Mitochondria and AMP-activated Protein Kinase-dependent Mechanism of Efferocytosis*

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Background: Billions of cells undergo apoptosis in the human body every day, and the removal of dying cells (efferocytosis) is essential for tissue homeostasis.

Results: Energy demand during efferocytosis results in AMPK activation followed by enhancement of macrophage chemokinesis and efferocytosis.

Conclusion: The AMPK pathway is intimately linked to cellular bioenergetics and efferocytosis.

Significance: A novel mechanism of mitochondria and AMPK-dependent stimulation of efferocytosis is proposed.

Defective clearance of apoptotic cells is frequently associated with perpetuation of inflammatory conditions. Our results show a rapid activation of AMP-activated kinase (AMPK) in macrophages upon exposure to apoptotic cells or lysophosphatidylcholine, a specific phospholipid that is produced and released from dying cells. AMPK activation resulted from inhibition of mitochondrial oxygen consumption and ATP production and further depended on Ca2+ mobilization and mitochondrial reactive oxygen species generation. Once activated, AMPK increased microtubule synthesis and chemokinesis and provided adaptation to energy demand during tracking and engulfment. Uptake of apoptotic cells was increased in lungs of mice that received lysophosphatidylcholine. Furthermore, inhibition of AMPK diminished clearance of apoptotic thymocytes in vitro and in dexamethasone-treated mice. Taken together, we conclude that the mitochondrial AMPK axis is a sensor and enhancer of tracking and removal of apoptotic cell, processes crucial to resolution of inflammatory conditions and a return to tissue homeostasis.

The effective clearance of dying cells by phagocytes, a process known as efferocytosis, plays an essential role in tissue homeostasis. The neutralization of apoptotic cells in a rapid and efficient manner is especially important during the resolution of inflammatory conditions (1–3). Early detection of apoptotic neutrophils prevents secondary necrosis, cytolysis, and release of many inflammatory mediators that are responsible for aggravated inflammation and increased severity of organ injury (3, 4). Impairment of efferocytosis is associated with unfavorable outcomes in severe infection, burns, hemorrhage, acute lung injury, or rheumatic arthritis (5–8). The ability of macrophages to track (“find me”) and subsequently engulf (“eat me”) apoptotic cells have an equally important contribution in phagocytosis (1–3). There are several specialized receptors, such as phosphatidylserine on apoptotic cells or TAM family (MER or Tyro3) receptors on phagocytes that are responsible for binding to apoptotic cells. In addition, bridging molecules, including MFG-E8 or GAS6, facilitate docking of apoptotic cells to macrophages (9). However, less is known about the regulatory mechanisms that affect the ability of macrophages to find apoptotic cells. For example, activation of the G-coupled receptors G2A, P2Y2, S1P-R, or CX3R have recently been proposed to initiate the migration of macrophages toward dying cells (3). In particular, the release of ATP and UTP or CX3CL1 from apoptotic cells were shown to activate macrophage chemotaxis in a P2Y2- or CX3R-dependent manner (10). Another attractant is lysophosphatidylcholine (lyso-PC),2 a bioactive phospholipid that is produced and released from apoptotic cells that acts as a find me signal through activation of the macrophage G2A receptor (11). A beneficial action of lyso-PC was reported in a mouse model of sepsis (12). However, little is known about how lyso-PC affects intracellular signaling pathways associated with the tracking and uptake of apoptotic cells.

AMP-activated protein kinase is an important sensor of energy status and regulator of metabolism at the cellular and organism level. AMPK is a heterotrimeric enzyme consisting of two regulatory β and γ and one catalytic α subunit. AMPK activation typically takes place upon increased bioavailability of cellular AMP, situations frequently associated with stress conditions, such as a restricted supply of oxygen or nutrients (13–15). The first step in AMPK activation is the binding of AMP to the α regulatory subunit followed by α, β, and γ conforma-

2 The abbreviations used are: Lyso-PC, lysophosphatidylcholine; AMPK, AMP-activated protein kinase; ROS, reactive oxygen species; mAsV, mitochondrial membrane potential; NAC, N-acetyl cysteine; BAPTA-AM, 1,2-bis[2-aminophenoxy]-ethane-N,N,N’,N’-tetraacetic acid acetoxymethyl ester; TFFA, thenoyltrifluoroacetone; OCR, O2 consumption rate; ACC, acetyl-CoA carboxylase; BALs, bronchoalveolar lavages.
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tional changes from the open to the closed configuration (16–18). In the second step, such conformational changes make the α subunit available for Thr-172 phosphorylation by upstream kinases, including liver kinase B1 (LKB) and calmodulin-dependent protein kinase kinase (CaMKK) (17). Although subsequent binding of AMP to the γ subunit and phosphorylation of the α catalytic domain activate AMPK (18), other signaling events, such as glycogen binding to the AMPKβ subunit or direct oxidation of specific cysteine residues within the α subunit, can increase AMPK activity (19, 20). Upon activation, AMPK affects lipid synthesis, glucose uptake, protein synthesis, and other metabolic pathways to promote ATP synthesis or to limit energy expenditure (13, 18).

