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Regulation of cytokine-induced prostanoid and nitric oxide synthesis by extracellular signal-regulated kinase 1/2 in lung epithelial cells

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Abstract

The inflammatory cytokines, tumor necrosis factor alpha (TNF α) and interferon gamma (IFN γ), stimulate production of the inflammatory mediators prostaglandin (PG) E₂, PGI₂, and nitric oxide (NO) in cultured lung epithelial cells. Pre-treatment of these cells with the selective MEK1/2 inhibitor, U0126, blocked ERK1/2 activation and inhibited cytokine-induced production of these inflammatory mediators. Primary bronchiolar epithelial Clara cells treated with TNF α and IFN γ also produced increased PGE₂, PGI₂, and NO, and PG and NO production was decreased by MEK inhibition. U0126 differentially affected COX-1, COX-2 and iNOS expression in cell lines, however, suggesting that MEK1/2 regulates prostanoid and NO production by means other than inducing their biosynthetic enzymes. Functionally, inhibition of MEK1/2 caused G1 cell cycle arrest and decreased cyclin D1 expression, but these effects were not related to decreased prostanoid production. These results indicate separate pro-inflammatory and proliferative roles for ERK1/2 in lung epithelial cells. During lung tumor formation *in vivo*, ERK1/2 phosphorylation increased as lung tumors progressed. Since tumor-derived cells were more sensitive than non-tumorigenic cells to the anti-proliferative effects of U0126, MEK1/2 inhibition may serve as an attractive chemotherapeutic target.

Introduction

The lungs are particularly susceptible to inflammation since their massive surface area is continuously exposed to both airborne and blood-borne pro-inflammatory agents. Incomplete or inappropriate resolution of inflammation can lead to or exacerbate asthma, chronic obstructive pulmonary disease (COPD), and lung cancer. Deciphering signaling pathways regulating the production of, and cellular responsiveness to, inflammatory mediators during pulmonary inflammation will improve our understanding of how chronic inflammatory diseases develop, and may lead to better preventive and therapeutic interventions.

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Epidemiologic[1] and familial clustering studies in humans[2], and pharmacologic[3] or genetic[4–6] manipulation of inflammatory pathways in mouse models, suggest that inflammation plays a key role in initiation and maintenance of pulmonary neoplasia, the leading cause of cancer death in the USA[7]. Sustained and inappropriate release of pro-inflammatory mediators, including prostanoids and nitric oxide (NO), are early events during lung tumor development. Increased iNOS expression and enhanced production of NO occurs in lung cancer patients[8;9], and targeted deletion of the *NOS2* gene nearly abolishes carcinogen-induced lung tumor formation in mice[6]. Pharmacologic down-modulation of iNOS resulting from silibinin therapy is associated with reduced angiogenesis and lung tumor growth[10;11].

The cyclooxygenase enzymes, COX-1 and COX-2, catalyze the rate-limiting step in the conversion of arachidonic acid to prostanoids. COX-2 protein expression increases in human and mouse lung cancer compared to normal lung tissue[12;13]. Regular use of non-steroidal anti-inflammatory drugs (NSAIDs), which enzymatically inhibit COX-1 and COX-2, is associated with a decreased risk of lung cancer[14], and several NSAIDs protect against lung tumor development in animal models[3;15;16]. While COX expression has been extensively studied in lung and other cancers, less is known about the downstream pathways mediating pro-tumorigenic effects of COX (particularly COX-2) activation. Human and transgenic mouse studies suggest that some prostanoids are pro-tumorigenic while others display anti-tumor effects. For example, urinary PGE₂ expression increases in lung cancer patients compared to healthy controls[17], while expression of PGI₂ synthase (PGIS) and synthesis of its product, PGI₂ (prostacyclin), decrease in human[18] and mouse lung tumors[19;20]. Those few patients who maintain high lung PGIS expression exhibit better survival[20], and targeted PGIS over-expression or treatment with the PGI₂ analog, iloprost, protected against mouse lung tumor formation[4;5;21]. Pro-tumorigenic prostanoids contribute to lung tumor progression by stimulating angiogenesis, increasing tumor cell invasiveness, suppressing immune surveillance, and inhibiting apoptotic cell death of tumor cells[22].

The pro-inflammatory cytokines, tumor necrosis factor alpha (TNF α) and interferon gamma (IFN γ), induce COX-2 and iNOS expression which in turn enhances the synthesis of pulmonary prostanoids and NO *in vitro* and *in vivo*[19;23–25]. Tumorigenic and non-tumorigenic mouse lung epithelial cells differentially regulate prostanoid and NO pathways upon cytokine stimulation[19;26]. The extracellular signal-regulated kinase 1/2 (ERK1/2) pathway is activated by TNF α and IFN γ in some cell types and is upregulated in lung tumors[27–29]. Herein we examine ERK1/2-dependent signaling events in TNF α /IFN γ -mediated inflammatory responses by comparing prostaglandin and NO production with cell proliferation in non-transformed and tumor-derived mouse lung epithelial cell lines using a small molecule inhibitor of MEK1/2 (U0126), an upstream kinase that phosphorylates and activates ERK1/2. Understanding the regulatory properties of these pathways would provide attractive targets for novel anti-inflammatory therapies.

