Constitutive NADPH oxidase and increased mitochondrial respiratory chain activity regulate chemokine gene expression

Linda A. Tephly1 and A. Brent Carter1,2

1Department of Medicine, University of Iowa Roy J. and Lucille A. Carver College of Medicine, and 2Iowa City Veterans Administration Medical Center, Iowa City, Iowa

Submitted 23 March 2007; accepted in final form 15 August 2007

Tephly LA, Carter AB. Constitutive NADPH oxidase and increased mitochondrial respiratory chain activity regulate chemokine gene expression. Am J Physiol Lung Cell Mol Physiol 293: L1143–L1155, 2007. First published August 17, 2007; doi:10.1152/ajplung.00114.2007.—Alveolar macrophages, which generate high levels of reactive oxygen species, especially O2•−, are involved in the recruitment of neutrophils to sites of inflammation and injury in the lung, and the generation of chemotactic proteins triggers this cellular recruitment. In this study, we asked whether O2•− generation in alveolar macrophages had a role in the expression of chemokines. Specifically, we hypothesized that O2•− generation is necessary for chemokine expression in alveolar macrophages after TNF-α stimulation. We found that alveolar macrophages have high constitutive NADPH oxidase activity that was not increased by TNF-α, but TNF-α increased the activity of the mitochondrial respiratory chain. In addition, the mitochondrial respiratory chain increased O2•− generation if the NADPH oxidase was inhibited. O2•− generation was necessary for macrophage inflammatory protein-2 (MIP-2) gene expression, because inhibition of NADPH oxidase or the mitochondrial respiratory chain or overexpression of Cu,Zn-superoxide dismutase significantly inhibited expression of MIP-2. TNF-α activated the ERK MAP kinase, and ERK activity was essential for chemokine gene expression. In addition, overexpression of the MEK1/ERK pathway significantly increased IL-8 expression, and a small interfering RNA to the NADPH oxidase inhibited ERK- and TNF-α-induced chemokine expression. Collectively, these results suggest that in alveolar macrophages, O2•− generation mediates chemokine expression after TNF-α stimulation in an ERK-dependent manner.

macrophages; chemokines; superoxide anion; inflammation; reactive oxygen species

REACTIVE OXYGEN SPECIES (ROS) are oxygen-containing molecules that have a greater chemical activity than molecular oxygen. ROS are generated in many immune and inflammatory conditions in the lung and have been associated with progression of disorders by inducing cell injury, apoptosis, and fibrosis (3, 6, 16, 48, 65). The signaling pathways that lead to the generation of ROS have been best characterized in phagocytic cells, such as neutrophils and macrophages. These cells, including alveolar macrophages, generate superoxide anion (O2•−) from the NADPH oxidase complex (4, 5, 37). In addition, O2•− can also be generated by the mitochondrial respiratory chain (37, 54, 63).

TNF-α is a proinflammatory cytokine that plays an integral role in immune and inflammatory responses, and it has been shown to induce the generation of O2•− in phagocytic cells (18, 24, 44, 60). In fact, TNF-α has been shown to stimulate O2•− generation by activating both NADPH oxidase and mitochondria (44, 63). TNF-α activates NADPH oxidase by enhancing the assembly of the enzyme by inducing the expression of regulatory subunits, and it maintains the oxidase in an activated state (34, 69). This activation occurs through stimulation of tyrosine kinases (17, 24). TNF-α also increases the activity of sphingomyelinases, which increase the production of ceramide. The increased level of ceramide stimulates the generation of O2•− in the mitochondria (31, 36).

Although alveolar macrophages are the first line of defense, inflammatory responses in the lung are characterized by the recruitment of neutrophils to the site of inflammation. The prolonged presence of neutrophils is often associated with further inflammation and injury. Neutrophil recruitment is regulated by the expression of inflammatory mediators, such as CXC chemokines (23). Macrophage inflammatory protein-2 (MIP-2), which is a murine CXC chemokine, is a functional homolog of human CXC chemokines, such as IL-8, and is a potent neutrophil chemoattractant (9, 14, 23, 60, 66). TNF-α promotes progression of inflammation by increasing the expression of chemokines, such as MIP-2, in various cell types. Although ROS and TNF-α have been linked separately to chemokine expression, the relationship of TNF-α-induced ROS generation and chemokine expression has not been explored.

Mitogen-activated protein (MAP) kinases are a family of second messengers that are essential for transferring signals from the cell surface to the nucleus, and we have previously shown that MAP kinase activation is necessary for cytokine and chemokine gene expression in alveolar macrophages (10–12). Studies have shown that MAP kinases are activated by ROS (2, 13, 35, 39, 47, 61, 67, 75), and ROS-induced ERK kinase activation has been linked to cell growth, differentiation, and survival (2, 35, 39, 70). These studies also show that both O2•− and H2O2 act as mediators of MAP kinase activation. In contrast to some of these studies, we have demonstrated that high catalase and glutathione peroxidase activity in alveolar macrophages limits the effectiveness of H2O2 to induce inflammatory gene expression (13). We did not, however, determine the effect that O2•− generation had on signaling in alveolar macrophages. To our knowledge, no study has linked TNF-α-induced ROS generation and ERK kinase activation to the production of proinflammatory chemokines, such as MIP-2.

On the basis of these findings, we hypothesized that O2•− generation is necessary for MIP-2 expression after TNF-α...
stimulation in alveolar macrophages. Our study demonstrates that O$_2^-$ generation is increased in alveolar macrophages after stimulation with TNF-α, and TNF-α stimulation increased MIP-2 gene expression. Both the NADPH oxidase and the mitochondrial respiratory chain contributed to O$_2^-$ generation and activation of the ERK kinase. Furthermore, inhibition of O$_2^-$ generation decreased chemokine gene expression after TNF-α stimulation in an ERK-dependent manner.

