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The role of sphingosine 1-phosphate in the TNF- α induction of IL-8 gene expression in lung epithelial cells

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Abstract

Tumor necrosis factor- α (TNF- α) is an important cytokine involved in the pathogenesis of inflammatory diseases of the lung. Interleukin-8 (IL-8), a C-X-C chemokine, is induced by TNF- α and initiates injury by acting as a chemoattractant for neutrophils and other immune cells. Although sphingolipids such as ceramide and sphingosine 1-phosphate (S1-P) have been shown to serve as signaling molecules in the TNF- α inflammatory response, their role in the TNF- α induction of IL-8 gene expression in lung epithelial cells is not known. We investigated the role of sphingolipids in the TNF- α induction of IL-8 gene expression in H441 lung epithelial cells. We found that TNF- α induced IL-8 mRNA levels by increasing gene transcription, and the stability of IL-8 mRNA was not affected. Exogenous S1-P but not ceramide or sphingosine increased IL-8 mRNA levels and IL-8 secretion. Dimethylsphingosine, an inhibitor of sphingosine kinase, partially inhibited TNF- α induction of IL-8 mRNA levels indicating the importance of intracellular increases in S1-P in the IL-8 induction. S1-P induction of IL-8 mRNA was due to an increase in gene transcription, and the stability of IL-8 mRNA was not affected. S1P induction of IL-8 mRNA was associated with an increase in the binding activity of AP-1 but the activities of NF- κ B and NF IL-6 were unchanged. S1-P induced the phosphorylation of ERK, p38 and JNK MAPKs. Pharmacological inhibitors of ERK and p38 but not JNK partly inhibited S1-P induction of IL-8 mRNA levels. These data show that increases in the intracellular S1-P partly mediate TNF- α induction of IL-8 gene expression in H441 lung epithelial cells via ERK and p38 MAPK signaling pathways and increased AP-1 DNA binding.

Keywords

sphingolipids; transcription; lung injury; inflammation

1. Introduction

The respiratory epithelium contributes to normal lung function by maintaining alveolar stability through secretion of surfactant, clearance of inhaled particulates by mucociliary action, and facilitates phagocytosis of pathogens by secretory immunoglobulin and surfactant production. Additionally the respiratory epithelium plays important roles in the control of various aspects of inflammation such as initiation, amplification and down regulation as well as tissue repair through the elaboration of various mediators including cytokines (Standiford et al., 1990; Stadnyk, 1994) and surfactant (Crouch and Wright, 2001). Tumor necrosis factor- α (TNF- α), a cytokine expressed predominantly by the alveolar macrophages has been implicated

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in the pathophysiology of inflammatory diseases of the lung resulting from acute injury due to sepsis, infections, and pulmonary fibrosis (Tracey et al., 1988; Piguet, 1990; Suter et al., 1992). TNF- α levels are markedly elevated in the bronchoalveolar lavage fluid from patients with acute respiratory distress syndrome (ARDS) (Suter et al., 1992). Elevated TNF- α levels are associated with increased IL-8 levels, and TNF- α is a major inducer of IL-8 expression in lung epithelial cells (Standiford et al., 1990). IL-8, a member of the C-X-C family of chemokines is a potent chemoattractant and an activator of neutrophils, T cells and other immune cells (Kunkel et al., 1995). Neutralizing IL-8 antibodies prevented lung injury in animal models of lung disease, indicating that IL-8 is an important mediator of lung injury (Broaddus et al., 1994) (Modelska et al., 1999).

The sphingolipid ceramide has long been recognized as a signaling molecule in the inflammatory response. It acts as a second messenger in the signal transduction pathway activated by a host of stress agents including TNF- α (Kim et al., 1991), ionizing radiation (Haimovitz-Friedman et al., 1994) and platelet activating factor (PAF) (Goggel et al., 2004). Ceramide can be produced via de novo synthesis or hydrolysis of membrane sphingomyelin by sphingomyelinase enzymes. TNF- α increases intracellular levels of ceramide by activating lysosomal and plasma membrane-associated sphingomyelinases that display different pH and metal requirements. There is accumulating evidence to suggest that sphingolipid metabolites contribute to the pathophysiology of acute lung injury. Lung cells express high levels of sphingolipid enzymes and sphingolipid metabolites, and sphingolipid levels are elevated in acute lung injury. Intratracheal administration of TNF- α and ceramide into rats increases lung permeability and decreases surface tension lowering effects of surfactant (Ryan et al., 2003). In other studies, platelet activating factor (PAF) was found to cause lung edema in rats (Goggel et al., 2004). PAF induced edema was suppressed in acid sphingomyelinase deficient animals and was reduced by the non-specific acid sphingomyelinase inhibitors D609 and imipramine indicating that acid sphingomyelinase mediated production of ceramide is responsible for the edema (Goggel et al., 2004). Ceramide once produced can be metabolized further into sphingosine and S1-P by the sequential actions of ceramidase and sphingosine kinase respectively. These metabolites of ceramide play a variety of roles in diverse cellular activities such as cell growth and survival, cell motility and immunity (Spiegel and Milstien, 2003).