Although the beneficial effects of activated AMPK are mediated by the regulation of lipid and glucose metabolism, recent studies highlight the importance of AMPK in the inflammatory setting (21–25). For example, the AMPK activators metformin or 5-amino-4-imidazolecarboxamide ribonucleotide decrease neutrophil or macrophage proinflammatory action and diminish the severity of endotoxin-induced organ injury in the lungs and liver (22, 26). Recent studies have also shown that AMPK promotes migration of T cells and increases the phagocytic ability of neutrophils as well as macrophages (23, 27). Cytoskeletal reorganization induced by AMPK-dependent effects on actin and tubulin dynamics has been proposed recently as a relevant mechanism for the enhancement of cell migration and efferocytosis. For example, AMPK-mediated phosphorylation of CLIP-170 increases the rate of microtubule formation in epithelial cells and in macrophages (23, 28). Interestingly, the regulation of the AMPK pathway is intimately linked to cellular bioenergetics, and it has also been shown that lyso-PC-dependent cell signaling involves the production of mitochondrial ROS (29). On the basis of these data, we hypothesized that mitochondrial function plays a key role in the signaling pathways controlling efferocytosis.

Both lyso-PC and AMPK have been shown recently to enhance phagocytosis and also improve the outcomes in inflammatory conditions. In this study, we investigated the potential roles of lyso-PC, AMPK, and mitochondrial function in regulating macrophage chemotaxis and neutralization of apoptotic cells.

EXPERIMENTAL PROCEDURES

Mice—Male C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). Male mice, 8–10 weeks 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—Lyso-PC 18:0 and 16:0 were purchased from Sigma-Aldrich (St. Louis, MO). Compound C was purchased from Millipore (Billerica, MA). siRNA to the AMPKα1 subunit, scrambled siRNA, and Accel medium were purchased from Thermo Scientific/Dharmacon (St. Louis, MO). Anti-G2A antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for phospho-Thr-172-AMPK, total AMPK, phospho-Ser-79-ACC, and total ACC were from Cell Signaling Technology (Danvers, MA). Antibodies to AMPKα1 and AMPKα2 were obtained from R&D Systems (Minneapolis, MN). β-Actin antibody was from Santa Cruz Biotechnology. α-tubulin antibody and PKH26 were from Sigma-Aldrich. FITC-conjugated anti-CD11b was from BD Biosciences. N-acetyl cysteine (NAC), 1,2-bis(2-aminophenoxy)-ethane-N,N,N’,N’-tetraacetic acid acetoxy-methyl ester (BAPTA-AM), rotenone, antimycin A, and thionyltrifluoroacetone (TTFA) were purchased from Sigma-Aldrich. Emulsion oil solution containing DAPI was from Vector Laboratories (Burlingame, CA), and Hoechst dye and JC-1 were from Invitrogen. HEMA3 was from Fisher Scientific (Kalamazoo, MI).

Purification and Culture of Peritoneal Macrophages—Peritoneal macrophages were collected 4 days after intraperitoneal injection of 4% Brewer thioglycollate and seeded on plates in RPMI 1640 medium. After 2 h, the plates were washed with serum-free RPMI 1640 medium to remove non-adherent cells. Macrophages were further cultured in RPMI 1640 medium (FBS 5%) and used for experiments within 5 days.

siRNA Knockdown of AMPKα1—Macrophages were incubated with specific siRNA (1 μM) to AMPKα1, as described previously (23). Briefly, cells (5 × 10⁶/well) in 12-well plates were incubated in Accell medium (serum-free) containing scrambled siRNA (1 μM) or to AMPKα1 for 72 h. Cells were then subjected to AMPK Western blot analysis or trans-well migration assay.

