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Constitutive ERK MAPK Activity Regulates Macrophage ATP Production and Mitochondrial Integrity

Martha M. Monick,* Linda S. Powers,* Christopher W. Barrett,* Sara Hinde,* Dayna J. Groskreutz,* Toru Nyunoya,* Mitchell Coleman,† Douglas R. Spitz,† and Gary W. Hunninghake*

A unique feature of human alveolar macrophages is their prolonged survival in the face of a stressful environment. We have shown previously that the ERK MAPK is constitutively active in these cells and is important in prolonging cell survival. This study examines the role of the ERK pathway in maintaining mitochondrial energy production. The data demonstrate that ATP levels in alveolar macrophages depend on intact mitochondria and optimal functioning of the electron transport chain. Significant levels of MEK and ERK localize to the mitochondria and inhibition of ERK activity induces an early and profound depletion in cellular ATP coincident with a loss of mitochondrial transmembrane potential. The effect of ERK suppression on ATP levels was specific, since it did not occur with PI3K/Akt, p38, or JNK suppression. ERK inhibition led to cytosolic release of mitochondrial proteins and caspase activation. Both ERK inhibition and mitochondrial blockers induced loss of plasma membrane permeability and cell death. The cell death induced by ERK inhibition had hallmarks of both apoptotic (caspase activation) and necrotic (ATP loss) cell death. By blocking ERK inhibition-induced reactive oxygen species, caspase activation was prevented, although necrotic pathways continued to induce cell death. This suggests that mitochondrial dysfunction caused by ERK inhibition generates both apoptotic and necrotic cell death-inducing pathways. As a composite, these data demonstrate a novel mitochondrial role for ERK in maintaining mitochondrial membrane potential and ATP production in human alveolar macrophages. The Journal of Immunology, 2008, 180: 7485–7496.

Human alveolar macrophages survive for long periods in the lung (1). Survival occurs even in the face of exposure to chemical pollutants, reactive oxygen species (ROS),3 inflammatory mediators, and infectious agents (2). This ability to adapt to stress is crucial to their survival. We have shown that human alveolar macrophages have high constitutive activity of two survival pathways, PI3K/protein kinase B (Akt) and ERK MAPK (3, 4). The survival effects of Akt are well described and include inhibition of BH3-only proapoptotic proteins (5, 6), stabilization of antiapoptotic X-linked inhibitor of apoptosis (7), inactivation of protease activity of HtrA2/Omi (8), inactivation of caspase 9, and inactivation of transcription factor FoxOa (9, 10). The survival mechanisms of ERK activity are less well described and appear to be cell type and system specific. Although ERK has been linked to apoptosis in a few cases (11) in the majority of studies, ERK activity is linked to cell survival (3, 12–16).

ERK is a member of the MAPK family of serine/threonine kinases. It is activated by phosphorylation of a tyrosine × threonine motif by the upstream kinases, MEK1 and 2, and in turn phosphorylates downstream substrates containing a consensus proline-directed motif (PX(S/T)P) (17, 18). Among known ERK substrates are a number of proapoptotic proteins that are inhibited by ERK phosphorylation (caspase 9 and BimEL) (19, 20). ERK has also been shown to contribute to cell survival via other mechanisms (13–15, 21–28). We have recently shown that one mechanism of ERK-dependent alveolar macrophage survival is positive regulation of protein translation via an effect on the eukaryotic translation initiation factor eIF2α (4). It is likely that ERK maintains alveolar macrophage survival via a number of synergistic mechanisms.

ERK is a ubiquitously expressed kinase with more than 160 substrates identified to date (29). ERK has been shown to phosphorylate transcription factors, other kinases, phosphatases, cytoskeletal and scaffold proteins, receptors, and apoptosis-related proteins (29). These substrates are found all over the cell (nucleus, cytosol, and organelles). Some recent articles have localized a subset of ERK to the mitochondria (30–33). The studies demonstrating mitochondrial ERK were performed in neuronal cells and cardiomyocytes. For the cardiomyocytes, Baines et al. (33) found that in the mitochondria, ERK complexes with protein kinase Cε and plays a role in protection from ischemia reperfusion. In neuronal cells, Alonso et al. (34) found that ERK1 and 2 are present in brain mitochondria at the outer membrane/intermembrane space. Zhu et al. (35) found active ERK associated with mitochondrial proteins (e.g., manganese superoxide dismutase) and used ultrastructure immunogold studies to demonstrate the presence of active ERK in mitochondria from midbrain sections of patients with Parkinson’s disease and diffuse Lewy body disease. These studies found ERK

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3 Abbreviations used in this paper: ROS, reactive oxygen species; ETC, electron transport chain; mHMP, mitochondrial membrane potential; CCCP, carbonyl cyanide m-chlorophenylhydrazone; PVDF, polyvinylidene difluoride; PARP, poly(ADP-ribose) polymerase; VDAC, voltage-dependent anion channel; AIF, apoptosis-inducing factor; LDH, lactate dehydrogenase; GSH, reduced glutathione; GSSH, oxidized GSH; NAC, N-acetylcysteine; PDC, pyruvate dehydrogenase complex; EthD-1, ethidium homodimer.

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Mitochondria perform multiple functions in the cells (36, 37). In addition to generating energy by recycling ADP to ATP via oxidative phosphorylation, mitochondria break down sugars and long-chain fatty acids, synthesize steroids and lipids, replicate, transcribe, and translate proteins from mitochondrial DNA, produce ROS, and integrate survival/death signals (36, 37). ATP production relies on the electron transport chain (ETC) and ATP synthase. Movement of electrons down the ETC results in an electrochemical gradient (measured as mitochondrial membrane potential (mitΔΨ)). The pumping of protons from the intermembrane space to the matrix through the ATP synthase unit generates ATP from ADP, providing energy for the cell (37). In this study, the role of ERK in mitochondrial ATP production was examined.

