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Original article

Pseudomonas signal molecule 3-oxo-C12-homoserine lactone interferes with binding of rosiglitazone to human PPARγ

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Abstract

Peroxisome proliferator activated receptor (PPARγ) has been suggested as a target for anti-inflammatory therapy in chronic lung disease, including infection with *Pseudomonas aeruginosa*. However, the *P. aeruginosa* signal molecule N-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) has been reported to inhibit function of PPARs in mammalian cells. This suggests that binding of 3-oxo-C12-HSL to PPARs could increase inflammation during *P. aeruginosa* infection, particularly if it could compete for binding with other PPAR ligands. We investigated the ability of 3-oxo-C12-HSL to bind to a PPARγ ligand binding domain (LBD) construct, and to compete for binding with the highly active synthetic PPARγ agonist rosiglitazone. We demonstrate that 3-oxo-C12-HSL binds effectively to the PPARγ ligand binding domain, and that concentrations of 3-oxo-C12-HSL as low as 1 nM can effectively interfere with the binding of rosiglitazone to the PPARγ ligand binding domain. Because 3-oxo-C12 HSL has been demonstrated in lungs during *P. aeruginosa* infection, blockade of PPARγ-dependent signaling by 3-oxo-C12-HSL produced by the infecting *P. aeruginosa* could exacerbate infection-associated inflammation, and potentially impair the action of PPAR-activating therapy. Thus the proposed use of PPARγ agonists as anti-inflammatory therapy in lung *P. aeruginosa* infection may depend on their ability to counteract the effects of 3-oxo-C12-HSL.

Keywords: PPAR; Pseudomonas; Inflammation; Homoserine lactones

1. Introduction

The interaction between pathogenic microbes and the mammalian hosts they infect is complex. It is clear that these interactions are bidirectional, with both effects of host products on microbes, and effects of microbial products on host cells having been identified. The recognition of microbial products by mammalian pathogen-associated molecular pattern (PAMP) recognition receptors, including the toll-like receptors (TLRs) is well established. However, it is becoming increasingly clear that host–pathogen interactions are not confined to these well-described receptors.

A number of bacterial species produce small molecules that act as signals for quorum sensing, the coordinated, population wide control of gene expression. While these molecules have important roles in controlling bacterial functions, it has been recently reported that at least some of these quorum sensing signal molecules (QSSMs) also directly affect host cell metabolism and function. The *Pseudomonas aeruginosa* quorum sensing signal molecule (QSSM) *N*-3-(oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) has been demonstrated to modulate the function of a wide range of mammalian cell types, including lymphocytes [1–4], macrophages/monocytes [5], neutrophils [5–7], respiratory epithelial cells and fibroblasts [8–12], and vascular smooth muscle

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cells [13]. Recently, some insight into the mechanism of these effects has been provided by the demonstration of Jahoor et al. [14] that 3-oxo-C12-HSL can modulate the function of host transcriptional regulators peroxisome proliferator activated receptors (PPARs), and by Kravchenko et al. [15] that 3-oxo-C12-HSL can modulate inflammatory pathways involving NF-κB.

PPARs are a family of nuclear membrane-associated transcriptional regulators that act as lipid sensors and play important roles in mammalian lipid metabolism [reviewed in [16]] and inflammation [17–23]. Inhibition of PPAR function, particularly of PPARγ, has a net proinflammatory effect. We hypothesized that interaction of 3-oxo-C12-HSL with PPARγ in the lung during infection could exacerbate the infection-induced inflammation by inhibiting PPAR activation, particularly in cystic fibrosis patients whose cells express low levels of PPARs [24,25], and that treatment with PPAR agonists could alleviate this inflammation, an hypothesis that is supported by the report of Perez et al. who showed that troglitazone, a PPAR agonist, could reduce inflammation in CF mice during *P. aeruginosa* lung infection [25]. However, the relative effects of 3-oxo-C12-HSL and pharmacological PPAR agonists on PPARγ have not been investigated.

In this study, we used an in vitro reporter system in human bronchial epithelial cells to explore the ability of 3-oxo-C12-HSL to bind to the PPARγ ligand binding domain and its ability to compete for binding with a high-affinity synthetic PPARγ agonist, rosiglitazone, with the aim of determining the potential usefulness of PPAR agonist therapy in *P. aeruginosa* lung infection.