Measurement of Cellular Bioenergetics—The bioenergetics of macrophages was determined using the XF24 analyzer from Seahorse Bioscience, which measures O₂ consumption and proton production (pH) in intact cells, as performed previously (30). In particular, the O₂ consumption rate (OCR) is correlated with oxidative phosphorylation, and proton production (extracellular acidification rate) can be related to glycolysis. Measurements were performed using macrophages (2 × 10⁵) that were plated on XF24 plates, after which they were treated with the compounds of interest. The plate was then washed with XF assay buffer (DMEM, 5% FBS supplemented with 5.5 mM, D-glucose, 4 mM L-glutamine, and 1 mM pyruvate (pH 7.4)) and incubated in XF buffer for 30–60 min before the assay. After the assay, the cells were lysed with radioimmune precipitation assay buffer, and protein concentration was determined by DC Lowry assay. All results were corrected to protein levels in individual wells.

Trans-well Migration Assay—A trans-well migration assay was performed using 24-well cell plate BD Falcon™ cell culture inserts with 8.0-μm pore size of the membrane matrix (translucent polyethylene terephthalate membrane, BD Biosciences). Briefly, peritoneal macrophages or Raw 264.7 cells (10⁶ cells/well) in 300 μl of DMEM (8% FBS) were added to the upper chamber, whereas lyso-PC (0 or 50 μM) in culture medium was placed in the lower reservoir. The migration assay was conducted for 2.5 or 5 h. Next, cells were scraped off from the upper side of the membrane, whereas cells remaining on the lower membrane surface were stained with HEMA3 and then subjected to microscopy. The cell number was determined by a blinded observer by counting for eight to 10 fields on the lower side of the membrane. Bright light images were acquired using
a Leica microscope (magnification ×20, Leica Microsystems Inc.). In selected experiments, cells were preincubated with compound C (20 μM), NAC (20 mM), or anti-G2A antibody (5 mg/ml) for 30 min before inclusion of the cells into the upper chamber.

Detection of Intracellular ROS Formation—The intracellular level of superoxide generation by macrophages was measured using mitoSOX or a dichlorofluorescein-diacetate probe in conjunction with confocal microscopy, as described previously (29, 31). In selected experiments, mitoSOX fluorescence was also determined using flow cytometry (32).

Western Blot Analysis—Western blot analysis was performed as described previously (20, 22, 34). Each experiment was carried out two or more times using peritoneal macrophages obtained from separate groups of mice.

Measurement of Mitochondrial Membrane Potential (mΔΨ)—Peritoneal macrophages were seeded in a 4-well chambered coverslip until 70–80% confluent. The cells were treated with lyso-PC (0 or 50 μM) for 60 min. JC-1 dye (10 μM) in RPMI medium was added to the cell culture for 60 min. Microscopy was performed using a confocal laser-scanning microscope (model LSM 710 confocal microscope, Carl Zeiss MicroImaging, Jena, Germany). Images and three-dimensional graphs that show fluorescent intensity were processed using IPlab Spectrum. In additional experiments, mitochondrial membrane potential was also measured using the JC-1 probe in conjunction with flow cytometry (35).

Imaging Tubulin in Macrophages—Cells were subsequently incubated with 4% paraformaldehyde in PBS for 30 min at room temperature, washed with PBS, and permeabilized with 1% Triton X-100/PBS for 4 min. Next, cells were incubated with 3% BSA in PBS for 1 h, followed by the addition of α-tubulin-specific antibody for 60 min and then fluorescent anti-mouse antibody (Alexa Fluor 555) for an additional 60 min at room temperature. After rinsing with PBS, cells were mounted with emulsion oil solution containing DAPI to visualize the nuclei. Microscopy was performed using a confocal laser-scanning microscope (model LSM 710 confocal microscope, Carl Zeiss MicroImaging) provided by the high-resolution imaging facility at the University of Alabama at Birmingham.

In Vitro Scratch Assay—An in vitro scratch assay was performed as described previously, with minor modifications (36). Isolated peritoneal macrophages were cultured in DMEM (5% FBS) and plates (60 mm) at 4 × 10⁶ cells/well to allow for high-density cell adhesion. After 2 h of incubation, floating cells were removed, and adherent cells were cultured for an additional 24 h. The cell monolayer (~85% confluent) was scratched with a thin pipette tip, and then images were acquired using a phase-contrast microscope (magnification ×5, Leica Microsystems Inc.).

Measurement of Cell Velocity—Macrophage motility was determined using video microscopy as described previously (28). In brief, peritoneal macrophages were cultured in coverglass-bottomed wells that were placed in a humid, CO₂- and temperature-controlled chamber (Pathology Devices, Inc., Westminster, MD). Images were acquired using time-lapse, phase-contrast microscopy (magnification ×10, Nikon Eclipse Ti). The subsequent images were taken with 10-min intervals for a total of 1.5 h and after inclusion of compound C (20 μM) for an additional 1.5 h. Cell velocity was determined using NIS Element software (Nikon Instruments Inc., Melville, NY). A series of time-lapse images were converted to video format.

