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Active ERK Contributes to Protein Translation by Preventing JNK-Dependent Inhibition of Protein Phosphatase 1

Martha M. Monick,² Linda S. Powers, Thomas J. Gross, Dawn M. Flaherty, Christopher W. Barrett, and Gary W. Hunninghake

Human alveolar macrophages, central to immune responses in the lung, are unique in that they have an extended life span in contrast to precursor monocytes. We have shown previously that the ERK MAPK (ERK) pathway is constitutively active in human alveolar macrophages and contributes to the prolonged survival of these cells. We hypothesized that ERK maintains survival, in part, by positively regulating protein translation. In support of this hypothesis, we have found novel links among ERK, JNK, protein phosphatase 1 (PP1), and the eukaryotic initiation factor (eIF) 2α. eIF2α is active when hypophosphorylated and is essential for initiation of protein translation (delivery of initiator tRNA charged with methionine to the ribosome). Using [35S]methionine labeling, we found that ERK inhibition significantly decreased protein translation rates in alveolar macrophages. Decreased protein translation resulted from phosphorylation (and inactivation) of eIF2α. We found that ERK inhibition increased JNK activity. JNK in turn inactivated (via phosphorylation) PP1, the phosphatase responsible for maintaining the hypophosphorylated state of eIF2α. As a composite, our data demonstrate that in human alveolar macrophages, constitutive ERK activity positively regulates protein translation via the following novel pathway: active ERK inhibits JNK, leading to activation of PP1, eIF2α dephosphorylation, and translation initiation. This new role for ERK in alveolar macrophage homeostasis may help to explain the survival characteristic of these cells within their unique high oxygen and stress microenvironment. The Journal of Immunology, 2006, 177: 1636–1645.

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uman alveolar macrophages survive for long periods in the lung (1). Survival occurs even in the face of exposure to chemical pollutants, reactive oxygen species, inflammatory mediators, and infectious agents (2). This ability to adapt to stress is crucial to their survival. We have shown that human alveolar macrophages are characterized by high constitutive activity of two survival pathways, PI3K/protein kinase B (Akt) and ERK MAPK (3). The survival effects of Akt are well described and include inhibition of BH3-only proapoptotic proteins, caspases, and the transcription factor, FoxOa (4, 5). 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 (6), in the majority of studies, ERK activity is linked to cell survival, and the specific mechanisms of its survival activity are unknown (3, 7–11).

ERK is a member of the MAPK family of serine/threonine kinases. It is activated by phosphorylation of a tyrosine × threonine motif (TEY) and in turn phosphorylates downstream substrates containing a consensus proline-directed serine or threonine site (PX(S/T)P) (12, 13). Among known ERK substrates are a number of proapoptotic proteins that are inhibited by ERK activity (caspase 9 and BimEL) (14, 15). ERK has also been shown to contribute to cell survival via other mechanisms. In one case, ERK has been shown to regulate Hdm2 protein expression (Hdm2 inhibits p53 accumulation) by enhancing association of Hdm2 transcripts with polyribosomes (16). This effect of ERK on protein translation led to our present hypothesis that in human alveolar macrophages constitutive ERK activity positively regulates protein translation and survival.

Ongoing protein translation is important for the continued health of cells and is regulated at a number of points, including initiation, elongation, and termination. The primary rate-limiting step is initiation of translation, and this is regulated at multiple checkpoints (17). Stresses, including DNA damage, misfolded proteins, nutrient deprivation, and viral infection, induce a temporary shutdown of the translation machinery (17). In addition to the temporary shutdown of translation induced by stress, a prolonged shutdown of protein translation accompanies apoptosis generated by many stimuli (18, 19). The prolonged survival of alveolar macrophages suggests ongoing functional translation machinery.

The two main pathways leading to initiation of translation are the eukaryotic initiation factor (eIF)4E pathway that brings mRNAs with 5’ caps to the ribosome and the eIF2 pathway that is responsible for bringing the initiator methionyl-tRNA (Met-tRNA [met]) to the start site. ERK activity is already linked in some systems to translation initiation via a described effect on Mnk1 phosphorylation of eIF4E (20). In contrast to the described link between ERK and eIF4E, there is no described link between ERK

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2 Address correspondence and reprint requests to Dr. Martha M. Monick, Division of Pulmonary, Critical Care, and Occupational Medicine, Room 100, Eckstein Medical Research Building, Carver College of Medicine, University of Iowa, Iowa City, IA 52242. E-mail address: martha-monick@uiowa.edu

³ Abbreviations used in this paper: eIF, eukaryotic initiation factor; Met-tRNA [met], initiator methionyl-tRNA; Cdk, cyclin-dependent kinase; ER, endoplasmic reticulum; GCN2, general control nonderepressing 2 kinase; HRI, heme-regulated inhibitor kinase; MKP, MAPK phosphatase; PARP, poly(ADP-ribose) polymerase; PERK, protein kinase R-like ER kinase; PKR, protein kinase R; PP1, protein phosphatase 1; PVDF, polyvinylidene difluoride.
signaling and the eIF2 pathway (17, 21–23). In preliminary studies, we found no effect of ERK on the eIF4E pathway in human alveolar macrophages, but we did find that ERK activity modulated the phosphorylation state of eIF2α (dephosphorylation of serine 51 is required for activity).