2. Materials and methods

2.1. Cell lines and cultures

BEAS-2B cells (human bronchial epithelial cell line) were obtained from ATCC, and are cultured in Dulbecco's Minimal Essential Medium (DMEM, Gibco, Gaithersburg, MD) supplemented with 10% v/v heat-inactivated fetal calf serum (FCS, JRH Biosciences, Victoria, Australia) and 2 mM L-Glutamine (Gibco) (DMEM10%FCS).

2.2. Reagents

3-oxo-C12-HSL was synthesized as previously described [26]. C4-HSL was purchased from Sigma–Aldrich (Castle Hill, NSW Australia). The PPARγ agonist rosiglitazone was purchased from Cayman Chemical (Ann Arbor, MI).

2.3. Reporter systems

2.3.1. Assay of PPAR-dependent signaling

PPAR-dependent signaling was assessed using A-Ox3-TKSL-PPRE and pcDNA3.1-hPPARγ1-Flag (both gifts from V.K.K. Chatterjee, Department of Medicine, University of Cambridge Clinical School).

Binding of a ligand to the PPARγ encoded by pcDNA3.1-hPPARγ1 results in binding of that PPARγ to the PPAR response element (PPRE) of A-Ox3-TKSL-PPRE, resulting in luciferase expression. In all experiments, cells were cotransfected with pcDNA3.1-CMVβgal to enable normalization for transfection efficiency.

2.3.2. Assay of binding to PPARγ ligand binding domain (PPARγLBD)

A reporter system using two interacting plasmids was used to determine the ability of ligands to bind to the PPARγLBD. pGAL4-PPARγLBD [27] contains the DNA sequence encoding residues 173–475 of the PPARγLBD cloned into pSG424. This results in a fusion protein composed of the PPARγLBD and the DNA binding domain (DBD) of Gal4. If a ligand is present and binds the PPARγLBD, the Gal4 DBD is released from interaction with the PPARγLBD and is then able to bind Gal4 recognition sequences in the target gene, resulting in gene transcription. pUAS-TKLUC [28] contains Gal4 recognition sequences and the thymidine kinase promoter in pA_3LUC [29], which codes for luciferase. If the Gal4 DBD is released from interaction with PPARγLBD, it is able to bind the Gal4 recognition sequence, inducing expression of the luciferase gene. Luciferase activity is thus proportional to the level of binding of PPARγ ligand to the PPARγLBD.

In all experiments, cells were cotransfected with pcDNA3.1-CMVβgal, a β-galactosidase reporter plasmid (gift from Shane Grey, Garvan Institute, Sydney Australia) to enable normalization for transfection efficiency.

2.4. Transient transfection of BEAS-2B cells

BEAS-2B cells were plated in 12 well plates (BD Falcon, BD Biosciences, Sydney Australia) at 2×10^5 cells/ml/well in DMEM10%FCS. Cells were incubated at 37 °C, 5% CO_2 overnight until cells were confluent.

For transfections using the PPRE reporter, each well was transfected with 50 ng pcDNA3.1-CMVBgal, 1 μg p(A-Ox3)-TKSL-PPRE and 50 ng pcDNA3.1-hPPARγ1-Flag.

For transfections with the PPARγLBD reporter, each well was incubated with 50 ng pcDNA3.1-CMVBgal, 20 ng pSG424-GAL4-PPARγLBD and 1 μg of pA_3LUC-UAST-KLUC. For each well, plasmid DNA was made up to 100 μl with OptiMEM (Gibco) and incubated for 5 min at room temperature. Separately, 4 μl of Lipofectamine 2000 (Invitrogen, Carlsbad, California) was added to 96 μl OptiMEM (Invitrogen) and the mixture incubated for 5 min at room temperature. Plasmid DNA and Lipofectamine 2000 mixtures were combined and incubated at room temperature for 20 min. Two hundred microliters of this mixture was added per well, and cells were incubated 5–6 h at 37 °C, 5% CO_2. Media was then removed and replaced with DMEM supplemented with 0.5% FCS and cells were incubated overnight at 37 °C, 5% CO_2.

