IL-8 Production in Human Lung Fibroblasts and Epithelial Cells Activated by the *Pseudomonas* Autoinducer *N*-3-Oxododecanoyl Homoserine Lactone Is Transcriptionally Regulated by NF-κB and Activator Protein-2[1]

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The destructive pulmonary inflammation associated with *Pseudomonas aeruginosa* colonization is caused, in part, by the production of the chemokine IL-8, which recruits neutrophils into the lung. The *Pseudomonas* autoinducer, *N*-3-oxododecanoyl homoserine lactone (3-O-C12-HSL), is a small lipid-soluble molecule that is essential in the regulation of many *P. aeruginosa* virulence factors, but little is known about how it affects eukaryotic cells. In this report we demonstrate that 3-O-C12-HSL is a potent stimulator of both IL-8 mRNA and protein from human fibroblasts and epithelial cells in vitro. The IL-8 produced from these 3-O-C12-HSL-stimulated cells was found to be functionally active by inducing the chemotaxis of neutrophils. To determine a mechanism for this IL-8 induction, deletion constructs of the IL-8 promoter were examined. It was found that the DNA region between nucleotides −1481 and −546 and the transcription factor NF-κB were essential for the maximal induction of IL-8 by 3-O-C12-HSL. This was confirmed by EMSAs, where 3-O-C12-HSL induced a shift with both AP-2 and NF-κB consensus DNA. The activation of NF-κB and subsequent production of IL-8 were found to be regulated by a mitogen-activated protein kinase pathway. These findings support the concept that the severe lung damage that accompanies *P. aeruginosa* infections is caused by an exuberant neutrophil response stimulated by 3-O-C12-HSL-induced IL-8. Understanding the mechanisms of 3-O-C12-HSL activation of lung structural cells may provide a means to help control lung damage during infections with *P. aeruginosa*. *The Journal of Immunology*, 2001, 167: 366–374.

Pseudomonas aeruginosa is an opportunistic pathogen that causes a wide range of acute and chronic infections. However, the most prominent are the pulmonary infections. Due to the ubiquitous nature of *P. aeruginosa* and its ability to develop resistance to many antibiotics, it has become the major cause of nosocomial pneumonia (1, 2). Conditions such as bronchiectasis, glucocorticoid therapy, acute respiratory distress syndrome, cystic fibrosis, certain malignancies, HIV infection, broad-spectrum antibiotic therapy, and use of respiratory equipment predispose patients to *P. aeruginosa* lung infections (3). *P. aeruginosa* pneumonia is an emerging problem and in many cases is fatal in patients with advanced HIV infections (4, 5) and ventilator-associated pneumonia (6–8). This ability that *P. aeruginosa* has to induce a wide range of infections is largely due to the many virulence factors that it produces. The synthesis of several of these virulence factors is regulated by a cell-to-cell signaling mechanism referred to as quorum sensing (9). This mechanism enables *P. aeruginosa* to regulate genes in a density-dependent manner by the production of small diffusible molecules called *Pseudomonas* autoinducers. *P. aeruginosa* predominately makes two autoinducers, *N*-3-oxododecanoyl homoserine lactone (3-O-C12-HSL,[3] also called PAI-1) and *N*-butyryl-L-homoserine lactone (C4-HSL, also called PAI-2) (10, 11). *P. aeruginosa* cell-to-cell signaling uses a LuxR/LuxI-type system, which was originally described in *Vibrio fisheri* (12). In this system 3-O-C12-HSL and C4-HSL complex with their cogent transcription factors, LasR and RhlR, respectively, and induce the transcription of genes that lead to the production of bacterial virulence factors (for a review of *P. aeruginosa* cell-to-cell signaling and virulence see Ref. 13). The effects of these autoinducer-regulated virulence factors directly contribute to the persistence and dissemination of the bacteria, which leads to induction of intense inflammation during infection.

The early stages of *P. aeruginosa* colonization of the lung are hallmarked by the entry of neutrophils. This neutrophil recruitment is most likely due to the production of the chemokine IL-8. In support of this concept, animal models of *P. aeruginosa* lung infection demonstrate concomitant increases in IL-8 and neutrophils in the airways (14–16). When bronchoalveolar lavage fluid from *P. aeruginosa*-infected humans was examined, IL-8 levels were significantly increased over those of uninfected patients. *P. aeruginosa*-infected patients also had highly significant increases in the number of lung neutrophils (17–19). Increases in IL-8 protein leads to massive infiltration of neutrophils, which results in the

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[3] Abbreviations used in this paper: 3-O-C12-HSL, *N*-3-oxododecanoyl-homoserine lactone; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; MEK, MAP kinase/ERK; C4-HSL, *N*-butyryl-L-homoserine lactone; h, human.

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production of proteolytic enzymes, such as elastase, and subsequently tissue destruction and pulmonary failure. Persistent infection with *P. aeruginosa* leads to exuberant production of IL-8 and neutrophil migration into the lungs and results in chronic inflammation and tissue destruction.

Supernatants from *P. aeruginosa* contain a small, nonprotein, non-LPS molecule that stimulates bronchial epithelial cells to produce IL-8 (20). This phenomenon was further examined by DiMango et al. who demonstrated that human epithelial cells incubated with 3-O-C12-HSL in vitro produced significant levels of IL-8 (21). It was also shown that the *P. aeruginosa* mutant PAOR1 (22), which produces miniscule amounts of the autoinducer 3-O-C12-HSL, had a decreased ability to stimulate IL-8 when compared with the wild-type strain PAO1 (14). The production of *Pseudomonas* autoinducers have been shown to be essential for the infection and pathology found in both acute and chronic infections (23–25). In these models it was observed not only that the degree of pathology was decreased but that the infiltration of neutrophils to the site of infection was also reduced with autoinducer mutant strains of *Pseudomonas*. These data suggest that 3-O-C12-HSL is instrumental in the initial recruitment of neutrophils to the lung.

In this study we examined whether 3-O-C12-HSL could activate human lung cells to produce the neutrophil chemotactic molecule IL-8. We also demonstrate the role of mitogen-activated protein (MAP) kinases and transcription factors in the production of IL-8 with activation 3-O-C12-HSL in structural cells.

Materials and Methods

Cell lines and culture conditions

L828, a nontransformed normal human lung fibroblast strain (26), A549, human type II-like alveolar epithelial cells (ATCC, Manassas, VA), and primary human foreskin fibroblasts (27), were maintained in MEM (Life Technologies, Gaithersburg, MD) supplemented with 10% FBS (HyClone Laboratories, Logan, UT) and 50 U/ml gentamicin (Life Technologies). The 16HBE, human bronchial epithelial cell line, was grown on collagen/fibronectin-coated culture dishes in MEM with 10% FBS and 50 U/ml gentamicin (28). Cells were passed every 4–5 days using 0.05% trypsin with 0.1% EDTA to dissociate adherent cells. Fibroblasts were used between passages 5 and 20.

3-O-C12-HSL synthesis

3-O-C12-HSL was synthetically produced as previously described (10). This molecule is structurally and functionally identical with the natural 3-O-C12-HSL produced by *P. aeruginosa*. No detectable levels of endotoxin were found in preparations of 3-O-C12-HSL using a *Limulus* Amebocyte Lysate assay (Cape Cod Associates, Falmouth, MA).