**MATERIALS AND METHODS**

**Cells.** Human alveolar macrophages and whole blood were obtained by bronchoalveolar lavage and phlebotomy, respectively, from normal volunteers with approval from the Human Subjects Review Board of the University of Iowa Carver College of Medicine. Normal volunteers had to meet the following criteria: 1) aged between 18 and 45 yr, 2) no history of cardiopulmonary disease or other chronic disease, 3) no prescription or nonprescription medication except oral contraceptives, 4) no recent or present evidence of infection, and 5) a lifetime nonsmoker. The volunteers underwent bronchoscopic bronchoscopy with bronchoalveolar lavage after receiving intramuscular atropine (0.6 mg) and local anesthesia. Three separate subsegments of the lung were lavaged with five 20-ml aliquots of normal saline, and the first aliquot in each was discarded. The percentage of alveolar macrophages was determined by Wright-Giemsa stain and varied from 90 to 98%. Cells were plated in serum-free RPMI 1640 (Gibco, Carlsbad, CA) and allowed to adhere for 1 h before experiments. For certain experiments, murine alveolar macrophage (MH-S) cell line was used, and these cells were obtained from American Type Cell Culture (Manassas, VA). MH-S cells were maintained in RPMI 1640 containing 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/l glucose, 1.5 g/l bicarbonate, 2 mM l-glutamine, and 10% fetal bovine serum (HyClone, Logan, UT).

Human neutrophils were isolated from human venous blood by density gradient centrifugation on a Polymorphprep (Nicomed Pharma, Oslo, Norway) for 30 min at room temperature. Erythrocytes were removed by hypotonic lysis, and the isolated neutrophils were plated in the upper compartment of the Transwell filter plate for the transmigration assay, as previously described (45, 51). Briefly, supernatants obtained from human alveolar macrophages were added to the lower compartment of the Transwell at the indicated concentration in a total volume of 750 μl. Neutrophils were added to the Transwell (upper chamber) in a volume of 250 μl. Neutrophils were allowed to migrate over a 3-h period at 37°C, after which migrated cells were collected from the lower chamber for counting in a Coulter counter in triplicate.

**Plasmids, small interfering RNA, and transfections.** A luciferase reporter plasmid driven by the IL-8 5′-flanking sequence of the promoter was constructed using the pBH-luc vector (a gift from Dr. Christian Stratowa, Boehringer Ingelheim, Vienna, Austria). IL-8 5′-flanking sequence from -1480 to +45 was generated by PCR using human genomic DNA as a template and primers with 5′-restriction enzyme site extensions. The IL-8 sequence was ligated into pBH-luc. The University of Iowa DNA Facility verified insert orientation and sequence integrity. The pCMV-MEK1 plasmid (a generous gift from Dr. Roger Davis, University of Massachusetts, Worcester, MA) has been described previously (72). Plasmid transfections were performed utilizing Fugene 6 (Roche, Indianapolis, IN) according to the manufacturer’s instructions. The scrambled and gp91phox small interfering RNAs (siRNAs) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Transfection of siRNAs was performed utilizing Lipofectamine 2000 (Invitrogen, Carlsbad, CA) or DharmaFECT 2 (Dharmacon, Lafayette, CO), according to the manufacturer’s instructions. For luciferase assays, a renilla luciferase plasmid was co-transfected to normalize for transfection efficiency. Luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI), according to the manufacturer’s instructions. Briefly, firefly luciferase expression was measured and the reaction stopped after 10 s. Renilla luciferase activity was then measured for transfection efficiency. Luciferase activity, which is expressed as fold increase from control, is normalized to renilla luciferase expression.

**Adenovirus infection.** Cells were plated in serum-free RPMI 1640 and infected with either an Ad.5-CMVempty, Ad.5-CuZnSOD, or Ad.5-Catalase (Viraiquest, North Liberty, IA) at a multiplicity of infection of 500. After 3 h, serum was added to cells to make a final concentration of 10%. Experiments were performed 48 h after infection in serum-free media.

**Western blot analysis.** Whole cell lysates were prepared by harvesting the cells after culturing in the presence or absence of TNF-α (10 ng/ml) for 3 h. The cells were harvested and resuspended in lysis buffer (1% NP-40, 0.15 M NaCl, 0.05 M Tris, pH 7.4, and protease and phosphatase inhibitors). Samples were separated by SDS-PAGE, and gels were transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ) as previously described (11). The p-MEK1 and p-ERK monoclonal and MAP kinase (MEK) and ERK polyclonal antibodies (Santa Cruz Biotechnology) were used at dilutions of 1:500 and 1:1,000, respectively. The gp91phox monoclonal antibody (BD Biosciences, San Jose, CA) was used at a dilution of 1:1,000. The endogenous mouse gp91phox is expressed as a 58-kDa band because of fewer glycosylation sites in the mouse sequence compared with the human sequence (8). The Cu,Zn-superoxide dismutase (Cu,Zn-SOD) sheep polyclonal antibody (Calbiochem, La Jolla, CA) was used at a dilution of 1:1,000. The p22phox and VDAC1 polyclonal antibodies (Santa Cruz Biotechnology) were used at 1:250. When used, the MEK inhibitor U0126 (Calbiochem) was used at 5 μM and added 30 min before TNF-α.

**Electron spin resonance spectroscopy.** Human alveolar macrophages were placed in chelated PBS (pH 7.4) and stimulated with TNF-α (10 ng/ml) for 15 min before addition of the spin trap, 5,5-dimethyl-1-pyrroline N-oxide (DMPO), at a final concentration of 50 mM. The samples were incubated for the designated amount of time and then scraped and injected into a Bruker EMX electron spin resonance (ESR) spectrometer, and spectra were collected for determination of HOO' generation with the following settings: receiver gain, 1 × 106; modulation amplitude, 1.0 G; modulation frequency, 100.0 kHz; sweep width, 80.0 G; microwave power, 40.1 mW; and frequency, 9.775 GHz. Spectra were the result of eight signal-averaged scans collected over 15 min.

**Extracellular measurement of H$_2$O$_2$.** Measurement of H$_2$O$_2$ release from alveolar macrophages was performed as previously described (13, 56). This method takes advantage of the fact that H$_2$O$_2$ reacts with horseradish peroxidase (HRP), forming compound I, which in turn reacts with p-hydroxyphenyl acetic acid (pHPA), forming a stable fluorescent dimer, (pHPA)$_2$. Cells were cultured in the presence or absence of TNF-α (10 ng/ml) in phenol red-free HBSS (1.0 ml) supplemented with glucose (6.5 mM), HEPES (1 mM), sodium bicarbonate (6 mM), pHPA (1.6 mM), and HRP (95 μg/ml). The amount of H$_2$O$_2$ released into the medium was followed spectrophotometrically every 20 min over a period of 80 min at excitation and emission wavelengths of 323 and 400 nm, respectively. The fluorescent intensity of each sample was corrected for changes in pH and compared with standard concentrations of H$_2$O$_2$ determined by absorption at 240 nm.