Cells were then treated with combinations of DMSO (Sigma Chemical Co., Perth, Western Australia), rosiglitazone, 3-oxo-C12-HSL or C4-HSL. Unless otherwise noted,

rosiglitazone was used at 100 ng/ml, 3-oxo-C12-HSL and C4-HSL at 10 μM and DMSO at 0.1%. Cells were incubated for 8 h at 37 °C, 5% CO_2. Media was then removed and cells rinsed with 1 ml Dulbecco's Ca^{++} and Mg^{++} free phosphate buffered saline (PBS). Cell culture lysis reagent (Promega Corp, Sydney, Australia) was diluted to working concentration according to manufacturer's instructions, and 150 μl added/well. Cell lysates were collected, vortexed for 15 s and centrifuged at 15,000 rpm for 2 min at 4 °C. Supernatants were stored at −80 °C until assay.

2.5. Luciferase assay

Luciferase assays were performed using a Packard TopCount scintillation counter (LKB Instruments, Mt Waverley Vic, Australia). In a white opaque 96-well plate (Perkin Elmer Pty Ltd., Melbourne, Australia) 5 μl sample was added to 50 μl luciferase substrate (Promega) and luminescence was assayed immediately.

2.6. β-Galactosidase assay

Galacto-Star™ β-Galactosidase detection system (Applied Biosystems, Scoresby, Australia) was used for β-galactosidase assays according to manufacturer's instructions. Galacto-Star substrate was diluted to working concentration with reaction buffer diluent. In a white opaque 96-well plate (Perkin–Elmer) 5 μl sample and 80 μl substrate were added. The plate was incubated at room temperature for 45 min out of direct light and luminescence was assayed on a TopCount scintillation counter. All data are normalized to β-galactosidase expression and presented as a ratio of luciferase/β-Galactosidase activity.

3. Results

3.1. 3-Oxo-C12-HSL can transactivate PPRE

Jahoor et al. reported that 3-oxo-C12-HSL could trigger binding of PPARs to the PPAR response element (PPRE) in isolated DNA [14]. To confirm that we could detect PPAR-dependent signaling in BEAS-2B cells, the cells were transiently transfected with both PPARγ construct and the PPRE reporter, and then treated with a range of concentrations of 3-oxo-C12-HSL. The results of this experiment are shown in Fig. 1, and clearly demonstrate that 3-oxo-C12-HSL can transactivate expression of the PPRE reporter at concentrations between 100 nM and 10 μM. At 50 μM 3-oxo-C12-HSL, activation of the construct is apparently inhibited below control levels, although the β-galactosidase reporter levels were not significantly lower than in other samples.

3.2. 3-Oxo-C12-HSL binds to PPARγLBD

To confirm that 3-oxo-C12-HSL was directly binding to PPARγLBD, we investigated the ability of 3-oxo-C12-HSL and C4-HSL, another *P. aeruginosa* quorum sensing signal

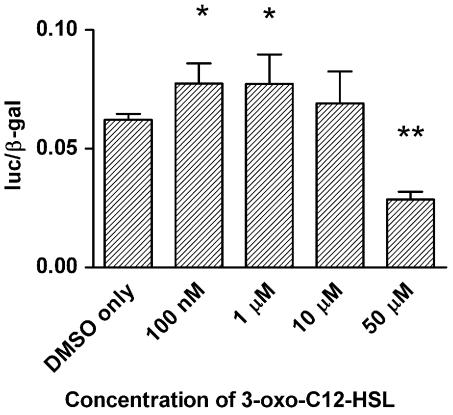

Fig. 1. 3-oxo-C12-HSL weakly transactivates PPAR-dependent signaling. BEAS-2B cells were transiently transfected with A-Ox3-TKSL-PPRE (expresses luciferase under control of 3 copies of PPAR response element (PPRE)) and pcDNA3.1-hPPARγ1-Flag (expresses PPARγ) and pcDNA3.1-CMVβgal (normalization plasmid), then treated with the indicated concentrations of 3-oxo-C12-HSL for 8 h. Results expressed as ratio of luciferase (luc) to β-galactosidase (β-gal) expression. Mean ± SD of six replicates. Data analyzed with one-way ANOVA with Dunnett's post-test. $*p < 0.05$; $**p < 0.01$ relative to DMSO control.