RNase protection assays

16HBE and L828 cells were grown in 100-mM plates until confluent monolayers formed. Cells were serum-starved for 18 h and then stimulated with 100 μM 3-O-C12-HSL for varying amounts of time. Cells were extracted with Tri Reagent (Molecular Research Center, Cincinnati, OH), and total RNA was quantified by spectrophotometry. DNA templates for human chemokines (PharMingen, San Diego, CA) were used to make ^{32}P-labeled RNA probes. Probes were mixed with 2–8 μg of sample RNA, and a RiboQuant kit (PharMingen) was used to perform RNase protection assays as per the manufacture's procedures. Protected RNA fragments were resolved on a 5% polyacrylamide gel and quantified by densitometry. GAPDH controls were used to standardize the quantification of RNA samples.

IL-8 measurements

Cell lines were grown in 96-well plates until confluent ($\sim 5 \times 10^4$ cells/well) and then serum starved for 18 h before stimulation. Cells were treated with 25–100 μM 3-O-C12-HSL or C4-HSL for 18 h and the amount of IL-8 in the supernatants determined using an IL-8 ELISA as previously described (29). In MAP kinase experiments, 50 μM of the MAP kinase/extracellular signal-regulated kinase (MEK) inhibitor PD98059 (Calbiochem, La Jolla, CA) was added to cultures along with a titration of 3-O-C12-HSL. Cells were incubated for 18 h, and IL-8 in the supernatants was measured.

Granulocyte chemotaxis assays

Granulocytes were isolated from healthy human plasma by centrifugation over a Ficoll-Hypaque density gradient. Residual erythrocytes were removed via hypotonic lysis. The resulting population routinely contained 80–90% neutrophils and 1–15% eosinophils and basophils. Using the ChemoTx System (Neuroprobe, Cabin John, MD), 3.25-mm diameter, 3-μm pore polycarbonate membranes were placed over the wells of a corresponding 96-well tissue culture plate. Each well contained 28 μl of MEM or supernatant from 16HBE cultures, which were nonstimulated or induced with 100 μM 3-O-C12-HSL for 18 h. Some wells contained 10 μg/ml mouse anti-human IL-8 mAb (R&D Systems, Minneapolis, MN) or control mouse IgG. As a positive control for migration, a standard curve of 0–5000 ng/ml of recombinant human IL-8 (rhIL-8) (R&D Systems) was added to some wells. Repeatedly, maximal migration was found with 5–500 ng/ml rhIL-8. Granulocytes (2.5×10^5) in 25 μl of DMEM, 10% FBS, 50 μg/ml L-glutamine, 50 U/ml gentamicin were added to the upper side of the membrane, and the plate was incubated at 37°C for 2 h. Granulocytes in the lower chamber were quantified with a FACScan (BD Biosciences, San Jose, CA). Forward vs side scatter was used to identify the granulocytes that were in the migrating population. Chemotactic index was calculated by dividing values for cells migrating to supernatants by values for cells migrating to medium controls (background migration). A chemotactic index of 1 reflects no specific migration. A standard curve was constructed using known numbers of input granulocytes; this was used to calculate the total percentage of the input cell population that migrated to the supernatants.

Transient transfections

Luciferase reporter constructs of the IL-8 promoter consisted of a full-length 5′ unencoded region of the IL-8 promoter (−1481→+44) and deletions of this construct of (−546→+44) and (−133→+44). Site-directed mutations were also made in the binding sites for NF-κB, NF-IL-6, and AP-1 in the −133 construct (see Fig. 5*A*) (30). Transfections were performed by adding 16HBE cells to collagen/fibronectin-coated six-well plates (BD Labware, Franklin Lakes, NJ) at 2×10^6 cells/well in 1 ml MEM supplemented with 2% FBS and 50 U/ml gentamicin. DNA was prepared by mixing 5 μg of the IL-8 promoter constructs in 20 mM HEPES buffer with 20–30 μl DOTAP (Boehringer Mannheim, Indianapolis, IN) at room temperature for 15 min. The DNA/DOTAP mixture was added to the 16HBE cells and incubated overnight at 37°C. Transfection with a vector expressing β-galactosidase was used to determine efficiency of transfections. Transfected cells were serum-starved for 18 h before being stimulated with 100 μM 3-O-C12-HSL or 50 ng/ml hTNF-α + 200 U/ml hIL-1β (Genzyme, Cambridge, MA) for an additional 12 h. Cells were assayed for luciferase activity using a luciferase assay kit (Promega, Madison, WI) and light intensity determined with a luminometer (Packard, Meriden, CT). Data were expressed as fold increase over nonstimulated cells transfected with the same construct.

EMSA

16HBE and L828 cells were grown to confluent monolayers in six-well plates ($\sim 2 \times 10^6$ cells/ml). Cells were serum-starved for 18 h before stimulation with 100 μM 3-O-C12-HSL, 50 ng/ml hTNF-α (Genzyme), or 10 ng/ml PMA (Sigma, St. Louis, MO) for 30 min or 2 h at 37°C. Cells with MEM only were used as a control for induction. Nuclear protein extracts were prepared as previously described (31). Briefly, cells were removed from plates and washed in cold PBS. Cell pellets were resuspended in 400 μl of an ice-cold hypotonic buffer (10 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5% Nonidet P-40, and 0.2 mM PMSF) and incubated on ice for 10 min. Lysates were vortexed for 10 s and centrifuged at 13,000 rpm in a microfuge for 30 s. Supernatants containing cytoplasmic proteins were removed, and pellets were resuspended in 50 μl cold hypertonic buffer (20 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF). Samples were incubated on ice for 20 min then centrifuged for 2 min at 4°C. Nuclear protein-containing supernatants were removed and quantified by bicinchoninic acid protein assay (Pierce, Rockford, IL). Consensus sequences for the AP-1 DNA binding site (5′-CGCTTGATGAGT CAGCCGGAA-3′), AP-2 DNA binding site (5′-GATCGAACTGAC CGCCCGCGGCCCGT-3′), and NF-κB DNA binding site (5′-AGTT GAGGGGACTTTCCCAGGC-3′) (Promega) were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase for 30 min at 37°C. Labeled DNA was purified over a G-25 column to remove unbound nucleotides. Nuclear protein extracts, at a concentration of 300–5 μg, were incubated at room temperature for 20 min with ~50,000 cpm (~0.06 pmol) of the

labeled oligonucleotide suspended in binding buffer (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, and 0.05 mg/ml poly(dI:dC)). Samples were resolved on a 4% nondenaturing polyacrylamide gel at 100 V and exposed to film. Cold competition assays were performed by adding a 100-fold excess of unlabeled oligonucleotides to nuclear protein extracts 10 min before the addition of the labeled oligonucleotides. Supershift experiments were completed by adding 2 μg of the anti-AP-2 Ab C18 (Santa Cruz Biotechnology, Santa Cruz, CA) to the binding reaction.