Thymocyte Isolation and Induction of Apoptosis—Apoptotic thymocytes are widely used as source cell to model efferocytosis (10, 37, 38). Thymocyte isolation from the thymus and induction of apoptosis were performed as described previously (39, 40). In brief, apoptosis in thymocytes was induced by treating the cells with dexamethasone (1 μM) for 16 h. Cells were then washed three times with RPMI 1640 medium to remove dexamethasone. Annexin/propidium iodide staining and flow cytometry showed that ≥ 90% of the thymocytes were apoptotic.

In Vitro Efferocytosis Assay—Peritoneal macrophages were pretreated with or without compound C (20 μM) for 30 min, and in vitro efferocytosis assays were then performed as described previously (41). Briefly, 10⁶ apoptotic thymocytes suspended in RPMI medium containing 5% FBS were cocultured with 2.5 × 10⁵ macrophages on glass coverslips for the times indicated in the figure legends. Next, coverslips were washed three times with ice-cold PBS, and cells were stained with HEMA3. Phagocytosis was evaluated by two observers, including a blinded observer who counted five to six randomly selected fields per slide. The phagocytosis index was calculated as the percentage of macrophages containing at least one engulfed thymocyte. In selected experiments, real-time efferocytosis was performed after the addition of apoptotic thymocytes to cultures of peritoneal macrophages. Bright light images were acquired within 5-min intervals using a Leica microscope (magnification ×20, Leica Microsystems Inc.).

Clearance of Apoptotic Cells in the Thymus—Cell clearance in the thymus was performed as described previously, with some modifications (42). Briefly, mice (9 weeks old) were injected intraperitoneally with compound C (0 or 5 mg/kg body weight in 500 μl of PBS) (43), and, after 2 h, mice received dexamethasone (10 mg/kg intraperitoneally) or vehicle (control, 300 μl of PBS, intraperitoneally). Thymuses were dissected 6 h after dexamethasone injection. To determine the percentage of apoptotic cells, the isolated thymocytes were stained with annexin V and propidium iodide (annexin V-FITC apoptosis detection kit, Calbiochem), and then subjected to flow cytometry.

Statistical Analysis—Statistical significance was determined by the Wilcoxon rank sum test (independent two-group Mann-Whitney U test) as well as Student’s t test for comparisons between two groups. Multigroup comparisons were performed using one-way analysis of variance with Tukey’s post hoc test. p < 0.05 was considered significant. Analyses were performed on SPSS version 16.0 (IBM, Armonk, NY) for Windows (Microsoft Corp., Redmond, WA).

RESULTS

Lyso-PC Induces Rapid Activation of AMPK in Macrophages—In the first series of experiments, RAW 264.7 cells were dose- and time-dependently treated with lyso-PC followed by Western blot analysis for total and phospho-Thr-172 AMPK as well as total and phospho-Ser-79 acetyl-CoA carboxylase, an AMPK downstream target (18). As shown in Fig. 1, A and B, inclusion of lyso-PC resulted in an increased and persistent phosphory-
AMPK and ACC. Similar to the results obtained with RAW 264.7 cells, exposure of mouse peritoneal macrophages to lyso-PC increased phosphorylation of AMPK (Fig. 1C). Fatty acids containing 16 or 18 carbons are the major bioactive forms of lyso-PC (12, 29). However, exposure to lyso-PC (18:0) but not lyso-PC (16:0) resulted in activation of AMPK (Fig. 1D).

**AMPK Inhibition Diminishes Migration of Macrophages toward a Lyso-PC Concentration Gradient**—To further characterize the relationship between lyso-PC and AMPK, we examined the ability of lyso-PC to affect macrophage chemotaxis using a transmigration system in which peritoneal macrophages or RAW 264.7 cells were loaded into the upper chamber, whereas a lyso-PC attractant was placed in the lower chamber. As shown in Fig. 2, A and B, representative Western blot analyses and quantitative analysis showing the amount of phospho-Thr-172-AMPK and total AMPK or phospho-Ser-79-ACC and ACC or β-actin obtained from Raw 264.7 cells after dose- (A) or time-dependent (B) treatment with lyso-PC 18:0. Data are mean ± S.D. (n = 3). *, p < 0.05; **, p < 0.01 compared with untreated cells. C, peritoneal macrophages were treated with lyso-PC 18:0 (0, 5, 10, 20, 30, 40, or 50 μM) for 90 min, followed by Western blot analysis of AMPK and ACC phosphorylation. D, AMPK and ACC phosphorylation status was determined in Raw 264.7 cells after exposure to lyso-PC 16:0 (0, 10, 30, or 50 μM) for 90 min. Representative Western blot analyses and quantitative data are shown. Data are mean ± S.D. (n = 3). p = not significant.