The study was performed in human alveolar macrophages obtained from normal volunteers. We found that inhibition of constitutive ERK activity (with dominant negative mutations of the upstream kinase MEK, cell-permeable inhibitory peptides, and chemical inhibitors) led to a rapid loss of mitΔΨ and a decrease in ATP levels. These events were an early response to ERK inhibition and were followed at later time points by cytosolic release of mitochondrial proteins, caspase activation, rupture of the plasma membrane, and cell death. The apoptotic/necrotic nature of the cell death was at least partially dependent on a requirement for ROS for the caspase activation. These data suggest the existence of a previously unknown mitochondria localized prosurvival role for ERK MAPK in human alveolar macrophages.

Materials and Methods

Materials

Chemicals including antinymycin A (A8674), N-acetylcysteine (NAC; A8199), and rotenone (R8875) were obtained from Sigma-Aldrich. The MEK inhibitor UO126 (662005), p38 inhibitor SB203580 (559389), JNK inhibitor II (SP600125 420191), ERK activation inhibitor peptide I (328000), oligomycin (495455), and carbonyl cyanide m-chlorophenylhydrazone (CCCP; 215911) were obtained from Calbiochem. The dominant negative MEK adenoviral vector (mutated at serine 217 and serine 221) was obtained from Cell Biolabs. Ethidium homodimer (EtH-1; E1169) was obtained from Molecular Probes. JC-1 was obtained from Guava Technologies. Western blotting reagents include phosphatase inhibitor mixture (524625; Calbiochem), complete mini-tub protease inhibitors (1183610001; Roche Diagnostics), and ECL (RPN2106) and ECL Plus (RPN2132) chemiluminescent detection reagents (Amersham Biosciences). Acrylamide (161-0158), buffers (161-0798 and 161-0799), polyvinylidene difluoride (PVDF) membranes (162-0174), and the Bradford protein assay from Bio-Rad. Abs used in this study were obtained from Santa Cruz Biotechnology. JC-1 was obtained from Guava Technologies. Western blotting reagents include phosphatase inhibitor mixture (524625; Calbiochem), complete mini-tub protease inhibitors (1183610001; Roche Diagnostics), and ECL (RPN2106) and ECL Plus (RPN2132) chemiluminescent detection reagents (Amersham Biosciences). Acrylamide (161-0158), buffers (161-0798 and 161-0799), polyvinylidene difluoride (PVDF) membranes (162-0174), and the Bradford protein assay reagent (500-0006) were obtained from Bio-Rad. Abs used in this study were obtained from a variety of sources. Phosphorylation-specific ERK Ab (9101), cytochrome c (4280), cleaved caspase 7 (9491), cleaved caspase 9 (9509), and cleaved poly(ADP-ribose) polymerase (PARP; 9541) Abs were obtained from Cell Signaling. Voltage-dependent anion channel (VDAC; sc-8828), Apoptosis-inducing factor (AIF; sc-5586), α-tubulin (sc-5568), and HRP-conjugated Abs anti-rabbit (sc-2004), anti-mouse (sc-2005), and anti-goat (sc-2020) were all obtained from Santa Cruz Biotechnology. β-Actin (A5316) Ab was obtained from Sigma-Aldrich. Culture medium used in experiments was serum-free RPMI 1640 medium (61870-036) from Invitrogen. Kits used in this study included a Cell Titer-Glo Luminescent Cell Viability Assay (G7571) from Promega and a BD Biosciences Oxygen Biosensor System.

Isolation of human alveolar macrophages

Alveolar macrophages were obtained from normal nonsmoking volunteers, as previously described (38). Briefly, normal volunteers with a lifetime nonsmoking history, no acute or chronic illness, and no current medications underwent bronchoalveolar lavage. The cell pellet was washed twice in irrigation saline and suspended in complete RPMI 1640 medium with Glutamax (Invitrogen) and added gentamicin (80 μg/ml). Cells were cultured in special nonadherent plates from Costar (3471 and 3472) to minimize the effect of adherence on signaling. All experiments were conducted in serum-free conditions. Differential cell counts were determined using a Wright-Giemsa-stained cytocentrifuge preparation. All cell preparations had between 90 and 100% alveolar macrophages. This study was approved by the Committee for Investigations Involving Human Subjects at the University of Iowa.

Fibroblast and epithelial cell cultures

Primary human lung fibroblasts (C-12360; Promo Cell) were cultured at 37°C and in DMEM with 10% FBS, 1% sodium pyruvate, 1% t-glutamine, 40 μg/ml gentamicin, and 25 μg/ml Fungizone (complete medium). The complete medium was changed every 2 days and the cells were subcultured every 4–5 days. The normal fibroblasts were used between the third and eighth passages. For primary human tracheobronchial epithelial cells, all protocols were approved by the University of Iowa Institutional Review Board. Human tracheobronchial epithelial cells were obtained as previously described (39). Epithelial cells were isolated from tracheal and bronchial mucosa by enzymatic dissociation and cultured in laboratory of carcinogenesis-8e medium on plates coated with collagen/albumin for study up to passage 10.

JC-1 stain

JC-1 is a cationic dye that accumulates in mitochondria with intact mitΔΨ. Fluorescence shifts within the mitochondria from green (590 nm) to red (525 nm). For these experiments, alveolar macrophages were seeded onto two chamber Titrertek microscope slides at 1 million cells/ml. Experimental groups were incubated with U0126 (20 μM), ERK inhibitory peptide (50 μM), or infected with dnMEK Ad vector (multiplicity of infection, 10–100). After various incubation times, live cells were stained with JC-1 (3 μM). The cells with stain were incubated for 30 min at 37°C and then evaluated by fluorescence microscopy or, in some cases, by confocal microscopy (University of Iowa Microscopy Facility). Photomicrographs were obtained showing orange/red intact mitochondria or diffuse green staining in cells with depolarized mitochondria. For quantitation, alveolar macrophages were seeded in 96-well plates at 30,000 cells/well. After incubation with ERK inhibitors, the cells were stained with JC-1 (30 min at 37°C) and then fluorescence read in a Tecan Safire Fluorescent plate reader. Red aggregates were recorded with an excitation of 485 nm and an emission of 590 nm. Green monomers were red with an excitation of 485 nm and an emission of 535 nm. Data are shown as arbitrary aggregate (red) fluorescent units (average ± SE) of three separate experiments.