Immunoblots

Cell lysates were obtained from 16HBE cells as previously described for EMSA. Proteins were quantified by bicinchoninic acid protein assay and 20 μg of protein was mixed with an equal volume of 2× SDS sample buffer and boiled for 5 min. Denatured proteins were separated by electrophoresis on a SDS-polyacrylamide gel along with control proteins (nonphosphorylated ERK-2 and phosphorylated ERK-2) (Cell Signaling Technology, Beverly, MA). Proteins were transferred to Hybond-C extra nitrocellulose membrane (Amersham, Piscataway, NJ) in 20 mM Tris base, 150 mM glycine, and 20% v/v methanol overnight at 35 V. Nonspecific binding was blocked by incubating the blot with 10% skim milk in PBS with 0.1% Tween 20 for 2 h at room temperature. Immunoreactive proteins were detected by incubating the blot with specific Abs (phospho-p44/42 mAb E10 or phospho-p38) overnight at 4°C (Cell Signaling Technology). These Abs only recognize the active phosphorylated form of the kinase. Between each step the nitrocellulose was washed four times for 5 min with PBS/0.1% Tween 20. Bound Abs were detected with either an anti-mouse IgG or an anti-rabbit IgG conjugated to HRP. Specific bands were visualized with ECL reagents (Amersham, Piscataway, NJ) then exposed to Kodak X-OMAT film (Kodak, Rochester, NY).

Nuclear mobilization of NF-κB

Cells were grown in eight-well Permanox chamber slides (Nalge Nunc, Naperville, IL) at 1–5 × 10^4 cells/well in MEM with 10% FBS. Cells were serum-starved for 18 h before incubation with medium only or with 100 μM 3-O-C12-HSL for 2 h. Some wells were pretreated with 50 μM PD98059 for 1 h before stimulation. Immunocytochemistry was used to determine the localization of NF-κB in cells. Cells were washed several times with PBS + 0.05% Tween 20, and endogenous peroxidase activity was quenched with 3% H_2O_2 for 20 min. Cells were blocked with 2% horse serum for 1 h at room temperature before overnight incubation at 4°C with 2 μg/ml anti-NF-κB p65 Ab (Santa Cruz Biotechnology) or an isotype control mouse IgG (Caltag, South San Francisco, CA). Cells were washed and incubated at room temperature for 30 min with 7 μg/ml of a biotin-labeled anti-mouse IgG secondary Ab (Vector Laboratories, Burlingame, CA). Cells were washed with PBS/Tween and incubated for 1 h at room temperature with a 1/1000 dilution of streptavidin HRP (Jackson Immuno-Research Laboratories, West Grove, PA). AEC (Zymed Laboratories, San Francisco, CA), which reacts with the bound HRP to form a red precipitate that can be visualized in the cells, was used to detect anti-NF-κB Ab binding.

Statistical tests

A two-tailed Student *t* test was used to analyze the statistical differences between samples.

Results

3-O-C12-HSL stimulation of human cells induces the production of IL-8

Cultures of the 16HBE cells (Fig. 1) or L828 fibroblasts (Fig. 2) in MEM without serum induced little IL-8 mRNA or protein. Stimulation of these cells with an optimal dose of 100 μM 3-O-C12-HSL dramatically increased the levels of IL-8 mRNA over that of nonstimulated cells within 2–4 h. 3-O-C12-HSL had no toxic effects on these cells at any of the concentrations used as assessed by both viability counts with trypan blue and MTT assays (data not shown). In kinetic studies of 3-O-C12-HSL activation of 16HBE cells, a 15-fold increase in IL-8 mRNA was stimulated after only 2 h (Fig. 1*A*). This induction dissipated after 4 h, and IL-8 mRNA returned to background levels by 24 h. Supernatants from 3-O-C12-HSL-stimulated 16HBE cells were also tested in an IL-8 ELISA. Production of IL-8 protein was found between 4 and 24 h of stimulation, at which point the amount of IL-8 in the supernatant

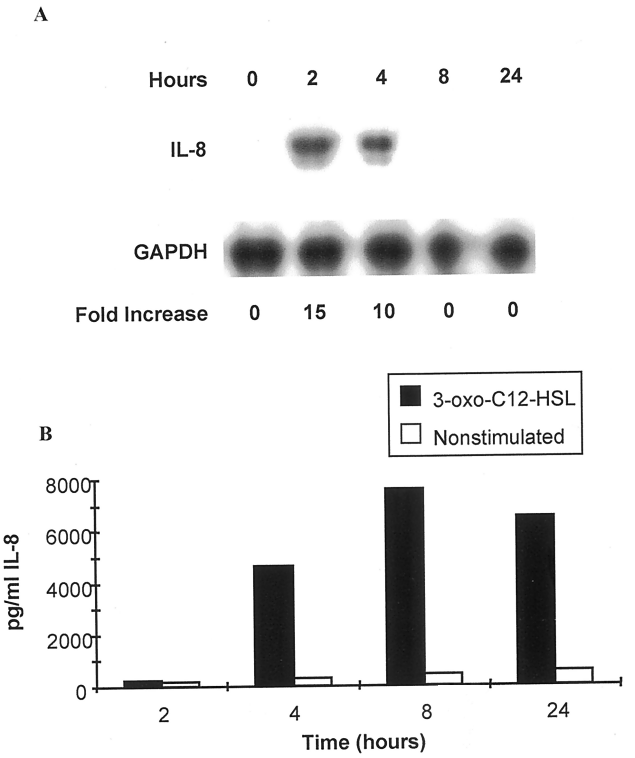

FIGURE 1. 3-O-C12-HSL induces IL-8 mRNA and protein in 16HBE human bronchial epithelial cells. *A*, RNase protection for IL-8 was performed using RNA extracted from 16HBE epithelial cells stimulated with 100 μM 3-O-C12-HSL for 0, 2, 4, 8, or 24 h. Levels of mRNA were determined by densitometry and normalized to GAPDH mRNA. Fold induction of mRNA was determined by comparing the relative levels of IL-8 mRNA in resting cells to 3-O-C12-HSL-stimulated cells. *B*, IL-8 in culture supernatants was quantified using an IL-8-specific ELISA. □, Supernatant from nonstimulated cells; ■, cells stimulated with 100 μM 3-O-C12-HSL. These data are representative of three independent experiments.

was starting to decrease. After 8 h of stimulation with 3-O-C12-HSL, a maximal 14-fold induction in IL-8 protein was detected (Fig. 1*B*). Although not as dramatic, a similar induction in IL-8 mRNA and protein was found with 3-O-C12-HSL-stimulated L828 fibroblasts (Fig. 2). Interestingly, although maximal induction of IL-8 mRNA in L828 cells was found after 4 h of stimulation with 3-O-C12-HSL, levels of IL-8 mRNA persisted out to at least 24 h.