Four kinases have been described that can phosphorylate serine 51 on eIF2α and decrease protein translation after stress (24). Protein kinase R (PKR) is activated by dimerization following exposure to dsRNA (25). Heme-regulated inhibitor kinase (HRI) is activated by low heme levels (26). PKR-like endoplasmic reticulum kinase (PERK) senses endoplasmic reticulum (ER) stress (27) and general control nonderepressing 2 kinase (GCN2) responds to amino acid deprivation (28). Activation of any of these kinases can lead to eIF2α phosphorylation on serine 51 and decreased protein translation. The exceptions to this block in translation are a few stress-related proteins whose translation actually goes up in the setting of eIF2α phosphorylation (17). As well as the described kinases, the phosphorylation and activity of eIF2α are also regulated by the activity of protein phosphatase 1α (PP1α) phosphatase (29). The balance between upstream kinase activity and PP1α phosphatase activity determines the translation outcome (21).

This study was performed in human alveolar macrophages obtained from normal volunteers. Alveolar macrophages are a distinct cell population with a unique environment. They are both similar to and distinct from other macrophage populations. We have recently shown differences in survival pathways between alveolar macrophages and their precursors, blood monocytes (3, 30).

In both studies, we found that alveolar macrophages have constitutive activity of ERK and the PI3K/Akt pathways that is not present in monocytes. The role of the baseline ERK activity is the focus of this study. There are other studies that define important differences between alveolar macrophages and other tissue macrophages, especially the studies by Curtis and colleagues (31) and Peters-Golden et al. (32–37). This study focuses on human alveolar macrophages and does not attempt to define which other macrophages exhibit the described phenotype. We consider this to be an important component of further studies.

In this study, we investigated the novel hypothesis that in unstimulated human alveolar macrophages, long-term viability is maintained via ERK-dependent effects on the phosphatase, PP1α, and protein translation. We demonstrate that inhibition of ERK results in decreased protein translation, JNK-dependent inactivation of PP1α, and phosphorylation of eIF2α. The end result of these events (downstream of ERK inhibition) is shortened survival of alveolar macrophages, due, at least in part, to decreased protein translation.

**Materials and Methods**

**Materials**

Chemicals were obtained from Sigma-Aldrich. The MEK inhibitor U0126 (662005), p38 inhibitor SB203580 (559389), JNK inhibitor II (SP600125 (420119)), PP1α inhibitor tautomycin (580551), ERK activation inhibitor peptide II (328005), autophosphoryation inhibitor (D)-arginine (114666), and recombinant rabbit muscle PP1α isofrom (539493) were obtained from Calbiochem. Recombinant human active JNK2α2 peptide (PHO3011) was purchased from BioSource International. Radionuclides [35S]methionine (NEG-709A) and [γ-32P]ATP Easitides (NEG-502Z) were purchased from PerkinElmer. Ethidium homodimer (E1169) was obtained from Molecular Probes. Western blotting reagents include Calbiochem’s phosphatase inhibitor mixture (524625), complete minitab protease inhibitors (11836170001) from Roche, ECL (RPN2106) and ECL⁺ (RPN2132) chemiluminescent detection reagents from Amersham Biosciences, and acrylamine (161-0158), buffers (161-0798 and 161-0799), polyvinylidene difluoride (PVDF) membranes (162-0174), and Bradford protein assay reagent (500-0006) from Bio-Rad. Abs used in this study were obtained from a variety of sources. Phosphorylation-specific Abs to eIF2α (9721), PP1α (2581), and ERK (9101) were obtained from Cell Signaling Technology, and JNK (559309) from Calbiochem. Cleaved caspase 7 (9401) and 9 (9509) and cleaved poly(ADP-ribose) polymerase (PARP) (9541) and eIF2α (nonphospho) (9722) Abs were also obtained from Cell Signaling Technology. PP1α (sc-6105), JNK2 (7345), PP1α agarose conjugate (sc-6105-AC), HRP-conjugated Abs anti-rabbit (sc-2004), anti-mouse (sc-2005), and anti-goat (sc-2020) were all obtained from Santa Cruz Biotechnology. We tried a number of MAPK phosphatase-7 (MKP-7) Abs, including ones from Abcam (ab15698), Imgenix (IMX-3042), and an Ab provided by M. Collins (Royal Veterinary College, UK). Phosphorylation-specific Abs used in this study were obtained from a variety of sources. Abs used in this study were obtained from a variety of sources.

