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Peroxisome Proliferator-Activated Receptors Mediate Host Cell Proinflammatory Responses to *Pseudomonas aeruginosa* Autoinducer▽

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The pathogenic bacterium *Pseudomonas aeruginosa* utilizes the 3-oxododecanoyl homoserine lactone ($3OC_{12}$-HSL) autoinducer as a signaling molecule to coordinate the expression of virulence genes through quorum sensing. $3OC_{12}$-HSL also affects responses in host cells, including the upregulation of genes encoding inflammatory cytokines. This proinflammatory response may exacerbate underlying disease during *P. aeruginosa* infections. The specific mechanism(s) through which $3OC_{12}$-HSL influences host responses is unclear, and no mammalian receptors for $3OC_{12}$-HSL have been identified to date. Here, we report that $3OC_{12}$-HSL increases mRNA levels for a common panel of proinflammatory genes in murine fibroblasts and human lung epithelial cells. To identify putative $3OC_{12}$-HSL receptors, we examined the expression patterns of a panel of nuclear hormone receptors in these two cell lines and determined that both peroxisome proliferator-activated receptor beta/delta (PPARβ/δ) and PPARγ were expressed. $3OC_{12}$-HSL functioned as an agonist of PPARβ/δ transcriptional activity and an antagonist of PPARγ transcriptional activity and inhibited the DNA binding ability of PPARγ. The proinflammatory effect of $3OC_{12}$-HSL in lung epithelial cells was blocked by the PPARγ agonist rosiglitazone, suggesting that $3OC_{12}$-HSL and rosiglitazone are mutually antagonistic negative and positive regulators of PPARγ activity, respectively. These data identify PPARβ/δ and PPARγ as putative mammalian $3OC_{12}$-HSL receptors and suggest that PPARγ agonists may be employed as anti-inflammatory therapeutics for *P. aeruginosa* infections.

Inflammation is a complex biological reaction of the innate immune system in response to harmful stimuli, such as pathogens, damaged cells, or irritants (1). This inflammatory response serves to destroy, dilute, or contain the injurious agent and sets into motion events that promote tissue repair. Although fundamentally a protective response, inflammation may contribute to a host of disease processes (1). Chronic inflammation underlies several degenerative diseases, such as rheumatoid arthritis, atherosclerosis, and lung fibrosis, and acute inflammation is responsible for life-threatening hypersensitivity reactions to insect bites, drugs, and toxins (16, 29, 30, 55). In chronic lung infections, tissue repair by fibrosis may lead to remodeling and loss of function (29). For example, cystic fibrosis (CF) patients are colonized by the gram-negative pathogen *Pseudomonas aeruginosa*, which may become highly resistant to antibiotics (4). Persistent inflammation and infection cause the dilation and destruction of the bronchioles, resulting in airway obstruction and death from respiratory failure (38).

In chronically infected CF patients, *P. aeruginosa* may adopt a sessile biofilm lifestyle that is resistant to antimicrobial treatment (34). The communities of bacteria coordinate changes in gene expression through a cell-to-cell signaling mechanism termed quorum sensing (QS) (14, 54). QS systems consist of small soluble signaling molecules called autoinducers and receptors that act as transcriptional regulators (20). As the bacterial cell density increases, *P. aeruginosa* augments the production of virulence factors in response to the increased production of autoinducers, such as the acyl homoserine lactone (AHL) *N*-(3-oxododecanoyl)-L-homoserine lactone ($3OC_{12}$-HSL) (22). $3OC_{12}$-HSL functions as a ligand for a transcriptional regulator in *P. aeruginosa*, but recent studies indicate that $3OC_{12}$-HSL can also alter mammalian cell responses (56). For example, $3OC_{12}$-HSL can promote the upregulation of proinflammatory cytokines and chemokines and the induction of apoptosis (44). These changes may potentiate inflammatory responses in the host tissue and lead to the exacerbation of disease symptoms and permanent organ damage in chronic infections. Although several independent studies have shown that $3OC_{12}$-HSL can elicit changes in mammalian gene transcription, little is known about the mechanism(s) through which autoinducers influence mammalian cells.

We propose that $3OC_{12}$-HSL alters host cell responses by interacting with endogenous receptor proteins and exploiting mammalian signaling pathways in a process termed interkingdom signaling (41, 43). Several studies have identified proteins, such as the transcriptional regulators nuclear factor kappa B (NF-κB) and activator protein-2 (AP-2), as mammalian components of $3OC_{12}$-HSL interkingdom signaling pathways (45); however, no direct targets of $3OC_{12}$-HSL in mammalian cells have yet been identified. Based on the observations that

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TABLE 1. Primers used in this study