Although TNF- α induction of IL-8 gene expression in lung epithelial cells has been studied previously, information is lacking on the role of sphingolipid metabolites in the induction process. Considering that the respiratory epithelium plays key roles in the initiation and amplification of inflammatory responses and that sphingolipid metabolites are recognized as important signaling molecules in the inflammatory response, we sought to understand the roles of sphingomyelin metabolites in the TNF- α induction of IL-8 mRNA. In this investigation, we studied the effects of TNF- α and sphingolipids on IL-8 gene expression in lung H441 cells, a cell line with characteristics of bronchiolar (Clara) epithelial cells. We found that TNF- α induced IL-8 mRNA levels by increasing gene transcription without altering IL-8 mRNA stability. Of the sphingolipid metabolites tested, S1-P but not ceramide or sphingosine induced IL-8 mRNA and protein levels. TNF- α induction of IL-8 mRNA levels was partly reduced by dimethylsphingosine, an inhibitor of sphingosine kinase, indicating the importance of elevated levels of intracellular S1-P in the induction. Consistent with the effects of dimethylsphingosine, SiRNA-mediated inhibition of sphingosine kinase partially blocked TNF- α induction of IL-8 mRNA. S1-P induction of IL-8 mRNA was associated with an increase in AP-1 DNA binding activity, however NF- κ B binding activity was unchanged. S1-P induction of IL-8 mRNA expression was partly reduced by MAPK inhibitors, SB203580 and PD98059 indicating the involvement of ERK and p38 MAPKs in the S1-P regulation of IL-8 expression.

2. Methods

2.1 Cell culture

NCI-H441 cells [American Type Culture Collection (ATCC) HTB-174], a human lung adenocarcinoma cell line of bronchiolar (Clara) cell lineage were grown on plastic tissue culture dishes in RPMI 1640 medium containing 10% fetal bovine serum, penicillin (100 u/ml), streptomycin (100 µg/ml) and amphotericin B (0.25 µg/ml). In all experiments the medium was changed to RPMI 1640 without serum for 24 h before the start of the experiment. In experiments to test the effects of S1-P on IL-8 expression, BSA (0.4 % w/v) was added to the culture medium.

2.2. Materials

TNF- α was purchased from R & D systems (Minneapolis, MN). TRI Reagent was from Molecular Research Center (Cincinnati, OH). Actinomycin D, bovine serum albumin [low endotoxin (≤ 0.1 ng/ml) and fatty acid free], *Staphylococcus aureus* sphingomyelinase and sphingosine were from Sigma (St. Louis, MO). 5,6-dichloro-1-b-D-ribofuranosyl-benzimidazole (DRB) was from Calbiochem. C2-ceramide, sphingosine-1-phosphate, N,N-Dimethyl-D-erythro-sphingosine (DMS) were obtained from Avanti (Alabaster, AL). S1-P was dissolved in a mixture of methanol-water (95:5) at 0.5 mg/ml by heating at 45°C for 10–15 min followed by sonication for 10 s each time for three times. Solubilized S1-P was dried under nitrogen and reconstituted in cell culture medium containing 0.4% bovine serum albumin.

2.3. RNA isolation and Northern blot analysis

Experimental procedures for total RNA isolation and Northern blotting analysis are as described previously (Boggaram and Margana, 1994). Cytosolic RNA was isolated according to published protocol (Greenberg and Bender, 1997). IL-8 and GAPDH RNA bands were quantified with a PhosphorImager using Quantity One Image Acquisition and Analysis Software (BioRad) and IL-8 mRNA levels were normalized to 18S rRNA levels to correct for variations in the quantification, loading and transfer of RNA. The expression of GAPDH mRNA was assessed as an internal control. Plasmids encoding human IL-8 cDNA were kindly provided by Drs. Edmund Miller and Usha Pendurthi, University of Texas Health Center at Tyler, Tyler, TX.

2.4. Determination of IL-8

IL-8 levels in cell medium were determined by enzyme-linked immunosorbent assay (ELISA) using a matched antibody pair according to the manufacturer's protocol (R & D Systems, Minneapolis, MN).

2.5. Isolation of nuclei and nuclear run-on transcription assay

Methods for the isolation of nuclei and run-on transcription assay are as described previously (Greenberg and Bender, 1997) (Boggaram and Margana, 1994). Total RNA from labeled nuclei was isolated and equal amounts of radioactive RNAs ($10\text{--}30 \times 10^6$ counts/min) were hybridized to nitrocellulose membranes containing immobilized plasmid DNAs containing human IL-8 and GAPDH cDNAs and pBluescript. After washing, radioactivity bound to the filters was quantified with a PhosphorImager. Radioactivity bound to pBluescript was considered as background.

2.6. Plasmid DNA isolation

Luciferase reporter plasmids containing –546/+44 and –133/+44 bp sequences of human IL-8 gene were kindly provided by Dr. Naofumi Mukaida, Cancer Research Institute, Kanazawa

University, Kanazawa, Japan. Plasmid DNAs were amplified in *Escherichia coli* top 10 strain (Invitrogen, Carlsbad, CA) and purified by anion exchange chromatography using QIA filter plasmid purification kit (Qiagen, Valencia, CA).

2.7. Transient transfection and reporter gene assay

Plasmid DNAs were transiently transfected into cells by liposome-mediated DNA transfer with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. β -galactosidase and luciferase reporter activities in cell extracts were measured by chemiluminescence assays (Tropix, Bedford, MA and Promega, Madison, WI).