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Human alveolar macrophages were cultured with an Ab from Calbiochem (MKP-7; PC726). β-Actin (A5316) was obtained from Sigma-Aldrich. Culture medium used in experiments was serum-free RPMI 1640 tissue culture medium plus Glutamax (Invitrogen Life Technologies) and added gentamicin (80 μg/ml). Cells were cultured in special nonadherent plates from Costar (3471, 3473) to minimize effect of adherence on signaling. 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.

**Methionine labeling**

Alveolar macrophages were put into culture in low adherent 100-mm plates (10⁶ cells/ml RPMI 1640 with glutamax and gentamicin). Actinomycin D (Calbiochem) was added to prevent any new transcription of proteins. After a time period for isolation recovery, the cells were transferred to methionine-free RPMI 1640 (Invitrogen Life Technologies) and added gentamicin (80 μg/ml). Cells were cultured in special nonadherent plates from Costar (3471, 3473) to minimize 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.

**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 HBSS without Ca²⁺ and Mg²⁺ and suspended in complete RPMI 1640 medium with glutamax (Invitrogen Life Technologies) and added gentamicin (80 μg/ml). Cells were cultured in special nonadherent plates from Costar (3471, 3473) to minimize effect of adherence on signaling. All experiments were conducted in serum-free conditions. Differential cell counts were determined using a Wright-Giemsa-stained cytospin 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.

**Immunoprecipitation of cellular proteins**

For immunoprecipitation, lysates were combined with 25 μg of PP1α agarose conjugate (Santa Cruz Biotechnology) overnight rotating at 4 degrees. Beads were washed three times with lysis buffer. For Western blot analysis, the beads were suspended in 50 μl of 2× sample buffer and heated for 5 min at 95 degrees. The beads were spun down and proteins were analyzed by SDS-PAGE. Western blot protocol was followed, as described below. Coimmunoprecipitations were determined by staining the resulting blots for proteins other than the one pulled down. Equal loading was determined by stripping the blots and reprobing for the immunoprecipitated protein.

**TCA precipitation of labeled proteins**

Some of the protein from the 35S-labeling experiments was used to obtain relative 35S counts of the labeled proteins. A total of 20 μl of the 35S-labeled cell suspension was added to 100 μl of BSA (1 mg/ml) and sodium nitrate (NaN₃, 0.02%). One milliliter of ice-cold 10% w/v TCA was added to each tube, and the samples were vortexed vigorously. The solution was filtered through a 2.5-cm glass microfiber filter (Millipore) and the filters were washed twice with 5 ml of ice-cold 10% TCA. The discs were washed two more times in ethanol, dried, and placed in vials with 5–10 ml of scintillation mixture, and radioactivity was determined with a scintillation counter set on a wide open channel. Starting values (total) were determined by spotting 20 μl of the original cell lysate onto a filter and counting. Calculations were as follows: sample counts/total counts × 100 yields percentage of 35S incorporation. Fold increase was determined by dividing experimental sample values by control values.

**Whole cell protein isolation**

Whole cell protein was obtained by lysing the cells on ice for 20 min in 200 μl of lysis buffer (0.05 M Tris (pH 7.4), 0.15M NaCl, 1% Nonidet P-40, with added protease and phosphatase inhibitors: 1 protease mini tab (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, and 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.

**FIGURE 2.** Inhibition of ERK increases phosphorylation of eIF2α on serine 51. A. Three sets of human alveolar macrophages were cultured with U0126 (20 μM) for various lengths of time. Whole cell lysates were obtained and Western blot analysis was performed using an Ab specific for eIF2α phosphorylated on serine 51. Composite densitometry of the three experiments is shown on the right. B. Human alveolar macrophages were exposed to varying doses of U0126 (0.2, 2, and 20 μM) for 1 h. Whole cell lysates were obtained and Western blot analysis was performed for phosphorylated eIF2α (serine 51) and active ERK. Equal loading of the proteins is demonstrated by staining of the blots for β-actin. C. Human alveolar macrophages were cultured with a cell-permeable ERK inhibitory peptide (50 μM) for various lengths of time. Whole cell lysates were obtained and Western blot analysis was performed using an Ab specific for eIF2α phosphorylated on serine 51. D. Human alveolar macrophages were cultured with an ERK inhibitor (U0126, 20 μM) for prolonged time periods. Whole cell lysates were obtained and Western blot analysis was performed using an Ab specific for eIF2α phosphorylated on serine 51.
Western blot analysis