Gene product	Sequence (5′ to 3′) of:	
	Forward primer	Reverse primer
Murine gene products		
GAPDH	AAGGTCGGAGTCAACGGATT	TTGATGACAAGCTTCCCGTT
COX-2	CCCCCACAGTCAAAGACACT	GAGTCCATGTTCCAGGAGGA
KC	GCTGGGATTCACCTCAAGAA	TGGGGACACCTTTTAGCATC
IL-6	ACTTCACAAGTCCGGAGAGG	GTTGAAGATATGAATTAGAG
IL-1α	CGTCAGGCAGAAGTTTGTCA	GTGCACCCGACTTTGTTCTT
PPARα	TGCAAACTTGGACTTGAACG	AATCCCCTCCTGCAACTTCT
PPARβ/δ	GCAGCCTCTTCCTCAATGAC	GTACTGGCTGTCAGGGTGGT
PPARγ	CTGGCCTCCCTGATGAATAA	ACGTGCTCTGTGACGATCTG
RXRα	CTCCTATCAGCACCCTGAGC	TGTTGTCTCGGCAGGTGTAG
RXRβ	CTCCTCATTGCGTCCTTCTC	CCTGCTGCTCAGGGTACTTC
RXRγ	AATGCTCTTGGCTCTCCGTA	TGAAGAAGCCTTTGCAACCT
FXR	GGCAGAATCTGGATTTGGAA	GTGAGCGCGTTGTAGTGGTA
PXR	TTTCAGAAGGGCCATGAAAC	CCTGCAGAAACTCTGGAAGC
CAR	GGAGGACCAGATCTCCCTTC	GTTCAGAATCAGCGCCATTT
Human gene products		
L19	GCTGATCAAGGATGGGCTGA	CGGGAATGGACAGTCACAGG
COX-2	TGAAACCCACTCCAAACACA	AACTGATGCGTGAAGTGCTG
IL-8	TCTGCAGCTCTGTGTGAAGG	ATTGCATCTGGCAACCCTAC
IL-6	AAAGAGGCACTGGCAGAAAA	AAAGCTGCGCAGAATGAGAT
IL-1α	GTAAGCTATGGCCCACTCCA	AGCAGCCGTGAGGTACTGAT
PPARα	CTGGAAGCTTTGGCTTTACG	GTTGTGTGACATCCCGACAG
PPARβ/δ	GTGTGGAAGCAGTTGGTGAA	GCGTTGAACTTGACAGCAAA
PPARγ	TTCAGAAATGCCTTGCAGTG	CACCTCTTTGCTCTGCTCCT
RXRα	TTCTCCACCCAGGTGAACTC	CTTGGTGAAGGAAGCCATGT
RXRβ	CCTTCTCACACCGATCCATT	CCTGCTGCTCAGGGTACTTC
RXRγ	TGTGGTCAACAGTGTCAGCA	CCTCACTCTCAGCTCGCTCT
FXR	TTGCTTTGCTGAAAGGGTCT	GAGGATTTTCAGGCTGGTGA
PXR	CTGATGGACGCTCAGATGAA	AGGGAGATCTGGTCCTCGAT
CAR	GCAGCTGTGGAAATCTGTCA	AGGCCTAGCAACTTCGCATA

$3OC_{12}$-HSL elicits effects similar to those of some endogenous mammalian hormones (44, 46, 51) and that autoinducers can enter and retain their functions within mammalian cells (56), we hypothesize that $3OC_{12}$-HSL crosses cell membranes and utilizes intracellular mammalian receptors to alter gene transcription. Lipidic signaling compounds commonly elicit responses in mammalian cells by acting as ligands for transcriptional regulators of the nuclear hormone receptor (NHR) superfamily (10). NHRs and LuxR-type proteins (bacterial AHL receptors) function as ligand-regulated transcription factors, and both undergo conformational changes upon ligand binding. Although LuxR proteins and NHRs are not encoded by orthologous genes, their functional similarities have identified NHRs as candidate mammalian AHL receptors. The NHR superfamily is composed of 48 distinct members in humans (26), several of which are associated with the regulation of proinflammatory genes. For example, the peroxisome proliferator-activated receptors (PPARs) are a subset of NHRs with documented anti-inflammatory effects (15, 25, 48), and several $3OC_{12}$-HSL-activated genes are also PPARγ targets (24). Here, we demonstrate that $3OC_{12}$-HSL modulates the transcriptional and DNA binding activities of PPARβ and/or PPARγ and that, thus, these proteins may mediate some of the actions of $3OC_{12}$-HSL within mammalian cells.

MATERIALS AND METHODS

Cell culture and reagents. The murine fibroblast cell line NIH 3T3 (ATCC, Rockville, MD) was cultured in Dulbecco's modified Eagle's medium (BioWhittaker, Walkersville, MD) supplemented with 10% Cosmic calf serum (HyClone, Logan, UT) and 100 U of penicillin ml^{-1} and 100 μg of streptomycin ml^{-1} (BioWhittaker, Walkersville, MD). The human alveolar epithelial cell line A549 (ATCC, Rockville, MD) was cultured in Kaighn's modification of Ham's F-12K medium (Mediatech Inc., Herndon, VA) supplemented with 10% fetal bovine serum, 100 U of penicillin ml^{-1}, and 100 μg of streptomycin ml^{-1}. For reverse transcription (RT)-PCR analysis, cells were seeded onto six-well tissue culture plates (1.5×10^5 cells per well). Cells were stimulated with autoinducer in the appropriate medium lacking fetal bovine serum for 4 h.

Autoinducer preparation. *N*-Butyryl-homoserine lactone (C_4-HSL) was purchased from Sigma (St. Louis, MO) and dissolved in ethyl acetate containing 0.001% glacial acetic acid for stable storage at −20°C. 3-*O*-Dodecanoyl homoserine lactone ($3OC_{12}$-HSL) was synthesized as described previously (11, 42) and was also dissolved in ethyl acetate containing 0.001% glacial acetic acid for stable storage at −20°C. For addition to cell cultures, autoinducers were placed under a gentle stream of nitrogen gas to evaporate the ethyl acetate solvent and were dissolved in the cell culture medium immediately prior to being added to the cell culture.