**FIGURE 1.** Lyso-PC induces rapid activation of AMPK. A and B, representative Western blot analyses and quantitative analysis showing the amount of phospho-Thr-172-AMPK and total AMPK or phospho-Ser-79-ACC and ACC or β-actin obtained from Raw 264.7 cells after dose- (A) or time-dependent (B) treatment with lyso-PC 18:0. Data are mean ± S.D. (n = 3). *, p < 0.05; **, p < 0.01 compared with untreated cells. C, peritoneal macrophages were treated with lyso-PC 18:0 (0, 5, 10, 20, 30, 40, or 50 μM) for 90 min, followed by Western blot analysis of AMPK and ACC phosphorylation. D, AMPK and ACC phosphorylation status was determined in Raw 264.7 cells after exposure to lyso-PC 16:0 (0, 10, 30, or 50 μM) for 90 min. Representative Western blot analyses and quantitative data are shown. Data are mean ± S.D. (n = 3). p = not significant.

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AMPK Activation Is Associated with Enhanced Tubulin Formation and Macrophage Motility—The lyso-PC-dependent activation of AMPK and stimulation of chemotaxis was likely associated with cytoskeletal rearrangement, including enhancement of AMPK-mediated tubulin assembly (23, 45). As shown in Fig. 3A, representative images demonstrate an enhanced tubulin network formation after exposure of macrophages to lyso-PC when compared with untreated (control) cells. Such a lyso-PC-mediated enhancement of tubulin assembly was diminished when cells were preincubated with the AMPK inhibitor com-
pound C. These results suggest that AMPK activation plays an important role in lyso-PC-mediated tubulin assembly and, therefore, affects cell motility (chemokinesis). To distinguish between the role of AMPK in enhancing macrophage directional (chemotaxis) from non-directional movement (chemokinesis), a scratch assay was performed (28, 36). As shown in Fig. 3B, pretreatment with compound C (20 μM) for 30 min markedly inhibited migration of macrophages across the gap. In additional experiments, time-lapse microscopy was performed to determine free cell movement (chemokinesis) before and after inclusion of compound C. The macrophage motility was significantly diminished upon exposure to compound C. In particular, a reduced level of migration is shown in Fig. 3C, whereas the average macrophage velocity is demonstrated in D and is inhibited by inclusion of AMPK inhibitor. It is important to note that the macrophages are differentiated at this stage and that proliferation of the cells is not contributing to the closure of the scratch. These results indicate that AMPK plays a key role in regulating lyso-PC-induced tubulin synthesis and macrophage chemokinesis.