Whole cell protein isolation

Whole cell protein was obtained by lysing the cells on ice for 20 min in 200 μl of lysis buffer of 0.05 M Tris (pH 7.4), 0.15 M NaCl, 1% Nonidet P-40, with added protease and phosphatase inhibitors: 1 protease miniblot (Roche Biochemicals)/10 ml and 100 μl of 100× phosphatase inhibitor mixture (Calbiochem)/10 ml. The lysates were sonicated for 20 s, kept at 4°C for 30 min, spun at 15,000 × g for 10 min, and the supernatant was saved. Protein determinations were made using the Bradford protein assay from Bio-Rad. Cell lysates were stored at −70°C until use.

Mitochondria isolation

Following experimental incubations, alveolar macrophages were pelleted in ice-cold conditions (all steps of the mitochondrial isolation were performed on ice or in refrigerated centrifuges). Cells were resuspended in cold homogenizing buffer with added protease inhibitors (0.25 M sucrose, 0.2 mM EDTA, and 10 mM Tris-HCl) and homogenized 20–40 times with a cold Dounce homogenizer. Unlysed cells and nuclei were removed by centrifuging the sample at 1000 × g (3000 rpm) in a refrigerated table top microfuge. The supernatant containing the mitochondria was centrifuged at 10,000 × g (12,000 rpm) for 10 min at 4°C. The supernatant from this spin was removed and saved as the cytosolic portion. The pellet containing the mitochondria was resuspended in protein lysis buffer (see Whole cell protein isolation) and sonicated for 10 s (mitochondrial fraction). Debris from the mitochondrial fraction was removed with a 5-min spin at 12,000 × g (14,000 rpm). Protein measurements were performed using the Bradford protein assay from Bio-Rad.

Western blot analysis

Western blot analysis for the presence of particular proteins or for phosphorylated forms of proteins was performed as previously described (40). Briefly, 30 μg of protein was mixed 1:1 with 2× sample buffer (20% glycerol, 4% SDS, 1% 2-ME, 0.05% bromphenol blue, and 1.25 M Tris (pH 6.8)) and loaded onto a 10 or 12% SDS-PAGE gel and run at 110 V for 2 h. Cells were transferred to PVDF membranes with a Bio-Rad semidy transfer system according to the manufacturer’s instructions.
Equal loading of the protein groups on the blots was evaluated using Ponceau S (Sigma-Aldrich), a staining solution designed for staining total proteins on PVDF membranes. The PVDF was then blocked with 5% milk in TBST for 1 h, washed, and then incubated with the primary Ab at dilutions of 1/500 to 1/20,000. Immunoreactive bands were developed using a chemiluminescent substrate, ECL Plus or ECL (Amersham Biosciences). An autoradiograph was obtained with exposure times of 10 s to 2 min. Protein bands were quantified using a FluorS scanner and Quantity One software for analysis (Bio-Rad). The data were analyzed and statistics performed using GraphPad software. Densitometry is expressed as fold increase (experimental value/control value).

Adenoviral vector infection
To infect alveolar macrophages with an adenoviral vector expressing with GFP or dominant negative MEK, cells were seeded onto 24-well plates (1 million cells/well) in RPMI 1640 medium with no FCS. A suspension of vector (AdGFP or Ad dnMEK; multiplicity of infection, 10) (10 million PFUs) was mixed with RPMI 1640 (167 µl) and Viralution Adenovirus Transduction Reagent (2 µl; Cell Biologics) and allowed to sit at room temperature for 10 min. The medium was removed from the alveolar macrophages and replaced with the transduction mixture (169 µl) and incubated at 37°C for 4 h. Without removing the virus, an additional 400 µl of RPMI 1640 was added and the culture continued. GFP expression was checked at 16–18 h and averaged 50–70%.

Cell survival analysis
For analysis of cell survival, alveolar macrophages were cultured in 6-well tissue culture plates for 6 h without preliminary incubation in a Tecnam Safire II plate reader. ATP was measured by bringing the plate to room temperature and fluorescence using a Leica DM2B microscope equipped with a Qimaging RETICA 1300 digital camera and imaging system. After obtaining images, the percentage of EthD-1–positive cells was determined. Quantification was by direct cell count. Two hundred cells were counted from a minimum of four different fields. In some cases, viability was assessed using trypan blue permeability. Cell samples were exposed to 20% trypan blue in medium and the percentage of dead cells was calculated. At least 300 cells were counted for each sample from a minimum of six fields. In other cases, plasma membrane integrity was assessed by examining release of lactate dehydrogenase (LDH). This was done using a kit from Promega (CytoTox-ONE) that measures LDH release via a coupled fluorescent assay. Experiments were performed according to the manufacturer’s instructions.

ATP assay
ATP levels were monitored using a CellTiter-Glo Luminescent Cell Viability Assay from Promega. Alveolar macrophages were cultured (1 million alveolar macrophages treated with U0126 (20 µM), ERK activation inhibitor peptide I (50 µM) for the described times. Most cultures were performed in standard RPMI Glutamax medium (11 mM glucose levels) with no added serum. Triplicate cultures were performed on all experiments. After the incubation period, the cells were stained with EthD-1 (Molecular Probes) at 8 µM and images were obtained of both bright-field and fluorescence using a Leica DM2B microscope equipped with a Qimaging RETICA 1300 digital camera and imaging system. After obtaining images, the percentage of EthD-1–positive cells was determined. Quantification was by direct cell count. Two hundred cells were counted from a minimum of four different fields. In some cases, viability was assessed using trypan blue permeability. Cell samples were exposed to 20% trypan blue in medium and the percentage of dead cells was calculated. At least 300 cells were counted for each sample from a minimum of six fields. In other cases, plasma membrane integrity was assessed by examining release of lactate dehydrogenase (LDH). This was done using a kit from Promega (CytoTox-ONE) that measures LDH release via a coupled fluorescent assay. Experiments were performed according to the manufacturer’s instructions.