RT-PCR. RNA was isolated from cells using the Versagene RNA cell kit according to the specifications of the manufacturer (Gentra Systems, Minneapolis, MN). cDNA was prepared by combining 2 μg of total RNA, 400 U of SuperScript RT (Invitrogen, Carlsbad, CA), and 500 ng of oligo(dT) and incubating the mixture at 37°C for 1 h. Specific primer sets for genes encoding murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cyclooxygenase-2 (COX-2), interleukin-6 (IL-6), keratinocyte-derived cytokine (KC; an orthologue of IL-8), and IL-1α and human L19, COX-2, IL-8, IL-6, and IL-1α (Table 1) were used to amplify DNA templates in a T3 thermocycler (Biometra, Goettingen, Germany) with *Taq* DNA polymerase (New England Biolabs, Ipswich, MA). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). Specific murine and human primer sets for the PPARα, PPARγ, PPARβ/δ, retinoid X receptor α (RXRα), RXRβ, RXRγ, pregnane X receptor (PXR), farnesoid X receptor (FXR), and constitutive androstane receptor (CAR) genes (Table 1) were used to amplify DNA templates in a RapidCycler air thermocycler (Idaho Technology, Idaho Falls). PCR products were run on 1.5% agarose

gels containing 10 ng of ethidium bromide (Fisher Biosciences, Lafayette, CO), and the gels were visualized under UV light.

Transcriptional assays. A *Renilla* luciferase vector, pRL-TK (Promega, Madison, WI), was included in all experiments as a transfection efficiency control. Firefly and *Renilla* luciferase activities were assayed using the dual luciferase reporter assay kit according to the instructions of the manufacturer (Promega, Madison, WI). Luminescence was measured with a Modulus single-tube luminometer (Turner Biosystems, Sunnyvale, CA). Transfections were performed using Polyfect according to the instructions of the manufacturer (Qiagen, Valencia, CA). For luciferase assays, NIH 3T3 cells were transfected with plasmids expressing the NHR of interest (either PPARγ or PPARβ/δ) and the PPAR binding partner RXRα, as well as a receptor-specific luciferase reporter plasmid. The reporter plasmid for PPARγ contained a fragment from the rat phosphoenolpyruvate carboxykinase promoter encompassing nucleotides −1130 to +69 (18, 28) in the pGL3-basic plasmid (Promega). The reporter for PPARβ/δ was the 2X Cyp4A6Z pal thymidine kinase (TK)-luciferase plasmid, which contains a TK basal promoter fragment upstream of two copies of the Cyp4A6Z motif from the reporter plasmid pBL-CAT8+ (28) in the pGL2-basic plasmid (Promega). Twenty-four hours after transfection, cells were treated with increasing concentrations of $3OC_{12}$-HSL or C_4-HSL with or without rosiglitazone for 4 h and then assayed for luciferase activity. The pSG5-PPARβ/δ, pSPORT-PPARγ, pSPORT-RXRα, and phosphoenolpyruvate carboxykinase-luciferase reporter plasmids were generous gifts from Elmus Beale (Texas Tech University Health Sciences Center).

Nuclear extracts and Western blotting. Nuclear extracts were prepared from A549 and NIH 3T3 cells as described previously (7). All steps in the nuclear extract preparation were carried out at 4°C or on ice. The cells were washed twice with phosphate-buffered saline, harvested in ice-cold lysis buffer (Dignam buffer A: 10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 2 μg of a protease inhibitor cocktail [Cal Biochem, San Diego, CA]/ml), and incubated on ice for 20 min. The cells were lysed by passing eight times through a 21-gauge needle, and the lysates were centrifuged at 13,000 × *g* for 45 s to pellet the nuclei. The nuclear pellet was resuspended in Dignam buffer C (20 mM HEPES [pH 7.9], 25% [vol/vol] glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 2 μg of a protease inhibitor cocktail/ml), and the resulting suspension was rotated at 4°C for 30 min. The suspension was then centrifuged at 13,000 × *g* to pellet nuclear debris, and the supernatant containing the nuclear extract was collected and stored at −80°C. The protein concentration was determined utilizing a protein assay reagent according to the instructions of the manufacturer (Bio-Rad Laboratories, Hercules, CA). A 10-μg sample of nuclear proteins was separated on sodium dodecyl sulfate–8% polyacrylamide gel, and the proteins were electroblotted onto nitrocellulose membranes. Membranes were preblocked in a mixture of phosphate-buffered saline–Tween 20 and 4% milk and probed using anti-PPARγ (Cell Signaling, Danvers, MA) or anti-PPARβ/δ (Santa Cruz) antisera at 1:1,000 dilutions. Membranes were then stripped with 0.1 M glycine at pH 2.6 and reprobed using an antilamin antibody (Santa Cruz) at a 1:1,000 dilution as a loading control.

EMSA. Double-stranded oligonucleotides containing a consensus PPARγ response element were end labeled with [-^{32}P]ATP using T4 polynucleotide kinase (Promega), purified in Microspin G-25 columns (Sigma), and used as probes for an electrophoretic mobility shift assay (EMSA). Recombinant PPARγ and RXRα proteins were synthesized using the TNT T7 quick translation-transcription system (Promega), and programmed lysate or unprogrammed lysate was incubated with the radioactively labeled wild-type oligonucleotide carrying the consensus PPARγ response element. All binding reactions were performed at 23°C with a 25-μl mixture containing 2 μl of the programmed or unprogrammed lysate, 6% (vol/vol) glycerol, 4% (wt/vol) Ficoll, 10 mM HEPES (pH 7.9), 10 mM dithiothreitol, 0.25 μg of bovine serum albumin, 0.06% bromophenol blue, 1 μg of poly(dI-dC), and 1.25 ng of the probe. For competition assays, a 10× or 100× molar excess of unlabeled oligonucleotides (both wild-type and mutated forms) was included in the reaction mixture, along with the radiolabeled probe. The effects of AHLs on DNA binding activities were assessed by incubating programmed lysates with 100 μM $3OC_{12}$-HSL or C_4-HSL during the binding reaction. Gels were dried after electrophoresis and subjected to autoradiography.