Materials and Methods

Cell culture

The cell lines used in these studies have been described in detail[30]. C10 and E10 cells were originally derived from a normal BALB/c mouse lung explant, are non-tumorigenic, contact-inhibited, had alveolar type 2 cell features at early passage, and are maintained in CMRL1066 medium[31]. LM1 and LM2 cells were derived from solid and papillary urethane-induced A/J mouse lung tumors, respectively, and are maintained in MEM α medium. LM1 and LM2 cells are tumorigenic in syngeneic mice and not contact inhibited *in vitro*[30]. LM2 cells contain a codon 61 *Kras* initiating mutation (CAA $\rightarrow$ CGA) that yields

a constitutively active Kras protein, while LM1 cells have wild-type Kras[32]. Cell culture media was supplemented with 10% fetal bovine serum (FBS; Gemini Bioproducts, Woodland, CA), 100 U/ml penicillin, 100 µg/ml streptomycin, and 100 µg/ml amphotericin B (Invitrogen). Cells were grown in a humidified atmosphere of 5% CO₂ in air at 37°C, and passaged twice weekly by trypsinization.

Cytokine administration

Cells were grown (3 plates/condition) to confluence, serum-deprived for 24h, and treated with serum-free media containing 10 ng/ml each of mouse TNFα and mouse IFNγ (R&D Systems, Minneapolis, MN) for 24 h. Control cells were serum-deprived for 24h and then incubated with 0.05% dimethyl sulfoxide (DMSO) vehicle in serum-free media. U0126 (Calbiochem, San Diego, CA) was diluted into serum-free media from a 25 mM stock solution dissolved in DMSO immediately prior to use and applied to cells 1 h prior to cytokine exposure. Serum-deprived LM1 and LM2 cells were very sensitive to U0126 treatment, but doses of 1 and 5 µM were sufficient to inhibit ERK1/2 phosphorylation without causing cell death. LM1 and LM2 cells maintained in the presence of FBS were viable at 10 and 25 µM U0126. Serum-deprived and serum-fed non-tumorigenic C10 and E10 cells were viable in 25 µM U0126.

To test effects of MEK1/2, COX, and iNOS inhibition on the cell cycle, LM2 cells were grown to confluence and treated with 10 µM U0126, 1 µM indomethacin (COX inhibitor), or 1 µM aminoguanidine (AMG, iNOS inhibitor) in serum containing media. Cells were counted using a hemocytometer after 48 h of treatment or fixed for cell cycle analysis 24 h after treatment (see below). To test the effects of prostaglandin exposure on proliferation, LM2 cells were treated with 10 and 1000 ng/ml PGE₂ and/or the stable PGI₂ analog, iloprost. Cells were counted using a hemocytometer 48 h later.

PGE₂, PGI₂, and NO determination

Culture media samples (1 ml) were collected prior to cell harvest and stored at -80°C. ELISA immunoassays (Cayman Chemical, Ann Arbor, MI) for PGE₂ and 6-keto PGF_{1α}, the stable metabolite of PGI₂[33], were performed in duplicate according to manufacturer's instructions. NO production by cells was determined by measurement of nitrite and nitrate metabolites by colorimetric NO metabolite detection assay (Cayman Chemical, Ann Arbor, MI), according to the manufacturer's instructions. Samples were diluted so that values were in the linear portion of standard curves.

Primary Clara cell isolation and culture

Clara cells were isolated as described previously[34]. The newly isolated Clara cell population was greater than 90% pure. Cells were plated in 12 well culture dishes at 100,000 cells/well in MEMα media supplemented with 10% FBS and antibiotics as described above. After Clara cells adhered to the culture plates (48 h at 10% plating efficiency), media was replaced with serum-free MEMα for 48 h to discourage contaminating fibroblast cell growth. U0126 at 0, 1, 5, 10, and 25 µM doses was added in fresh serum-free MEMα 1 h prior to cytokine treatment (as described above for cell lines). Primary cultures were viable at all 4 U0126 doses, and plated cells displayed epithelial cell morphological characteristics throughout treatment. Twenty-four hours later, media was harvested from cells and assayed for PGE₂, 6-keto PGF_{1α}, and NO as described above.

Sample preparation for immunoblotting

Cell homogenates were prepared by washing cells twice with cold phosphate buffered saline (PBS), scraping, and pelleting by centrifugation at 2,400 × g for 5min, as described

previously [19]. Cell pellets were lysed in extraction buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1mM NaF, 20 mM Na₄P₂O₇, 2 mM Na₃VO₄, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1mM PMSF, 60 µg/ml aprotinin, 10 µg/ml leupeptin, 1 µg/ml pepstatin) for 30 min. on ice with mixing at 10 min. intervals. Lysates were sonicated then centrifuged at 16,000 × g for 10min at 4°C, and supernatants collected. Protein concentrations in each sample were determined by the method of Lowry et al.[35] prior to mixing 1:1 with 2× sample loading buffer (100 mM Tris, pH 6.8, 0.4% sodium dodecyl sulfate, 2% β-mercaptoethanol, 20% glycerol, 0.3% pyronine Y).

Western immunoblotting

Cell samples (100 µg total protein/lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred onto an Immobilon-P PVDF membrane (Millipore, Bedford, MA). Phospho-ERK1/2 (sc-7383), total ERK1/2 (sc-94), COX-1 (sc-1754), and COX-2 (sc-1747) primary antibodies and all secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); iNOS (610332) primary antibody was from BD Biosciences (San Jose, CA); and cyclin D1 primary antibody (2978) from Cell Signaling Technology (Danvers, MA).