Western blot analysis for the presence of particular proteins or for phosphorylated forms of proteins was performed, as previously described (39). Briefly, 30 μg of protein was mixed 1:1 with 2X sample buffer (20% glycerol, 4% SDS, 10% 2-ME, 0.05% bromophenol blue, and 1.25 M Tris (pH 6.8)), loaded onto a 10% SDS-PAGE gel, and run at 110 V for 2 h. Cell proteins were transferred to PVDF membranes with a Bio-Rad semi-dry transfer system, according to the manufacturer’s instructions. Equal loading of the protein groups on the blots was evaluated using Ponceaus S (Sigma-Aldrich), a staining solution designed for staining total proteins on PVDF membranes. The PVDF was then blocked with 5% milk in TTBS (TBS with 0.1% Tween 20) for 1 h, washed, and then incubated with the primary Ab at dilutions of 1/500 to 1/2,000 overnight. The blots were washed four times with TTBS and incubated for 1 h with HRP-conjugated anti-IgG Ab (1/5,000 to 1/20,000). Immunoreactive bands were developed with a chemiluminescent substrate, ECL Plus or ECL (Amersham Biosciences). An autoradiograph was obtained, with exposure times of 10 s to 2 min. Protein levels were quantified using a FluorS scanner and Quantity One software for analysis (Bio-Rad). The data were analyzed, and statistics were performed using Graphpad software. Densitometry is expressed as fold increase (experimental value/control value).

Phosphatase activity assay

Phosphatase activity was measured using a pNPP phosphatase assay kit from BioAssay Systems. To measure PP1α phosphatase activity, PP1α was immunoprecipitated from whole cell lysates (human alveolar macrophages) using microcystin-Sepharose beads or PP1α Ab/protein A-Sepharose beads. Serial 2-fold dilutions of the washed beads were made in 100 mM Tris-HCl (pH 7.5) and 10 mM MgCl₂. A total of 50 μl of each dilution was placed into a 96-well plate. pNPP substrate solution (50 μl) was then added to each well. After 5–30 min, the absorbance was read at 405 nM. Dilutions that fit into a linear range of purified standard (PP1α) were used to acquire data. Data are presented as OD units.

Cell survival analysis

For analysis of cell survival, alveolar macrophages were cultured in six-well tissue culture plates with or without pathway inhibitors (ERK, U0126 at 20 μM or ERK activation inhibitor peptide II (Calbiochem) at 50 μM) for the described times. Triplicate cultures were performed on all experiments. After the incubation period, the cells were stained with ethidium homodimer (Molecular Probes) at 8 μM, and images were obtained of both bright field and fluorescence using a Leica DMRB microscope equipped with a Qimaging RETICA 1300 digital camera and imaging system. After obtaining images, the percentage of EthD-1-positive cells was determined. The percentage of EthD-1-positive cells was determined using an Ab specific for eIF2α phosphorylated on serine 51. Densitometry shown as arbitrary units is on the right.

Quantification was by direct cell count. Two hundred cells were counted from a minimum of four different fields. Average viability was determined and statistics were performed using GraphPad software.

ATP assay

Cell survival was also monitored by using CellTiter-Glo Luminescent Cell Viability Assay from Promega. Alveolar macrophages were cultured (1 million alveolar macrophages treated with either U0126 or ERK Activation Inhibitor Peptide II for 6 or 24 h) in 24-well plates. ATP, signaling the presence of metabolically active cells, was measured by bringing the plate to room temperature and adding CellTiter-Glo directly to each well. The plate was mixed on an orbital shaker to induce cell lysis and then the sample was read in a luminometer (integration time of 1 s). Data are presented as mean ± SE of luminescent readings from three separate experiments.

In vitro kinase assay

To evaluate PP1α phosphorylation, 250 ng of recombinant human active JNK2α peptide and 10 μg of recombinant rabbit muscle PP1α isoform were combined with 5 μCi of ATP (32) to evaluate the ability of JNK2α-activatable PP1α. The PP1α inhibitor tautomycin (1 μM) was included in the reaction buffer (20 mM MOPS, 25 mM glycerol phosphate, 1 mM NaVO₄, 5 mM EGTA, and 1 mM DTT). The experiment was conducted with JNK2α peptide alone; PP1α isoform alone; JNK2α peptide and PP1α isoform; and JNK2α peptide, PP1α isoform, and the JNK inhibitor SP600125 at 1 μM. Samples were incubated at 30 degrees for 20 min. Equal volume of 2X sample buffer was added at the end of the incubation, and samples were run on a 10% acrylamide gel, transferred to PVDF membrane, and visualized on a phosphor imager (Bio-Rad; Quantity One software).