RESULTS

$3OC_{12}$-HSL modulates mRNA levels for inflammatory mediators in mammalian cells. We previously demonstrated that $3OC_{12}$-HSL increases mRNA levels for IL-6, COX-2, and KC in mouse fibroblasts, and several other reports have described similar effects in human fibroblasts and epithelial cells (44–46). However, as demonstrated by previous apoptotic studies, $3OC_{12}$-HSL may have various cell type-specific effects (50). To determine whether $3OC_{12}$-HSL elicits similar effects in murine and human cells, we have examined mRNA levels for an expanded panel of proinflammatory genes in mouse NIH 3T3 fibroblasts and human A549 lung epithelial cells. These cell lines are representative of cell types likely to be exposed to elevated levels of *P. aeruginosa* autoinducers during lung and wound infections. Cells were exposed to a range of concentrations of $3OC_{12}$-HSL for 6 h, RNA was harvested and converted into cDNA, and PCR was performed utilizing primers for several candidate inflammatory mediators (Fig. 1). The range of autoinducer concentrations necessary to elicit changes in cytokine mRNA levels in the A549 human cells was lower than that in the hardier NIH 3T3 mouse fibroblasts. IL-1α, IL-6, IL-8/KC, and COX-2 mRNA levels exhibited dose-dependent increases in both cell lines and were unaffected by the exposure of cells to C_4-HSL, the second *P. aeruginosa* autoinducer, which was used to demonstrate AHL specificity (Fig. 1). These data demonstrate that $3OC_{12}$-HSL increases mRNA levels for several inflammatory mediators in both human and murine cells. Thus, we have identified a common response to $3OC_{12}$-HSL in cell lines from two distinct mammalian species by using corresponding panels of inflammatory cytokines.

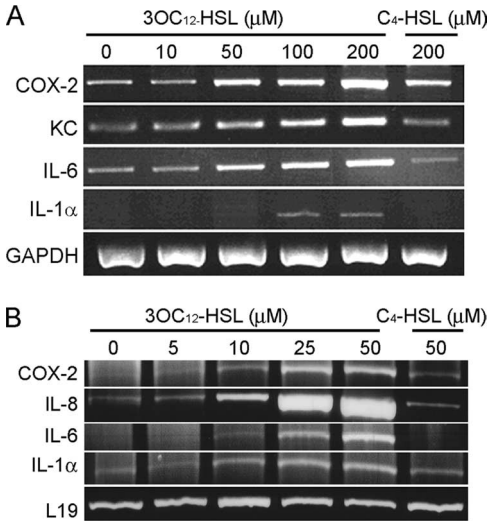

FIG. 1. $3OC_{12}$-HSL modulates proinflammatory gene expression in vitro. NIH 3T3 (A) and A549 (B) cells were incubated in the absence or presence of increasing concentrations of $3OC_{12}$-HSL or C_4-HSL for 6 h, and RNA was extracted and converted into cDNA. mRNA levels for genes encoding the inflammatory mediators COX-2, IL-8, IL-6, and IL-1α were assessed by RT-PCR. GAPDH and L19 mRNAs were used as loading controls.

Mammalian NHRs are expressed in $3OC_{12}$-HSL-sensitive cell lines. Despite growing evidence that AHLs directly influence mammalian responses, endogenous mammalian receptors have not been identified. Our previous studies suggested that at least two distinct $3OC_{12}$-HSL receptors may be present in mammalian cells (41, 44). One receptor that mediates a Ca^{2+} signaling-based apoptotic response appears to be located at or close to the cell membrane, while a second, uncharacterized receptor may be responsible for the proinflammatory effects of $3OC_{12}$-HSL. We

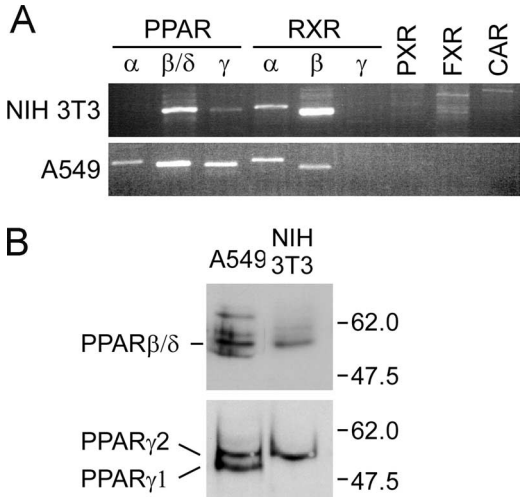

FIG. 2. NHRs are expressed in $3OC_{12}$-HSL-responsive cell lines. RNA from cell lines that exhibit $3OC_{12}$-HSL-dependent changes in proinflammatory mRNAs was extracted and converted into cDNA. (A) RT-PCR was used to screen for a panel of NHRs in A549 cells. (B) Western blot analysis revealed PPARγ and PPARβ/δ proteins in the nuclear extracts of A549 and NIH 3T3 cells.