2.8. Transfection of SiRNA oligonucleotides

SiRNA duplex oligonucleotides (siGENOME SMARTpool reagent) targeting human sphingosine kinase (Human SPHK1) and non-targeting SiRNA duplex oligonucleotides were purchased from Dharmacon RNA Technologies (Lafayette, CO). The SMARTpool SiRNA oligonucleotides comprise of four SiRNAs combined into a single pool. The non-targeting SiRNA served as a negative control having no perfect matches to known human or mouse genes. H441 cells (30–50% confluent) plated on T25 tissue culture plastic dishes were transfected with 100 nM SiRNA oligonucleotide and 20 μ l Oligofectamine (Invitrogen) per dish according to the manufacturer's protocol. Transfected cells were cultured for 72 h in order to achieve maximum silencing effects and then subjected to treatments.

2.9. Preparation of nuclear extracts and electrophoretic mobility shift analysis (EMSA)

Nuclear extracts were prepared according to the methods described previously (Schreiber et al., 1989) (Singh and Aggarwal, 1995). Protein concentrations of nuclear extracts were determined by Bradford's method using Bio-Rad protein assay reagent.

Synthetic oligonucleotides were annealed by heating equimolar concentrations of sense and antisense oligonucleotides in 10 mM Tris-HCl, pH 7.5 containing 10 mM $MgCl_2$ and 50 mM NaCl at 95°C for 5 min and then allowed to cool to room temperature over a period of 1 h. The sense strand sequences of the human IL-8 promoter oligonucleotides (binding sites are underlined) used in EMSA are as follows:

NF- κ B: 5'-AATCGTGGAATTCCTGA-3' (−84/−65 bp)

AP-1: 5'-AGTGTGATGACTCAGGTTTG-3' (−133/−114 bp)

NF IL-6: 5'-TCCATCAGTTGCAAATCGTGGA-3' (−94/−76 bp)

Double stranded oligonucleotides were 5'end labeled using [$\gamma^{32}P$] and T4 polynucleotide kinase. EMSAs were performed as described previously (Berhane and Boggaram, 2001) by incubating 0.5–1.0 ng (100,000 cpm) of the labeled oligonucleotide with 5 μ g of nuclear protein in 20 μ l of binding buffer [13 mM Hepes, pH 7.9 containing 13% glycerol, 80 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA and 1 μ g of poly (dI-dC) as non-specific competitor DNA] at 30°C for 20 min. After electrophoresis, the gel was dried and exposed to an X-ray film.

2.11. Immunoblotting analysis

Cells were rinsed twice with cold phosphate buffered saline and incubated in lysis buffer (50 mM Tris.Cl, pH 7.4 containing 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM sodium vanadate, 2.5 mM sodium pyrophosphate, 2 μ g/ml leupeptin and aprotinin, 1 mM PMSF and 15% glycerol) for 15 min on ice. The cell lysate was cleared by centrifugation at 14,000 rpm for 10 min and the supernatant used for Western immunoblotting analysis. SDS-PAGE separation and transfer of proteins to membrane were carried out with an XCell II Mini-Cell

apparatus (Novex, San Diego, CA) according to the manufacturer's instructions. Equal amounts of cellular protein (10 µg) were separated by SDS-PAGE on 10% Bis-Tris gels with MOPS running buffer and electrophoretically transferred to PVDF membranes. Membranes were successively incubated with rabbit polyclonal antibodies against phosphorylated and total p38, p44/42 and JNK MAPKs (Cell Signaling, Beverly, MA) at 1:1000 dilution overnight at 4°C followed by goat anti-rabbit alkaline phosphatase conjugated-secondary antibody (Cell Signaling) at 1:2000 dilution for 1 h at room temperature. Protein bands were visualized by the Enhanced Chemifluorescence (ECF) detection method (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. Membranes were scanned with a fluorescence scanner for visualization of protein bands and the intensity of bands was quantified using Quantity One Image Acquisition and Analysis Software (BioRad).

2.12. Statistics

Data are shown as means $\pm$ SD/SE. In experiments where IL-8 mRNA levels in control cells were arbitrarily set at 100%, statistical significance was analyzed by one-sample *t*-test. For other samples, unpaired *t*-test was used to analyze statistical significance. One-tailed *P* values of <0.05 were considered significant.

3. Results

3.1. TNF- α increases IL-8 mRNA in H441 lung epithelial cells in a time-dependent manner

H441 cells are human lung epithelial cells that possess many of the characteristics of Clara cells such as morphologic and ultrastructural features as well as expression of lung-specific surfactant proteins (SP)-A, B and D making them better suited as a cell line representing the distal lung epithelium. We studied the effects of TNF- α on the time-course of induction of IL-8 mRNA. We found that IL-8 mRNA was barely detectable in untreated cells and was induced rapidly by TNF- α to maximum level at 1 h of incubation (Figs. 1 A and B). Elevated IL-8 mRNA levels in TNF- α treated cells were sustained for extended periods of time. The levels of GAPDH mRNA were unaffected by TNF- α (Fig. 1A).