molecule with a 4-carbon side chain rather than the 12-carbon side chain of 3-oxo-C12-HSL, to bind to PPARγLBD construct. To confirm that 3-oxo-C12-HSL does not nonspecifically trigger Gal4 binding to the luciferase reporter, we transiently transfected BEAS-2B cells with pUAS-TKLUC and pcDNA3.1-CMVβgal without the pGAL4-PPARγLBD construct. Cells were treated with 10 μM 3-oxo-C12-HSL in 0.1% DMSO (*v*/*v*) or 0.1% DMSO (*v*/*v*) alone for 8 h before being lysed and luciferase and β-Galactosidase assays performed. There was no significant difference (unpaired *t*-test) between the luciferase/β-galactosidase ratio for DMSO control (0.0015 ± 0.0001, $n = 4$) and that for 3-oxo-C12-HSL treated cells (0.0018 ± 0.0002, $n = 4$), showing that 3-oxo-C12-HSL does not initiate Gal4 dependent luciferase expression in the absence of the PPARγLBD construct. When the PPARγLBD construct was included, Fig. 2 shows that 3-oxo-C12-HSL but not C4-HSL induced luciferase expression by binding to PPARγLBD. To better illustrate the extent of PPARγLBD binding by 3-oxo-C12-HSL, we compared its effects to those of the synthetic strong PPARγ agonist rosiglitazone, and to one known weak endogenous agonist, arachidonic acid. Fig. 3 shows that the effect of 3-oxo-C12-HSL on PPARγLBD is comparable to that of arachidonic acid, and approximately 5–10 times less than that of rosiglitazone.

3.3. 3-Oxo-C12-HSL can compete with rosiglitazone for binding to PPARγ

We next investigated the ability of 3-oxo-C12-HSL to compete with the PPARγ agonist, rosiglitazone, for binding to the PPARγLBD. We selected rosiglitazone for these experiments because it has a higher affinity for PPARγ than troglitazone or ciglitazone. BEAS cells were transfected with the PPARγLBD construct pUAS-TKLUC and pcDNA3.1-CMVβgal as before, and in separate experiments, treated with

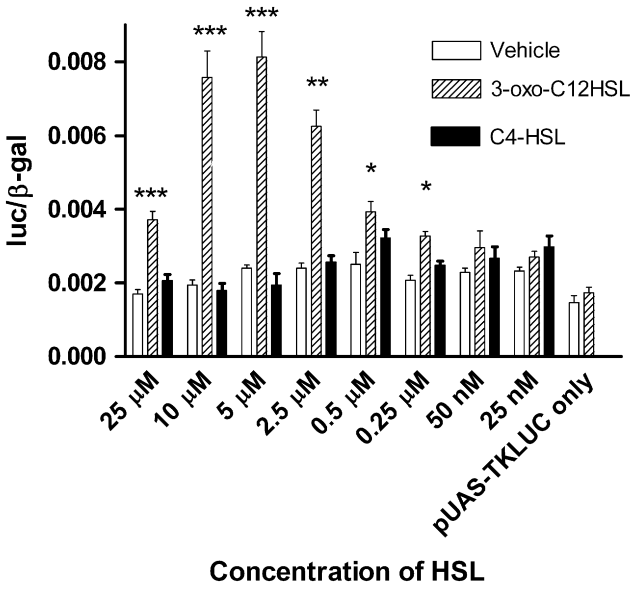

Fig. 2. 3-oxo-C12-HSL but not C4-HSL binds to PPARγ ligand binding domain construct. BEAS-2B cells were transiently transfected with pGAL4-PPARγLBD (PPARγ ligand binding domain construct linked to Gal-4), pUAS-TKLUC (luciferase reporter under control of Gal-4 binding), and pcDNA3.1-CMVβgal (normalization plasmid), then treated with the indicated concentrations of 3-oxo-C12-HSL or C4-HSL for 8 h. Vehicle controls were treated with DMSO at the same concentration as in the corresponding HSL treatment between 0.1% at 10 μM HSL to 0.001% at 0.1 μM HSL. pUAS-TKLUC only: cells transfected with pUAS-TKLUC and pcDNA3.1-CMV-β but not PGAL4-PPARγLBD, and treated with 0.1% DMSO or 10 μM OdDHL. Results expressed as ratio of luciferase (luc) to β-galactosidase (β-gal) expression. Mean ± SD of six replicates. Data analyzed with one-way ANOVA with Dunnett's post-test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ relative to relevant vehicle-treated control.

either a range of concentrations of rosiglitazone alone or in combination with 10 μM 3-oxo-C12-HSL (Fig. 4A), or with a range of concentrations of 3-oxo-C12-HSL in combination with 100 nM (optimum) concentration of rosiglitazone (Fig. 4B). The results of these experiments show that 3-oxo-C12-HSL can interfere with rosiglitazone binding to PPARγLBD, and that concentrations of 3-oxo-C12-HSL down to 1 nM can reduce the binding of 100 nM rosiglitazone to the PPARγ ligand binding domain by 50–70%.