Immunoblotting was performed as previously described[19] for phospho (ph)-ERK1/2 (1:500 dilution), total (pan)-ERK1/2 (1:5000 dilution), COX-1 (1:1000 dilution) and COX-2 (1:1000 dilution) proteins. Immunoblotting for iNOS (1:1000 dilution) was performed using a modification of Kisley et. al.[6]. PVDF membranes were incubated with primary antibody, washed, incubated in secondary antibody (1:20,000 dilution), and washed again. Cyclin D1 protein (1:4000 dilution) was detected similarly except that secondary antibody was used at a 1:10,000 dilution. After treatment with Super Signal West HRP-substrate (Pierce Biotechnology; Rockford, IL), membranes were exposed to X-ray film and protein bands quantified by densitometry using Un-Scan-It software (Silk Scientific Corporation, Orem, UT). To confirm even protein loading of the gels, the membranes were stained with 0.1% Ponceau S (Fisher Biotech, Fair Lawn, NJ) in 5% acetic acid prior to being stripped and re-probed with panERK1/2 antibody.

Cell proliferation assay

Cell proliferation was examined using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS, Promega, Madison, WI) according to the manufacturer's instructions. Briefly, cells were trypsinized and resuspended in media at a concentration of 2×10⁶ cells per ml, and 50 µl of cell suspension placed into 96-well cell culture microtiter plates. Cells were allowed to adhere overnight and then exposed to various concentrations of U0126 or vehicle control (DMSO) in serum containing media. After 48 h, media was replaced with 100 µl of phenol-free RPMI-1640 containing 10% FBS, and 20 µl of MTS reagent/well. After 1 h, absorbance was measured at 490 nm with a microtiter plate reader (Molecular Devices, Palo Alto, CA).

Flow cytometry

Cell cycle analysis was performed by the University of Colorado Cancer Center Flow Cytometry Core Laboratory. Cells were treated with 10 µM U0126 in serum-containing media and trypsinized 24h after treatment, washed in PBS, pelleted at 2000 × g, and incubated in Krishan stain[36] at 4°C for 24h. Cell cycle analysis was performed using a Beckman Coulter Epics XL flow cytometer. Doublets were excluded from analysis using the peak vs. integral gating method, and ModFit LT software (Verity Software House, Topsham, ME) yielded cell cycle parameter. Three independently-treated samples were analyzed for each treatment group/repetition.

Apoptosis by morphologic determination and caspase-3 activity assays

Apoptosis and viability were quantified as previously described[37] by staining cells with acridine orange and ethidium bromide, and then assaying for nuclear morphology, a hallmark of apoptosis[38]. To perform caspase-3 activity assays[39], protein lysates (50 µg) were incubated for 30min at 37°C in caspase assay buffer (50 mmol/L Hepes, pH 7.4, 100 mmol/L NaCl, 2 mmol/L EDTA, 10% sucrose, 0.1% CHAPS, (3-[(3-cholamidopropyl)dimethyl ammonio]-1-propanesulfonate (Meriden, CN) 10 mmol/L dithiothreitol) containing 10 µmol/L of DEVD-AMC (caspase 3 substrate peptide conjugated to aminomethylcoumarin (AMC)). Enzymatic assays and AMC standard curves were carried out in duplicate using a fluorescent plate reader (Packard Instruments, Meriden, CT) with excitation and emission wavelengths of 360 nm and 460 nm, respectively.

Animal studies

Male A/J mice purchased from Jackson Laboratories were maintained on hardwood bedding with a 12 h light/dark cycle, and given chow (Teklad) and water *ad libitum* under an Institutional Animal Care and Use Committee approved protocol. Lung tumors were induced by a 1 mg/g IP injection of urethane when mice were 6–8 wks old. Seven and 11 months after urethane treatment, lung tumors were harvested, dissected from surrounding tissue (uninvolved lung), and measured. Tumors from mice in the 7 month group were divided into small (< 1mm diameter) and large (> 1mm diameter) groups; tumors in the 11 month group were larger than 1mm in diameter. Control, uninvolved lung, and tumor samples were homogenized in 20 mM HEPES (pH 7.2), 2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 10% glycerol, 5 µg/ml aprotinin, 10 µM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1mM NaF, 20 mM Na₄P₂O₇, 2 mM Na₃VO₄, and 1% Triton X-100. Samples were centrifuged at 16,000×g to remove cellular debris and protein concentration determined by Lowry[35] prior to immunoblotting as described earlier.

Statistical analysis

Graphical data is presented as means ± standard errors. Data was analyzed by one-way analysis of variance (ANOVA) followed by student Newman-Keuls post-hoc test using Graph-Pad Prism software (version 4.02, San Diego, CA). Statistical significance was accepted at $p < 0.05$.

Results

Inhibition of MEK1/2 blocks cytokine-induced prostaglandin and nitric oxide production

TNF α and IFN γ induce inflammatory mediators such as PGE₂, PGI₂, and NO in lung epithelial cells[19]. To assess the role of ERK1/2 activation in this induction, non-tumorigenic (C10 and E10) and tumorigenic (LM1 and LM2) lung epithelial cells were pre-treated with the selective MEK1/2 inhibitor, U0126 [40] or DMSO vehicle, prior to treatment with TNF α and IFN γ (TNF α /IFN γ). TNF α /IFN γ treatment stimulated production of PGE₂ and PGI₂ in all cell lines (Fig. 1A) in agreement with our previous work[19]. The extent of prostaglandin induction was cell line dependent, but not a function of neoplasticity, since non-tumorigenic C10 and tumorigenic LM2 cells were most responsive to mediator induction by TNF α /IFN γ . Pre-treatment with U0126 resulted in a dose-dependent inhibition of cytokine-induced PGE₂ and PGI₂ production towards control levels in all 4 cell lines (Fig. 1A).