Lyso-PC-induced AMPK Activation Is Independent from Stimulation of G2A Receptor—Lyso-PC has been shown previously to induce chemotaxis through activation of the G protein-coupled receptor G2A (11, 46). Consistent with previous studies, culture of macrophages with anti-G2A (blocking) antibody diminished lyso-PC-mediated chemotaxis (Fig. 4A). However, as shown in Fig. 4, B and C, despite inclusion of a G2A blocking antibody, lyso-PC was able to activate AMPK. These results suggest that, although stimulation of the G2A receptor was essential for the directional movement of macrophages, the mechanism of AMPK activation by lyso-PC was G2A-independent.
Effects of Lyso-PC on Cellular Bioenergetics and Glycolysis in Macrophages—Although AMPK activation was independent from stimulation of G2A receptor by lyso-PC, another possibility is that AMPK activation was a result of alteration in cellular metabolism. To determine whether lyso-PC affects cellular bioenergetics, the Seahorse Extracellular Flux analyzer was used to measure oxygen consumption rates as well as glycolysis (30). Peritoneal macrophages were treated with or without lyso-PC (50 μM) for 60 min, and then the OCRs were monitored over time and after application of oligomycin (1 μg/ml), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (0.5 μM), and antimycin A (10 μM). As shown in Fig. 5, A and B, lyso-PC significantly decreased macrophage basal oxygen consumption, ATP-linked OCR, proton leakage, and the maximal respiration rate (A and B). Although these data show that lyso-PC significantly diminished basal and maximal cellular respiration, the mitochondrial electron transport chain was not damaged because the uncouplers stimulated OCR to a similar extent from the basal measurement, resulting in the same level of bioenergetic reserve capacity in control and lyso-PC treated macrophages. In the same experiment, the extracellular acidification rate was also measured, which is related to glycolysis (30). As shown in Fig. 5C, treatment of macrophages with lyso-PC significantly increased glycolysis under conditions where basal respiration was suppressed. These data are consistent with an effect of lyso-PC on the ability of
Although activation of AMPK can result from an increased AMP/ATP ratio, recent studies also revealed a role for mitochondria, including the ability of mitochondrially derived hydrogen peroxide to activate AMPK (20). To test this possibility, cells were loaded with mitoSOX, a mitochondria-targeted dihydroethidium probe that is associated with increased mitochondrial superoxide formation. As shown in Fig. 6A, an increase in the level of mitoSOX fluorescence was found in lyso-PC-treated peritoneal macrophages. Additional results obtained from experiments with flow cytometry showed that mitochondria to couple ATP synthesis to oxygen consumption (47).
lyso-PC dose-dependently increased the level of mitoSOX fluorescence (Fig. 6D). A lyso-PC-induced increase in macrophage ROS formation was also found by using 2,7-dichlorodihydrofluorescein diacetate, a compound that is oxidized to DCF green fluorescence upon ROS formation, including hydrogen peroxide. As shown in Fig. 6B, culture of macrophages with lyso-PC (50 μM) resulted in an increased level of DCF fluorescence to a similar level found with rotenone (5 μM).

Next, the ability of lyso-PC to alter mitochondrial function was determined regarding mitochondrial membrane potential. Macrophages were incubated with a JC-1 probe followed by treatment with or without lyso-PC. The JC-1 monomer fluoresces green and accumulates in the mitochondria in response to the membrane potential to form red fluorescent JC-1 aggregates. As shown in Fig. 6, C and E, exposure of peritoneal macrophages to lyso-PC resulted in a significant decrease in the JC1 red/green fluorescence ratio, indicative of a decreased mitochondrial membrane potential.

Next, we determined whether lyso-PC-dependent activation of AMPK was mediated by redox-dependent changes. As shown in Fig. 7A, pretreatment with NAC (20 mM), which suppresses thiol-dependent redox signaling pathways, for 30 min diminished AMPK activation by lyso-PC. Previous studies conducted in endothelial cells have shown that, in addition to ROS scavengers, lyso-PC-dependent ROS formation dissipates upon inclusion of BAPTA-AM, a cytosolic Ca²⁺ chelator (29). Indeed, pretreatment with BAPTA-AM (20 μM) for 30 min completely diminished the ability of lyso-PC to activate AMPK (Fig. 7B).

To further explore the mechanisms of AMPK activation, Raw 264.7 macrophages were treated with inhibitors targeting the mitochondrial electron transport chain complexes. Although inhibitors of complex I or III (rotenone or antimycin A) did not inhibit lyso-PC-dependent stimulation of AMPK phosphoryla-
tion (data not shown), inclusion of the complex II inhibitor TTFA resulted in a diminished lyso-PC-mediated activation of AMPK and macrophage chemotaxis (Fig. 7, E and F). To determine whether the effect of the mitochondrial inhibitors were dependent on inhibition of electron transport, basal OCR was determined at the concentrations used to assess the effects on AMPK. Of note, basal oxygen consumptions were diminished in lyso-PC- or rotenone- but not TTFA-treated macrophages (Fig. 7G). These results suggest that mitochondrial complex II was involved in the activation of AMPK after exposure to lyso-PC.

AMPK Activation Enhances Uptake of Apoptotic Cells by Macrophages In Vitro and Clearance of Apoptotic Thymocytes In Vivo—Recent studies indicate that AMPK activation is associated with cytoskeletal rearrangement, followed by enhancement of cell mobility and phagocytosis (23, 27, 28). As shown in Fig. 8A, direct exposure of peritoneal macrophages to apoptotic thymocytes (including culture medium taken from apoptotic thymocytes) resulted in an increased level of phospho-AMPK and phospho-ACC. In turn, the culture of macrophages with viable cells had no effect on AMPK activation (Fig. 8A).