**Real-time PCR.** Total RNA was isolated using the Absolutely RNA RT-PCR Miniprep Kit (Stratagene, La Jolla, CA), according to the manufacturer’s instructions. Total RNA (1 μg) was reverse transcribed, and the cDNA was subjected to PCR when 2 μl of cDNA were added to 48 μl of SYBR Green Supermix (Bio-Rad) according to the manufacturer’s instructions. Amplification was then performed in a Bio-Rad iCycler iQ Fluorescence Thermocycler as follows: 3 min at 95°C, followed by 45 cycles of 20 s at 95°C, 20 s at 60°C, and 10 s at 72°C.
amplimer. Fluorescence data were captured to ensure that primer dimers were not contributing to the fluorescence signal generated with SYBR Green I DNA dye. Specificity of the amplification was confirmed using melting curve analysis. Data were collected and recorded by Bio-Rad iCycler iQ software and expressed as a function of threshold cycle (Ct), which is the cycle at which the fluorescence intensity in a given reaction tube rises above background. The gene-specific Ct for each sample was corrected by subtracting the Ct for hypoxanthine phosphoribosyltransferase (HPRT) (ΔCt). Untreated controls were chosen as the reference samples, and the ΔCt for all experimental samples was subtracted by the ΔCt for the control samples (ΔΔCt). Finally, sample mRNA abundance relative to control mRNA abundance was calculated by the formula 2−(ΔΔCt). Specific primer sets used for the murine MIP-2 and the HPRT housekeeping gene are as follows: MIP-2 sense, 5′-AACATTGCGGCTGGGCGGTGTA-3′; MIP-2 antisense, 5′-CCATCAACTGGATGAC-3′; HPRT sense, 5′-CCTCATGGACTGATTATGGAC-3′; HPRT antisense, 5′-CAGATTCACTTGCGGCTCATC-3′. Primers were selected based on nucleotide sequences downloaded from the National Center for Biotechnology Information data bank and designed with software by Integrated DNA Technologies (Coralville, IA).

Mitochondrial and plasma membrane isolation. Cells were cultured in the presence or absence of TNF-α (10 ng/ml) for 30 min. Mitochondria were then isolated by lysing the cells in a mitochondria buffer containing 10 mM Tris, pH 7.8, 0.2 mM EDTA, and protease inhibitors. Lysates were homogenized using a Kontes Pellet Pestle Motor and centrifuged at 1,000 g for 10 min at 4°C. The supernatant was removed and kept at 4°C, and the pellet was lysed, homogenized, and centrifuged again. The two supernatants were pooled and centrifuged at 12,000 g for 15 min at 4°C. After the supernatant was discarded, the pellet was then resuspended in mitochondria buffer. For membrane isolation, cells were lysed in a buffer containing 50 mM Tris·HCl, pH 8.0, 10 mM EDTA, and protease inhibitors. Lysates were homogenized using a Kontes Pellet Pestle Motor and centrifuged at 3,000 rpm for 3 min at 4°C. Supernatants were centrifuged at 100,000 g for 1 h. After removal of the supernatant, the membrane pellet was resuspended in lysis buffer and incubated on ice for 30 min.

Inhibitors. Diphenylene iodonium (DPI; 10 μM), apocynin (500 μg/ml) (Fluka, St. Louis, MO), antimycin A (10 μM), rotenone (25 μM), 2-thiophenylfluorocetone (TFA) (25 μM) (Antioxidant Enzyme Core, Univ. of Iowa), and U0126 (5 μM) were not toxic to the cells, as determined by an ATP assay.

Lucigenin assay. Membrane or mitochondrial protein (10 μg) was diluted in 1× PBS to a final volume of 1 ml. Lucigenin (5 μM) and NADPH (100 μM) (Sigma-Aldrich, St. Louis, MO) were added to each sample, and luminescence was recorded every 30 s for 10 min.

IL-8 and MIP-2 protein expression. Human alveolar macrophages or MH-S cells were cultured for 24 h in the presence or absence of TNF-α (10 ng/ml). The supernatants of the cells were harvested after 24 h, and IL-8 and MIP-2 protein levels were measured by ELISA for human alveolar macrophages and MH-S cells, respectively (DuoSet Kit; R&D Systems, Minneapolis, MN).

Statistical analysis. Statistical comparisons were performed using an unpaired one-tailed t-test. Values in figures are expressed as means with standard error, with the probability of P < 0.05 considered to be significant.

RESULTS

Alveolar macrophages express chemokines and generate \(O_2^-\) after stimulation with TNF-α. TNF-α, which acts by both autocrine and paracrine pathways, stimulates release of chemokines in epithelial and endothelial cells and fibroblasts. We determined whether TNF-α also increased chemokine gene expression in human alveolar macrophages. ELISA was utilized to measure IL-8 protein expression in human alveolar macrophages stimulated with TNF-α (10 ng/ml) for 24 h. Alveolar macrophages had a high expression of IL-8 at baseline, but TNF-α increased IL-8 release significantly (Fig. 1A).

To demonstrate that IL-8 was biologically active, we used a Transwell system to determine neutrophil migration using IL-8 as a chemoattractant. IL-8 was obtained from the supernatant of human alveolar macrophages stimulated with TNF-α (10 ng/ml) for 24 h. Neutrophils were allowed to migrate for 3 h at 37°C. The no. of neutrophils in the lower compartment of the Transwell were counted at 37°C. The no. of neutrophils in the lower compartment of the Transwell were counted by a Coulter counter. MH-S cells were cultured for 3 (C) or 24 h (D) with TNF-α (10 ng/ml). For A and D, cell supernatants were harvested, and IL-8 and macrophage inflammatory protein-2 (MIP-2) were measured by ELISA, respectively. C: total RNA was isolated and reverse transcribed to cDNA. PCR amplification was performed as described in MATERIALS AND METHODS. Data are expressed in pg/ml (A and D) and as fold induction from control (C). For statistical analysis in A, C, and D: *comparison of control with TNF-α. For statistical analysis in B: *comparison of 1 with 50 nM concentration of IL-8.
ing IL-8 (Fig. 1B). We used concentrations of IL-8 that have been published previously (45).