TNF α /IFN γ treatment stimulated production of nitric oxide (NO) in these cells, as determined by measuring levels of nitrate and nitrite metabolites in the culture media (Fig. 1B). Although all four cell lines produced NO in response to cytokine treatment, media from LM1 and LM2 cells contained significantly more NO. Since a confluent plate of LM1 or

LM2 cells contains up to ten times more cells than a confluent plate of C10 or E10 cells, these differences in NO production among the cell lines may be a function of cell number. U0126 suppressed cytokine-induced NO production in each cell line (Fig. 1B). Since LM1 and LM2 cells are not viable at 10 and 25 μ M U0126 in serum-free conditions, we were unable to assess whether NO production could be completely ablated at the higher U0126 concentrations, but 1 and 5 μ M U0126 significantly inhibited cytokine-induced NO production in these lines. (Fig. 1B).

Effect of MEK inhibition on primary cell PG and NO production

Freshly isolated bronchiolar Clara cells were treated with TNF α and IFN γ in the presence or absence of U0126. Because of the low cell densities, the production of PG and NO is less than that of the cultured cells, but the effects of treatment are similar. Non-transformed primary Clara cells produce more PGI₂ than PGE₂ basally and in response to cytokines (Fig. 2A, B). PG production increased with TNF α /IFN γ treatment. Both basal and cytokine-induced PG production were inhibited by U0126 pretreatment (Fig. 2A, B). NO production was very low in these cells but followed the same basic trends as seen in the cell lines; NO production increased after cytokine treatment, and U0126 inhibited both basal and induced NO production (Fig. 2C).

Inhibition of MEK1/2 with U0126 differentially regulates expression of COX-1, COX-2, and iNOS proteins in C10, E10, LM1, and LM2 cells

Pre-treatment with U0126 blocked TNF α /IFN γ -induced ERK1/2 phosphorylation in C10, E10, and LM2 cells (Fig. 3A, Table I. Although ERK1/2 phosphorylation did not increase in response to cytokine treatment in LM1 cells (in fact, ERK1/2 phosphorylation decreased), U0126 inhibited basal ERK1/2 phosphorylation at the highest dose (Table 1). The increase in cytokine-induced PGE₂ and PGI₂ production correlated with increased COX-2 expression in TNF α /IFN γ -treated C10, E10, and LM1 cells, and higher expression of both COX-1 and COX-2 in LM2 cells[19]. Pre-treatment with U0126 did not block cytokine-induced COX-1 or COX-2 expression C10 cells, but significantly inhibited cytokine-induced expression of both COX-1 and COX-2 proteins in LM2 cells and COX-2 in LM1 cells (Fig. 3 B, C, and Table 1).

Cytokine-induced NO production correlated with iNOS induction in all 4 cell lines[19]. In C10 cells, pre-treatment with 10 and 25 μ M U0126 inhibited iNOS protein expression and NO production to baseline levels, and iNOS expression and NO production in E10, LM1, and LM2 were significantly reduced although not to basal levels (Fig. 3D, Table 1).

MEK1/2 inhibition induces cell cycle arrest and is associated with decreased expression of cyclin D1 protein

Cellular proliferation is the increase in cell number resulting from increased cell division and/or decreased cell death. The effects of U0126 on C10, E10, LM1, and LM2 cell proliferation were examined by MTS assay and cell cycle analysis. Proliferation was significantly inhibited at 25 μ M U0126 (Fig. 4A) in all cell lines, but LM2 cells were more sensitive than C10, E10, and LM1. U0126 (10 μ M) treatment caused a moderate, but significant, accumulation of C10, E10, and LM1 cells in G1 phase. LM2 cells, which were most sensitive to growth inhibition by U0126, were also most sensitive to G1 arrest and displayed a dramatically decreased percentage of cells in S phase (Table I). U0126 treatment for 24h significantly decreased basal expression of cyclin D1 protein in all 4 cell lines and TNF α /IFN γ -induced expression in LM1 and LM2 cells, consistent with arrest in G1 phase (Fig. 4B, C). We have previously shown that cyclin D1 concentrations are a sensitive predictor of proliferative activity in these cell lines[41]. U0126 treated and control C10, E10, LM1, and LM2 cells were examined for apoptotic cell death 1, 2, and 5 days after

treatment using acridine orange and ethidium bromide staining. Stained cells were examined under a fluorescent microscope for morphological signs of apoptotic cell death, but no increase in apoptosis above control rates was observed at U0126 concentrations up to 25 μ M in any of these cell lines (data not shown). Consistent with this observation, caspase-3 activation, an enzymatic indicator of apoptosis, was not detected.

Pro-inflammatory mediators do not directly affect lung epithelial cell proliferation

Treatment of tumorigenic and non-tumorigenic cells with TNF α and IFN γ increased the production of PGE₂, PGI₂, and NO in all cell lines, and ERK1/2 phosphorylation in 3 of the 4 cell lines examined. Inhibiting ERK activation decreased cytokine-induced PGE₂, PGI₂, and NO production (Fig. 1) as well as cyclin D1 protein expression (Fig 4B). To determine if these inflammatory mediators directly influenced cell proliferation, LM2 cells were treated with a physiologically relevant range of PGE₂ and/or iloprost (stable PGI₂ analog) doses. Neither prostanoid had any effect on proliferation (Fig. 5A), implying that the increased PG production observed with cytokine-mediated ERK activation was not sufficient to increase cell number. In addition, treatment with the COX-1/2 inhibitors indomethacin or aspirin (data not shown), or the COX-2 specific inhibitor NS-398 (data not shown) at levels which inhibit PG production did not affect LM2 cell cycle (data not shown) or cell number (Fig. 5B). Interestingly, treatment of LM2 cells with the iNOS-inhibitor, aminoguanidine, caused an accumulation of cells in S-phase (Fig. 5C), an effect opposite to the decrease of cells in S-phase caused by U0126 (Table 2). These results indicate that the inhibition of prostanoid and NO production could not account for the decreased proliferation seen with U0126.

