *E. coli* (2 × 10^8) for an additional 6 h. (E) Representative images showed the number of *E. coli* colonies formed from viable bacteria that were recovered from peritoneal lavages. (F, G) Average of viable bacteria (F) and neutrophils (G) obtained from peritoneal lavages (mean ± SEM, $n \geq 5$). *P < 0.05, **P < 0.01. RFU, relative fluorescence unit; ctr, control; met, metformin; CFU, colony-forming unit; com. C, compound C.

in the level of actin polymerization was found in LPS-treated neutrophils, such inhibitory effects of LPS were prevented by exposure of the neutrophils to metformin or AICAR. As shown in Figures 7A and 7B, inclusion of metformin or AICAR effectively diminished LPS-induced IκBα degradation. Of note, pretreatment with the specific IKK inhibitor PS-1145 also resulted in inhibition of IκBα degradation as well as prevented neutrophil immobilization by LPS (Figures 7C–E). Although mTORC1 activation is essential for the enhancement of the proinflammatory properties of LPS-treated neutrophils, metformin did not affect LPS-mediated phosphorylation of rpS6 (Figure 7F). Of note, inclusion of rapamycin diminished mTORC1 activation (Figures 7G, H).

DISCUSSION

In the present studies, we found that activation of AMPK enhanced neutrophil chemotaxis and bacterial uptake, both essential components of bacterial killing. Recent studies, including results obtained from our laboratory (39), have shown that metformin or other AMPK activators enhance cell mobility and also phagocytosis. For example, AMPK activation was associated with enhanced T cell chemotaxis or migration of epithelial cells (38,50). AMPK activation has also been shown to enhance the phagocytic ability of macrophages, including uptake of bacteria, synthetic beads or apoptotic cells (39). These results are consistent with an ability of activated AMPK to facilitate microbial eradication through mechanisms that involve enhancement of neutrophil chemotaxis and/or bacterial uptake. Consistent with these previously reported findings, our present *in vivo* results showed that metformin effectively improved bacterial killing *in vivo*.

Severe sepsis is characterized by alterations in immunologic and host defense functions that include downregulation of neutrophil chemotaxis and phagocytosis (2,16,17,51,52). Because AMPK activation in metformin-treated neutrophils prevented LPS-mediated inhibition of

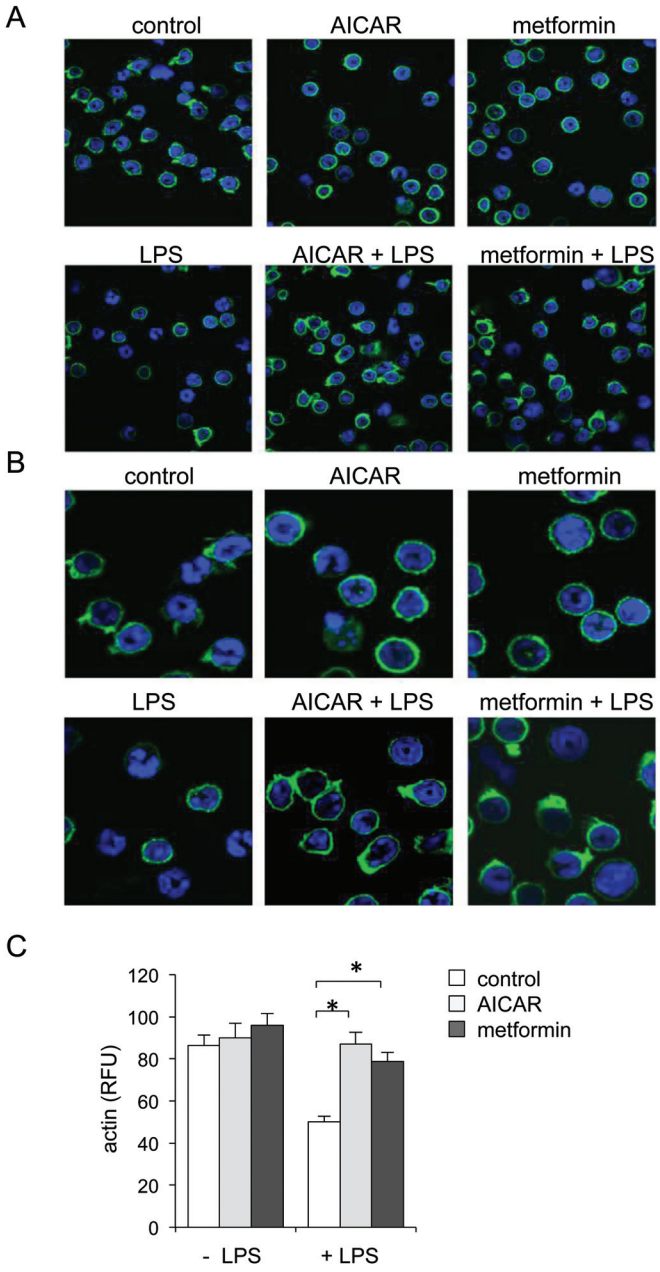

Figure 6. AMPK activation induces neutrophil cytoskeletal rearrangement and leading edge formation. Neutrophils were pretreated with AICAR (0 or 250 μmol/L) for 90 min or metformin (0 or 500 μmol/L) for 2.5 h followed by inclusion of LPS (0 or 1 μg/mL) for additional 90 min. (A,B) Representative images show actin (green) and nuclei (blue) staining (A), whereas (B) shows magnified region of interest. (C) Average of actin fluorescence is shown. Mean ± SD actin fluorescence intensity ($n < 3 \sim 5$), $^*P < 0.05$.