We next determined whether murine alveolar macrophages express MIP-2, which is the functional homolog of IL-8, after stimulation with TNF-α, since some of the studies presented here use these cells. MH-S cells were stimulated with TNF-α for 3 h to evaluate mRNA expression by real-time PCR. TNF-α increased MIP-2 gene expression >35-fold (Fig. 1C). To assure that the increase in mRNA expression reflected an increase in protein, we measured MIP-2 protein release after TNF-α stimulation. Like human alveolar macrophages, MH-S cells had a high expression of the chemokine in control cells, but cells stimulated with TNF-α had a significant increase (*P < 0.0001) in MIP-2 release compared with control cells (Fig. 1D). These data demonstrate that TNF-α-stimulated alveolar macrophages have increased expression of CXC chemokines.

Since TNF-α is known to increase the generation of ROS in various cell types by activating NADPH oxidase and the mitochondrial respiratory chain, we next determined whether human alveolar macrophages have increased O₂•− generation after TNF-α stimulation. We utilized ESR spin trapping to detect HO• generation, because we have previously shown that HO• is primarily derived from O₂•− via the Haber-Weiss reaction in alveolar macrophages (13). Cells were cultured in chelated PBS to avoid any potential iron contamination and then stimulated with TNF-α. The spin trap, DMPO, was added to the culture 15 min after TNF-α. Alveolar macrophages were found to spontaneously generate HO•, and cells stimulated with TNF-α had a significant increase in HO• adducts of DMPO (Fig. 2A). These HO• adducts of DMPO remained increased after longer periods of TNF-α stimulation (data not shown).

In some studies, apocynin, which is a specific inhibitor of the p47phox component of NADPH oxidase, or Cu,Zn-SOD was used to inhibit O₂•− generation or increase dismutation, respectively. This generation of HO• was significantly inhibited, below control levels, by both apocynin and Cu,Zn-SOD (Fig. 2A).

We performed similar studies to determine the effect of mitochondrial O₂•− generation. Human alveolar macrophages were cultured in the presence or absence of antimycin A, an inhibitor of complex III in the mitochondrial respiratory chain. Alveolar macrophages cultured in the presence of TNF-α and antimycin A generated HO• adducts of DMPO that were significantly lower than cells stimulated with TNF-α alone.

**Fig. 2.** TNF-α induces superoxide anion (O₂•−) generation. Human alveolar macrophages were cultured in chelated PBS and stimulated with TNF-α (10 ng/ml) for 30 min. The spin trap, DMPO, was added after 15 min of TNF-α. When used, apocynin (Apo; 500 μg/ml) or Cu,Zn-superoxide dismutase (Cu,Zn-SOD; 1,000 U) (A) or antimycin A (Ant A; 10 μM) (B) was added 30 min before stimulation with TNF-α. Electron spin resonance (ESR) was performed as described in MATERIALS AND METHODS. All ESR spectra are presented with the same ordinate scale.
Taken together, these data demonstrate that alveolar macrophages stimulated with TNF-α have a significant increase in O$_2$•$^-$ generation and MIP-2 gene expression, but the relative contribution of the NADPH oxidase and the mitochondrial respiratory chain to O$_2$•$^-$ generation and MIP-2 production remains undetermined.

Since inhibition of both the NADPH oxidase and the mitochondrial respiratory chain inhibited O$_2$•$^-$ generation, and to determine the relative contribution of each enzyme, we next determined the kinetics of each enzyme activity separately in the membrane or mitochondrial fractions. Both membrane and mitochondrial fractions were isolated from human alveolar macrophages cultured for 30 min in the presence or absence of TNF-α. O$_2$•$^-$ generation was measured by incubating membrane or mitochondrial protein with lucigenin (5 μM) and NADPH (100 μM), as an electron donor, in PBS. After a 2-s delay, luminescence was immediately measured over a 10-min period in increments of 30 s. We found that O$_2$•$^-$ generation, as measured by luminescence, was significantly greater in the membrane fraction ($P < 0.0001$) compared with the mitochondrial fraction, but TNF-α did not increase the activity in the membrane fraction (Fig. 3A). O$_2$•$^-$ generation also increased over the 10-min period in mitochondria obtained from control cells, and this activity was significantly enhanced in the mitochondria obtained from alveolar macrophages stimulated with TNF-α (Fig. 3B). To ensure that the isolated cellular fractions contained NADPH oxidase and mitochondria, we performed Western blot analysis for p22phox and voltage-dependent anion channel-1 (VDAC1) from membrane and mitochondrial protein fractions. We found that p22phox expression in membrane fractions did not change with TNF-α stimulation (Fig. 3A, inset). Similarly, we found VDAC1 in the mitochondrial fractions, and TNF-α did not change the protein expression (Fig. 3B, inset). The membrane fractions contained small amounts of VDAC1, but the mitochondrial fractions did not contain p22phox, indicating a fairly pure isolation of each cellular fraction.

We next investigated whether there was cross talk between the NADPH oxidase and the mitochondrial respiratory chain in relation to O$_2$•$^-$ generation. Human alveolar macrophages were cultured in the presence or absence of apocynin or antimycin A for 30 min before being stimulated with TNF-α for 30 min. The mitochondrial fraction was isolated from cells cultured with apocynin, and the membrane fraction was isolated from cells cultured with antimycin A, as described above. We found that inhibition of complex III of the mitochondrial respiratory chain had no significant effect on O$_2$•$^-$ generation in the membrane fraction (Fig. 3C). In contrast, inhibition of p47phox with apocynin significantly enhanced O$_2$•$^-$ generation in the mitochondrial fraction to a level similar to that seen in the membrane fraction alone (Fig. 3D). As a negative control, the vehicle, DMSO, was used in all experiments, and there was no effect on O$_2$•$^-$ generation. In aggregate, these data demonstrate that alveolar macrophages stimulated with TNF-α generate O$_2$•$^-$ by activating the mitochondrial respiratory chain and that the activity of the NADPH oxidase is constitutive, although its overall activity is significantly greater than that of the mitochondrial respiratory chain. In addition, these data demonstrate for the first time that O$_2$•$^-$ generation increases significantly in the mitochondria if the NADPH oxidase is inhibited in alveolar macrophages stimulated with TNF-α. That is, the mitochondrial respiratory chain appears to compensate for the lack of NADPH activity. The relationship of TNF-α-induced ROS generation and chemokine expression, however, is not clearly linked based on this data.