4. Discussion

The results reported here confirm the report of Jahoor et al. [14] that 3-oxo-C12-HSL can bind to and modulate signaling through PPARs, in particular PPARγ. In contrast to Jahoor et al. who suggested that 3-oxo-C12-HSL was a PPARγ antagonist, our data suggest that at concentrations between 1 μM and 10 μM, it is able to weakly promote signaling. This provides one potential mechanism for the immunomodulatory effects of these concentrations of 3-oxo-C12-HSL as reported by ourselves and others [1–5,7,8,30–35]. Jahoor et al. used 50 μM 3-oxo-C12-HSL in most of their study, and it is perhaps relevant that we see an apparent inhibition of PPAR signaling and binding at 25 μM 3-oxo-C12-HSL, which since the

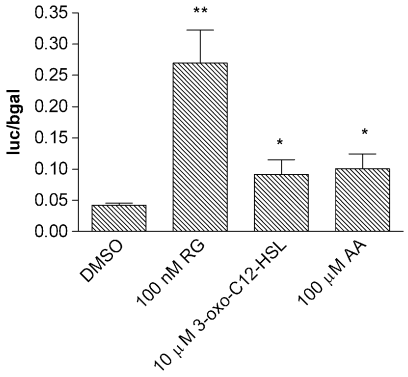

Fig. 3. Effect of 3-oxo-C12-HSL on PPARγLBD construct is comparable to that of arachidonic acid and less than that of rosiglitazone. BEAS-2B cells were transiently transfected with pGAL4-PPARγLBD (PPARγ ligand binding domain construct linked to Gal-4), pUAS-TKLUC (luciferase reporter under control of Gal-4 binding), and pcDNA3.1-CMVβgal (normalization plasmid), then treated with the indicated concentrations of rosiglitazone (RG), 3-oxo-C12-HSL or arachidonic acid (AA) for 8 h. Results expressed as ratio of luciferase (luc) to β-galactosidase (β-gal) expression. Mean ± SD of six replicates. Data analyzed with one-way ANOVA with Dunnett's post-test. *$p < 0.05$; **$p < 0.01$ relative to DMSO control.

β-galactosidase reporter is not inhibited, is unlikely to be caused by nonspecific toxicity or cell death.

Importantly the results shown in Fig. 4 indicate that 3-oxo-C12-HSL not only binds to the PPARγ ligand binding domain, but can interfere with binding of rosiglitazone, possibly by at least partially occupying the ligand binding pocket. Ten micromolar 3-oxo-C12-HSL is not surprisingly able to efficiently inhibit binding of the optimal concentration (100 nM) of rosiglitazone, but the potentially most significant result we obtained is the demonstration that concentrations of 3-oxo-C12-HSL as low as 1 nM can reduce by 50–70% the effect on the PPARγLBD construct of a hundred-fold excess (100 nM) rosiglitazone, suggesting that 3-oxo-C12-HSL binds very strongly to the PPARγLBD construct. This raises the possibility that in vivo, 3-oxo-C12-HSL could interfere with binding to PPARγ of pharmacological and endogenous ligands. Thus, while strong binding of a weak agonist such as 3-oxo-C12-HSL during an inflammatory response to infection could have a small activating effect on PPARγ, it could also "override" the effects of stronger endogenous agonists that would normally reduce inflammation by fully activating PPARγ. This would have the net effect of impairing full activation of PPARγ, thus having a proinflammatory effect. Such a reduction of PPARγ activity resulting from binding of 3-oxo-C12-HSL in the presence of other stronger PPARγ agonists could explain some of the contradictions between the reported activities of 3-oxo-C12-HSL on mammalian cells in vitro and in vivo. In vitro, where only 3-oxo-C12-HSL is present, it could operate as a weak PPARγ agonist, whereas in vivo, particularly during inflammation where a number of endogenous or pharmacological agonists are present, the net effect of 3-oxo-C12-HSL binding could be to reduce overall stimulation of PPARγ activity. Thus, the net effect of 3-oxo-C12-HSL on PPARs probably depends on context, the presence of other PPARγ ligands and the state of activation of