Production of IL-8 protein was stimulated in cells at levels of 3-O-C12-HSL as low as 25 μM, which in some cell lines was sufficient to give a 2- to 3-fold induction over cells with medium only. When cells were activated with 100 μM 3-O-C12-HSL, a 7- to 8-fold induction in IL-8 protein was observed (Fig. 3). When cells were cultured with IL-1β or TNF-α, known potent inducers of IL-8, 7,000–15,000 pg/ml IL-8 were measured in culture supernatants. Interestingly, when A549, a type II-like alveolar epithelial cell line, and foreskin fibroblasts were stimulated with 25 or 100 μM 3-O-C12-HSL a similar induction in IL-8 was observed (Fig. 3). This indicates that 3-O-C12-HSL can interact with multiple cell types, which include epithelial cells and fibroblasts from different sites in the body, and stimulates a similar IL-8 response. When 100 μM C4-HSL, a second *P. aeruginosa* autoinducer, was added to cultures of epithelial or fibroblast cells, no IL-8 was detected above that of nonstimulated cells (data not shown). This indicates that the activation of epithelial cells and fibroblasts with 3-O-C12-HSL is structurally specific. The structural specificity of

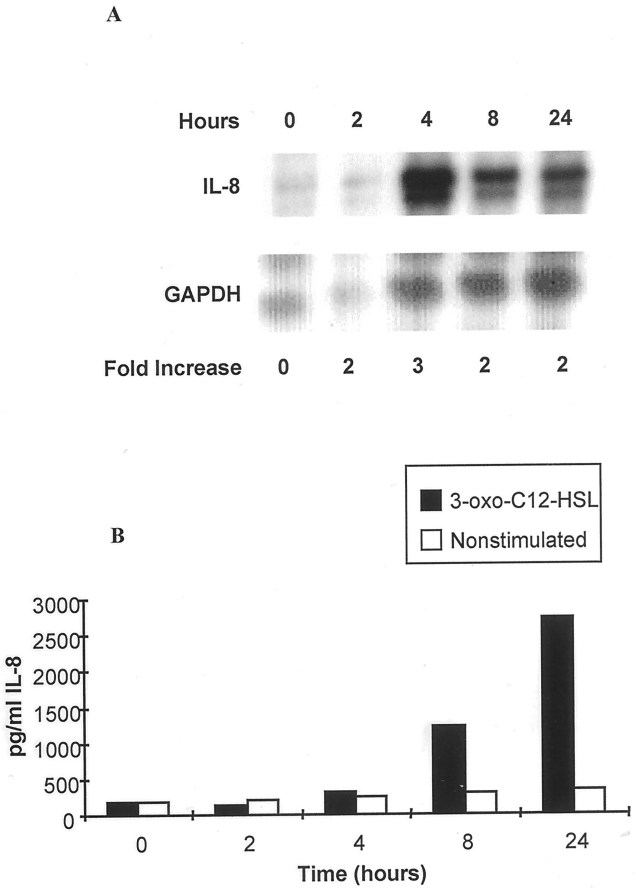

FIGURE 2. 3-O-C12-HSL induces IL-8 mRNA and protein in L828 human fibroblasts. Experiments were performed as described in Fig. 1. *A*, IL-8 mRNA from L828 cells after stimulation with 3-O-C12-HSL. *B*, IL-8 protein was measured from culture supernatants of L828 cells stimulated with 3-O-C12-HSL. These data are representative of three independent experiments.

homoserine lactone activation of IL-8 was also demonstrated by other investigators (21).

Supernatants from 3-O-C12-HSL-stimulated cells induces neutrophil chemotaxis

Having shown that 3-O-C12-HSL induced the production of both IL-8 mRNA and protein in multiple cell types, we next wanted to determine whether this IL-8 was functionally active and whether it could stimulate the migration of neutrophils. Supernatants from 16HBE cells that were incubated with medium only or medium containing 100 μM 3-O-C12-HSL were added to a neutrophil chemotaxis assay. When medium alone, medium containing 3-O-C12-HSL, or supernatant from nonstimulated 16HBE cells was added to the assay, ~20% of the neutrophils migrated though the chamber; this reflects background migration and was assigned a chemotactic index of 1. When supernatant from 3-O-C12-HSL-stimulated cells was added to the assay, a chemotactic index of 3.2 was obtained, which represents the migration of 63% of the cells (Fig. 4). This level of migration was equal to the maximal migration stimulated by rhIL-8. To demonstrate specificity of this migration to IL-8, an anti-IL-8 Ab was added to culture supernatants. When anti-IL-8 was added to rhIL-8 controls, the migration of neutrophils was inhibited to background levels. Interestingly, when anti-IL-8 was added to supernatants from 3-O-C12-HSL-stimulated cells, migration was only partially inhibited (~70%), indicating that 3-O-C12-HSL is possibly stimulating an additional chemotactic factor(s) that is inducing migration. Using RNase protection assays, no significant increases were found with the chemotactic factors RANTES, IP-10, macrophage-inflammatory protein-2α, macrophage-inflammatory protein-2β, and monocyte chemoattractant protein-1 with 3-O-C12-HSL stimulation of 16HBE cells or L828 fibroblasts (data not shown). Similar chemotactic activities were found with supernatants from 3-O-C12-HSL-stimulated L828 cells (data not shown).

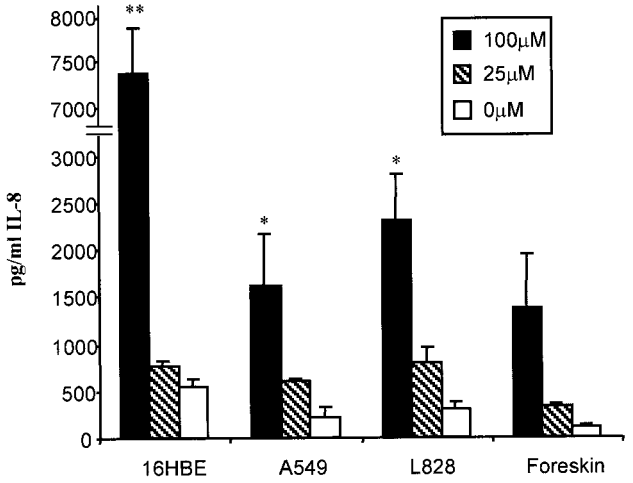

FIGURE 3. 3-O-C12-HSL stimulates production of IL-8 protein from several types of epithelial and fibroblast cells. Epithelial and fibroblast cells were untreated (□), stimulated with 25 μM (▨) or 100 μM 3-O-C12-HSL (■) for 18 h. An IL-8 specific ELISA was used to quantify levels of IL-8 protein in culture supernatants. **, $p < 0.0001$; *, $p \leq 0.005$ compared with untreated cultures.