To further confirm the role of AMPK in the engulfment of apoptotic cells, peritoneal macrophages were treated with or without compound C (20 μM) for 30 min, followed by inclusion of apoptotic thymocytes. As shown in Fig. 8, B and C, there was a time-dependent uptake of apoptotic cells by macrophages. In particular, about 8% of the macrophages were positive for engulfment of apoptotic cells after incubation for 15 min, whereas nearly 50% were found to be positive after exposure for 90 min. Importantly, the phagocytic ability of macrophages was diminished after treatment with compound C (Figs. 8, B and C). Similar to the effects of lyso-PC on mitochondrial function (Fig. 5), exposure of peritoneal macrophages to apoptotic thymocytes resulted in a decreased maximal oxygen consumption rate (Fig. 8D).

To determine whether AMPK contributes to the clearance of apoptotic cells in vivo, we utilized a method described previously to induce apoptosis of thymocytes after administration of dexamethasone (42) with or without compound C (43). Of note, clearance of apoptotic thymocytes is a widely used method to model efferocytosis (10, 37, 38). As shown in Fig. 9, A and B, application of dexamethasone (10 mg/kg body weight, intraperitoneally) caused a reduction in the size of the thymus along
with a decrease in thymus cellularity and an increase in the percentage of apoptotic cells. Interestingly, injection of compound C (5 mg/kg) 2 h prior to dexamethasone resulted in an enlarged thymus (Fig. 9A), as evidenced by total cell number and a greater percentage of apoptotic cells (B) when compared with mice that received dexamethasone alone.

To determine whether the mechanisms outlined above occur in lungs, we examined the effects of lyso-PC on efferocytosis in mice exposed to BSA (control group) or lyso-PC at 18 h, followed by a second injection of BSA or lyso-PC 90 min prior to intratracheal administration of apoptotic thymocytes. To monitor efferocytosis, fluorescently labeled apoptotic thymocytes (10^7 in 70 μl of PBS) were instilled for 2 h followed by acquisition of BALs. As shown in Fig. 9, C and D, a significant increase in efferocytosis was found in macrophages obtained from BAL fluids of mice subjected to administration of apoptotic thymocytes and lyso-PC compared with BAL macrophages obtained from mice that received apoptotic thymocytes and BSA (D, left panel). Although administration of lyso-PC resulted in enhanced uptake of apoptotic cells, a similar number of macrophages was found in BALs of control or lyso-PC-treated mice (Fig. 9D, right panel).

DISCUSSION

In this study we have shown that lyso-PC-induced activation of AMPK and enhancement of the migratory ability of macrophages is dependent on mitochondrial function. In particular, we found that exposure of macrophages to lyso-PC promoted phosphorylation of AMPK and ACC, a downstream target of AMPK. Furthermore, our results demonstrate that pretreatment with the AMPK inhibitor compound C or a specific siRNA approach to knock down AMPK expression diminished

FIGURE 8. AMPK affects the clearance of apoptotic cells in vitro. A, apoptotic or viable thymocytes (4 × 10^6 cells/well, 300 μl) were incubated with peritoneal macrophages (10^6) for 0, 15, 30, 60, or 90 min. Next, cells were washed, and then the levels of p-AMPK, AMPK, p-ACC, ACC, and β-actin were determined using Western blot analysis. p-ACC/ACC ratio values are indicated below the representative Western blot analyses. A second experiment provided similar results. B, representative images show a time-dependent uptake of apoptotic thymocytes by peritoneal macrophages. Macrophages were incubated with compound C (0 or 20 μM) for 30 min prior to inclusion of apoptotic cells at the indicated time points. Dotted lines indicate macrophage perimeters. C, peritoneal macrophages were pretreated with compound C (0 or 20 μM) for 30 min, followed by inclusion of apoptotic thymocytes (10^6 cells/well, 300 μl) at the indicated time points. Phagocytic indices were determined using flow cytometry as described under “Experimental Procedures.” D, the OCRs were determined before and after incubation of peritoneal macrophages with apoptotic thymocytes.
the migration of macrophages toward lyso-PC find me signaling. Although lyso-PC is a naturally occurring bioactive lipid molecule thought to be important in the pathophysiology of atherosclerosis (48, 49), lyso-PC is also produced and released from dying cells (50, 51). In particular, during apoptosis, the calcium-independent phospholipase A2 (iPLA2) is activated and, in turn, hydrolyzes phosphatidylcholine to lyso-PC, which is subsequently externalized and released (51). In addition to lyso-PC, other lipids can activate AMPK, including lysophosphatidic acid, which has been shown recently to promote AMPK activity and the motility of ovarian cancer cells (52), or nitrated oleic acid (OA-NO2), which has been shown to activate AMPK in endothelial cells (53). Our study suggests that, although AMPK participates in lipid metabolism, specific lipids are also capable of modulating AMPK activity and that this, in turn, will likely affect tracking and engulfment of apoptotic cells.