Results

Inhibition of ERK decreases protein translation

Human alveolar macrophages were placed in culture with and without the MEK inhibitor (U0126) for 2 h. During the last 30 min of culture, the cells were moved to methionine-deficient medium for 30 min (U0126 was replaced where appropriate). Cells were then treated with [35S]methionine and actinomycin D (to block transcription) and cultured for additional 2 h. Whole cell lysates were obtained and an aliquot was run out on a 10% SDS-PAGE gel. The gel was dried and an autoradiogram was obtained. Fig. 1 demonstrates that ERK inhibition results in decreased translation.
of multiple proteins. Also shown are data from three separate experiments in which TCA-precipitable proteins were isolated from the lysate and 35S counts were analyzed. Data are presented as fold difference (U0126/control). The graph demonstrates that in conditions in which transcription is frozen (actinomycin treatment), U0126 decreases the amount of new protein production. To confirm the presence of baseline ERK activity, three sets of macrophages were cultured with U0126 for 1 h. Whole cell lysates were obtained and ERK activity was monitored by Western blot analysis for the phosphorylated form of the protein. Fig. 1B demonstrates significant baseline ERK activity in human alveolar macrophages. As a composite, these data suggest that in alveolar macrophages there is constitutive ERK activity that plays an important role in maintaining ongoing protein translation. To examine the specificity of U0126, we treated alveolar macrophages with U0126 for 1 h and examined activity of the MAPK, p38, and the PI3K pathway component, Akt. Fig. 1C demonstrates that 1 h of U0126 completely blocks baseline ERK activity, has some negative effect on p38 activity, and has no effect on the activity of Akt. The effect of U0126 on JNK activity is not shown in Fig. 1C, as the activation of JNK by U0126 is a part of this study, and is shown in Figs. 5 and 6. Inhibition of ERK increases phosphorylation of eIF2α. Protein translation is primarily regulated at initiation. One major regulatory pathway is the eIF4F pathway, which recruits mRNAs with 5' caps to the ribosome. The other major pathway is the eIF2 pathway, which brings the start methionine (Met-tRNA<sub>iMet</sub>) to the ribosome. When we examined the eIF4F pathway (eIF4E, 4EBP-1, eIF4A, eIF4B, and eIF4G) for alterations in activating and inhibiting phosphorylations after ERK inhibition, we found little change (data not shown). This is despite the described role of ERK as an upstream activator of Mnk1, which phosphorylates eIF4E. However, when we examined phosphorylation of eIF2α on serine 51, an inhibiting phosphorylation, we found significant and prolonged phosphorylation of eIF2α after U0126 (Fig. 2A). Densitometry from the three separate experiments is also shown. It is important to note that there is individual variation among the donors. In some cases, the eIF2α phosphorylation began as early as 15 min after U0126 and continued out to 24 h. In other cases, the time of eIF2α inhibition was more restricted. One possible cause of the discrepancies may be varied induction of GADD34, a PP1 regulator (and negative feedback mechanism (21, 29)) induced following eIF2α phosphorylation. We found various levels of GADD34 induction in the different donors (data not shown). This would contribute to altered duration of the eIF2α phosphorylation. In all cases, U0126 caused a prolonged phosphorylation of eIF2α (see Fig. 2D).

We treated normal alveolar macrophages with varying amounts of U0126. Fig. 2B demonstrates that U0126 causes significant phosphorylation of eIF2α over multiple concentrations. The inhibitory effect of U0126 on ERK phosphorylation is shown as a control. To determine that the U0126 observation was not due to an artifact of the chemical inhibitor, we performed the same experiment using a small peptide ERK inhibitor. This inhibitor is a 13-aa peptide from the ERK upstream kinase, MEK, fused to the HIV-TAT membrane translocation peptide via a glycine linker (HGGRKKRRQRRR-G-MPKKKPPIQ LNP-NH2). The peptide is membrane permeable and interferes with ERK/MEK interactions. Fig. 2C demonstrates that, as with U0126, the ERK inhibitor peptide also increased eIF2α phosphorylation on serine 51. Finally, we asked whether the increased eIF2α phosphorylation generated by ERK inhibition was a transient or prolonged event. Fig. 2D shows that the increase in eIF2α phosphorylation on serine 51 after ERK inhibition is a prolonged event (extending out to 24 h). Consistent with the discussion in the paragraph above, there was some variability in the duration of the eIF2α. However, in most cases, U0126 caused prolonged phosphorylation of eIF2α.