5′ upstream regions of the IL-8 promoter are essential for 3-O-C12-HSL activation

Induction of IL-8 synthesis by cytokines usually involves *cis*-acting elements that stimulate the transcription of IL-8 (32). Fig. 5*A*

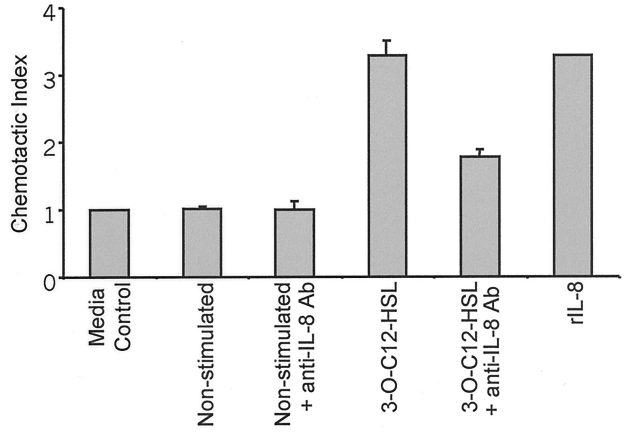

FIGURE 4. Supernatants from 3-O-C12-HSL-stimulated 16HBE cells induce chemotaxis of neutrophils. 16HBE cells were incubated with medium alone (nonstimulated) or with 100 μM 3-O-C12-HSL. Supernatants from these cells were added to a neutrophil chemotaxis assay. Data are expressed as chemotactic index, which is the percentage of migrating cells divided by medium control migration (background). A value of 1 represents no specific migration. To show specificity of the migration to IL-8, 10 μg/ml of anti-human IL-8 Ab was added to supernatants. Controls included the addition of medium only or 5 ng/ml recombinant IL-8 to the chemotaxis assay. 3-O-C12-HSL alone showed no chemotaxis over that of medium control.

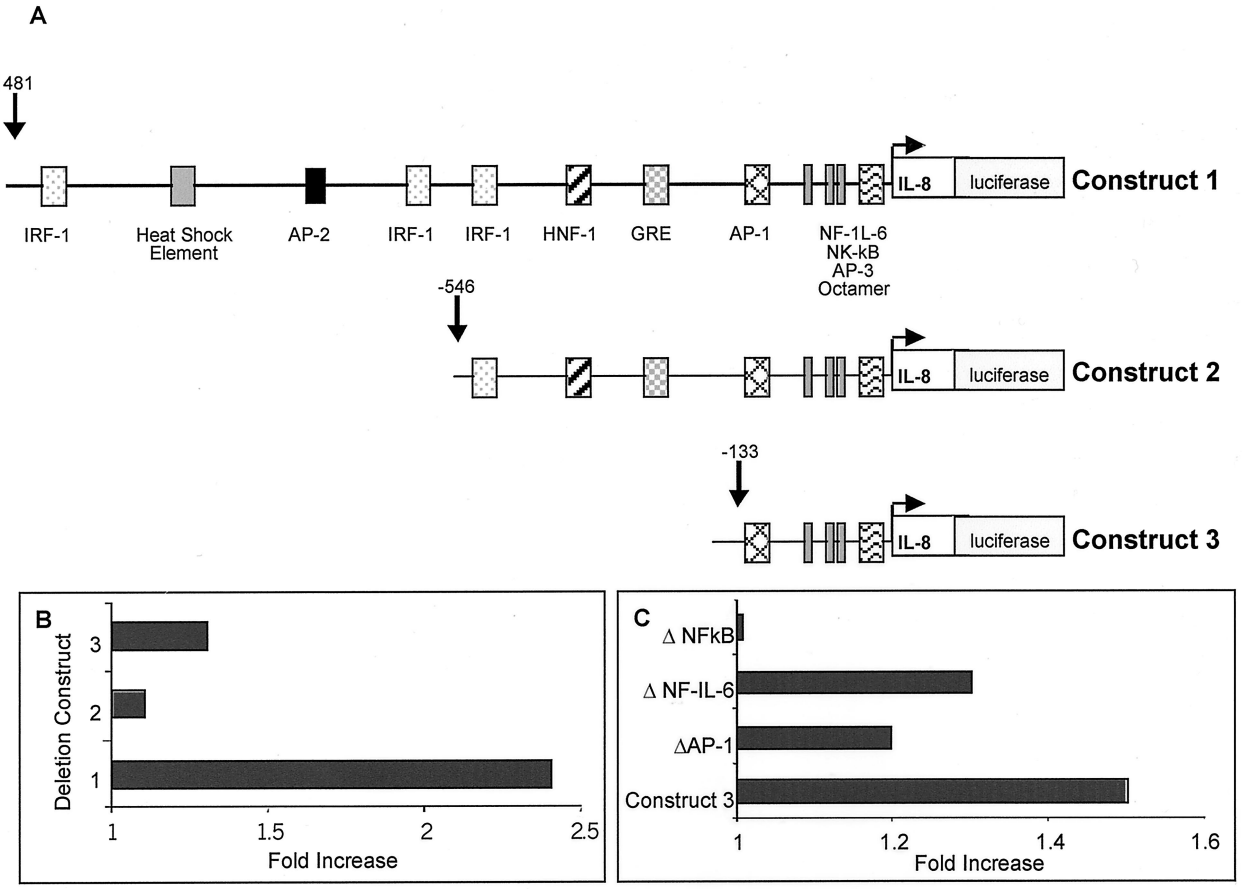

FIGURE 5. Upstream regions of the IL-8 promoter are essential for the maximal induction of IL-8 by 3-O-C12-HSL. *A*, Schematic of the IL-8 promoter region indicating transcription factor binding site locations and luciferase reporter. Construct 1 is the full-length IL-8 promoter (−1481 to +44), constructs 2 and 3 are deletion constructs that contain nucleotides (−546 to +44) and (−133 to +44), respectively. Arrows indicate the location of deletions made in the promoter. *B* and *C*, Full-length and deletion luciferase constructs (*B*) or constructs containing site-directed mutations in the AP-1, NF-IL-6, or NF-κB DNA binding sites (*C*) were transfected into 16HBE cells, and luciferase activity was measured when cells were stimulated with 100 μM 3-O-C12-HSL. Fold increase in luciferase activity was determined by comparing relative light units from 3-O-C12-HSL-stimulated cells with nonstimulated cells transfected with the same IL-8 luciferase construct. These studies are representative of three separate experiments.

shows diagrams of the 5′ region of the IL-8 promoter and promoter deletion constructs. To test whether 3-O-C12-HSL regulation of IL-8 was due to the activation of transcription factors, luciferase reporter constructs of the IL-8 promoter were transiently transfected into 16HBE cells (Fig. 5*A*). Transfected cells were stimulated with 3-O-C12-HSL or a mixture of TNF-α and IL-1β as a control. Stimulation with TNF-α and IL-1β induced the production of luciferase with all the constructs (2.5- to 4-fold over background) except for the NF-κB-deleted construct, indicating that NF-κB is essential for activation of IL-8 with these cytokines (data not shown). When transfected cells were stimulated with 3-O-C12-HSL, maximal induction of luciferase production was seen with the −1481 to +44 full-length construct (construct 1). When a deletion was made at −546 (construct 2), a decrease in activity of 75% was repeatedly found. An additional deletion at −133 (construct 3) gave no further significant decrease in luciferase activity (Fig. 5*B*). This indicates that the deletion of DNA between −1481 and −546 eliminated sites that were important for 3-O-C12-HSL induction of maximal IL-8 transcription. Site-directed mutations in the AP-1, NF-IL-6, and NF-κB DNA binding sites were made in construct 3 and tested in transcription assays. When compared with construct 3, there was a 60% reduction in luciferase activity with the construct that contained a mutation in the AP-1 DNA binding site and a 40% reduction with the NF-IL-6 mutated construct (Fig. 5*C*). A mutation in the NF-κB binding site resulted in virtually no luciferase activity with 3-O-C12-HSL activation (reduction of 99% when compared with construct 3). These data indicated that NF-κB was essential for activation of IL-8 with 3-O-C12-HSL stimulation of 16HBE cells and that the area between −1481 and −546 was needed for maximal IL-8 induction.