$O_2$•$^-$ generation is necessary for MIP-2 gene expression after TNF-α stimulation. We first determined the relationship of O$_2$•$^-$ generation and MIP-2 gene expression using DPI, a nonspecific inhibitor of flavoproteins such as NADPH oxidase, myeloperoxidase, and nitric oxide synthase, in MH-S cells.
which can be infected with adenoviral vectors or transfected with plasmids or siRNA, unlike human alveolar macrophages, to maintain consistency throughout these experiments. Cells were cultured in the presence or absence of DPI (10 μM) before stimulation with TNF-α for 3 h. The cells stimulated with TNF-α had a significant increase in MIP-2 gene expression (P < 0.0002), while cells cultured with DPI before TNF-α stimulation had a significant decrease (P < 0.0005) to near control levels (Fig. 4A).

To evaluate whether the effect of DPI was due to decreased O$_2^•^−$ generation from NADPH oxidase, we performed similar experiments with apocynin. Apocynin inhibited MIP-2 gene expression significantly (P < 0.0107) compared with alveolar macrophages stimulated with TNF-α alone (Fig. 4B), indicating that the constitutive activity of the NADPH oxidase is important for TNF-α signaling. We also measured myeloperoxidase activity and performed experiments with l-NNMA, an inhibitor of nitric oxide synthase. We found no significant myeloperoxidase activity in alveolar macrophages, and l-NNMA had no significant effect on the ability of alveolar macrophages to express MIP-2 (data not shown).

To further confirm the role of NADPH oxidase and avoid the nonspecific effects of the pharmacological inhibitor, we transfected MH-S cells with a scrambled or a gp91$^{phox}$ siRNA. After 48 h, cells were cultured in the presence or absence of TNF-α for 3 h, and whole cell lysates were obtained. We performed appropriate controls by confirming gene knockdown in cells transfected with gp91$^{phox}$ siRNA. Cells transfected with the scrambled siRNA had expression of gp91$^{phox}$ protein, which was detected at 58 kDa, consistent with previous data, because of fewer glycosylation sites in the mouse sequence compared with the human sequence (8). Cells transfected with the gp91$^{phox}$ siRNA had a significant reduction in protein expression (Fig. 4C). To determine the effect of gp91$^{phox}$ siRNA on MIP-2 expression, we obtained total RNA after 3 h of TNF-α stimulation. We found that TNF-α significantly increased MIP-2 gene expression in cells transfected with the scrambled siRNA, while MIP-2 expression was significantly inhibited to near control levels (P < 0.0315) in cells transfected with the gp91$^{phox}$ siRNA (Fig. 4C). Similar results were obtained in cells transfected with a p47$^{phox}$ siRNA (data not shown). These data demonstrate that NADPH oxidase has a role in regulating chemokine gene expression in alveolar macrophages stimulated with TNF-α.

Since TNF-α increased activation of the mitochondrial respiratory chain, we asked whether mitochondrial O$_2^•^−$ generation had a role in MIP-2 gene expression. To maintain consistency with the above data, we cultured MH-S cells in the presence or absence of antimycin A, an inhibitor of complex III in the mitochondrial respiratory chain, before stimulation with TNF-α. As shown in the above data, TNF-α significantly increased MIP-2 gene expression, but MIP-2 gene expression was significantly inhibited (P < 0.0025) in cells exposed to antimycin A before TNF-α (Fig. 5A). We performed similar experiments with the complex I inhibitor rotenone and the complex II inhibitor TTFA. Both rotenone and TTFA inhibited TNF-α-induced MIP-2 gene expression to a similar extent as antimycin A (Fig. 5, B and C). These data demonstrate that activation of the mitochondrial respiratory chain is necessary for TNF-α to induce chemokine expression and suggest that O$_2^•^−$ generation is important for MIP-2 gene expression. Taken together, these data suggest that both NADPH oxidase and the mitochondrial respiratory chain contribute to the regulation of chemokine gene expression in TNF-α-stimulated alveolar macrophages.

To further confirm our hypothesis that O$_2^•^−$ generation is necessary for MIP-2 gene expression, we infected MH-S cells with a replicative-deficient adenovirus vector expressing either an empty vector or human Cu,Zn-SOD. After 48 h, cells were stimulated with TNF-α, and whole cell lysates were separated by SDS-PAGE. Western blot analysis confirmed that cells expressing Cu,Zn-SOD had increased SOD protein expression.
Cells infected with these adenoviral vectors were also stimulated with TNF-α/H9251 for 3 h, and MIP-2 gene expression was determined by real-time PCR. MIP-2 mRNA levels were increased after TNF-α/H9251 in cells expressing the empty vector, while cells expressing Cu,Zn-SOD had a significant reduction (P < 0.0350) in MIP-2 expression (Fig. 6B). Cu,Zn-SOD is found both in the cytosol and in the intermembrane space of the mitochondria. Thus these data suggest that overexpression of Cu,Zn-SOD inhibits MIP-2 expression secondary to a reduction in mitochondrial O2•− generation in the intermembrane space. Overexpression of Mn-SOD had no significant effect on MIP-2 gene expression (data not shown). Taken together, these data demonstrate that O2•− generation is necessary for chemokine gene expression in alveolar macrophages stimulated with TNF-α. This also supports the hypothesis that both the NADPH oxidase and mitochondrial respiratory chain regulate chemokine gene expression.