3.2. TNF- α increases IL-8 gene transcription rate in H441 cells

TNF- α induction of IL-8 gene expression is subject to regulation at the transcriptional and mRNA stabilization levels. To determine the roles of transcriptional and posttranscriptional mechanisms, we analyzed the effects of TNF- α on IL-8 gene transcription rate and IL-8 mRNA levels by transcription run-on assay and Northern blotting, respectively. We found that in cells treated with TNF- α IL-8 transcription increased after 0.5 h and declined thereafter (Fig. 2 A and C, dot # 1). Northern blot analysis of cytosolic RNA isolated from the same cells showed that, in agreement with our earlier data, the steady state IL-8 mRNA levels increased to maximum levels after 1 h of treatment (Fig. 2 B). TNF- α induction of IL-8 gene transcription (~ 10-fold relative to control at 1 h) was similar to increase of cytosolic IL-8 mRNA levels (~ 10-fold relative to control at 1 h) indicating that the inductive effects of TNF- α are exerted primarily at the transcriptional level. GAPDH transcription was unaffected by TNF- α (Fig. 2 A, dot # 2). We further evaluated the effect of TNF- α on the posttranscriptional regulation of IL-8 mRNA by determining its effect on the stability of IL-8 mRNA. Results (Fig. 3 B) showed that the half-life of IL-8 RNA was ~ 2 h in control cells and was unaffected by TNF- α indicating that TNF- α does not influence the stability of IL-8 mRNA. Similar results were obtained when 5,6-dichlororibofuranosylbenzimidazole (DRB) was used as a transcriptional inhibitor (data not shown). The stability of GAPDH mRNA was not affected by TNF- α . Taken together the data of transcription run-on assays and the RNA half-life experiments showed that TNF- α increases IL-8 mRNA levels in H441 cells primarily by increasing gene transcription.

In agreement with transcription run-on experiments, TNF- α increased luciferase reporter gene expression from IL-8 minimal promoter (-133/+44 bp) in transient transfection assays (data not shown). TNF- α induction of IL-8 mRNA was associated with an induction of NF- κ B DNA binding activity, however AP-1 binding activity was not changed (data not shown).

3.3. Sphingomyelin metabolite(s) mediate TNF- α induction of IL-8 mRNA levels

Sphingomyelin metabolites have been shown to play important roles in mediating the inflammatory responses in the lung. To determine the role of sphingomyelin metabolites in the regulation of IL-8 gene expression, we investigated the effects of exogenously added sphingomyelinase, ceramide, sphingosine and S1-P on IL-8 mRNA and IL-8 secretion in H441 cells. As S1-P was reconstituted in serum-free medium containing 0.4% BSA, we determined the effects of 0.4% BSA on IL-8 mRNA and IL-8 secretion and found that BSA did not alter IL-8 expression (data not shown). Results (Fig. 4 A) showed that at 1 h incubation, sphingomyelinase (1 u/ml) and S1-P (1 μ M) increased IL-8 mRNA levels by ~ 3.5 and 4.8 fold, respectively, compared to control whereas ceramide (10 μ M) and sphingosine (10 μ M) did not cause any increase. Preliminary experiments to test the effects of different concentrations of ceramide (1–100 μ M) did not show any inductive effects on IL-8 mRNA levels after 1 h incubation. As expected, TNF- α was highly effective in increasing IL-8 mRNA levels. Measurement of IL-8 levels by ELISA in the medium after 6 h of incubation showed that S1-P and TNF- α significantly increased IL-8 levels (Fig. 4 B). A small increase in IL-8 levels in cells treated with ceramide was also found (control = 4.64 ± 0.5 pg/mg protein, ceramide = 8.15 ± 1 pg/mg protein) (Fig. 4 B). A time point of 6 h was chosen to allow sufficient time for changes in protein synthesis and secretion to occur as S1-P maximally stimulates IL-8 mRNA levels at 1 h of incubation. The inductive effect of S1-P on IL-8 secretion was significantly greater than its effect on IL-8 mRNA levels. H441 cells were treated with ceramidase inhibitors, D-erythro-2-(N-myristoylamino)-1-phenol-1-prop (MAPP) and N-oleoyl-ethanolamine (NOE), for 1–24 h to increase the intracellular accumulation of ceramide, and their effects on IL-8 mRNA levels were determined. Results (Fig. 5) showed that treatment of H441 cells with ceramidase inhibitors for 1–24 h did not increase IL-8 mRNA levels consistent with the lack of inductive effects of exogenously added ceramide.

The inductive effect of S1-P on IL-8 mRNA levels was time-dependent with maximum effect at 1 h of incubation (Fig. 6). The inductive effects of S1-P were concentration-dependent with the compound at 0.5 and 1 μ M causing maximum induction after 1 h of treatment (data not shown). S1-P similarly increased IL-8 mRNA levels in A549 cells, a cell line derived from alveolar type II epithelial cells (Sparkman, L. and Boggaram, V., unpublished observations). To determine if sphingomyelinase induction of IL-8 gene expression is due to the generation of intracellular S1-P or not, the effects of inhibition of sphingosine kinase by DMS on the sphingomyelinase induction of IL-8 mRNA was determined. Results showed that DMS caused a dose-dependent inhibition of sphingomyelinase induction of IL-8 mRNA (control = 1, sphingomyelinase = 9.78 ± 0.80 , DMS (10 μ M) + sphingomyelinase = 7.49 ± 0.55 , DMS (20 μ M) + sphingomyelinase = 4.47 ± 0.46 , mean $\pm$ SEM, n = 3) indicating the involvement of S1-P in the induction.