The data in this study demonstrate, consistent with the effects of ERK inhibition on translation, that eIF2α phosphorylation is regulated by the combination activity of upstream kinases (PKR, PERK, GCN2, and HRI). It is also regulated by the dephosphorylating activity of PP1α. We initially examined the effect of ERK inhibition on the upstream kinases and

Inhibition of ERK decreases PP1α activity

EIF2α phosphorylation is regulated by the combined activity of upstream kinases (PKR, PERK, GCN2, and HRI). It is also regulated by the dephosphorylating activity of PP1α. We initially examined the effect of ERK inhibition on the upstream kinases and
did not find any significant effect (data not shown). We then examined the effect of ERK inhibition on PP1α activity. Immuno-precipitated PP1α was evaluated for in vitro phosphatase activity using pNPP as a substrate. ERK inhibition significantly decreased PP1α activity (Fig. 3A). To further examine whether phosphatase inhibition was sufficient to increase phosphorylation of eIF2α, we treated human alveolar macrophages with the PP1-specific inhibitor, tautomycin. Fig. 3B shows that inhibition of PP1 (tautomycin) results in phosphorylation of eIF2α. This suggests that inhibiting PP1 is sufficient to increase eIF2α phosphorylation in human alveolar macrophages and that there is enough baseline activity in the upstream kinases to assure phosphorylation of eIF2α in conditions in which PP1 is inhibited. As a composite, these data suggest that ERK inhibition in alveolar macrophages leads to a decrease in PP1α activity, which is sufficient to increase the phosphorylation of eIF2α at serine 51.

**ERK inhibition increases PP1α phosphorylation**

To determine a possible mechanism by which ERK regulates PP1 activity, we evaluated the effect of ERK inhibition on an inhibiting phosphorylation of the α catalytic subunit of PP1 (threonine 320). Fig. 4A demonstrates that in alveolar macrophages, ERK inhibition increases phosphorylation of PP1α, a phosphorylation event that inhibits activity of PP1α. The phosphorylation site on PP1α (threonine 320) is a consensus MAPK substrate site (PX(S/T)P)(PP1α: 315 GGRPIPTPRNS 325). The MAPK consensus sequence is underlined and in italics.

The threonine phosphorylation site is shown in bold. Because ERK was inhibited in these studies, it is not a potential upstream kinase. The only described upstream kinases for this site are the cell cycle regulatory kinases, cyclin-dependent kinase 1 (Cdk1) and Cdk2 (40, 41). We could find no sign in any change in Cdk1 or Cdk2 activity with ERK inhibition (data not shown). This is consistent with a recent study by Romerio and Zella (42), demonstrating that IFN-α decreases ERK activity leading to decreased Cdk2 activity. To investigate other possible upstream kinases of the PP1α site, we evaluated the effect of inhibiting other MAPKs together with the ERK inhibition. Fig. 4B demonstrates that inhibiting p38 (SB203580) had no effect on the U0126-induced eIF2α phosphorylation. In contrast, blocking JNK activity at the same time as U0126 resulted in a loss of the U0126-induced phosphorylation of eIF2α. As a composite, these experiments demonstrate that inhibition of baseline ERK activity in alveolar macrophages results in phosphorylation of PP1α on threonine 320 and that JNK activity is required for the ERK inhibition-dependent phosphorylation of eIF2α.

**ERK inhibition results in activation of JNK and a decrease in the JNK phosphatase, MKP-7**

We next asked whether ERK inhibition increased JNK activity. In Fig. 5A, we demonstrate that ERK inhibition by either U0126 or an ERK inhibitory peptide significantly increased JNK activity. In Fig. 5B, we examine the effect of ERK inhibition on amounts of MKP-7. ERK inhibition decreased total MKP-7 protein amounts.
These data suggest that at baseline in alveolar macrophages, active ERK decreases JNK activity by phosphorylating and protecting from degradation the JNK phosphatase, MKP-7.

**JNK phosphorylates PP1α in vitro and complexes with eIF2α and PP1α**

To test the hypothesis that JNK could be a PP1α threonine 320 kinase, we performed an in vitro assay using purified active JNK and purified catalytic domain of PP1α. Isolated proteins were mixed with a kinase buffer and [32P]ATP. The samples were then run on an SDS-PAGE gel, the gel was transferred to PVDF membrane, and radioactivity was determined using a phosphor imager. Fig. 6A demonstrates that in vitro, JNK can phosphorylate PP1α on threonine 320. We next asked whether ERK inhibition would bring PP1α and JNK together in a complex. Alveolar macrophages were treated with an ERK inhibitor and then PP1α immunoprecipitated. The protein complex was then analyzed for JNK binding. Fig. 6B demonstrates that JNK is recruited to PP1α under conditions of ERK inhibition. It is also clear that it is the active form of JNK2 that is associating with PP1α after ERK inhibition.