In initial experiments, the application of a specific antibody to block the G2A receptor diminished lyso-PC-mediated chemotaxis. However, this approach did not prevent the ability of lyso-PC to activate AMPK (Fig. 4). Because inhibition of either AMPK or the G2A receptor results in inhibition of chemotaxis, there exists the possibility that two independent yet required signaling pathways are activated by lyso-PC during efferocytosis. Our results indicate that this alternative pathway involves mitochondrial retrograde signaling. In support of this, we found that lyso-PC inhibited mitochondrial respiration, ATP production, and enhanced ROS formation that was responsible for activation of AMPK (Fig. 10). Interestingly, direct exposure of macrophages to apoptotic cells decreased maximal respira-
nisms that involve an increased cellular AMP:ATP ratio and stimulation of regulatory AMPKα subunits or by direct oxidation of specific cysteine thiols in the AMPKα subunit (14, 15, 18, 20). Although recent studies have found that mitochondrial status can affect efferocytosis, our results revealed a new mechanistic link between lyso-PC find me signaling initiated by dying cells and mitochondrially induced AMPK activation in phagocytes. In particular, AMPK is known to enhance glycolysis as a response to cellular energy demand, and such an energy demand is associated with macrophage motility and engulfment of apoptotic cells.

Our new findings indicate that, in addition to engulfment (23), AMPK also facilitates efferocytosis through a novel mechanism that involves enhancement of chemokinesis (Fig. 10). Chemokinesis is an important component of cell non-directional motility that is associated with cytoskeletal rearrangement (54, 55). In particular, actin and tubulin dynamics allow for generation of pseudopodia, efficient motility, and formation of phagosomes, processes important for effective efferocytosis (56, 57). The effects of lyso-PC on AMPK activation and microtubule formation are consistent with the ability of activated AMPK to enhance tubulin and actin network formation in macrophages (23). AMPK activation has also been shown to enhance chemokinesis through mechanism that involves microtubule synthesis in epithelial cells (28), T cells, and ovarian cancer cells (52, 58). Taken together, our results evidence that AMPK-dependent enhancement of tubulin synthesis and chemokinesis is essential for lyso-PC-induced chemotaxis. Both chemotaxis (find me signaling) and uptake of apoptotic cells (eat me signaling) are equally important for efferocytosis.

More recent studies have indicated that mitochondria significantly contribute to the phagocytic ability of neutrophils and macrophages (12, 42). In particular, mitochondrial membrane potential ($\Delta\Psi$) has the ability to affect efferocytosis (42). For example, a decrease in $\Delta\Psi$ was found during engulfment of dying cells and interventions that decrease $\Delta\Psi$ improve clearance of apoptotic cells (42). A decrease in mitochondrial membrane potential was also found after exposure to lyso-PC, suggesting that a similar mechanism exists in macrophages. This would also be consistent with an increased Ca$^{2+}$ uptake by mitochondria and is supported by the inhibitory effects of BAPTA-AM on AMPK signaling. Of note, previous studies have shown that lyso-PC facilitated phagocytosis, particularly microbial eradication by neutrophils, including killing bacteria and improved survival of mice subjected to cecal ligation and puncture-induced sepsis (12).

Efficient neutralization of apoptotic cells during the early stage of cell death prevents secondary necrosis, cytolsis, and release of inflammatory mediators. For example, release of histones, high-mobility group box 1 (HMGB1) proteins, mitochondrial DNA and proteins (8), and other members of danger-associated molecular pattern molecules perpetuate inflammatory conditions and increase the severity of organ injury (59). The appearance of extracellular HMGB1 and histones has been shown recently to diminish efferocytosis (60). Our ongoing studies are testing whether lyso-PC can decrease the appearance of extracellular danger-associated molecular patterns.

AMPK is emerging as a major regulator linking inflammation and metabolism (21). Although AMPK is known to regulate glucose and lipid metabolism, recent studies revealed that activated AMPK also diminishes inflammatory conditions associated with severe infection- or hemorrhage-induced organ injury, including acute lung injury (22, 33). Our study provides important insight into a new mechanism through which specific phospholipids such as lyso-PC affect AMPK activity as well as the ability of AMPK to enhance the clearance of apoptotic cells in vivo. In addition to the known anti-inflammatory function of AMPK, its mitochondria-dependent activation appears to be a suitable target to improve recovery and lung repair, particularly because of the enhanced neutralization of dying cells.

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