We further assessed the role of S1-P in the TNF- α induction of IL-8 mRNA expression using N,N-dimethylsphingosine (DMS), an inhibitor of sphingosine kinase. DMS reduced TNF- α induction of IL-8 mRNA levels in a dose-dependent manner indicating that intracellular increase in S1-P levels contributes to the TNF- α induction of IL-8 mRNA levels (Fig 7 A and B). To further prove that TNF- α induced increase in the intracellular S1-P is involved in the induction of IL-8 mRNA, we used RNA-mediated interference to reduce sphingosine kinase expression and studied its effects on TNF- α induction of IL-8 mRNA expression. RNA-mediated interference results in gene-specific silencing providing superior advantage over

chemical inhibitors. SiRNA duplex oligonucleotides targeting sphingosine kinase was transfected into H441 cells and the effects of TNF- α on IL-8 mRNA induction was determined. Results (Fig. 7 C and D) showed that in agreement with the results of DMS, SiRNA inhibition of sphingosine kinase partially reduced TNF- α induction of IL-8 mRNA. The effects of silencing was apparent in cells exposed to TNF- α for 1 h (data not shown) and more pronounced after 24 h of treatment. We were unable to detect sphingosine kinase levels by Western blotting in control or SiRNA transfected cells.

3.4. S1-P induction of IL-8 gene expression is regulated at the transcriptional level

Our data showed that S1-P induced IL-8 mRNA levels and IL-8 secretion in H441 cells. To understand molecular mechanisms that mediate S1-P induction of IL-8 mRNA levels, we determined its effects on IL-8 gene transcription and mRNA stability in H441 cells. Analysis of the effects of S1-P on IL-8 gene transcription rate and mRNA stability showed that S1-P increased IL-8 gene transcription rate (Fig. 8 A, dot # 1) and IL-8 promoter activity (Fig. 8 C) without any effect on the half-life of IL-8 mRNA (Fig. 8 B) indicating that the inductive effects are mediated primarily at the transcriptional level. GAPDH transcription was not significantly increased by S1-P (Fig. 8A, dot # 2).

3.5. Effects of S1-P on NF- κ B and AP-1 DNA binding activities

Our data showed that TNF- α induced intracellular S1-P levels play important roles in the induction of IL-8 mRNA. Our data also showed that S1-P increased IL-8 mRNA levels by increasing gene transcription. As NF- κ B and AP-1 transcription factors play key roles in the induction of IL-8 expression, we were interested to determine the effects of S1-P on NF- κ B and AP-1 DNA binding activities. Results (Fig. 9) showed that NF- κ B DNA binding activity was undetectable in control cells (**lane 2**) and exposure of cells to S1-P (**lane 3**) did not increase NF- κ B DNA binding activity, however AP-1 DNA binding activity was modestly increased (**lanes 4 and 5**) and NF IL-6 binding activity remained unchanged (**lanes 6 and 7**). Similar results were obtained when cells were incubated with S1-P for different periods of time ranging from 10 min to 6 h (data not shown).

3.6. Involvement of MAPKs in the S1-P induction of IL-8 mRNA

Sphingolipids are known to activate MAPK signaling pathways in a variety of cells. We studied the involvement of MAPK signaling pathways in the S1-P induction of IL-8 mRNA expression. S1-P stimulated the phosphorylation of p38, ERK and JNK MAPKs in a time-dependent manner in H441 cells; enhanced phosphorylation was observed as early as 10 min after exposure to S1-P and leveled off to control levels after 2 h (Fig. 10 A). The stimulatory effect of S1-P on p46 form of JNK was more apparent than on p54 form of JNK. These data indicated that MAPK signaling pathways may be involved in the S1-P induction of IL-8 mRNA expression. We ascertained the involvement of MAPKs in the S1-P induction of IL-8 mRNA by determining the effects of pharmacological inhibitors of MAPKs on S1-P induction of IL-8 mRNA. ERK, p38 and JNK MAPKs were inhibited with PD98059, SB203580 and JNK II inhibitor respectively and the effects of S1-P on IL-8 mRNA expression were determined. Results (Fig. 10 B and C) showed that inhibition of p38 and ERK inhibited S1-P induction of IL-8 mRNA levels by approximately 50 and 40 %, respectively indicating the involvement of p38 and P44/42 MAPK signaling pathways. In contrast JNK II inhibitor increased IL-8 mRNA levels and additively enhanced S1-P induction of IL-8 mRNA.

4. Discussion

IL-8 gene expression is induced by a wide variety of agents including cytokines, growth factors, bacterial and viral products, oxidants and others (Roebuck, 1999). Induction of IL-8 gene expression is subject to both transcriptional and posttranscriptional regulation in a cell/tissue-