previously showed that *P. aeruginosa* autoinducers can enter and function in mammalian cells, suggesting that the second receptor may be an intracellular protein (56). As many hydrophobic signaling compounds can pass through cell membranes and directly affect transcription by interacting with members of the NHR superfamily, we next sought to identify NHRs that may function as $3OC_{12}$-HSL receptors (17). We extracted endogenous mRNA from untreated NIH 3T3 and A549 cells and performed RT-PCR to identify mammalian NHRs common to both cell lines. RT-PCR assays were performed using primers specific for three PPAR (PPARα, PPARβ/δ, and PPARγ) genes (NR1C1, NR1C2, and NR1C3) (36), three RXR (RXRα, RXRβ, and RXRγ) genes (NR2B1, NR2B2, and NR2B3), and the PXR (NR1I1), FXR (NR1H4), and CAR (NR1I3) genes (Fig. 2A). These genes were examined based on the associations between their products and inflammatory or xenobiotic reactions and the fact that several exhibit flexibility in accommodating structurally diverse ligands. Strong signals for PPARβ/δ, PPARγ, RXRα, and RXRβ were detected in RNAs from both NIH 3T3 and A549 cells. PPARα mRNA was detected only in A549 cells, while RXRγ, PXR, FXR, and CAR mRNAs were not detected in either cell line. Nuclear extracts from NIH 3T3 and A549 cells were subjected to immunoblotting analysis to confirm that PPARβ/δ and PPARγ proteins were also expressed in these cell lines. Both PPARγ1 and PPARγ2 isoforms were detected in A549 cells, while only the PPARγ1 isoform was detected in NIH 3T3 cells (Fig. 2B). PPARβ/δ was also detected in NIH 3T3 and A549 cells (Fig. 2B).

$3OC_{12}$-HSL affects the transcriptional activities of PPARβ and PPARγ. The PPAR family consists of three isoforms with high degrees of sequence and structural similarities, PPARα, PPARγ, and PPARβ. All PPARs form heterodimers with the RXR and bind to the same DNA response element after activation. This sequence, containing multiple AGGTCA repeats, is termed the specific PPAR response element (PPRE) and is found within the promoters of target genes. Recent evidence has credited PPARs with the ability to decrease inflammation through the transrepression of transcription factors involved in the inflammatory response (13). Transcriptional activation assays were performed to test whether $3OC_{12}$-HSL affected the activity of PPARβ/δ and PPARγ. NIH 3T3 cells were transfected with plasmids expressing either PPARβ or PPARγ and the PPAR binding partner RXRα, along with a receptor-specific luciferase reporter plasmid. Twenty-four hours after transfection, cells were stimulated with various concentrations of $3OC_{12}$-HSL and cultured for a further 24 h, and luciferase activity in the cell extracts was assayed. C_4-HSL was again used as an AHL specificity control. The transcriptional activity of PPARγ was specifically inhibited by $3OC_{12}$-HSL in a dose-dependent manner both in the presence and in the absence of the potent PPARγ agonist rosiglitazone (Fig. 3A). In contrast, the activity of PPARβ-RXRα was specifically

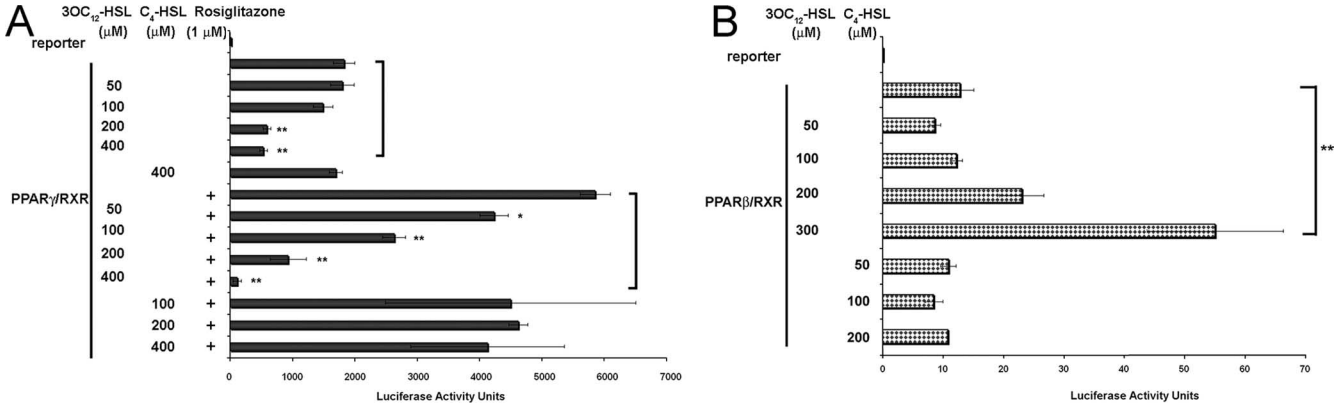

FIG. 3. $3OC_{12}$-HSL modulates the transcriptional activities of NHRs. NIH 3T3 cells were transfected with expression vectors for either PPARγ and RXRα (A) or PPARβ/δ and RXRα (B), along with the appropriate PPAR-responsive luciferase reporter plasmid and the TK-*Renilla* luciferase control plasmid. Twenty-four hours after transfection with the expression vectors, cells were stimulated with $3OC_{12}$-HSL or C_4-HSL in the presence (+) or absence of rosiglitazone as indicated and incubated for 6 h. Cell lysates were prepared and assayed for luciferase activity. Each bar represents the average of results obtained from three transfections, and each experiment was performed at least three times. Statistical analysis was performed using a one-way analysis of variance with the Tukey-Kramer multiple-comparisons test. The error bars represent standard errors of the means. *, $P < 0.01$; **, $P < 0.001$.