chemotaxis, it is possible that metformin treatment may preserve neutrophil chemotaxis in the setting of sepsis and facilitate the ability of the host to clear invading pathogens. Our results showed that metformin can reverse the inhibitory actions of LPS on chemotaxis, and also prevent LPS-induced degradation of IκBα. Of note, exposure of neutrophils to the IKK inhibitor PS-1145 (53,54) also diminished IκBα degradation and prevented inhibition of neutrophil chemotaxis by LPS, suggesting that the effects of AMPK activation in this setting may also be due to its ability to inhibit IκBα degradation. Although previous studies have shown that metformin and other AMPK activators are capable of inhibiting mTORC1 function (55,56), in the present experiments, exposure of neutrophils to metformin did not prevent LPS-dependent activation of mTOR. These results suggest that AMPK activation enhances neutrophil chemotaxis through mechanisms that involve suppression of TLR4-associated signaling pathways other than those involving mTOR. Although our results suggest that AMPK activation has beneficial effects on neutrophil function related to microbial clearance, it will be important to determine how AMPK activation affects such functions in additional cell populations, and also if AMPK activation can restore the diminished monocyte and T cell responses frequently found in sepsis (57).

Previous studies and results obtained from our laboratory have described anti-inflammatory effects mediated by activated AMPK. Metformin, AICAR or berberine all were shown to diminish neutrophil and macrophage proinflammatory activation, as well as to decrease the severity of endotoxin- or ventilator-induced acute lung injury (29,31,58). Exposure of macrophages to antiinflammatory mediators, such as IL-10 or TGF-β, resulted in activation of AMPK followed by transition of the cells from the M1 to M2 phenotype (33). Recent studies have shown that activated AMPK can also modulate the resolution of inflammatory conditions due to enhancement of the phagocytic ability of macrophages and neutrophils. In particular, AMPK activation increased the uptake of bacteria and enhanced efferocytosis, an essential process in the resolution of inflammation in which apoptotic cells are ingested and cleared by phagocytic cells (39,59,60). Beneficial effects of AMPK activation also were related to improvement of vascular integrity in mice models for