H2O2 does not mediate MIP-2 gene expression. Since O2•− spontaneously dismutates to H2O2 at a rapid rate, and Cu,Zn-SOD increases the rate of dismutation (29, 30, 46), we wanted to determine whether H2O2, instead of O2•−, was the mediator of MIP-2 gene expression. We performed real-time PCR for MIP-2 mRNA accumulation in MH-S cells, which can be infected with adenoviral vectors, unlike human alveolar macrophages, to maintain consistency throughout these experiments. Cells were stimulated with exogenous H2O2 or cultured with aminotriazole (ATZ), an irreversible inhibitor of catalase, for 3 h. Compared with cells stimulated with TNF-α alone, MIP-2 gene expression in cells stimulated with H2O2 or cultured with ATZ was no different than control (Fig. 7A). To confirm that the effects of H2O2 did not occur at an earlier time point, MH-S cells were stimulated with exogenous H2O2 for 60, 90, and 120 min. Similar to the 3-h time point, MIP-2 gene expression in cells exposed to H2O2 was no different than control (Fig. 7A, inset).

Since it is known that catalase is highly expressed in alveolar macrophages (13, 42), we wanted to confirm that H2O2 does not decrease after TNF-α stimulation. We cultured MH-S cells (Fig. 6A). Cells infected with these adenoviral vectors were also stimulated with TNF-α for 3 h, and MIP-2 gene expression was determined by real-time PCR. MIP-2 mRNA levels were increased after TNF-α in cells expressing the empty vector, while cells expressing Cu,Zn-SOD had a significant reduction (P < 0.0350) in MIP-2 expression (Fig. 6B). Cu,Zn-SOD is found both in the cytosol and in the intermembrane space of the mitochondria. Thus these data suggest that overexpression of Cu,Zn-SOD inhibits MIP-2 expression secondary to a reduction in mitochondrial O2•− generation in the intermembrane space. Overexpression of Mn-SOD had no significant effect on MIP-2 gene expression (data not shown). Taken together, these data demonstrate that O2•− generation is necessary for chemokine gene expression in alveolar macrophages stimulated with TNF-α. This also supports the hypothesis that both the NADPH oxidase and mitochondrial respiratory chain regulate chemokine gene expression.
in the presence or absence of TNF-α for various amounts of time to determine the average amount of H₂O₂ released from the cell as a function of time. We found that TNF-α stimulation had no significant effect on H₂O₂ generation (Fig. 7B), and overexpression of Cu/Zn-SOD augmented H₂O₂ generation (data not shown). More importantly, the endogenous catalase did not decrease H₂O₂ generation. These data support the notion that H₂O₂ does not mediate MIP-2 gene expression.

To further confirm that H₂O₂ does not mediate TNF-α-induced MIP-2 gene expression, MH-S cells were infected with a replication-deficient adenovirus expressing either an empty vector or a catalase expression vector. After 48 h, the cells were stimulated with TNF-α for 3 h, and whole cell RNA was isolated. MIP-2 gene expression was determined by real-time PCR. There was no significant effect on TNF-α-induced MIP-2 gene expression between cells expressing catalase, a peroxide-removing enzyme, and cells expressing the empty vector (Fig. 7C). These data indicate that H₂O₂ does not mediate MIP-2 gene expression in alveolar macrophages stimulated with TNF-α.

Finally, to support our hypothesis that O₂⁻⁻ rather than H₂O₂ regulates chemokine gene expression, we performed ESR spin trapping at a later time point to determine whether O₂⁻⁻ was still generated by TNF-α stimulation. Human alveolar macrophages were cultured in chelated PBS to avoid any potential iron contamination and then stimulated with TNF-α for 3 h, which was the time point we used to perform real-time PCR. The spin trap, DMPO, was added to the culture 15 min after TNF-α. We found that HO• was spontaneously generated, but cells stimulated with TNF-α for 3 h had a significant increase in HO• adducts of DMPO (Fig. 7D). Thus, these data suggest that O₂⁻⁻ generation is maintained in alveolar macrophages stimulated with TNF-α, and that the generated O₂⁻⁻ regulates the expression of chemokine genes.

**ERK kinase activation by TNF-α depends on NADPH oxidase and mitochondrial respiratory chain activity.** MAP kinases are essential for transferring signals from the cell surface to the nucleus, so we evaluated the role of MAP kinase activation in alveolar macrophages following TNF-α stimulation. We used MH-S cells, which can be transfected with siRNA, unlike human alveolar macrophages, to maintain consistency throughout these experiments. Cells were stimulated with TNF-α at designated time points, and ERK kinase activation was evaluated by Western blot analysis using an antibody specific for p-ERK. TNF-α increased ERK activity, as measured by p-ERK at 15 min after stimulation, and it was maximally activated at 3 h (Fig. 8A). ERK activation remained increased above control levels at all time points between 15 min and 3 h. We found that both the ERK1 and ERK2 MAP kinases were strongly activated by TNF-α, but the ERK2 MAP

---

**Fig. 7.** H₂O₂ does not mediate MIP-2 gene expression. A: MH-S cells were cultured in the presence or absence of aminotriazole (ATZ; 20 mM), an irreversible inhibitor of catalase, for 30 min and then stimulated with TNF-α (10 ng/ml) or H₂O₂ (25 μM) for 3 h. B: MH-S cells were cultured in the presence or absence of TNF-α (10 ng/ml) in phenol red-free HBSS supplemented with glucose, HEPES, sodium bicarbonate, p-hydroxyphenyl acetic acid (pHPA), and horseradish peroxidase. The amount of H₂O₂ released into the medium was followed spectrofluorometrically by measuring the formation of the fluorescent dimer, (pHPA)₂, at excitation and emission wavelengths of 323 and 400 nm, respectively. C: MH-S cells were infected with Ad.CMV vector or Ad.Cat at 500 MOI. After 48 h, cells were cultured in the presence or absence of TNF-α (10 ng/ml) for 3 h. A, inset; and C: total RNA was isolated and reverse transcribed to cDNA. PCR amplification was performed as described in MATERIALS AND METHODS. Data are expressed as fold induction of MIP-2 mRNA from control. *comparison of control with TNF-α (Ad.Cat). **comparison of control with TNF-α (Ad.Cat).
ROS generation regulates ERK expression

Since MEK is the upstream kinase that activates ERK and ROS have been shown to activate ERK by the classically described mechanism (2, 35, 39, 70), we determined whether TNF-α increased ERK by activating MEK. MH-S cells were stimulated with TNF-α for 3 h, and MEK1 activation was evaluated by Western blot analysis with an antibody specific for p-MEK1. TNF-α increased MEK1 activity, as measured by p-MEK1 (Fig. 8B).