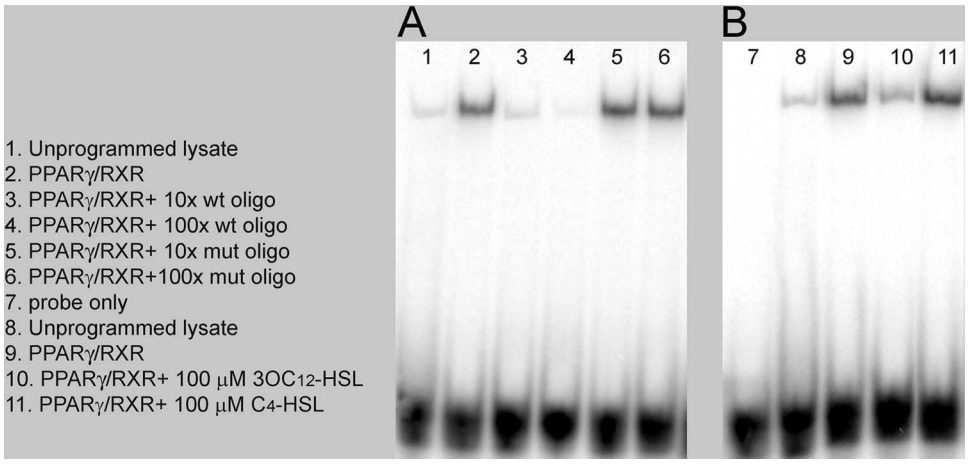

FIG. 4. $3OC_{12}$-HSL affects the DNA binding activity of PPARγ. Recombinant PPARγ and RXRα proteins were synthesized in vitro utilizing the TNT in vitro transcription-translation kit. Equal amounts of programmed or unprogrammed lysates were incubated with a radioactively labeled wild-type oligonucleotide carrying a consensus PPARγ-responsive element. (A) Competition assays were performed using 10× or 100× molar excesses of unlabeled wild-type (wt) or mutated (mut) oligonucleotides (oligo; lanes 3 to 6). (B) The effects of autoinducers on PPARγ DNA binding activity were assessed by adding 100 μM $3OC_{12}$-HSL or C_4-HSL to the binding reaction mixtures in lanes 10 and 11.

enhanced in a dose-dependent manner (Fig. 3B). Importantly, the activities of both PPARs were not significantly altered in cells exposed to C_4-HSL in the absence or presence of rosiglitazone, indicating that these responses are dependent on a specific AHL.

$3OC_{12}$-HSL inhibits the DNA binding activity of PPARγ. An EMSA was performed to determine whether $3OC_{12}$-HSL affects the ability of PPARγ to bind an oligonucleotide containing a PPRE. Recombinant PPARγ and RXRα were synthesized in reticulocyte lysates and added to binding reaction mixtures. Strong binding was observed in samples containing programmed lysates but was essentially absent in reactions using unprogrammed lysates (Fig. 4A, compare lanes 1 and 2). The specificity of this binding reaction was confirmed by competition with an excess of unlabeled PPRE oligonucleotide and a lack of competition with an excess of a mutated form of the oligonucleotide (Fig. 4A, compare lanes 3 and 4 with lanes 5 and 6). Importantly, the preincubation of the programmed lysate with 100 μM $3OC_{12}$-HSL resulted in a decrease in binding activity, while binding activity was essentially unaffected when the lysate was preincubated with 100 μM C_4-HSL (Fig. 4B, compare lanes 10 and 11). We interpret these data to indicate that $3OC_{12}$-HSL is potentially a ligand for PPARγ which induces a conformational change that diminishes the DNA binding ability of PPARγ.

$3OC_{12}$-HSL antagonizes the transrepression of cytokine expression by a PPARγ agonist. Ligand-bound PPARγ possesses an anti-inflammatory function mediated by the ability of PPARγ to directly inhibit transcription from the promoters of proinflammatory genes. For example, PPARγ has been proposed to inhibit the transcription of NF-κB-dependent genes, such as the inducible nitric oxide synthase gene, by stabilizing corepressor complexes and preventing coactivator recruitment to the target promoter (25, 37, 39). Additionally, ligand-bound PPARγ can inhibit the transcription of AP-1-regulated genes by competing for coactivator complexes (49). Collectively, these inhibitory functions of PPARγ are termed transrepression and presumably represent a mechanism for dampening inflammatory responses. We therefore tested whether the potent PPARγ agonist rosiglitazone could prevent the $3OC_{12}$-HSL-dependent increase in proinflammatory mRNA levels in A549 cells. Separate cultures of A549 cells were exposed to a constant concentration of $3OC_{12}$-HSL and increasing concentrations of rosiglitazone, and RNA was prepared for RT-PCR analysis. As before, IL-1α, IL-6, IL-8, and COX-2 mRNA levels were elevated in cells exposed to 25 μM $3OC_{12}$-HSL (Fig. 5, compare lanes 1 and 2). The levels of each mRNA were essentially unaffected in cells cultured in the presence of up to 25 μM rosiglitazone but were decreased nearly or completely to the unstimulated levels in cells cultured in 50 μM rosiglita-