EMSA confirms that 3-O-C12-HSL induces NF-κB and AP-2 but not AP-1

Other investigators have shown that AP-1 is important for IL-8 induction in lung cells (32). In transcriptional reporter assays, when AP-1 was mutated there was a 60% decrease in luciferase induction, when compared with construct 3 (Fig. 5*C*). EMSA was performed to determine whether AP-1 was induced in 16HBE cells with 3-O-C12-HSL stimulation. Using a consensus sequence for the AP-1 binding site revealed constitutive expression of AP-1 even in nonstimulated cell extracts (data not shown). Therefore, stimulation of 16HBE cells with 3-O-C12-HSL did not induce a significant increase in binding to the AP-1 DNA probe. A similar constitutive expression of AP-1 was found by other investigators examining IL-8 expression in A549 epithelial cells (33). These data indicate that the 3-O-C12-HSL stimulation of IL-8 is not dependent on induction of the transcription factor AP-1. Examination of the genomic DNA sequence of the 5′-IL-8 promoter region identified many consensus sequences for transcriptional activator

binding sites (Fig. 5*A*). Based on transcription reporter assays, interest was focused on the sites located between −1481 and −546. Using these data we identified a DNA consensus sequence that could bind the transcription factor AP-2. Therefore, we examined whether 3-O-C12-HSL could induce the transcription factor AP-2. Nuclear extracts were made from L828 and 16HBE cells incubated with or without 100 μM 3-O-C12-HSL for 30 min. Nuclear extracts (5 μg) were added to a ^{32}P-labeled oligonucleotide with a consensus sequence for the AP-2 DNA binding site. When this hybridized mixture was run on a nondenaturing gel, a shift from unbound DNA was clearly seen with extracts from cells that were stimulated with 3-O-C12-HSL compared with the slight shift observed with extracts from nonstimulated cells (Fig. 6*A*). Nuclear extracts from cells stimulated with PMA, a known inducer of AP-2, were used as a positive control. When a 100-fold excess of unlabeled AP-2 oligonucleotide was added to 16HBE extracts, most of the AP-2 shift was competed away, but 100-fold excess of a nonspecific oligonucleotide had no effect on the shift (Fig. 6*A*). To further prove the specificity of this AP-2 shift, supershifts were performed using an AP-2 specific Ab. When this Ab was added to 16HBE extracts along with the labeled AP-2 oligonucleotide, an additional shift in size was observed (Fig. 6*B*). These data confirm that 3-O-C12-HSL induces the transcription factor AP-2. When EMSAs were performed using a NF-κB consensus oligonucleotide, a shift was observed in extracts from 16HBE cells that were stimulated for 2 h with 3-O-C12-HSL (Fig. 7*A*). This shift could be competed away with a 100-fold excess of unlabeled NF-κB DNA but not a nonspecific DNA sequence (Fig. 7*B*). We also demonstrated that when 16HBE cells were stimulated with 100 μM 3-O-C12-HSL, NF-κB translocated from the cytoplasm to the nucleus (Fig. 8*C*). These data show that 3-O-C12-HSL induces the transcription factor NF-κB. Similar shifts for AP-2 and NF-κB were found when L828 fibroblasts were stimulated with 3-O-C12-HSL (data not shown).

3-O-C12-HSL activation of MAP kinase is important for IL-8 production

To better understand the activation of NF-κB and the subsequent production of IL-8 in these cells, we evaluated a potential role for MAP kinases. When 16HBE cells were stimulated with 3-O-C12-HSL, the active and phosphorylated forms of the ERK1/2 kinases were induced (Fig. 8*A*). Kinetic studies of 3-O-C12-HSL activation revealed ERK induction as early as 10 min with maximal expression at 15 min. Addition of the MEK inhibitor PD98059, which blocks the activation of ERK, completely inhibited induction. To determine whether this induction of MAP kinases was specific to the ERK pathway, samples were also tested for induction of the p38 kinase. When cellular extracts from 3-O-C12-HSL-stimulated cells were tested using a p38-specific Western blot, there was no significant induction over that found with nonstimulated cells (data not shown). Having shown that ERK was induced by 3-O-C12-HSL stimulation, we next determined what effect this may have on IL-8 and NF-κB induction. When 16HBE cells were cocultured with 3-O-C12-HSL and 50 μM of PD98059, the production of IL-8 protein was reduced by 80–90% (Fig. 8*B*). The MEK inhibitor was not found to have toxic effects at this concentration; therefore, the reduction in IL-8 was not due to cell death (data not shown). To determine whether the inhibition of IL-8 production was due to the inhibition of transcription factors, NF-κB nuclear mobilization experiments were performed. In nonactivated cells, NF-κB is held in the cytoplasm by inhibitory proteins called IκB. When cells become stimulated, NF-κB is released from IκB and translocates into the nucleus where it activates the transcription of multiple genes. When 16HBE cells were cultured with medium only, NF-κB was found mainly in the cytoplasm, but upon activation with 3-O-C12-HSL, NF-κB accumulated in the

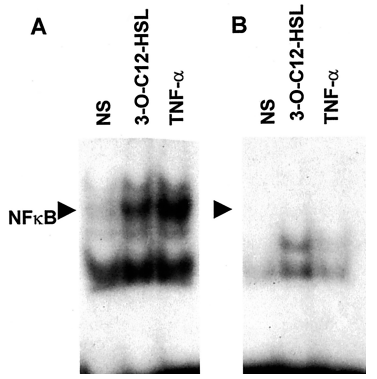

FIGURE 7. The transcription factor NF-κB is induced by 3-O-C12-HSL stimulation of 16HBE cells. 16HBE cells were either nonstimulated (NS) or stimulated with either 100 μM 3-O-C12-HSL or 50 ng/ml TNF-α for 2 h. *A*, Nuclear extracts (5 μg) were incubated with ^{32}P-labeled NF-κB oligonucleotides and run on a 4% nondenaturing gel. *B*, Cold (unlabeled) NF-κB oligonucleotide was added to the binding reactions. Arrows indicate the locations of shifted bands. These data are representative of three separate experiments.