ERK1/2 is activated in a mouse model of lung cancer

Urethane-induced murine lung tumors display molecular and histological similarities to human NSCLC[42]. PhERK1/2 and panERK expression were compared in naive lung, uninvolved lung from tumor bearing lung, and urethane-induced pulmonary tumors from A/J mice. Control A/J lung lysates displayed relatively low levels of ERK1/2 phosphorylation. These levels increase significantly in large tumors harvested 7 months after urethane treatment and more so in even larger tumors harvested 11 months after urethane treatment (Fig. 6 A, B). Adjacent, uninvolved lung tissue from this latter time point displayed a relatively low level of ERK1/2 phosphorylation similar to control lung lysates. The increase in ERK1/2 phosphorylation in lung tumors is expected in this model as 80% of urethane-induced lung tumors in A/J mice carry *Kras* mutations[43].

Discussion

Increasing evidence supports a role for cytokines including TNF α and IFN γ in the development of pulmonary inflammation and lung cancer[44]. Herein, we find that ERK1/2 signaling mediates TNF α /IFN γ induction of pro-inflammatory prostanoids and nitric oxide, and that inhibiting ERK1/2 activity arrests lung epithelial cell proliferation in the G1 cell cycle phase. Since inhibiting PGE₂ and PGI₂ production downstream of ERK1/2 does not inhibit cell proliferation, these PGs are presumably not responsible for the regulation of cell cycle progression. We provide additional evidence for this hypothesis by showing that direct application of prostanoids does not stimulate cell proliferation. NSAIDs can inhibit lung tumor formation *in vivo* and are thought to be chemopreventive in humans. These studies indicate that PG production may not directly affect tumor cell proliferation, but may act instead on stromal cells such as macrophages to stimulate production of pro-angiogenic or growth factors that promote tumor growth.

Exogenous TNF α /IFN γ activates ERK1/2 and increases inflammatory mediator production in cultured lung epithelial cell lines. TNF α elicits its biochemical effects by binding 2 different cell surface receptors, TNFR1 (p55) and TNFR2 (p75). Both TNFR1 and TNFR2 contain intracellular sequences capable of recruiting adaptor proteins that link TNF α binding to stimulate many signaling pathways including ERK1/2. TNF receptors can activate ERK1/2 by several mechanisms, including neutral sphingomyelinase-dependent activation of Raf[45], and direct interaction with the Grb2 and SOS exchange factors which activate Ras[46]. IFN γ binds to the IFN γ receptor complex, which consists of 2 different chains, IFN γ R1 and IFN γ R2, and is thought to activate ERK1/2 signaling through receptor-mediated activation of JAK-STAT transcription factors[47;48]. However, IFN γ failed to activate ERK1/2 phosphorylation in primary human bronchial epithelial cultures[49]. In another study, IFN γ stimulated autocrine production of epidermal growth factor receptor (EGFR) ligands in immortalized human bronchial epithelial cells[50]. This should enhance ERK1/2 signaling via Grb2 and SOS interactions with phosphorylated EGFR, but ERK1/2 activation was not examined in this report[50]. Our results indicate that TNF α /IFN γ stimulate prostanoid and NO production in primary Clara cells in an ERK1/2-dependent manner, however, the precise signaling mechanism by which TNF α /IFN γ treatment leads to ERK1/2 activation in lung epithelial cells is unknown.

Blocking ERK1/2 activation inhibits cytokine-induced production of PGE₂ and PGI₂ in the lung epithelial cells examined, but this may result from different mechanisms in individual cell lines. U0126 strongly suppresses COX-1 and/or COX-2 expression in LM1 and LM2, but not C10 or E10 cells. ERK1/2 phosphorylates and activates cPLA₂ to catalyze release of arachidonic acid from the perinuclear membrane[51]. ERK1/2 activation also triggers downstream signals that activate transcription factors including Elk-1 and AP-1 to induce immediate early genes such as COX-2[52]. The inhibition of cytokine-induced expression of COX-1 and COX-2 proteins in LM2 cells resulting from U0126 treatment suggests that ERK1/2 regulates PGE₂ and PGI₂ through induction of COX enzymes in this cell line. However, U0126 did not affect COX-2 induction by cytokines in C10 and E10 cells, suggesting that U0126 inhibits prostaglandin production by an alternative mechanism. ERK1/2 phosphorylation may activate PGE₂ and PGI₂ synthases downstream of COX, or inhibit the expression and/or activity of prostaglandin dehydrogenase catabolic enzymes. Since these enzymatic activities can also be regulated by post-translational modifications or shifts in sub-cellular localization, protein expression levels alone may be insufficient to account for changes in mediator synthesis by these enzymes. Taken together, ERK1/2 appears to regulate prostaglandin production in a cell line-dependent, and possibly transformation state-dependent manner.