We next asked whether the activity of NADPH oxidase regulated ERK activation. Cells were cultured in the presence or absence of apocynin before stimulation with TNF-α for 3 h. TNF-α increased activation of ERK, whereas ERK activity was significantly inhibited to control levels by apocynin, as measured by the presence of p-ERK (Fig. 8C).

To further confirm the role of NADPH oxidase and avoid the nonspecific effects of the pharmacological inhibitor, we transfected MH-S cells with a scrambled or a gp91phox siRNA. After 48 h, cells were cultured in the presence or absence of TNF-α for 3 h, and whole cell lysates were obtained. We performed appropriate controls by confirming gene knockdown in cells transfected with the scrambled siRNA. Cells transfected with the scrambled siRNA had expression of gp91phox protein, while cells transfected with the gp91phox siRNA had a significant reduction in protein expression (Fig. 8D). Cells transfected with the scrambled siRNA had an increase in ERK MAP kinase activation after TNF-α stimulation. In contrast, cells transfected with gp91phox siRNA had a decrease in ERK activation in both control and TNF-α-stimulated cells (Fig. 8D). These results demonstrate that the constitutive activity of NADPH oxidase regulates ERK activation in alveolar macrophages stimulated with TNF-α.

To evaluate the role of the mitochondrial respiratory chain, alveolar macrophages were cultured in the presence or absence of antimycin A before stimulation with TNF-α for 3 h. ERK was activated in cells stimulated with TNF-α alone, whereas ERK activity was significantly inhibited in cells cultured with the complex III inhibitor antimycin A (Fig. 8E). In aggregate, these data suggest that both the NADPH oxidase and the mitochondrial respiratory chain play a critical role in regulating ERK activation in TNF-α-stimulated alveolar macrophages. These data demonstrate that the inactivation of NADPH oxidase or the mitochondrial respiratory chain inhibits ERK activation in TNF-α-stimulated alveolar macrophages. The ERK MAP kinase, however, did not regulate O2•− generation from the mitochondria (data not shown).

Because of the fact that TNF-α activated the ERK kinase, we asked whether ERK kinase activity had any effect on MIP-2 gene expression. MH-S cells were cultured in the presence or absence of U0126, a specific inhibitor of MEK1, which is the upstream kinase that activates ERK, before stimulation with TNF-α. We first confirmed that ERK kinase activation was inhibited by U0126 (data not shown). We utilized real-time PCR and found that MIP-2 gene expression was significantly reduced to near control levels (P < 0.0010) in cells exposed to U0126 (Fig. 9A).

Since pharmacological inhibitors may have nonspecific effects, we determined the role of ERK on chemokine gene expression by overexpressing constitutively active MEK1,
renilla luciferase, was significantly increased in TNF-α/H9251 expression vector. After 24 h, cells were stimulated with either an empty vector or the constitutively active MEK1 transfected with a luciferase driven by the IL-8 promoter and transiently activates ERK (10). MH-S cells were transiently co-previously demonstrated that this expression vector constitutively activates ERK (10). We have previously shown that alveolar macrophages spontaneously generate high levels of ROS, especially O2•−, and that high catalase and glutathione peroxidase activity in alveolar macrophages limits the effectiveness of H2O2 to act as a signaling mediator (13). In this study, we asked whether O2•− generation in alveolar macrophages had a role in the expression of inflammatory genes. Specifically, we hypothesized that O2•− generation is necessary for chemokine gene expression in alveolar macrophages after TNF-α stimulation. We found that TNF-α increased O2•− generation and induced the expression of MIP-2 in alveolar macrophages. TNF-α-induced O2•− generation was necessary for MIP-2 gene expression because inhibition of the constitutively active NADPH oxidase, inhibition of the mitochondrial respiratory chain, or overexpression of Cu/Zn-SOD significantly inhibited expression of MIP-2. In addition, TNF-α-induced ROS generation was necessary for ERK MAP kinase activation, and the activation of the MEK→ERK pathway was necessary and sufficient for chemokine gene expression in alveolar macrophages stimulated with TNF-α. These results suggest that in alveolar macrophages, O2•− generation from both NADPH oxidase and the mitochondrial respiratory chain mediates MIP-2 gene expression after TNF-α stimulation in an ERK-dependent manner.

**DISCUSSION**

We have previously shown that alveolar macrophages spontaneously generate high levels of ROS, especially O2•−, and that high catalase and glutathione peroxidase activity in alveolar macrophages limits the effectiveness of H2O2 to act as a signaling mediator (13). In this study, we asked whether O2•− generation in alveolar macrophages had a role in the expression of inflammatory genes. Specifically, we hypothesized that O2•− generation is necessary for chemokine gene expression in alveolar macrophages after TNF-α stimulation. We found that TNF-α increased O2•− generation and induced the expression of MIP-2 in alveolar macrophages. TNF-α-induced O2•− generation was necessary for MIP-2 gene expression because inhibition of the constitutively active NADPH oxidase, inhibition of the mitochondrial respiratory chain, or overexpression of Cu/Zn-SOD significantly inhibited expression of MIP-2. In addition, TNF-α-induced ROS generation was necessary for ERK MAP kinase activation, and the activation of the MEK→ERK pathway was necessary and sufficient for chemokine gene expression in alveolar macrophages stimulated with TNF-α. These results suggest that in alveolar macrophages, O2•− generation from both NADPH oxidase and the mitochondrial respiratory chain mediates MIP-2 gene expression after TNF-α stimulation in an ERK-dependent manner.

One hallmark of the cellular response to inflammation and injury, such as acute lung injury, is the recruitment of neutrophils to sites of inflammation. The generation of neutrophil chemotactic proteins, such as MIP-2, triggers neutrophil recruitment, which often promotes further inflammation (71, 73). In addition, the human functional homolog of MIP-2, IL-8, has been associated with the development of acute lung injury (1, 15, 22, 32). Many cell types, including inflammatory cells, endothelial and epithelial cells, and fibroblasts, produce chemokines. Our data demonstrate that neutrophil recruitment occurs in a concentration-dependent manner, suggesting that these alveolar macrophages play an integral role in neutrophil recruitment.