**ERK inhibition shortens survival of human alveolar macrophages**

Our initial hypothesis was that baseline ERK activity in alveolar macrophages is an important survival pathway. Having demonstrated that ERK inhibition leads to a decrease in global protein translation, we then evaluated the effect of ERK inhibition on alveolar macrophage survival. Fig. 7 demonstrates that inhibition of ERK increases markers of apoptosis and death in alveolar macrophages. Fig. 7A demonstrates that ERK inhibition with U0126 results in cleaved caspase 7, cleaved caspase 9, and cleaved PARP, peaking at 24 h of U0126 exposure. Fig. 7B demonstrates that U0126 increases the membrane permeability of alveolar macrophages, as demonstrated by ethidium homodimer uptake (shown both by a photomicrograph and by cell counts from three separate experiments). In Fig. 7C, we evaluated ATP depletion as a marker of viability. Exposure to both U0126 and the ERK inhibitory peptide caused a significant drop in ATP levels, from 40 to 70% of control at 6 h and from 2 to 5% of control at 24 h. The ATP data are consistent with the enhanced caspase and PARP cleavage we saw at 24 h of U0126 exposure. As a composite, the data demonstrate that baseline ERK activity is crucial for the survival of human alveolar macrophages.

**Discussion**

The alveolar macrophage maintains baseline activity of two pathways that contribute to prolonged survival. We have shown previously that both the PI3K/Akt and ERK pathways are activated at baseline in alveolar macrophages (3). The prosurvival effects of Akt are well described and were not the subject of these studies. In contrast, little is known about the survival effects of ERK (10, 43–45). In this study, we examined the role of ERK activity in maintaining adequate levels of protein translation, a necessary condition for cell survival. We found that inhibition of ERK significantly decreased protein translation in alveolar macrophages. Due to the rich oxidant and pathogen exposure in the lung, it is our hypothesis that this is a lung-specific effect. However, we have not yet performed the macrophage comparative experiments that will allow for that conclusion. We have shown previously that survival pathway activity is different between human alveolar macrophages and their precursor cells, blood monocytes (3, 30). We have not yet compared alveolar macrophages and other tissue macrophages. These experiments are part of ongoing projects.

Regulation of translation primarily occurs at the initiation step (17, 46). One important pathway is recruitment of ribosomes to mRNA by the eIF4F complex. Despite described links between ERK and eIF4E (20, 47), we found no obvious effect of ERK inhibition on this complex in alveolar macrophages. Another important pathway in the initiation process is binding the initiator Met-tRNA<sup>Met</sup> to the P site of the small ribosomal subunit by the eIF2 complex (17). When we examined the effect of ERK inhibition on the eIF2 complex, we found a significant change in the phosphorylation status of eIF2α (active eIF2α is hypophosphorylated on serine 51). Phosphorylation of eIF2α normally occurs as part of a stress response (nutrient deprivation, viral infection, ER stress, and low heme levels). In the case of ERK inhibition, we observed no changes in the activity of the stress-related upstream kinases (data not shown). We did, however, observe marked changes in activity of the phosphatase, PP1α, which is responsible for maintaining eIF2α in a hypophosphorylated state (29)).

ERK inhibition resulted in decreased PP1α activity that was due to an increase in an inhibitory phosphorylation at a site on the catalytic unit of PP1α (threonine 320). Threonine 320 is a MAPK substrate consensus sequence. Therefore, we looked at other MAPKs as potential upstream kinases responsible for PP1α phosphorylation. We found that ERK inhibition increased activity of the MAPK, JNK. In addition, we found that active JNK can phosphorylate PP1α in vitro and that JNK inhibition reverses the increase in eIF2α phosphorylation seen with ERK inhibition. Finally, using a number of methods, we show that ERK inhibition significantly shortens survival of alveolar macrophages. (Fig. 8 contains a diagram demonstrating our hypothesis on the relationship among ERK, eIF2α, JNK, and PP1α.)
Translation inhibition (specifically phosphorylation of eIF2α) and apoptosis have been linked in other systems. One recent paper links apoptosis induced by proteosome inhibition to the phosphorylation of eIF2α by upstream kinases (48). These authors found a correlation between an increase in eIF2α phosphorylation and caspase activation and apoptosis. This is consistent with our observations.

In contrast to the proapoptotic effect of eIF2α phosphorylation, there are also data linking eIF2α phosphorylation to activation of the prosurvival transcription factor, NF-κB (23, 48, 49). One study by Jiang et al. (50) examined the hypothesis that NF-κB activity during ER stress requires temporary translational arrest to prevent the rapid resynthesis of the inhibitory subunit IκBα. The authors suggest that eIF2α phosphorylation is necessary for NF-κB activation. Our system differs in a variety of ways. For one, we have no significant induction of the upstream kinases (PERK, PKR, GCN2, and HRI). For another, the duration of phosphorylation of eIF2α with ERK inhibition is extremely long, and this is different from the normal stress response. Although ER stress temporarily inhibits most translation, it induces translation of a subset of stress-related proteins. One of these stress-related proteins is instrumental in reactivating PP1α and ending the eIF2α phosphorylation. This feedback mechanism is not operative in our system. We found no induction of GADD34 (the stress-related protein that activates PP1α). In our case, eIF2α phosphorylation has no endogenous shutoff mechanism as long as ERK is inactive and the extended shutdown of the translation apparatus leads to cell death.