Oxygen consumption
Oxygen consumption was measured using the BD Biosciences Oxygen Biosensor System. The empty Biosensor plate was initially read at ~485 nm excitation and ~630 nm emission in a Tecnam Safire II plate reader. Cells were seeded into the Biosensor plate at 50,000 per well. Cells were incubated for 0–120 min in the presence of different ERK inhibitors and mitochondrial inhibitors (U0126, 20 µM; ERK inhibitory peptide, 50 µM; CCO, 10 µM; and antimycin A, 10 µM). Incubation was in the Tecnam Safire plate reader set at 37°C. Fluorescent measurements were made every 15 min over a in the medium quenching the fluorescent signal over the time of the experiment (120 min).

Reduced glutathione (GSH)/oxidized GSH (GSSG) measurements
Cells were scraped into cold PBS and centrifuged at 4°C for 5 min at 400 × g to obtain cell pellets. The pellets were frozen at −80°C. Pellets were thawed and homogenized in 50 mM potassium phosphate buffer (pH 7.8) containing 1.34 mM diethylenetriaminepentaacetic acid. Total glutathione content was determined by the method of Spitz and colleagues (41). GSH and GSSG were distinguished by addition of 2 µl of a 1:1 mixture of 2-vinylpyridine and ethanol per 50 µl of sample followed by incubation for 1 h and assay as described previously by Griffith (42). All glutathione determinations were normalized to the protein content of whole homogenates using the method of Lowry et al. (43).

Transmission electron microscopy
Samples were fixed overnight with 2.5% glutaraldehyde in 0.1 M cacodylate buffer. Postfixation was conducted for 1 h at room temperature with a buffered 1% osmium tetroxide solution reduced with 1.5% potassium ferrocyanide. Samples were en bloc stained with 2.5% uranyl acetate. Cells were then rinsed and dehydrated using gradually increasing concentrations of acetone to 100%. Infiltration of Spurr’s epoxy resin and acetone were conducted over several days to 100% resin and cured overnight in a 70°C oven. Sections of 100-nm thickness were cut using an Ultratome E ultramicrotome (Reichert-Jung). Grids were then counterstained with 5% uranyl acetate for 12 min and Reynolds’ lead citrate for 5 min. Samples were imaged using a Hitachi H-7000 transmission electron microscope.

Phagocytosis assay
To evaluate bacterial phagocytosis by alveolar macrophages, cells were cultured in chamber slides (Lab Tek 4 chamber slides) for 2 h with and without treatments and then exposed to GFP-tagged Escherichia coli at a ratio of 25 bacteria/1 cell. Cells and bacteria were incubated for an additional 30 min. Nonphagocytosed cells were washed off by vigorously washing with PBS six times. Images were obtained using an inverted fluorescent microscope (Zeiss) and then counts of bacteria per cell were performed on random fields (50 cells/group). In some cases, adherent but not phagocytosed bacteria were killed with gentamicin and then the remaining bacteria were quantified using bacterial plate counts. The data obtained from these studies were not different from those obtained using fluorescent analysis (data not shown).

Results
Alveolar macrophages depend on mitochondria and the electron transport change for ATP production
Results extending back almost a century have suggested that both macrophages and neutrophils depend on cytosolic glycolysis for the generation of ATP (44–47). This includes macrophages found at sites of inflammation or wound repair that often depend on anaerobic glycolysis for ATP production (44, 45, 48). To determine the source of ATP in human alveolar macrophages, we cultured newly isolated alveolar macrophages with and without a number of inhibitors of mitochondrial ATP production. Oligomycin is an inhibitor of the ATP synthase subunit (F1)F (0) (49). Rotenone inhibits complex I of the ETC (leading to generation of ROS) (50, 51). CCCP is an uncoupler that disperses the proton gradient that drives ATP synthase without interfering directly with the ETC (52). Alveolar macrophages were treated with oligomycin (0.5 µM), rotenone (2.5 µM), and CCCP (10 µM). Combined ATP levels from both intracellular and extracellular sources were measured with a chemiluminescence reagent at various time points. ATP levels rapidly disappeared with all three exposures (Fig. 1A). These data demonstrate that ATP levels in alveolar macrophages are maintained via an intact mitochondria and functioning oxidative phosphorylation system (ETC and ATP synthase). In Fig. 1B, we show, using transmission electron microscopy, that at baseline alveolar macrophages have multiple mitochondria.

Disruption of ATP production leads to death of alveolar macrophages
Loss of ATP occurs under conditions of stress and if not reversed can lead to cell death. To investigate the effect of blocking the ETC

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on alveolar macrophage survival, alveolar macrophages were treated with ETC blockers (rotenone (complex I inhibitor), CCCP (uncoupler), and oligomycin (ATP synthase inhibition)) and cell viability was evaluated using EthD-1 staining and trypan blue exclusion. Fig. 1C demonstrates that loss of mitochondrial-derived ATP leads to death of the alveolar macrophages.

Alveolar macrophage mitochondria contain members of the ERK MAPK family at baseline

A few studies in neuronal cells have found ERK within the mitochondria, both in the intramembrane space and in the matrix. To search for ERK and the upstream kinase MEK in alveolar macrophage mitochondria, we isolated mitochondria from newly harvested alveolar macrophages and used Western blot analysis of mitochondrial protein. Fig. 2A demonstrates partial mitochondrial localization of both MEK and ERK. VDAC and cytochrome c were also examined to control for the efficacy of the mitochondrial protein isolation. We next asked whether the ERK present in alveolar macrophage mitochondria was active. Fig. 2B shows that in alveolar macrophages from multiple donors (n = 3), active ERK (phosphorylated on both threonine 183 and tyrosine 185) is found in proteins from the mitochondrial fraction. Blots were reprobed for VDAC (mitochondrial marker) or α-tubulin (cytosolic marker) to verify protein fractions. The studies performed in this project rely on a number of ERK inhibitors, a dominant negative adeno-

viral MEK construct, inhibitory peptides that block the binding of ERK to MEK and U0126, and a specific chemical inhibitor of the activity of the upstream kinase MEK. To establish that we were really inhibiting the mitochondrial-localized ERK, we treated alveolar macrophages with U0126 for 1 h. Fig. 2C demonstrates that by 1 h of U0126 exposure both cytosolic and mitochondrial-localized ERK activity was significantly depressed. As a composite, these data demonstrate that without inflammatory stimuli both ERK and active ERK are localized to the mitochondria and that MEK inhibition (U0126) decreases the mitochondrial-localized ERK activity. The next question was whether ERK activity played a role in production of ATP in alveolar macrophages.