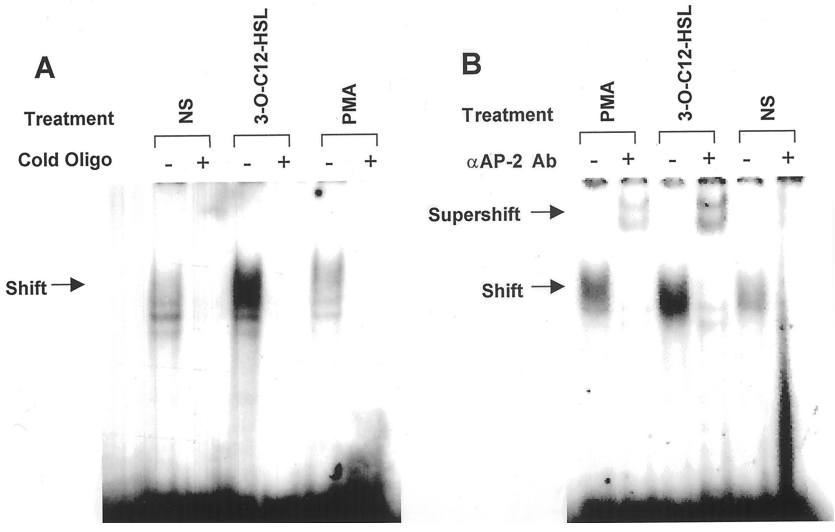

FIGURE 6. 3-O-C12-HSL stimulates the induction of the transcription factor AP-2 in 16HBE cells. 16HBE cells were either nonstimulated (NS) or stimulated with either 100 μM 3-O-C12-HSL or 10 ng/ml PMA for 30 min. Nuclear extracts were made and mixed with ^{32}P-labeled AP-2 oligonucleotide. *A*, Samples incubated with (+) or without (−) cold (unlabeled) AP-2 oligonucleotide and run on a 4% nondenaturing gel. *B*, Nuclear extracts incubated with (+) or without (−) the addition of anti-AP-2 Ab. Arrows indicate the locations of shifted and supershifted DNA. Shift assays were repeated three to four times with similar results.

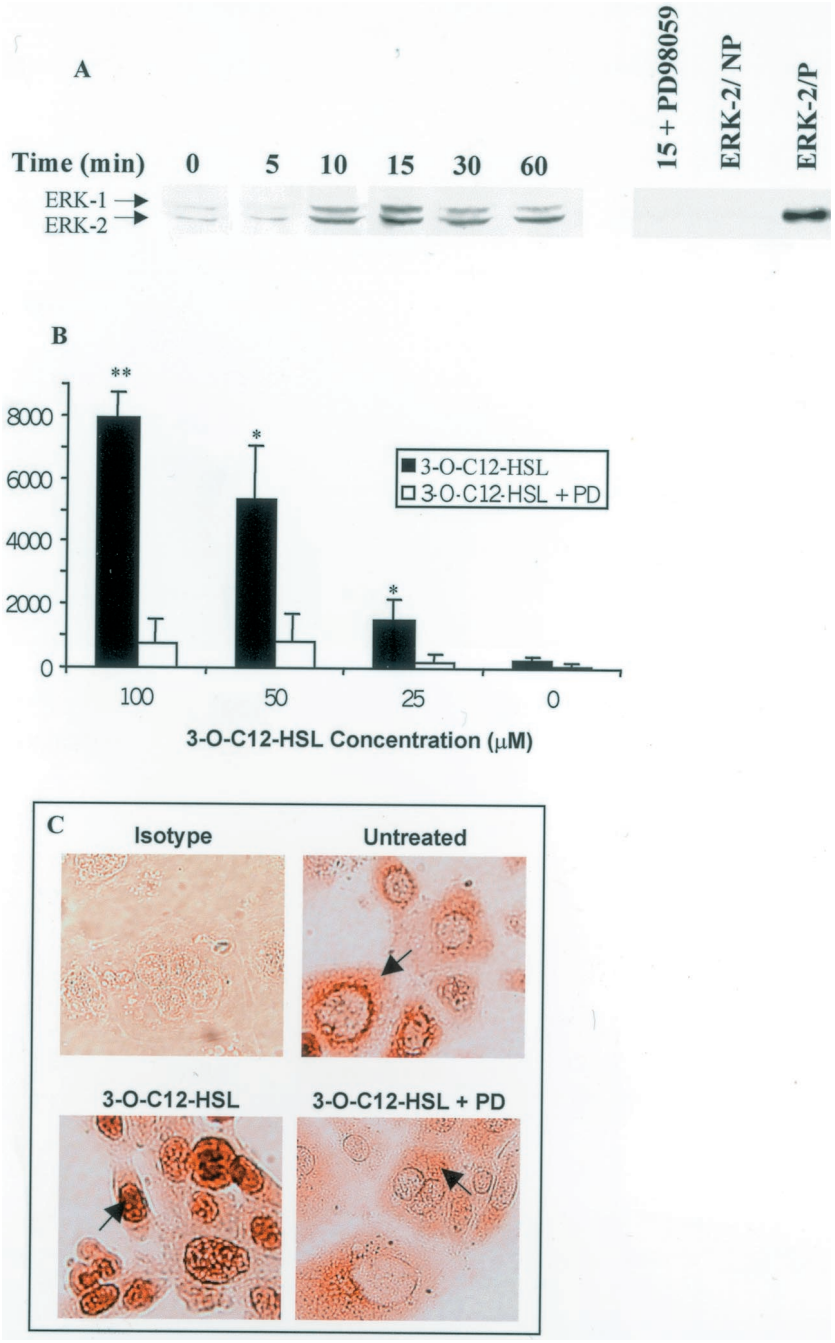

FIGURE 8. Activation of IL-8 and NF-κB occurs through ERK activation. *A*, Cytoplasmic protein extracts from 16HBE cells stimulated for 5–60 min with 3-O-C12-HSL were analyzed in an ERK1/2 specific Western blot. Specificity of the Western blot was confirmed by extracts from cells cocultured for 15 min with 3-O-C12-HSL and 50 μm PD98095 and the controls, 10 μg/ml nonphosphorylated ERK2 (ERK2/NP) and phosphorylated ERK2 (ERK2/P). *B*, 16HBE cells were cultured in medium containing 25–100 μM 3-O-C12-HSL, with (□) or without (■) 50 μM PD98059. Cells were incubated for 18 h, and IL-8 in the supernatants was measured. *, $p < 0.0005$; **, $p < 0.001$. *C*, 16HBE cells were stained with an anti-NF-κB Ab to determine the localization of NF-κB in cells that were cultured with medium only (untreated) or 3-O-C12-HSL. Some wells were preincubated for 1 h with PD98059. An isotype control (mouse IgG) was used to control for background staining. Arrows indicate the localization of NF-κB staining.

nucleus. The addition of a MEK inhibitor with 3-O-C12-HSL stimulation failed to induce nuclear mobilization of NF-κB (Fig. 8*C*). These data indicate that the induction of NF-κB by 3-O-C12-HSL occurs through the activation of a MAP kinase pathway.