and stimulus-specific manner (Roebuck, 1999) (Hoffmann et al., 2002). We found that in H441 cells TNF- α induced IL-8 mRNA levels primarily by increasing gene transcription. In A549 lung epithelial cells TNF- α was found to activate IL-8 promoter activity via recruitment of NF- κ B to a TNF- α response element consistent with a role for transcriptional mechanisms (Brasier et al., 1998) in the induction of IL-8 gene expression in lung epithelial cells. A relatively short sequence of DNA spanning -133/+41 bp is necessary and sufficient for the basal and TNF- α induction of IL-8 promoter activity (Yasumoto et al., 1992; Brasier et al., 1998). The transcriptional response region contains binding sites for NF-IL-6, NF- κ B and AP-1 that act independently and synergistically to activate IL-8 promoter in response to stimulatory agents in a cell type-specific manner [reviewed in (Roebuck, 1999)]. In this study we found that TNF- α and S1-P induced IL-8 expression by increasing gene transcription and without altering the stability of IL-8 mRNA. However, regulation of IL-8 mRNA stability has been found to play major roles in the control of IL-8 expression in different cells. Nitric oxide, lipopolysaccharide, adenovirus and Shiga toxin increase IL-8 mRNA expression in lung epithelial cells, THP cells, fibroblasts and A549 cells, respectively, by increasing the stability of IL-8 mRNA (Leland Booth and Metcalf, 1999; Thorpe et al., 2001; Ma et al., 2004; Sparkman and Boggaram, 2004). The half-life of IL-8 mRNA in untreated cells was in the range of 0.5 – 2 h and increased by several fold depending on the cell type and the stimulus.

Sphingomyelin metabolites are increasingly recognized as important mediators of inflammation in the lung, and ceramide has emerged as a putative lipid mediator in TNF- α signaling. Despite the important roles that sphingomyelin metabolites play in TNF- α signaling, little is known about their involvement in the TNF- α induction of IL-8 gene expression in lung cells. Our data showed that among the sphingomyelin metabolites, S1-P but not ceramide or sphingosine induced IL-8 mRNA levels and IL-8 secretion. Consistent with the lack of significant effects of ceramide, inhibition of ceramidase to increase intracellular ceramide levels did not increase IL-8 mRNA levels. We found that the inductive effect of S1-P on IL-8 level in the medium was significantly greater than its effects on IL-8 mRNA levels. Similarly we found that although ceramide did not increase IL-8 mRNA levels it caused a small increase in IL-8 levels. The observed discrepancy between IL-8 mRNA and IL-8 levels in S1-P and ceramide treated cells point to possible translational and/or posttranslational regulation of IL-8 expression. Together our data indicated that elevated intracellular S1-P generated as a result of activation of sphingosine kinase partly mediates TNF- α induction of IL-8 gene expression in H441 cells. Intracellular S1-P levels can also be modulated by the actions of sphingosine-phosphate lyase that catalyzes the irreversible cleavage of S1-P (Reiss et al., 2004). Whether TNF- α regulates sphingosine-phosphate lyase expression and/or activity to modulate intracellular S1-P levels in H441 cells is not known. In human umbilical vein endothelial cells (HUVEC) TNF- α was found to induce the expression of E-selectin and vascular adhesion molecule-1 (VCAM-1) via increased generation of S1-P by the activation of sphingosine kinase (Xia et al., 1998). Although TNF- α induced sphingomyelin breakdown and ceramide generation, ceramide failed to mimic the effects of TNF- α to induce E-selectin and VCAM-1 expression.

Changes in the levels of sphingolipid metabolites can occur via coordinate activation of the entire cascade of sphingolipid metabolizing enzymes as in the case of oxidized low density lipoprotein induced mitogenesis of smooth muscle cells (Auge et al., 1999) or via selective activation of one of the enzymes of the pathway as in the case of TNF- α activation of sphingosine kinase to inhibit apoptosis in HUVEC cells (Xia et al., 1999). In some cells the activation of ceramidase may be so robust that the levels of sphingosine and S1-P are vastly increased in the absence of substantial increases in ceramide levels (Kolesnick, 2002). Our experiments showed that exogenous ceramide and inhibition of ceramidase to increase intracellular ceramide levels failed to increase IL-8 mRNA and protein levels suggesting that the inability of ceramide to increase IL-8 may not be due to low intracellular ceramide levels.

TNF- α activates sphingomyelin hydrolysis to increase intracellular ceramide levels in lung (Ryan et al., 2003) and lung cells (Vivekananda et al., 2001). Sphingolipids generated in response to TNF- α inhibit the expression of CTP:phosphocholine cytidyltransferase (CCT α) (Vivekananda et al., 2001) the rate-limiting enzyme involved in the synthesis of phosphatidylcholine, an important component of lung surfactant, leading to the perturbation of surfactant lipid composition. These findings suggest that perturbations in surfactant lipid synthesis contribute to lung injury associated with inflammation and that sphingolipids play important roles in mediating these effects. Our findings of the inductive effects of S1-P on IL-8 mRNA levels reveal yet another pathway that potentially contributes to lung injury in inflammation. Increases in IL-8 levels can lead to increased recruitment of neutrophils into the lung contributing to lung injury.

The molecular mechanisms and signal transduction pathways by which S1-P induces the expression of IL-8 in H441 lung epithelial cells remains to be investigated. Our studies showed that S1-P increased AP-1 DNA binding activity in H441 cells suggesting that increase in AP-1 binding may be required for S1-P increase of IL-8 gene transcription. S1-P is known to enhance the DNA binding activity of AP-1 (Su et al., 1994).