ERK inhibition depletes ATP from alveolar macrophages

To study the effect of ERK inhibition on alveolar macrophage ATP levels, alveolar macrophages were cultured in medium without serum at 1 million cells/ml in 96-well tissue culture plates. First, we infected alveolar macrophages with an adenovirus vector expressing dominant negative MEK (Ad dnMEK). MEK is activated by phosphorylation of dual serines (serines 217 and 221). In the dominant negative mutant, both serines were mutated to alanine (A217/A221). The control cells were infected with an adenovirus vector expressing enhanced GFP. Ad dnMEK decreased ATP in alveolar
FIGURE 2. Protein isolations demonstrate mitochondrial localization of both ERK and MEK. A, Human alveolar macrophages have ERK and MEK protein in the mitochondria. Human alveolar macrophages collected by bronchoscopy were lysed and mitochondrial and cytosolic fractions were isolated. Western blot analysis was performed for the ERK (ERK 2 is the majority ERK species in human alveolar macrophages) and MEK. Mitochondrial isolation was controlled by staining for the mitochondrial proteins VDAC and cytochrome c. B, Active ERK (phosphorylated on Thr183 and Tyr187) is present in alveolar macrophage mitochondria. On the left is Western blot analysis of mitochondrial and cytosolic proteins for active ERK, VDAC (mitochondria control), and α-tubulin (cytosol control). On the right is composite densitometry from three samples. Significance at p < 0.01 was determined using a nonpaired t test. The data demonstrate constitutive ERK activity in mitochondria from human alveolar macrophages. C, U0126 inhibits active ERK in alveolar macrophage mitochondria. Human alveolar macrophages were placed in culture with and without U0126 (20 μM) for 1 h. Following incubation with U0126, mitochondrial proteins were isolated and Western blot analysis for active ERK was performed. The Western blot was probed for active ERK (phosphorylated on Thr183/Tyr185), VDAC (mitochondrial marker), α-tubulin (cytosolic marker), and Ponceau S for total protein levels. This is representative of three separate experiments.
FIGURE 3. ERK inhibition decreases ATP specifically in alveolar macrophages. A. Human alveolar macrophages were cultured (1 × 10^6/ml in 96-well tissue culture plates) at time 0, the cells were infected with Ad dnMEK as described in Materials and Methods. Control cells were infected with an adenovirus vector expressing GFP. At 6 h after infection, cells were lysed and total ATP levels were analyzed as described. The data represent three separate experiments. Significance was determined using a nonpaired t test. B. Human alveolar macrophages were cultured (1 × 10^6/ml in 96-well tissue culture plates) with and without a peptide ERK inhibitor (50 μM). At 3, 6, and 24 h after inhibition, cells were lysed and total ATP levels were analyzed as described. The data represent three separate experiments. Significance was determined using nonpaired t tests. C. Human alveolar macrophages were cultured (1 × 10^6/ml in 96-well tissue culture plates) with and without the MEK inhibitor U0126 (20 μM). At 3, 6, and 24 h after inhibition, cells were lysed and total ATP levels were analyzed as described. The data represent three separate experiments. Significance was determined using nonpaired t tests. D. Primary human tracheobronchial cells and primary human fibroblasts were grown in 96-well plates and then exposed to the ERK/MEK inhibitor U0126 (20 μM) for various times. ATP was measured as described and is presented as arbitrary bioluminescence numbers. The data represent three experiments.

macrophages at 6 h (Fig. 3A). Next, we examined two other methods of ERK inhibition at multiple time points. We used a membrane-permeable ERK inhibitory peptide (Ste-MEK113;Ste-MP KKKPTPI QLNP-NH2) that binds to ERK and prevents binding to MEK. The ERK inhibitory peptide decreased alveolar macrophage ATP in a time-dependent manner (Fig. 3B). Of note, by 3 h after treatment of cells with the inhibitory peptide, there was a significant decrease in ATP levels. ERK was also inhibited with U0126 and total ATP levels in the cell cultures were measured at various time points. Fig. 3C demonstrates that inhibition of ERK depletes alveolar macrophage ATP levels in a time-dependent manner. As a composite, these data demonstrate that inhibition of ERK rapidly depletes alveolar macrophage ATP in a time-dependent manner.

To determine whether the mitochondrial effect of ERK inhibition was unique to alveolar macrophages or also occurred in other cell types, we investigated primary fibroblasts and airway epithelial cells. Both cell types were cultured in 96-well tissue culture plates at 80% confluence. They were treated with and without U0126 or infected with the dominant negative MEK vector as described in Materials and Methods. Five and 24 h later, ATP levels were measured. Fig. 3D demonstrates that there was a minor decrease in ATP levels with the U compound at 24 h in the human tracheobronchial epithelial cells, but no change in the ATP levels of the primary lung fibroblasts. These data suggest that the significant ATP depletion with ERK inhibition may be a unique characteristic of alveolar macrophages. A definitive answer would require a more comprehensive survey of other cell types.

ATP depletion is specific to ERK inhibition

Other MAPKs (p38 and JNK) and the PI3K effector Akt have also been localized to the mitochondria (53–56). To see whether the rapid decrease in ATP with inhibition was specific to ERK, alveolar macrophages were treated with inhibitors for JNK (SP600125, 20 μM), p38 (SB203580, 10 μM), and PI3K/Akt (LY294002, 20 μM). Fig. 4 demonstrates decreased ATP levels after ERK inhibition but not after inhibition of p38, JNK, or PI3K/Akt.