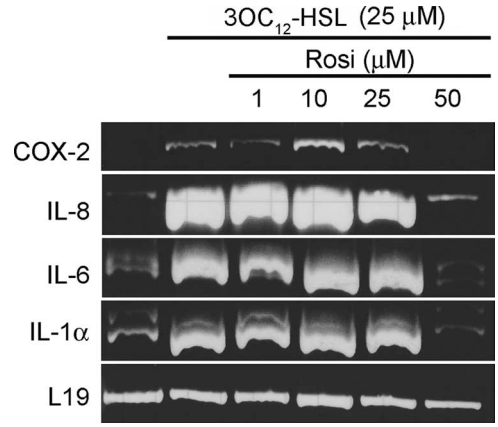

FIG. 5. Antagonistic effects of $3OC_{12}$-HSL and rosiglitazone on mRNA levels for proinflammatory genes. A549 cells were incubated in the absence or presence of 25 μm $3OC_{12}$-HSL, either alone or in the presence of various concentrations of rosiglitazone (Rosi) for 6 h. RNA was extracted and converted into cDNA, and mRNA levels for a panel of proinflammatory genes were assessed by RT-PCR. The mRNA encoding the ribosomal protein L19 was analyzed as a loading control.

zone (Fig. 5, lanes 3 to 6). These data suggest that $3OC_{12}$-HSL and rosiglitazone are mutually antagonistic in controlling the activity of PPARγ.

DISCUSSION

Eukaryotes and prokaryotes have coexisted for approximately 2 billion years, often existing in close apposition in a shared hormonal environment. We postulate that organisms from different kingdoms have acquired mechanisms to sense and respond to one another's signaling molecules. This cross-kingdom communication has been termed interkingdom signaling and has potentially important implications for the relationship between pathogenic organisms and their hosts. One example of interkingdom signaling emerged from the observation that the QS system of enterohemorrhagic *Escherichia coli* can be activated by epinephrine and norepinephrine produced by mammalian hosts (12). We have focused on a signaling pathway that appears to function in the opposite direction, in which AHLs produced by *P. aeruginosa* alter gene expression in mammalian cells. This pathway represents a previously uncharacterized aspect of host-pathogen interaction and innate immunity. In this report, we identify two members of the PPAR subfamily of NHRs as candidate mammalian receptors for the AHL $3OC_{12}$-HSL.

Several earlier studies have implicated transcriptional regulators and protein kinases as components of AHL signaling pathways in mammalian cells, but no specific receptors have been identified. For example, the NF-κB transcriptional regulator was implicated in the AHL-dependent activation of IL-8 and COX-2 transcription, and the AP-2 transcription factor was identified as a potential regulator of IL-8 (45, 46). Neither of these proteins is likely to interact directly with AHLs, and thus, these proteins are presumably targets of signaling pathways activated by AHLs. In addition, a number of protein kinases, including members of the mitogen-activated protein kinase family and Akt, were implicated previously as intermediates in AHL signaling; however, these kinases are also not likely to be direct targets of AHLs (23, 45). Subsequently, studies with genetically null mice revealed that the canonical Toll-like receptor pathways are not involved in AHL signaling (27). Together, these studies have highlighted the need to identify bona fide AHL receptors in order to clarify the mechanisms of action of these molecules in mammalian cells.

The concept that $3OC_{12}$-HSL interacts with multiple mammalian receptors emerged from our earlier studies on two distinct effects of $3OC_{12}$-HSL on mammalian cells, namely, apoptotic induction and increased mRNA levels for proinflammatory genes (44). These studies revealed that apoptotic induction is mediated by a calcium-dependent signaling pathway, apparently initiated by the interaction of $3OC_{12}$-HSL with an unknown receptor located at or close to the cell membrane, and that the inhibition of this pathway blocks apoptosis but not proinflammatory responses. Thus, we proposed that a second receptor was associated with the inflammatory response to AHLs. The candidacy of members of the NHR superfamily was supported by our previous data showing that autoinducers can enter and retain functionality in mammalian cells (56) and the recent direct demonstration that radiolabeled $3OC_{12}$-HSL can be detected in T cells (40). In addition, clear functional similarities exist between LuxR-AHL and NHR-ligand interactions, including the induction of conformational changes in the receptor protein upon ligand binding (3, 19). Although LuxR-type proteins and NHRs are not evolutionarily related (41), their functional similarities led us to propose NHRs as candidate AHL receptors (41, 43).

The PPARs were particularly attractive candidate AHL receptors for several reasons, particularly their responsiveness to ligands of relatively disparate structures and their association with inflammatory gene regulation (45). The PPAR family consists of three closely related gene products, PPARα, PPARγ, and PPARβ. All PPARs bind to DNA as obligate heterodimers with the RXR at specific PPREs within the promoters of target genes. In the absence of a ligand, they most likely bind to DNA and form complexes with corepressor proteins. Under these conditions, the transcription of target genes is repressed. Ligand binding induces a structural change that displaces corepressors, facilitates interaction with coactivators, and promotes the transcription of target genes (21). Of the three isotypes, PPARβ has the broadest expression pattern, and functions in the skin, gut, placenta, skeletal muscle, adipose tissue, and brain have been assigned to this isotype (5, 6, 31). PPARγ is expressed as two isoforms, PPARγ1 and PPARγ2, that differ at their N termini. PPARγ2 is found at high levels in the different adipose tissues (52), whereas PPARγ1 has a broader expression pattern that extends to settings such as the gut, brain, vascular cells, and specific kinds of immune and inflammatory cells (53, 57). Our data show that two members of the PPAR family, PPARβ/δ and PPARγ, as well as several of their heterodimeric partners from the RXR family, are expressed in cell types that are AHL responsive. Furthermore, we have shown that the transcriptional activities of both PPARβ/δ and PPARγ are affected by $3OC_{12}$-HSL, although in opposite directions. In theory, $3OC_{12}$-HSL may function as a ligand for either partner in these heterodimeric complexes. The RXRs were the first orphan nuclear receptors for which an endogenous ligand (9-*cis*-retinoic acid) was identified, and RXRs are also receptors for a variety of other dietary lipids, as well as some nonmammalian lipids (10). However, as RXRα was a common partner for PPARβ/δ and PPARγ in these transcriptional assays, we interpret the opposite effects of $3OC_{12}$-HSL on the two heterodimeric complexes as evidence that the PPAR partner mediates the effect of the AHL. Several independent assays, including direct binding assays with radiolabeled autoinducers, are currently under way to confirm this conclusion.