MAPKs regulate IL-8 expression and secretion in a variety of cells including lung epithelial cells (Hashimoto et al., 1999) (Matsumoto et al., 1998) and MAPK regulation of IL-8 expression occurs via transcriptional and mRNA stabilization mechanisms (Holtmann et al., 1999). Transcription factors NF- κ B and AP-1 play central roles in the transcriptional regulation of IL-8 expression. The involvement of ERK, p38 and JNK pathways in the regulation of IL-8 expression appears to be dependent on the cell type and the nature of the stimulus. Induction of IL-8 expression in BEAS-2B bronchial epithelial cells by S1-P (Wang et al., 2002) and lysophosphatidic acid (Saatian et al., 2006) required activation of p44/p42 whereas induction by *Streptococcus pneumoniae* (Schmeck et al., 2006) and zinc (Kim et al., 2006) involved activation of JNK and ERK plus JNK respectively. Our studies showed that S1-P activated p44/42, p38 and JNK phosphorylation in H441 cells, however, pharmacological inhibitors of ERK and p38 but not JNK MAPKs inhibited S1-P induction of IL-8 mRNA levels indicating that ERK and p38 signaling pathways are required for S1-P induction. S1-P increased AP-1 DNA binding activity suggesting a role for AP-1 in the induction of IL-8 mRNA expression. It remains to be determined if ERK and p38 MAPK pathways control AP-1 DNA binding activity to increase IL-8 expression. It is known that p38, p44/p42 and JNK MAPKs regulate AP-1 activity (Whitmarsh and Davis, 1996).

In summary, our studies have shown that TNF- α induces IL-8 gene expression in H441 lung epithelial cells by increasing gene transcription and that intracellular increases in S1-P levels play important roles in mediating TNF- α induction. S1-P induced IL-8 mRNA expression via activation of p38 and p44/42 MAPK signaling pathways and increase in AP-1 DNA binding activity. Thus TNF- α induces IL-8 gene expression in H441 lung epithelial cells via two pathways – one involving the activation of NF- κ B and the other via intracellular elevation of S1-P that results in an increase in AP-1 but not NF- κ B binding.

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Abbreviations

IL-8	interleukin-8
TNF-α	tumor necrosis factor-alpha
ARDS	acute respiratory distress syndrome
PAF	platelet activating factor
SSC	0.15 M NaCl/0.015 M Na ₃ citrate pH 7.6
ELISA	enzyme-linked immunosorbent assay
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
EMSA	electrophoretic mobility shift assay
DMS	N,N-dimethyl-D-erythro-sphingosine
DTT	dithiothreitol
MAP kinase	mitogen activated protein kinase
MAPP	D-erythro-2-(N-myristoylamino)-1-phenol-1-propanol
MOPS	[3-(N-morpholino) propanesulfonic acid]
NOE	N-oleoyl-ethanolamine
S1-P	sphingosine 1-phosphate

SiRNA	small inhibitor RNA
SP	surfactant protein
NF-κB	nuclear factor kappaB
AP-1	activator protein 1
NF-IL-6	nuclear factor IL-6
CCT-α	CTP:phosphocholine cytidyltransferase

Fig 1. TNF- α induces IL-8 mRNA levels in H441 lung epithelial cells

IL-8 and GAPDH mRNA levels in H441 cells incubated in control (C) or TNF- α containing medium (25 ng/ml) for the indicated periods of time were determined by Northern blotting

A. A representative autoradiogram showing the effects of TNF- α on IL-8, GAPDH mRNA levels. 18S rRNA levels visualized by ethidium bromide staining are also shown. **B.** IL-8 mRNA levels in control cells were arbitrarily set at 100, and the levels in treated cells relative to control cells are shown. Data are means $\pm$ SD of 2–3 independent experiments. * $P < 0.05$ and ** $P < 0.01$ for TNF- α treated samples compared to control.

Fig 2. TNF- α increases IL-8 gene transcription rate

IL-8 and GAPDH transcription rates and cytosolic IL-8 mRNA levels in H441 cells incubated in control (C) or TNF- α (25 ng/ml) containing medium was determined by transcription run-on assay and Northern blotting, respectively. **A.** A representative autoradiogram showing the effects of TNF- α on IL-8 and GAPDH transcription rates. 1, IL-8; 2, GAPDH; 3, pBluescript; 4, blank. **B.** A representative autoradiogram of the effects of TNF- α on cytosolic IL-8 mRNA levels. **C.** Radioactivity bound to pBluescript was considered as background and IL-8 gene transcription rate was normalized to GAPDH transcription rate. Data shown are means $\pm$ SD of 2 independent experiments.

Fig 3. TNF- α does not alter the stability of IL-8 mRNA

H441 cells were incubated in control (♦) or TNF- α (25 ng/ml) (■) containing medium for 1 h and then incubation continued in the presence of actinomycin D (5 μ M) for the indicated times. The levels of IL-8 and GAPDH mRNAs were determined by Northern blotting. **A.** A representative autoradiogram showing IL-8, GAPDH and 18 S rRNA bands. **B.** IL-8 mRNA level at 0 h was arbitrarily set at 100 and levels at other times are shown relative to 0 h. Data are means $\pm$ SD of 2 independent experiments.