ERK inhibition results in a rapid loss of mitΔΨ in human alveolar macrophages

As electrons move down the ETC, positive hydrogen atoms are pumped from the matrix into the intermembrane space. This creates a concentration gradient between the two spaces. It is the flow of protons back into the matrix through the ATP synthase unit that drives ATP production. Our initial ATP depletion data suggested that ERK inhibition affects ATP production as early as 1 h after exposure. We next asked whether changes in mitΔΨ happened within the same time frame. mitΔΨ can be measured using the cationic dye JC-1. In intact mitochondria with a healthy mitΔΨ, JC-1 aggregates in the mitochondria and fluoresces red (∼590 nm). With loss of membrane potential, the dye disaggregates, is seen in the cytosol, and fluoresces green (∼525 nm). We stained alveolar macrophages after exposure to U0126 for various times (as with the ATP assays, control cells were treated with equal amounts of DMSO). JC-1 staining was assessed using fluorescent
microscopy and quantified using a 96-well plate format and fluorescent plate reader. Fig. 5A (fluorescent images) shows that in the U0126-treated samples, there was a significant (although not complete) decrease in red staining and increase in green staining by 1 h. By 6 h, most of the red punctate staining was gone and by 24 h.

**FIGURE 4.** Inhibition of PI3K and other MAPKs does not decrease ATP in human alveolar macrophages. Human alveolar macrophages were cultured (1 × 10^6/ml in 96-well tissue culture plates) with and without various inhibitors: MEK inhibitor U0126 (20 μM), PI3K inhibitor LY294002 (20 μM), p38 inhibitor SB203580 (10 μM), and JNK inhibitor SP600125 (20 μM). Cells were cultured for 24 h and then lysed and ATP levels were determined as described. The data represent three separate experiments. Significance was determined using a non-paired t test.

**FIGURE 5.** ERK inhibition causes loss of mit∆Ψ in human alveolar macrophages. A, Human alveolar macrophages were cultured (1 × 10^6/ml in 6-well tissue culture plates or in two-chamber microscope slides) with and without U0126 (20 μM) or staurosporine (1 μM). At the end of the incubation period, the mitochondrial stain JC-1 was added as described in Materials and Methods. Cultures were incubated an additional 30 min and then examined with fluorescence microscopy. Photomicrographs were obtained. Red/orange stain denotes intact mitochondria with no disruption of membrane potential. Green staining denotes loss of mit∆Ψ. B, Identical experiments were performed in 96-well tissue culture plates and the red/orange stain was quantified using a fluorescence plate reader. The graph represents arbitrary units at an excitation of 535 nm and an emission of 590 nm.

**FIGURE 6.** ERK inhibition decreases oxygen consumption in human alveolar macrophages. Human alveolar macrophages were cultured (1 × 10^6/ml in 96-well BD Biosciences Oxygen Biosensor plate) with and without the MEK inhibitor U0126 (20 μM), an ERK inhibitory peptide (50 μM), CCCP (uncoupler; 10 μM), or antimycin A (complex III inhibitor; 10 μM). Fluorescent readings were taken every 15 min. The data are shown as arbitrary fluorescent units. Increased fluorescence denotes increased oxygen consumption (see Materials and Methods).
FIGURE 7. ERK inhibition induces both apoptotic and necrotic pathways. A, ERK inhibition leads to alveolar macrophage death. Human alveolar macrophages were cultured (1 × 10⁶/ml in 6-well tissue culture plates) with or without U0126 (20 μM) for 3, 6, or 24 h. At the end of each time point, EthD-1 entry was evaluated. Dead cells were counted by examining each cell for red nuclear staining. A total of 300 cells was examined for each data point. The data are graphed as percentage of cells that exclude EthD-1. The graph represents data from three separate experiments. Significance was determined using a nonpaired t test. B, ERK inhibition results in activation of necrotic pathways. Alveolar macrophages were cultured for 6 or 24 h (1 × 10⁶/ml in 6-well tissue culture plates) with or without U0126 (MEK inhibitor; 20 μM). The blots show mitochondrial and cytosolic protein fractions were analyzed (Western blot analysis) for cytochrome c. The 24-h blot was stained with VDAC as a control for the mitochondrial isolation. Also shown is transmission electron microscopy demonstrating loss of plasma membrane integrity after 24 h with an ERK inhibitory peptide (50 μM). Cells were fixed overnight with 2.5% glutaraldehyde in 0.1 M cacodylate buffer. They were then processed for transmission electron microscopy as described in Materials and Methods. C, ERK inhibition activates apoptotic pathways. Alveolar macrophages were cultured for 6 or 24 h (1 × 10⁶/ml in 6-well tissue culture plates) with or without U0126 (MEK inhibitor; 20 μM). The first blot shows mitochondrial and cytosolic protein fractions were analyzed (Western blot analysis) for cytochrome c. The other blots show total cell lysates stained for cleaved caspases 3, 7 and 9 and cleaved PARP. Equal loading was determined by staining identical blots for β-actin. D, ERK inhibition increases ROS in alveolar macrophages. Alveolar macrophages were cultured for 3 h (1 × 10⁶/ml in 6-well tissue culture plates) with or without U0126 (MEK inhibitor; 20 μM). Frozen cell pellets were used to measure GSH (nonoxidized GSH) and GSSG levels. Data are expressed as either total GSH or percentage GSSG (GSSG/GSH × 100). In addition, the effect of blocking oxidant increases with NAC (1 mM) on caspase activity evaluated by Western blot analysis of whole cell lysates at 6 and 24 h. E, Inhibition of caspase activity does not prevent ERK inhibition-induced loss of ATP and decreased survival. Human alveolar macrophages were cultured (1 × 10⁷/100 μl in 96-well tissue culture plates) with or without U0126 (20 μM) for 24 h. At the end of the incubation time, ATP levels were measured as described in Materials and Methods and LDH release (as described in Materials and Methods) was measured as a marker of plasma membrane permeability. NAC did not prevent either the loss of ATP or loss of plasma membrane integrity by ERK inhibition.
it was completely gone. As a positive control for mitochondrial dysfunction, staurosorine was used. Staurosorine rapidly converted punctate red staining to a diffuse green stain (3 h). The visual data obtained with fluorescent microscopy were confirmed with a 96-well quantitative assay (Fig. 5B). These data show that inhibition of ERK causes a rapid decrease in mitΔψ in alveolar macrophages.