MEK1/2 inhibition decreases NO production by lung epithelial cells to different extents, but iNOS activity is not completely ablated even when ERK1/2 phosphorylation is efficiently blocked. Inhibition of NO production by U0126 may be due to additional activities of this drug. U0126 blocked cytokine-induced iNOS expression in LM2 cells, but more U0126 was required to inhibit iNOS expression (25 μ M) than NO production (10 μ M) in C10 cells. iNOS expression is tightly controlled at the transcriptional level, and ERK1/2-dependent AP-1 activation regulates iNOS expression in A549 lung cancer cells[53]. Availability of L-arginine, the substrate for iNOS, can also affect NO production, and iNOS activity and subcellular localization is also be regulated by phosphorylation[54]. Many cytokines, including TNF α , co-induce L-arginine transport [55] along with iNOS expression, synergistically affecting NO production. If ERK1/2 regulates arginine transport or post-translational modification of iNOS protein, then ERK1/2 could affect NO production without significantly affecting iNOS protein expression.

Inhibition of ERK1/2 produces dramatic growth inhibitory effects in LM2 cells and less dramatic, but still significant effects in C10, E10 and LM1 cells. In contrast to the other cell lines, LM2 cells harbor a mutation in the *Kras* oncogene, which should increase ERK1/2 signaling. Our results suggest that cells with *Kras* mutations are highly dependent on ERK1/2 activation for cell proliferation, but these cells do not necessarily exhibit high levels of basal ERK1/2 phosphorylation. Growth of non-tumorigenic C10 and E10 cells, and of LM1 cells which express only wild type *Kras* [30], may be supported by several pro-proliferative pathways, and may not be as dependent on ERK1/2 signaling as cells expressing mutant *Kras*. In contrast, lung tumor cells with *Kras* mutations may be “addicted” to the signals provided by ERK1/2 activation and unable to proliferate in their absence[56]. U0126 decreased proliferation and induced apoptosis of H460 human NSCLC cells, but the relatively high concentrations of U0126 used (50–100 μ M) may have led to ERK-independent effects[57]. However, MEK1/2 inhibition did not affect cell survival of small cell lung cancer (SCLC) cells in culture[58]. In some cell types, ERK1/2 regulates expression of G1 checkpoint genes including cyclin D1, p21^{Cip1}, and p27^{Kip1}[59], and pharmacological inhibition of MEK1/2 induced G1 arrest and/or apoptosis in many cancer cell line[59]. Since direct inhibition of iNOS activity caused an increase in the percentage of LM2 cells in S-phase, this suggests that NO production regulates cell cycle progression itself. Our results indicate that ERK1/2 may regulate lung epithelial cell cycle progression in G1 at least in part by regulating cyclin D1 protein expression.

ERK1/2 phosphorylation increases in urethane-induced mouse lung tumors in a progression-dependent manner. This correlates with our *in vitro* LM2 data since ~80% of the urethane-induced tumors in A/J mice harbor a *Kras* mutation [43]. ERK1/2 phosphorylation also correlates with tumor progression in mouse lung tumors induced by the tobacco-specific nitrosamine, NNK[60]. ERK1/2 is activated in human NSCLC[23–25], and activation is associated with advanced tumor stage and progression[29]. Strategies to block ERK1/2 activation *in vivo* may prevent lung tumor growth by selectively preventing proliferation and/or inducing apoptosis in tumor but not normal lung cells. Treatment with MEK1/2 inhibitor AZD6244 caused lung tumor regression in an inducible mutant *Kras* mouse model[61]. AZD6244 and other MEK1/2 inhibitors are currently in early clinical development, and NSCLC patients represent an attractive target population for such treatments either as single agents or in combination with other chemotherapeutics[62]. Our results showing increased ERK1/2 phosphorylation during progression of urethane-induced lung tumors in mice further supports use of this model for pre-clinical development of cancer therapeutics. The chemopreventive agent, silibinin, which acts at least in part by inhibiting iNOS expression, decreases tumor cell proliferation and tumor neo-angiogenesis and causes lung tumor regression in the A/J urethane lung tumor model [10;11].

In conclusion, our data support separate pro-inflammatory and proliferative roles for ERK1/2 in cultured mouse lung epithelial cells. We show that TNF α /IFN γ not only stimulates PG and NO production in established lung epithelial cell lines but also in freshly isolated lung epithelial cells. Previous studies have shown that inflammatory mediator production increases in lung tumor tissue[19], and we show herein that ERK1/2 activity increases in tumor tissue. Together these results suggest that targeting the ERK1/2 pathway either alone or in combination with other therapies could be effective in treating both inflammatory lung disease and lung cancer.

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Abbreviations

COPD	chronic obstructive pulmonary disease
COX	cyclooxygenase
ERK1/2	extracellular signal-regulated kinase 1 and 2
IFNγ	interferon gamma
iNOS	inducible nitric oxide synthase
MEK1/2	MAP kinase/ERK kinase 1/2
MKP-1	MAP kinase phosphatase-1
NO	nitric oxide
NSCLC	non-small cell lung cancer
NSAID	nonsteroidal anti-inflammatory drug
PGE₂	prostaglandin E ₂
PGI₂	prostacyclin
PGIS	PGI ₂ synthase
TNFα	tumor necrosis factor alpha

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Figure 1. U0126 blocks cytokine-induced PGE₂, PGI₂, and NO production in nontumorigenic and tumorigenic lung cells

(A) PGE₂ (gray bars; left y-axis) and PGI₂ (black bars; right y-axis) concentrations in media from C10, E10, LM1, and LM2 cells treated with TNFα and IFNγ in the presence or absence of U0126. PG production was significantly decreased by U0126 basally in E10 and LM1 cells (#p < 0.05 vs. untreated cells (no cytokines or U0126)) and in all cytokine-treated cells (*p < 0.01 vs. TNFα/IFNγ treated cells in the absence of U0126). (B) Colorimetric determination of NO content in media from C10, E10, LM1, and LM2 cells treated with TNFα and IFNγ in the presence or absence of U0126. *p < 0.01 vs. TNFα/IFNγ treated cells in the absence of U0126. Graphs (A, B) represent means and standard errors for n = 3 samples and are representative of duplicate experiments. Data was analyzed by one-way ANOVA followed by Newman-Keuls post-hoc analysis.