Our data demonstrate a decrease in PP1α activity with ERK inhibition and the novel observation that this is linked to ERK inhibition-dependent activation of JNK. PP1α activity is regulated by its regulatory subunits. PP1α activity is also negatively regulated by phosphorylation at threonine 320 (40). There is only one previous study linking ERK to PP1. In a paper by Quevedo et al. (51), using neuronal cell cultures, insulin-like growth factor 1 was shown to induce PP1 activity via MEK/ERK activity. In this case, the endpoint is activation of eIF2B, not eIF2α. The Quevedo study does demonstrate that in another system, ERK can be upstream of PP1. Our data link ERK to PP1α via an effect on phosphorylation of PP1α on threonine 320 by JNK.

Our conclusion that PP1α phosphorylation is via JNK activation is supported by the following data: ERK inhibition by both chemical (U0126) inhibitor and cell-permeable inhibitory peptides results in JNK activation in alveolar macrophages. The threonine 320 site on PP1α is part of a consensus MAPK substrate sequence (PX(S/T)P). The kinases that preferentially phosphorylate this consensus sequence include: MAPKs (ERK, p38, and JNK isoforms), glycogen synthase-3, and Cdc2 (52). The upstream kinase is obviously not ERK as it is inhibited, and when we examined p38 we...
found that ERK inhibition in alveolar macrophages actually decreased p38 activity slightly. We also found no change in the activity of Cdk1, a previously described PP1α kinase (40). However, when we examined JNK activity, we found a significant increase in the level of JNK activity in ERK-inhibited alveolar macrophages. We also found that JNK could phosphorylate PP1α in vitro and that after ERK inhibition, JNK could be found in a complex with eIF2α and PP1α.

Our data suggest a mechanism for the increase in JNK activity. Previous studies have demonstrated a positive role for ERK activity in the stability of the dual phosphatase, MKP-7 (53, 54). The study by Katagiri et al. (53) demonstrates that ERK phosphorylates the JNK-specific dual phosphatase, MKP-7. The functional consequence of the ERK phosphorylation on serine 446 is to stabilize MKP-7 protein, allowing for its accumulation. MKP-7 has been demonstrated to preferentially dephosphorylate JNK5 (55). The role of dual specificity phosphatases in JNK activity is complicated. Along with the already discussed negative role of MKP-7 in JNK inactivation, Shen et al. (56) have described a novel dual specificity phosphatase, JSP-1, whose overexpression activates JNK. We don’t know at this point whether there is any link between ERK and JSP-1. Our data suggest a positive role for ERK activity in MKP-7 stability; however, due to the problems in visualizing the endogenous protein (see Ab information in Materials and Methods), a definitive link between ERK and MKP-1 in alveolar macrophages awaits future studies.

In human alveolar macrophages, we have found that ERK activity is a prosurvival phenomenon. That is not always the case. In the paper by Levinthal and DeFranco (57), glutamate-induced oxidative stress induces neuronal death in an ERK-dependent manner. These data suggest that the role of ERK in cell survival is stimulus and cell-type specific. JNK activity has also been linked to both cell survival and death. One study, consistent with our finding of a proapoptotic effect of JNK activity, is a study by Handley et al. (58). The Handley study demonstrates that oxidative stress-induced apoptosis requires JNK activity. Interestingly, they link JNK activity to inhibition of tyrosine phosphatases. Our study links JNK activation to inactivation of another class of phosphatases, the serine/threonine phosphatases (PP1α).

In contrast, a study by Himes et al. (59) shows that activation of JNK by CSF-1 is important in macrophage differentiation and survival. Although both our study and the Himes et al. (59) study are about macrophages and JNK, there are important differences between the two studies. The Himes et al. (59) study uses murine bone marrow-derived macrophages instead of human primary tissue macrophages (alveolar). They also are studying the effect of a growth factor, CSF-1, instead of homeostatic conditions. Furthermore, their inhibitor level is up to 10 times the amount used in our study (10–20 μM compared with 1 μM). We believe that the differences in species, model, and inhibitor use can explain the different survival outcomes in the two studies.

As a composite, our data demonstrate that in alveolar macrophages, baseline ERK activity maintains protein translation via the following novel pathway: ERK decreases JNK activity by stabilizing MKP-7. This, in turn, leads to activation of PP1α and maintenance of hypophosphorylated (active) eIF2α and translation. This positive effect of ERK on protein translation is consistent with the prolonged survival of human alveolar macrophages. Thus, this newly described role for ERK in alveolar macrophage homeostasis explains, in part, the survival characteristics of these cells.

Disclosures
The authors have no financial conflict of interest.

References