**ERK inhibition decreases oxygen consumption by human alveolar macrophages**

Alterations in mitΔψ and ETC function can alter rates of oxygen consumption. Uncouplers (that disperse the proton gradient without blocking ETC function) increase rates of oxygen consumption. Blockers of complex I or III in the ETC decrease oxygen consumption by blocking the free flow of electrons through the ETC. We measured oxygen consumption using a BD Biosciences Oxygen Biosensor System. To examine the effect of ERK inhibition, alveolar macrophages were treated with nothing (control 1), DMSO to match the amount in the U0126 preparation (control 2), the ERK inhibitory peptide U0126, and the mitochondrial targeted inhibitors CCCP (uncoupler) and antymycin A (complex III blocker). Sulfite (11 mM) was used as a positive control and plain medium was used as a negative control. The entire plate was pre-blanked and then alveolar macrophages with preadded inhibitors were seeded into the BD Biosciences Biosensor plate. The plate was inserted into a 37°C fluorescence plate reader and readings were taken every 15 min for 2 h. The data are presented as arbitrary fluorescence units. Fig. 6 demonstrates that alveolar macrophage oxygen consumption is blocked by both the ERK inhibitory peptide and U0126. This is consistent with a block in the ETC. As expected, the complex III inhibitor antimycin A blocked oxygen consumption and the uncoupler CCCP increased oxygen consumption. These data, combined with the early disruption of mitΔψ, suggest that the role of ERK in mitochondrial homeostasis is consistent with disruption of the ETC.

**ERK inhibition induces macrophage death**

To assess the effect of ERK inhibition on cell viability, a number of markers of cell death were examined. Alveolar macrophages were first examined at 3, 6, and 24 h after ERK inhibition for disruption of the plasma membrane. Fig. 7A demonstrates that cell death, as quantified by a leaky plasma membrane (EthD-1 staining), occurs by 24 h after ERK inhibition. In the next set of experiments, we examined markers of necrosis (AIF release from the mitochondria and disruption of the plasma membrane) and apoptosis (caspases and cytochrome c release from the mitochondria). AIF has been identified as a key player in caspase-independent cell death (57). AIF is a biphasic NADH oxidase involved in both mitochondrial respiration and cell death. Recently, Moubarak et al. (57) have described AIF as an inducer of programmed necrosis and as a part of a novel signaling pathway that distinguishes necrosis from apoptosis. Fig. 7B demonstrates that there are markers of necrosis induced by ERK inhibition. The Western blot demonstrates loss of AIF from the mitochondria and the electron photomicrograph demonstrates disruption of the plasma membrane after exposure to the ERK inhibitory peptide. We then examined the effect of ERK inhibition on cytochrome c release, caspase activation, and PARP inactivation, all of which are linked to apoptosis (Fig. 7C). In the same time frame as the loss of mitochondrial contents to the cytosol (24 h), we found activation of caspases (caspases 3, 7, and 9) and cleavage of PARP (Fig. 7C). The time frame of both necrosis (AIF release) and apoptosis (caspase activation) markers suggests that ATP loss and loss of mitΔψ precedes opening of the mitochondrial outer membrane pore rather than the other way around.

To pursue the relative role of the apoptotic pathway in cell death after ERK inhibition, we asked whether preventing caspase activation would block the ERK inhibition-induced cell death. If that were true, it would suggest that this is primarily an apoptotic process that with time results in disruption of the plasma membrane. We found that ERK inhibition increased ROS in the alveolar macrophages as measured by alterations in GSH:GSSH ratios. Non-oxidant-stressed cells have a surplus of GSH compared with the GSSG. A decrease in the ratio is indicative of increased ROS in the cells (58, 59). Treating alveolar macrophages with U0126 for 3 h induced a significant decrease in glutathione levels (control: 2.77 ± 0.87 compared with U0126: 1.30 ± 0.30) and an increase in the percentage of GSSG compared with GSH (Fig. 7D). When the increase in ROS was prevented with exogenous N-acetylcysteine (NAC), caspase activation by ERK inhibition was completely prevented (Fig. 7D). However, the lack of caspase activity did not protect the cells from ATP loss or plasma membrane rupture (as measured by LDH release) (Fig. 7E). We conclude that ERK inhibition induces two cell death pathways, one dependent on ROS and caspase activity and one dependent on ATP depletion and plasma membrane disruption. The balance between the two will be at least partially determined by the antioxidant status of alveolar macrophages. It is possible that individual variability in antioxidant status among alveolar macrophage donors can alter the balance between apoptotic and necrotic outcomes of ERK inhibition in alveolar macrophages.

**ERK inhibition decreases alveolar macrophage phagocytosis of GFP E. coli**

To evaluate the role of ERK in the innate immune function of alveolar macrophages, we evaluated the effect of ERK inhibition on phagocytosis. Phagocytosis is a high-energy using process that
could be impacted by the loss of ATP found with ERK inhibition. Using a GFP-tagged *E. coli.*, cells were treated with U0126 for 2 h (partial ATP depletion without the cell death that comes closer to 24 h after ERK inhibition) and then exposed to GFP-tagged *E. coli.* for 30 min. After washing off nonphagocytosed cells, fluorescent images were acquired and bacteria/cell counts obtained. The data shown in Fig. 8 demonstrate that ERK inhibition decreases bacte-
rial uptake by alveolar macrophages. As well as this newly de-
scribed link between ERK and mitochondria, the ERK MAPK has many other described biological effects. It is possible that the decrease in phagocytosis with ERK inhibition may be only a partial consequence of the loss of ATP. Other ERK-dependent processes may also play a role. However, considering the high energy needs of the phagocytic process, we believe that the ERK-related mito-
chondrial dysfunction plays an important role. The phagocytosis data suggest that the role of ERK in mitochondrial homeostasis may play an important part in the immune function of alveolar macrophages.