Fig 4. Effects of sphingomyelinase and sphingolipids on IL-8 mRNA and protein levels

IL-8 mRNA and IL-8 levels in medium in H441 cells incubated in control medium (C) or medium containing TNF- α (25 ng/ml), sphingomyelinase (Smase) (1 u/ml), C2-ceramide (10 μ M), sphingosine (10 μ M) or S1P (1 μ M) were determined by Northern blotting and ELISA, respectively. Cells were incubated for 1 and 6 h for Northern blotting (**A**) and ELISA (**B**), respectively. Data are means $\pm$ SEM of 3–4 independent experiments. Effects on IL-8 mRNA: ***P < 0.001 for TNF- α -treated cells vs control; *P < 0.05 for Smase and S1P-treated cells vs control. Effects on IL-8 protein: *P < 0.05 for ceramide and S1P-treated cells vs control; ***P < 0.001 for TNF- α –treated cells vs control.

Fig 5. Effects of ceramidase inhibitors on IL-8 mRNA levels

IL-8 mRNA levels in H441 cells incubated in control medium (C) or medium containing ceramidase inhibitors D-erythro-2-(N-myristoylamino)-1-phenol-1-propanol (MAPP) (5 μ M) or N-oleoyl-ethanolamine (NOE) (5 μ M) for the indicated times were determined by Northern blotting. Data are means $\pm$ SD of 2 independent experiments.

Fig 6. S1-P induces IL-8 mRNA levels

A. IL-8 mRNA levels in H441 cells incubated in control medium (C) or medium containing S1-P for the indicated times were determined by Northern blotting. **B.** Data are means $\pm$ SEM of 3 independent experiments. ** $P < 0.01$ for 30 min sample compared to control, * $P < 0.05$ for 1 h sample compared to control, ** $P < 0.01$ for 2 h sample compared to control.

Fig 7. Inhibition of sphingosine kinase reduces TNF- α induction of IL-8 mRNA levels

A. The effects of dimethylsphingosine (DMS), an inhibitor of sphingosine kinase, on TNF- α induction of IL-8 mRNA were determined by Northern blotting. H441 cells were first incubated in control medium (C) or medium containing DMS for 1 h and then treated with TNF- α for an additional 1 h. **B.** Data are means $\pm$ SD of 2–3 independent experiments. $^{**}P < 0.01$ for TNF- α compared to control, $^{*}P < 0.05$ for DMS (40 μ M) + TNF- α compared to TNF- α alone, $^{**}P < 0.01$ for DMS (30 μ M) + TNF- α compared to TNF- α alone, $^{***}P < 0.001$ for DMS (50 μ M) + TNF- α compared to TNF- α alone. **C.** SiRNA duplex oligonucleotides for sphingosine kinase were transfected into H441 cells by liposome mediated transfer with Oligofectamine. After 72 h, cells were treated with medium $\pm$ TNF- α (25 ng/ml) for 24 h and IL-8 and GAPDH mRNAs were analyzed by Northern blotting. 1 and 2, non-targeting SiRNA; 3, sphingosine kinase SiRNA. **D.** Data are means $\pm$ 3 independent experiments. $^{*}P < 0.05$ for TNF- α compared to control; $^{*}P < 0.05$ for sphingosine kinase SiRNA transfected cells compared to non-targeting SiRNA transfected cells.

Fig 8. S1-P increases IL-8 gene transcription and promoter activity. A. Effect on IL-8 gene transcription

IL-8 and GAPDH transcription rates in H441 cells incubated in control medium (C) or medium containing S1-P (1 μ M) for 1 h were determined by transcription run-on assay. The data is representative of 2 independent experiments. **B. Effect on IL-8 mRNA stability.** H441 cells were first incubated in control medium (C) or medium containing S1-P (1 μ M) ($\blacklozenge$, control; $\blacksquare$, S1-P) for 1 h and then incubation continued in the presence of actinomycin D (2.5 μ M) for the indicated times. IL-8 and GAPDH mRNA levels were determined by Northern blotting. Data are means $\pm$ SD of 2 independent experiments. **C. Effect on IL-8 promoter activity.** H441 cells were transiently transfected with pGL3luc plasmid containing -133/+44 bp of human IL-8 promoter DNA and then treated with medium $\pm$ S1P (1 μ M) for 6 h. Luciferase activity in cell extracts was determined and normalized to total cell protein. Data are means $\pm$ SD of 2 independent experiments.

Fig 9. Effects of S1-P on NF-κB, AP-1 and NF IL-6 DNA binding activities

H441 cells were incubated in control medium (C) or medium containing S1-P (1 μ M) for 1 h and nuclear extracts prepared. Binding activities of NF-κB, AP-1 and NF IL-6 to IL-8 promoter elements were analyzed by EMSA. The arrows indicate the mobility of protein-DNA complexes. Ns, non-specific band.

Fig 10. A. Effects of S1-P on MAPK activation

H441 cells were serum-starved for 24 h and then treated with S1-P (1 μ M) for the indicated periods of time. The levels of phosphorylated and total p44/42 MAP kinases were analyzed by Western immunoblotting. Similar results were obtained in 2 other independent experiments.

B and C. Effects of pharmacological inhibitors of MAPK activation on S1-P induction of IL-8 mRNA levels. H441 cells were first incubated in medium $\pm$ inhibitor (SB 203850, 10 μ M; PD98059, 50 μ M; JNK inhibitor II, 10 μ M) for 1 h and then treated with S1-P (1 μ M) for an additional 1 h. IL-8 mRNA levels were determined by Northern blotting analysis. A representative Northern blot is shown. Similar results were obtained in 2 other independent experiments.