TNF-α is a proinflammatory cytokine that has been linked to both injury and repair, and several studies have shown that TNF-α can activate NADPH oxidase (24, 34, 43, 69). TNF-α activates NADPH oxidase by enhancing the assembly of the enzyme by inducing the expression of regulatory subunits. The assembly after TNF-α stimulation appears to be regulated by activation of p47phox (17, 43). Although p47phox is phosphorylated on multiple serine residues by PKCs (7, 25, 26, 28), Akt (38), and MAP kinases (20, 21, 25, 27), TNF-α also stimulates the activation of tyrosine kinases (24). In particular, one study in lung endothelial cells has shown that p47phox undergoes tyrosine phosphorylation by directly associating with Src (17). Our data demonstrate that O2•− generation is significantly reduced with a specific p47phox inhibitor after TNF-α stimulation. Furthermore, our data demonstrate that the knockdown of gp91phox by transfection of an siRNA regulates activation of the ERK MAP kinase. The novel aspect of our study, however,
is that we show for the first time that this inhibitor, apocynin, and the knockdown of gp91-phox with transfection of siRNA also regulate the expression of ERK kinase, and that ERK kinase regulates CXC chemokine gene expression in alveolar macrophages after TNF-α stimulation.

Normal mitochondrial respiration is a major source of ROS, and O2•− is one of the primary by-products (54). TNF-α is known to increase mitochondrial O2•− generation (31, 33, 37, 50, 63, 64), and the mechanism has been shown to be secondary to the accumulation of ceramide (31, 36, 62). TNF-α-induced generation of ceramide results from the activation of neutral and acid sphingomyelinases (41, 57). Most of these studies showing generation of ROS from mitochondria demonstrated TNF-α-induced apoptosis or cytotoxicity. Only one study has shown an indirect relationship between TNF-α-induced gene transcription and mitochondrial ROS generation (64). This study showed that interference with the mitochondrial respiratory chain could inhibit NF-κB activation by TNF-α.

Both complex I and complex III generate O2•− during normal mitochondrial respiration. Only complex III, however, can generate O2•− on both sides of the inner mitochondrial membrane (53). The O2•− generated in the intermembrane space can cross the outer mitochondrial membrane into the cytoplasm (53). Thus Cu/Zn-SOD, which is located in the cytoplasm and the intermembrane space (55), can catalyze the dismutation of O2•−, as our data demonstrate. Our data demonstrate that TNF-α significantly increases the activity of the mitochondrial respiratory chain and that O2•− generation is significantly reduced by the inhibition of complex III in the mitochondrial respiratory chain.

Studies have shown that there is some interaction between NADPH oxidase and the mitochondrial respiratory chain. The gp91-phox subunit of NADPH oxidase in endothelial cells generates ROS, which have been linked to activation of the mitochondrial respiratory chain (52); other studies have shown that the mitochondria regulate Nox1 redox signaling (19, 40). Our data demonstrate, for the first time, that inhibition of NADPH oxidase results in increased O2•− generation by the mitochondrial respiratory chain. This increase, however, is not sufficient for chemokine gene expression. The mechanism by which mitochondrial O2•− generation is increased in the setting of an inactive NADPH oxidase can be the focus of future investigations.

Activation of the ERK MAP kinase by ROS, including O2•−, has been shown in many studies (2, 35, 39, 61, 70). Only one of these studies implicates O2•− generation by the mitochondria as being necessary for ERK kinase activation (61). In contrast to our findings, this study showed that Mn-SOD, which is located in the mitochondrial matrix, decreased ERK activation. Other studies have focused on ROS generated by NADPH oxidase. These studies indicate that ERK is activated by the classically described (Ras → Raf → MEK → ERK) mechanism (2, 35, 39, 70). One study also demonstrates that tyrosine kinase activation was necessary for ERK activation (70). In our study, we did not evaluate the activity of tyrosine kinases, Ras, or Raf, but we found that MEK was activated by TNF-α stimulation. We also found that overexpression of the MEK→ERK pathway increased CXC chemokine gene expression alone, and the expression was augmented with TNF-α stimulation. This suggests that ERK is activated by the classical mechanism. Furthermore, our data indicate that both NADPH oxidase and the mitochondrial respiratory chain regulate ERK activity and suggest that this activity was dependent on O2•− generation, since the production was maintained at 3 h, which was the time of maximal ERK phosphorylation.

The regulation of cytokine and chemokine gene expression by MAP kinases, including ERK kinase, has been extensively studied. We and others have found that activation of ERK kinase is necessary for inflammatory gene expression (12, 49, 58, 59, 68, 74), so it is not surprising that ERK regulated MIP-2 gene expression. Previous studies, however, have shown effects on cell growth, differentiation, and survival when linking O2•− (39, 70) or H2O2 (2, 35) generation to ERK. We have shown that alveolar macrophages have limited H2O2-mediated signaling because of high levels and activities of peroxide-removing enzymes (13). Thus a novel feature of the present study is that we show for the first time that O2•− generation regulates the expression of a chemokine in alveolar macrophages after TNF-α stimulation in an ERK-dependent manner. These results suggest that alveolar macrophages initiate the response to inflammatory conditions in the lung by expressing CXC chemokines to recruit neutrophils to sites of inflammation.

ACKNOWLEDGMENTS

We thank Sean Martin from the University of Iowa Carver College of Medicine Electron Spin Resonance Facility for assistance in performing some of the studies, Kevin Orcutt and Brett Wagner for technical assistance, Roger Davis for the pCMV-MEK1 plasmid, Christian Stratowa for the pBH vector, Dwight Look and Lori Manzel for the pBH-IL-8-luc plasmid, and Douglas Spitz for valuable discussions and review of the manuscript.

GRANTS

This work was supported by a Veterans Affairs Merit Review and an American Lung Association Career Investigator Award (both to A. B. Carter).

REFERENCES

ROS GENERATION REGULATES ERK EXPRESSION