Discussion

Pulmonary infections with *P. aeruginosa* are instrumental in initiating a cascade of IL-8 production. During these infections pulmonary structural cells such as epithelial cells and fibroblasts produce IL-8, which leads to the infiltration of neutrophils. The exposure of the lung to neutrophil-generated mediators, such as elastase and reactive oxygen species, leads to tissue destruction, obstructive pulmonary disease, and, ultimately, pulmonary failure. Several products of *P. aeruginosa* stimulate bronchial epithelial cells to produce IL-8, including pilin, nitrate reductase, and pyocyanin (33–35). In this study, we show that the *P. aeruginosa* autoinducer 3-O-C12-HSL is also a potent inducer of IL-8 production. The stimulation of bronchial epithelial cells (16HBE) with 3-O-C12-HSL induced a significant induction in IL-8 mRNA and protein (Fig. 1). During the initial colonization of the lung with *P. aeruginosa* it is thought that the bacteria adhere to the epithelium in the upper airways and that some of these bacteria are endocytosed by the epithelial cells (36). In this environment the epithelial cells would be in direct contact with *P. aeruginosa*. Because 3-O-C12-HSL freely diffuses and is actively pumped out of the bacteria, it would directly interact with epithelial cells (37). Previous data have shown that in the squid, where the symbiotic bacteria *V. fisheri* produces an autoinducer similar to *P. aeruginosa* (3-O-C6-HSL), this molecule was found not only in the area around the bacteria, but was detected also in the underlying epithelial cells (38). These observations support the hypothesis that 3-O-C12-HSL produced by *P. aeruginosa* interacts with the surrounding tissue.

This production of 3-O-C12-HSL could initiate inflammation in the lung and start a cascade of IL-8 production and, consequently, neutrophil infiltration. In cystic fibrosis patients, *P. aeruginosa* is found at high concentrations (10^8 CFU/ml of sputum) and grows in microcolonies or biofilms, which are clusters of bacteria embedded in a matrix of polysaccharide (39, 40). Although the concentration of 3-O-C12-HSL in the lungs of *P. aeruginosa*-infected patients remains unknown, mRNA encoding proteins involved in the regulation of 3-O-C12-HSL were found in the sputum of cystic fibrosis patients (41). These data indicate that a functional cell-to-cell signaling mechanism occurs in vivo and that 3-O-C12-HSL is most likely produced in these patients. Therefore, in the lung it is likely that *P. aeruginosa* attaches to epithelial cells and produces 3-O-C12-HSL, which stimulates surrounding cells. We also showed that 3-O-C12-HSL stimulates IL-8 production in L828, a primary human lung fibroblast strain (Fig. 2). During *P. aeruginosa* lung infections, where tissue destruction is prominent, the bacteria would also have direct contact with the underlying matrix of fibroblasts. The activation of L828 fibroblasts with 3-O-C12-HSL persisted longer than that found in 16HBE cells. This long-lasting activation may be a source of extended IL-8 production and may stimulate a steady infiltration of neutrophils into the lung, leading to extensive tissue damage.

The concentrations of 3-O-C12-HSL produced in vivo are unknown, just as the relevant in vivo concentrations for most cytokines are unknown. Studies with *V. fisheri* have shown that the concentration of *Vibrio* autoinducer isolated from the light organs of squids was up to 200-fold higher than that produced by the bacteria in culture (38). There is also evidence that when *Pseudomonas* is grown in a biofilm the concentration of autoinducers produced is significantly higher than that of planktonic bacteria. This indicates that the in vivo concentration of 3-O-C12-HSL in the *Pseudomonas*-infected lung may be significantly higher than the concentration attained in bacterial cultures ($\sim$10 μM). Throughout our studies we found that, in vitro, 3-O-C12-HSL works best at 25–100 μM, but we are also cognizant of the fact that lower concentrations of 3-O-C12-HSL may act in concert with other bacterial products and eukaryotic cytokines to synergistically activate resident pulmonary cells.

IL-8 production has been investigated in many different experimental systems and its regulation occurs at both transcriptional and posttranscriptional levels. Transcriptional regulation occurs through the differential activation and binding of inducible transcription factors. Based on the genomic sequence of the IL-8 gene, several transcription factor binding sites have been identified in the 5′ promoter region (Fig. 5*A*) (42). Using DNase footprinting and transcriptional fusion studies, the transcription factors AP-1, NF-IL-6, and NF-κB were found to be the most prominent activators of IL-8 in most cells (32). We show that 3-O-C12-HSL induction of IL-8 in 16HBE and L828 cells is dependent on the transcription factors AP-2 and NF-κB. When these same cells were stimulated with TNF-α and IL-1β, only the transcription factor NF-κB was necessary for maximal IL-8 induction. The transcription factor AP-2 has never been shown to play a role in the regulation of IL-8. However, other studies do show that AP-2 may be important in the regulation of other cytokines such as TNF-α and TGF-α (43, 44). Based on transcriptional data and EMSA (Figs. 5*B* and 6), we show that with 3-O-C12-HSL stimulation, AP-2 is necessary for maximal induction of IL-8. Inflammatory cytokines, such as IL-6, have been shown to induce AP-2 (45), but indirect stimulation of AP-2 by 3-O-C12-HSL, via IL-6 activation, is unlikely due to the fact that in the EMSA experiments AP-2 induction occurred within 30 min of activation.

Activation of NF-κB is tightly regulated by IκB proteins. These proteins bind to the nuclear localization signal in NF-κB and sequester it in the cytoplasm. When cells are activated, IκB is phosphorylated, which targets it for ubiquitination and subsequent degradation. NF-κB is then released and translocates to the nucleus where it induces the transcription of multiple genes (46). It has been shown that in some cases the Ras-Raf-MEK-MAP-pp90^{rsk} kinase pathway phosphorylates IκBα and initiates its degradation (47, 48). It has recently been shown that *P. aeruginosa* stimulation of mucin production in epithelial cells uses this pathway of activation (49). The activation of NF-κB and production of IL-8 found in 3-O-C12-HSL-stimulated cells may also occur through a similar mechanism. We demonstrate that 3-O-C12-HSL is able to specifically up-regulate both ERK1 and 2 in the cytoplasm of 16HBE cells. When a MEK inhibitor was added to these cultures, 3-O-C12-HSL stimulation of both IL-8 and NF-κB were inhibited. Although we have not shown the involvement of c-Ras and c-Raf in 3-O-C12-HSL activation of cells, we do know that by inhibiting MEK and the subsequent activation of MAP kinases we inhibit the nuclear mobilization of NF-κB and the production of IL-8.

3-O-C12-HSL induction of NF-κB and AP-2 and the subsequent up-regulation of IL-8 could directly contribute to the neutrophil infiltration and inflammation found in *P. aeruginosa* infections. Because the transcription factors NF-κB and AP-2 are pivotal in the production of IL-8 during *P. aeruginosa* infection, these transcription factors should be attractive targets for anti-inflammatory therapy. Blocking the activation of these transcription factors may prevent the early activation of IL-8 and neutrophil migration, therefore decreasing the inflammation induced by *P. aeruginosa* infection. An alternative approach would be to prevent the initial activation of IL-8 by regulating 3-O-C12-HSL production in the bacteria. The use of autoinducer analogs to 3-O-C12-HSL is a mechanism that is currently being developed. The use of analogs to inhibit the activation of R proteins has been demonstrated in many Gram-negative organisms (50–52). By directly inhibiting the production of 3-O-C12-HSL, the inflammation induced by *P. aeruginosa* may be greatly reduced and colonization of the lung repressed. Finally, these autoinducer analogs may also be useful in blocking the actions of native 3-O-C12-HSL on lung structural cells. If structural analogs can be found that antagonize the ability of 3-O-C12-HSL to induce IL-8, they may prove useful therapeutically in cases where exuberant neutrophil responses lead to tissue injury.

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