The observation that $3OC_{12}$-HSL inhibited DNA binding by recombinant PPARγ-RXRα heterodimers at a consensus PPRE is consistent with a model whereby $3OC_{12}$-HSL is directly recognized by PPARγ and induces a conformational alteration that negatively affects the DNA binding activity of PPARγ. However, as mentioned above, certain ligand-bound NHRs can also inhibit the expression of promoters that do not contain PPREs, via a phenomenon known as transrepression (8). In the case of PPARγ-dependent transrepression of NF-κB-regulated genes, ligand-bound PPARγ stabilizes repressor complexes associated with target genes and prevents the recruitment of coactivators in response to proinflammatory stimuli such as lipopolysaccharide (37) (Fig. 6A and B). We propose that $3OC_{12}$-HSL, as an antagonist of PPARγ, can

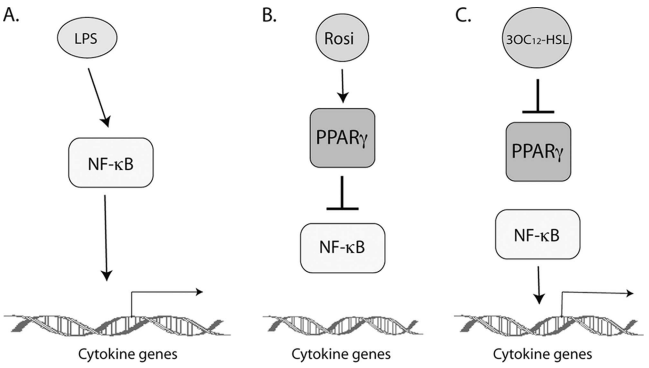

FIG. 6. Hypothetical model of mutual antagonism between $3OC_{12}$-HSL and rosiglitazone in the regulation of NF-κB-dependent genes. (A) Proinflammatory stimuli such as lipopolysaccharide (LPS) activate NF-κB and stimulate the transcription of several inflammatory mediators. (B) Ligand-bound PPARγ transrepresses NF-κB by stabilizing corepressor complexes bound at promoters of NF-κB-dependent genes. (C) $3OC_{12}$-HSL inhibits PPARγ activity, possibly by competing with rosiglitazone for binding within the PPARγ ligand binding domain, and relieves the transrepression of NF-κB activity.

compete with PPARγ agonists such as rosiglitazone, thereby inhibiting transrepression and potentiating the expression of proinflammatory genes (Fig. 6C). This exaggerated inflammation can lead to tissue destruction, which often precedes bacteremia. A similar scenario of mutual antagonism between $3OC_{12}$-HSL as a PPARγ antagonist and PPARγ agonists would also be consistent with other models of PPAR-dependent transrepression, such as the sequestration of coactivators of AP-1 on the COX-2 promoter (49).

Finally, it is important that PPARγ agonists are currently being employed as anti-inflammatory treatments for multiple diseases, such as inflammatory bowel disease (2), atherosclerosis (35), and asthma (47). The PPARγ agonists rosiglitazone and pioglitazone have protective effects against a variety of inflammation-related kidney injuries, such as diabetic nephropathy, hypertensive nephropathy, and ischemia-reperfusion injuries. PPARγ agonists have also been shown to decrease inflammation in injuries related to the digestive tract, lung, and heart. For example, the treatment of lung ischemia-reperfusion injuries in mouse models with the PPARγ agonist pioglitazone resulted in the inhibition of proinflammatory cytokines (tumor necrosis factor alpha and cytokine-induced neutrophil chemoattractant 1), the infiltration of neutrophils into the lung interstitium, and reduced pulmonary edema (32). Therefore, PPARγ agonists may be effective as anti-inflammatory agents in individuals with *P. aeruginosa* infections, including diabetic and CF patients.

Lastly, it is important that autoinducer concentrations ranging from 0 to 400 μM were utilized for the experiments presented in this paper. Currently, in vivo measurements of autoinducers in CF patient sputum have been in the nanomolar range (33), while experimental concentrations of synthetic autoinducers required to elicit effects in immortalized mammalian cell lines are in the micromolar range. However, these in vivo measurements most likely underestimate the local concentrations of autoinducers within the vicinity of a *P. aeruginosa* biofilm. AHLs are diluted in the sputum and lung fluid, and the local AHL concentration during an active *P. aeruginosa* lung infection remains unknown. Local and systemic concentrations may differ in vivo, where cells in close proximity to a high concentration of bacteria (e.g., a biofilm) are exposed to high concentrations of AHLs. In fact, AHL concentrations of up to 600 μM have been measured in the supernatants of biofilms grown in vitro (9). Also, researchers in our lab have shown that nonimmortalized cells are more sensitive to AHLs than immortalized cell lines (44). This work suggests that cells in vivo may be more sensitive to AHL concentrations than those previously studied in culture. Thus, the autoinducer concentrations utilized in these experiments may reflect the relative AHL concentrations to which primary cells are exposed in vivo. Unfortunately, this issue will remain uncertain until more accurate measurements of AHL concentrations in vivo are available.

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