**Discussion**

The MAPK ERK has many described functions. The majority of these functions are linked to increases in ERK activity subsequent to a stimulus (such as LPS or increased oxidants). We have been interested in the role of both stimulated and baselines levels of ERK in alveolar macrophage homeostasis. Earlier studies by our group (3, 4) identified significant levels of baseline ERK activity in human alveolar macrophages. This ERK activity was part of a significant prosurvival pathway. In one study (3), we linked up-
stream regulation of baseline ERK activity to novel sphingolipid pathways. In a second study (4), we linked cytosolic ERK activity to translation regulation via an effect on eIF2α (regulator of trans-
lation initiation). In this study, we identify ERK and its upstream kinase, MEK1/2, as mitochondrial-localized proteins in human al-
veolar macrophages. We found that ATP levels in alveolar mac-
rophages depended on mitochondrial respiration. Inhibition of ERK decreased ATP levels as early as 1 h postinhibition with significant depletion by 3 h. The ATP depletion was not due to an effect on cytosolic glycolysis as it could not be reversed by exog-
enous addition of pyruvate (data not shown). ERK inhibition caused a rapid decrease in mitΔψ and oxygen consumption, sug-
gest a role for ERK in ETC function.

By 24 h of ERK inhibition, there was substantial caspase activ-
ity and loss of plasma membrane integrity. The effects of ERK inhibition on survival, as demonstrated by this study, can be di-
vided into two phases: 1) early (loss of ATP and mitΔψ) and 2) late (permeability of the mitochondrial outer membrane, caspase activation, and loss of plasma membrane integrity). We found that both necrotic and apoptotic events were induced by ERK inhibition and that blocking caspase activation did not protect the cells from the necrotic component of the response. We conclude that in alveolar macrophages the loss of constitutive ERK activity results in cell death due to a combination of loss of ATP, release of mitochon-
drial proteins, activation of caspases, and loss of plasma membrane integrity (Fig. 9).

Our cultures are conducted in serum-free and nonadherent con-
ditions to most closely mimic the alveolar space. We have per-
formed some of these same experiments in adherent conditions and
found that the ATP decrease was similar. What is increased by adherence is the inflammation-induced activation of ERK (i.e., by LPS). We do not know at this time whether the upstream signals that regulate constitutive ERK activity are the same as the up-
stream signals that activate ERK during an inflammatory response. We are interested in addressing this question.

Reversible phosphorylation as a means of regulating mitochon-
drial function is an emerging topic of investigation. It is beyond the scope of this article to identify the mitochondrial site of ERK phos-
phorylation; however, there are a number of candidates. One known site of regulatory phosphorylation is the pyruvate dehy-
donase complex (PDC) in the mitochondrial matrix. The PDC catalyzes the conversion of pyruvate to acetyl CoA and is made up of a number of subunits. Searching the PDC subunits for potential proline-directed serine or threonines using Scan-
site (http://scansite.mit.edu/), we found that the E2 subunit has a possible ERK phosphorylation site at threonine 322. Another known site of regulatory phosphorylation is the ATP synthase unit. Two subunits of the F1 catalytic subunit of ATP synthase, δ and β, also have possible proline-directed sites. ATP synthase δ has two potential sites, threonines 280 and 290. In addition, there is an ERK-binding domain in the protein. ATP synthase β also contains two potential ERK phosphorylation sites at threonines 641 and 651 and a potential ERK docking site. Although inhibition of ATP synthase would generate the drop in ATP seen in our data, the block in oxygen consumption with ERK inhibition argues against this as a site of ERK regulation in alveolar macrophages.

It is of note that the only article to link ERK activity to ATP de-
pletion is a study examining ATP synthase activity. The study by Yung et al. (60) examined the effect of ERK inhibition in as-
trocytes. They found that ERK inhibition induced cell death via a decrease in F1F0 ATP synthase activity. Their data did not find a direct link between ERK and one of the ATP synthase subunits (60) and they concluded only that ERK inhibition might modulate ATP synthase function. These are only a few of many protein possibilities for the mitochondrial ERK target. The human mito-
chondrial database (http://bioinfo.nist.gov/hmmp/index.html) lists 1465 mitochondrial proteins, including both nuclear and mito-
chondrial-encoded proteins. We are at this time examining the database for possible novel ERK substrates that would explain the findings of this study. It is also possible that ERK does not alter ATP production by directly phosphorylating a mitochondrial protein but rather by phosphorylation of a regulatory intermediate. Identifica-

![Diagram of the study findings. In human alveolar macrophages, a decrease in ERK activity leads to loss of ATP and mitΔψ. This results in release of mitochondrial proteins to the cytosol, caspase activation, and cell death. Blocking caspase activation by preventing an increase in ROS does not prevent cell death.](http://www.jimmunol.org/DownloadedFrom)}
ERK inhibition-linked release of mitochondrial proteins, caspase activation, and cell death is totally the result of ATP depletion. It is more likely that the early ATP depletion and loss of mitΔψ synergize with other ERK-dependent changes to effect the subsequent caspase activation and cell death. As stated above, we have shown that ERK inhibition leads to a decrease in translation, which would affect the generation of antiapoptotic proteins like the IAPs (61). Another possible effector mechanism that could synergize would affect the generation of antiapoptotic proteins like the IAPs shown that ERK inhibition leads to a decrease in translation, which would affect the generation of antiapoptotic proteins like the IAPs (61). Another possible effector mechanism that could synergize would affect the generation of antiapoptotic proteins like the IAPs shown in this study, in the mitochondria. Endogenous regulators of the constitutive ERK activity include redox status of the cells, exposure to exogenous stimuli such as cigarette smoke, and age of the alveolar macrophage (i.e., Is it a more monocyte-like cell?). We have shown that hyperoxia causes prolonged activation of the ERK pathway, leading to enhanced survival (24, 62). It is not known whether the effect of the prolonged ERK in hyperoxic conditions is due to a protective effect on mitochondria. It is an interesting hypothesis to pursue.

In this article, we examined a role for baseline ERK activity in mitochondrial homeostasis. As a composite, the data demonstrate that inhibition of ERK leads to disruption of mitΔψ, loss of ATP, and eventual death of alveolar macrophages. We have previously shown that baseline ERK activity also regulates protein translation (4). The mitochondrial effects combined with the effects on translation suggest that ERK activity is an important survival pathway in alveolar macrophages. Compared with the short life span of alveolar macrophage precursors (blood monocytes survive for $\sim$24 h), alveolar macrophages can live for extended periods in the lung (up to 1 year) (63). Thus, this newly described role for ERK in alveolar macrophage homeostasis may explain, in part, the survival characteristics of these cells.

**Disclosures**

The authors have no financial conflict of interest.

**References**