Figure 2. U0126 inhibits basal and TNF α /IFN γ -induced PG and NO production in primary Clara cells

PGE₂ (A), PGI₂ (B), and NO (C) production by primary Clara cells in the presence (---▲---) or absence (—■—) of TNF α /IFN γ . PGE₂ production differed significantly ($p < 0.01$) between cells in the presence and absence of TNF α /IFN γ at 0 and 1 μ M U0126, and between cells treated or not treated with U0126 at all U0126 concentrations ($p < 0.01$). PGI₂ production differed significantly ($p < 0.05$) between TNF α /IFN γ -untreated and treated cells at 0, 1, and 10 μ M U0126, and between cells in the presence and absence of U0126 at all points except 1 μ M U0126 in the absence of TNF α /IFN γ ($p < 0.01$). There were no significant differences in NO production between TNF α /IFN γ -treated and untreated cells. NO production in TNF α /IFN γ -treated, 0 μ M U0126 Clara cells was significantly higher than that in cells treated with 5, 10, and 25 μ M U0126 in the presence of TNF α /IFN γ (* $p < 0.05$).

Figure 3. U0126 inhibits cytokine-induced phospho-ERK1/2, COX-1, COX-2, and iNOS expression in C10, E10, LM1 and LM2 cells

Immunoblot analysis of (A) pERK1/2, panERK1/2, (B) COX-1, (C) COX-2, or (D) iNOS in serum-deprived cells pretreated with U0126 or DMSO vehicle followed by exposure (+) or not (-) to TNFα/IFNγ. LD = low dose U0126; 1 μM in LM1, LM2 and 10 μM in C10, E10. HD = high dose U0126; 5 μM in LM1, LM2 and 25 μM in C10, E10. Relative protein levels were quantitated by densitometry and normalized to untreated cells (see Table 1).

Figure 4. U0126 inhibits cell proliferation and cyclin D1 expression

(A) Non-tumorigenic (C10, E10) and tumorigenic (LM1, LM2) cells were treated with vehicle, 1, 5, 10 or 25 μ M U0126 for 48h in the presence of serum-containing media. Cell viability was determined by MTS assay. Graph represents pooled means and standard errors from 2 independent experiments of $n = 8$ each. Data was analyzed by one-way ANOVA, using the Newman-Keuls post-hoc test. * $p < 0.05$, ** $p < 0.01$ vs. vehicle-treated control. (B) Cyclin D1 protein expression after U0126 and/or $\text{TNF}\alpha/\text{IFN}\gamma$ treatment in C10, E10, LM1 and LM2 cells. (C) Relative cyclin D1 expression was determined by densitometry and normalized to that of untreated (no $\text{TNF}\alpha/\text{IFN}\gamma$, no U0126) cells. Graph represents means and standard errors for $n = 3$ samples and is representative of duplicate experiments. * $p < 0.05$ vs. 0 μ M U0126, no cytokine groups, # $p < 0.05$ vs. 0 μ M U0126 + $\text{TNF}\alpha/\text{IFN}\gamma$.

Figure 5. Effects of prostaglandins and NO on ERK1/2-mediated proliferation

(A) Direct application of 10 (white bars) and 1000 (black bars) ng/ml PGE₂ and/or the stable PGI₂ analog, iloprost, to LM2 cells does not affect cell number. (B) Inhibition of COX with 1 μ M indomethacin does not inhibit LM2 cell proliferation. (C) The iNOS inhibitor, aminoguanidine (AMG), causes an increase in S phase and a decrease in G2/M in LM2 cell cycle. White bars, control; black bars, 1.0 μ M AMG. * p < 0.01 vs. control. Graph represent means and standard errors for $n=3$ samples and are representative of 2 independent experiments.

Figure 6. ERK1/2 phosphorylation increases during urethane-induced mouse lung tumorigenesis Lung tumors were induced in A/J mice with urethane; control mice received saline vehicle. Control, tumor, and uninvolved lung tissues were harvested at 7 and 11 months after urethane treatment. (A) Western blotting performed with phospho-ERK1/2 and pan-ERK1/2 antibodies. (B) Relative Phospho-ERK/PanERK ratios for each sample in (A). Graph represents means and standard errors for $n = 3-4$ samples. * $p < 0.05$ vs. control, 7 mo. small tumor, 7 mo. large tumor, and 11 mo. uninvolved; # $p < 0.05$ vs. 7 mo. large tumor; $^x p < 0.05$ vs. 7 mo. small tumor.

Table 1

ph-ERK1/2/panERK ratios and relative COX-1, COX-2, and iNOS levels (normalized to values from untreated cells) in TNF α /IFN γ and U0126 treated mouse lung epithelial cells.