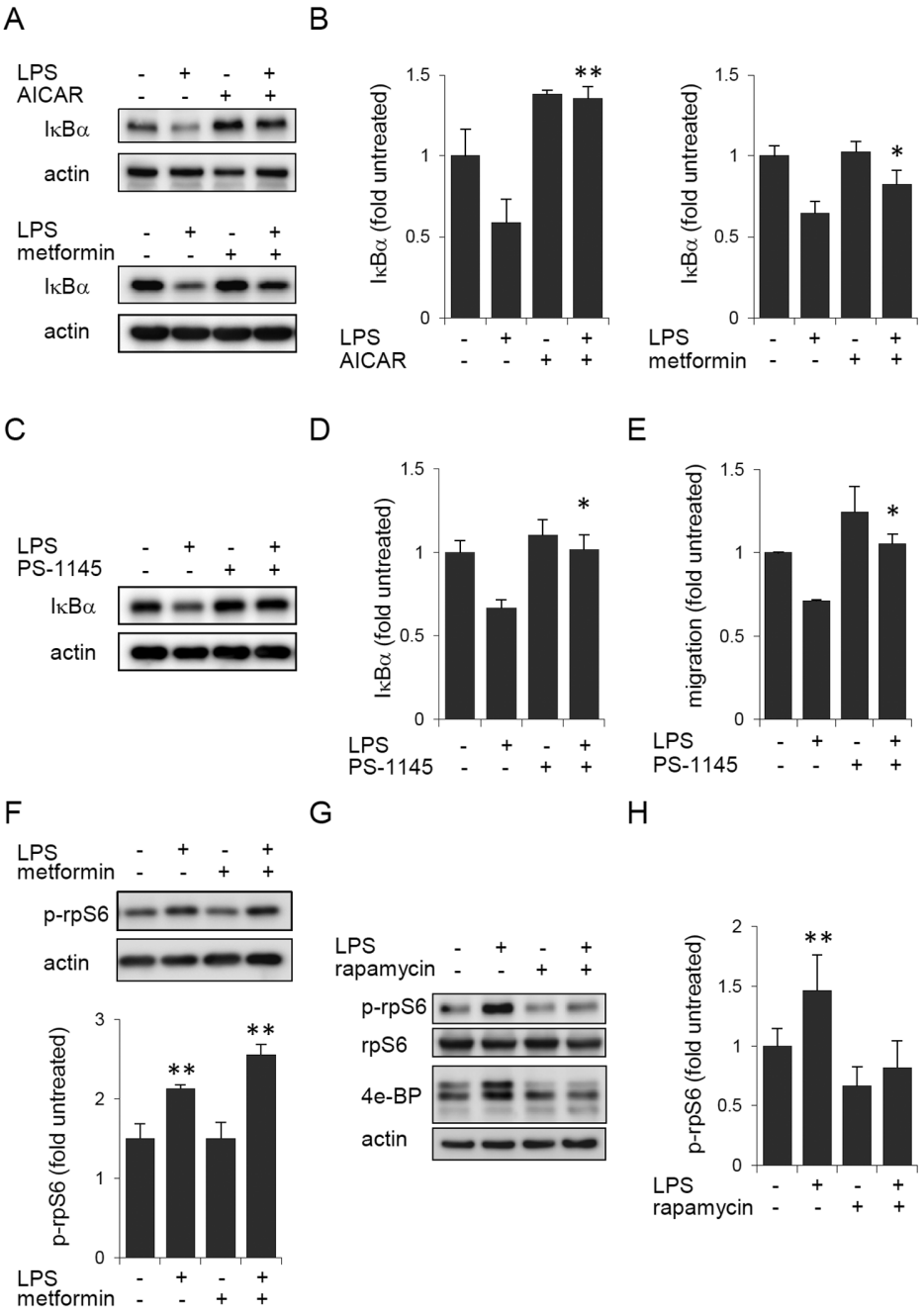

Figure 7. Effects of metformin, AICAR and IKK inhibitor on LPS-dependent activation of TLR4 or mTOR signaling pathways. Neutrophils were cultured with AICAR (0 or 500 μmol/L) for 2.5 h, metformin (0 or 500 μmol/L) for 2.5 h, IKK inhibitor PS-1145 (0 or 10 μmol/L) for 60 min or rapamycin (0 or 30 nmol/L) for 30 min. Next, neutrophils were cultured with LPS (0 or 300 ng/mL) for an additional 60 min. (A, C, F, G) Representative Western blots show amount of IκBα, total and phosphorylated ribosomal protein S6 (rpS6) (Ser$^{240/244}$), 4E-binding protein 1 (4E-BP1), and actin. Panels (B, D, F and H) show average of Western blots optical bend densitometry. Means ± SEM (n = 3); (B, D) *P < 0.05, **P < 0.01 compared with LPS only; (F) **P < 0.01 compared with control (untreated); (H) **P < 0.01 compared with control (untreated), LPS or rapamycin- and LPS-treated cells. Panel (E) shows the ability of IKK inhibitor PS-1145 to prevent LPS-mediated inhibition of neutrophil chemotaxis.

endotoxemia-induced ALI and to airway remodeling in asthma (34,61). In experimental models of diabetes, endothelial barrier function was preserved through mechanisms involving activation of AMPK (62).

Recent studies have shown that AMPK phosphorylation was diminished upon exposure of cells to LPS. In particular, culture with LPS significantly decreased AMPK activity in neutrophils, peritoneal macrophages, Raw 264.7 cells, and endothelial cells (33,34,45,63,64). The combination of LPS and saturated fatty acid palmitate also was shown to induce prolonged inactivation of AMPK in bone marrow macrophages (65). Our present results indicate that in spite of inhibitory action of LPS, inclusion of AICAR or metformin was able to partially increase AMPK phosphorylation, even when included in cultures after cellular exposure to LPS (Figure 3) (45).

Bacterial dissemination leading to multiorgan injury contributes to the high mortality rate associated with sepsis (10,11,19). Whereas many patients survive the initial stages of sepsis, many will develop later clinical complications including nosocomial infections that lead to prolonged hospitalization. In spite of improved understanding of the mechanisms responsible for complications in sepsis, pharmacological approaches to prevent secondary infection and decrease morbidity and mortality associated with late infection have not been well characterized (57). Metformin is approved for use in patients with diabetes with a well-established safety profile and known side effects and, for these reasons, may be considered for examination as a therapeutic approach in clinical trials of patients with sepsis. Although the early use of antibiotics is beneficial during sepsis, microbial products, as well as host-derived danger associated molecular pattern molecules (DAMPs) are still frequently present in the circulation and are likely to contribute to organ system dysfunction (66,67). For example, release of HMGB1, histones and mitochondrial proteins are known to increase the sever-

ity of acute inflammatory conditions and intensify the immunosuppressed state characteristic of late sepsis (68–71). Of note, AMPK activators, including metformin, were shown to diminish acute inflammatory injury of lung or liver as well as decrease the release of DAMPs and improve mortality in experimental models of LPS-induced sepsis (5,29,36). Although antibiotics are sufficient to kill bacteria, the combination of metformin and antibiotics may have additional benefit in sepsis, particularly as metformin-stimulated neutrophils have increased chemotaxis and bacterial uptake.

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DISCLOSURE

The authors declare that they have no competing interests as defined by Molecular Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.

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