Protein [*]	Cell Line	Untreated	LD U0126 ^{***}	HD U0126 ^{***}	TNF α /IFN γ	TNF α /IFN γ + LD U0126	TNF α /IFN γ + HD U0126
phERK/panERK	C10	1.0	0.57 $\pm$ 0.03 ^b	0.84 $\pm$ 0.12 ^b	3.27 $\pm$ 0.84 ^a	0.53 $\pm$ 0.21 ^b	0.20 $\pm$ 0.03 ^b
	E10	1.0	0.09 $\pm$ 0.02 ^{a,b}	0.08 $\pm$ 0.01 ^{a,b}	1.69 $\pm$ 0.10 ^a	0.17 $\pm$ 0.02 ^{a,b}	0.15 $\pm$ 0.06 ^{a,b}
	LM1	1.0	2.24 $\pm$ 0.53 ^{a,b}	0.47 $\pm$ 0.13 ^a	0.15 $\pm$ 0.03 ^a	0.12 $\pm$ 0.02 ^a	0.09 $\pm$ 0.06 ^a
	LM2	1.0	0.54 $\pm$ 0.16 ^{a,b}	0.32 $\pm$ 0.07 ^{a,b}	1.59 $\pm$ 0.25 ^a	0.38 $\pm$ 0.08 ^{a,b}	0.32 $\pm$ 0.24 ^{a,b}
COX-1	C10	1.0	1.64 $\pm$ 0.24	2.11 $\pm$ 0.61	1.56 $\pm$ 0.61	1.14 $\pm$ 0.07	1.79 $\pm$ 0.35
	E10	1.0	1.00 $\pm$ 0.12 ^b	0.95 $\pm$ 0.06 ^b	0.61 $\pm$ 0.10 ^a	0.92 $\pm$ 0.20 ^b	0.96 $\pm$ 0.09 ^b
	LM1	1.0	0.86 $\pm$ 0.11	0.99 $\pm$ 0.06	1.00 $\pm$ 0.07	1.14 $\pm$ 0.05	0.64 $\pm$ 0.51
	LM2	1.0	1.51 $\pm$ 0.19 ^b	1.60 $\pm$ 0.41 ^b	3.42 $\pm$ 0.94 ^a	2.34 $\pm$ 1.1	0.90 $\pm$ 0.18 ^b
COX-2	C10	1.0	0.97 $\pm$ 0.13	2.07 $\pm$ 0.40	1.97 $\pm$ 0.13	3.79 $\pm$ 0.84 ^{a,b}	8.03 $\pm$ 1.4 ^{a,b}
	E10	1.0	0.69 $\pm$ 0.16 ^b	0.47 $\pm$ 0.11 ^b	2.94 $\pm$ 0.17 ^a	3.24 $\pm$ 0.53 ^a	6.30 $\pm$ 0.15 ^{a,b}
	LM1	1.0	0.97 $\pm$ 0.06 ^b	1.19 $\pm$ 0.03 ^a	1.32 $\pm$ 0.02 ^a	1.36 $\pm$ 0.01 ^{a,b}	1.11 $\pm$ 0.11 ^b
	LM2	1.0	1.60 $\pm$ 0.10 ^b	2.35 $\pm$ 0.33 ^b	17.8 $\pm$ 2.8 ^a	9.10 $\pm$ 2.7 ^{a,b}	5.00 $\pm$ 0.99 ^b
iNOS	C10	1.0	0.83 $\pm$ 0.29 ^b	2.41 $\pm$ 3.4 ^b	206 $\pm$ 42 ^a	64.3 $\pm$ 57 ^b	3.23 $\pm$ 5.6 ^b
	E10	1.0	4.37 $\pm$ 5.09 ^b	5.60 $\pm$ 4.4 ^b	249 $\pm$ 7.2 ^{a,b}	133 $\pm$ 20 ^{a,b}	128 $\pm$ 13 ^{a,b}
	LM1	1.0	1.02 $\pm$ 0.11 ^b	1.30 $\pm$ 0.43 ^b	41.5 $\pm$ 8.4 ^a	31.5 $\pm$ 2.9 ^{a,b}	28.5 $\pm$ 4.0 ^{a,b}
	LM2	1.0	0.99 $\pm$ 0.26 ^b	1.11 $\pm$ 0.32 ^b	34.8 $\pm$ 2.2 ^a	24.1 $\pm$ 0.89 ^{a,b}	11.2 $\pm$ 1.6 ^{a,b}

* phERK/panERK, mean $\pm$ SD of ratio of relative densitometric units; COX-1, COX-2, iNOS mean $\pm$ SD of relative densitometric units

** LD=low dose U0126 = 1 μ M for LM1 and LM2 cells and 10 μ M for C10 and E10 cells, HD=high dose U0126 = 5 μ M for LM1 and LM2 cells and 25 μ M for C10 and E10 cells.

^a p < 0.05 vs. control

^b p < 0.05 vs. TNF α /IFN γ alone

Table 2

Lung epithelial cell cycle changes after U0126 treatment.

Cell Line	Treatment	%G1*	%S*	%G2/M*
C10	Vehicle	56.0 ± 1.8	33.5 ± 4.5	10.5 ± 3.1
	10 μM U0126	64.8 ± 1.2 ^a	19.9 ± 0.6 ^a	15.2 ± 0.8
E10	Vehicle	48.7 ± 5.4	38.1 ± 8.3	13.2 ± 4.1
	10 μM U0126	57.6 ± 1.3 ^a	30.6 ± 3.9	11.8 ± 3.1
LM1	Vehicle	27.2 ± 7.7	57.0 ± 12	15.8 ± 6.3
	10 μM U0126	37.6 ± 3.9 ^a	50.6 ± 7.2	11.8 ± 3.6
LM2	Vehicle	41.3 ± 0.5	51.5 ± 0.8	7.2 ± 0.7
	10 μM U0126	84.3 ± 2.7 ^a	11.1 ± 3.1 ^a	4.7 ± 1.2

* Values are mean ± SE of percent cells in cell cycle phase.

^a p < 0.01 vs. vehicle treated