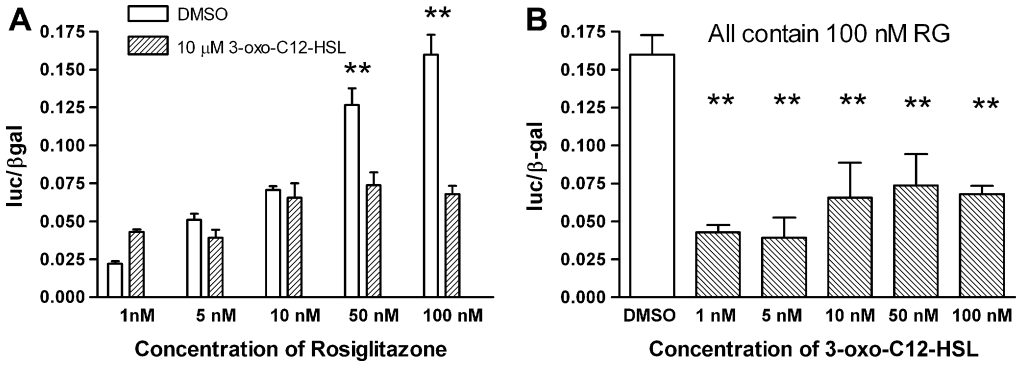

Fig. 4. 3-oxo-C12-HSL effectively interferes with rosiglitazone binding to PPARγ ligand binding domain. BEAS-2B cells were transiently transfected with pGAL4-PPARγLBD (PPARγ ligand binding domain construct linked to Gal-4), pUAS-TKLUC (luciferase reporter under control of Gal-4 binding), and pcDNA3.1-CMVβgal (normalization plasmid) then treated as indicated for 8 h. A. Cells treated with 10 μM 3-oxo-C12-HSL and the indicated concentration of rosiglitazone. B. Cells treated with 100 nM rosiglitazone and the indicated concentration of 3-oxo-C12-HSL. Results expressed as ratio of luciferase (luc) to β-galactosidase (β-gal) expression. Mean ± SD of six replicates. Data analyzed with one-way ANOVA with Dunnett's post-test. $^{**}p < 0.01$ relative to DMSO control.

other interacting pathways. An example of such context-dependent activity of 3-oxo-C12-HSL is the demonstration by Kravchenko et al. [9] that the effects of 3-oxo-C12-HSL on NF-κB activation are modulated by concurrent lipopolysaccharide-dependent signaling.

In cystic fibrosis patients and others, picomolar to nanomolar concentrations of 3-oxo-C12-HSL have been demonstrated to be present in sputum and in lung tissue [36–39] during *P. aeruginosa* infection. However, 3-oxo-C12-HSL is chemically labile under physiological conditions, so these concentrations are probably an underestimate of the in vivo concentrations. In addition, the molecule is unlikely to be evenly distributed within the lung tissue and airways. Local concentrations could be quite high, especially in the vicinity of biofilms: concentrations of up to 600 μM have been reported in laboratory biofilms of *P. aeruginosa* [40]. Thus, the demonstration that 1 nM 3-oxo-C12-HSL can block activation of PPARγ by rosiglitazone is particularly important, since the concentrations of 3-oxo-C12-HSL present in the lungs of cystic fibrosis patients may well be sufficient to at least partly inhibit activation of PPARγ by endogenous agonists, or in areas of high concentration, to directly inhibit PPARγ, and thus to exacerbate the already highly proinflammatory environment of the cystic fibrosis lung [41,42]. In addition, cystic fibrosis cells have been reported to express lower than normal levels of PPARγ [24,25,43], so that inhibiting an already marginally effective anti-inflammatory mechanism could have a significant pathological effect. PPARγ agonist therapy has been suggested as a strategy in a number of inflammatory lung diseases including cystic fibrosis, asthma and chronic obstructive pulmonary disease [44–47]. Our results suggest that at least in cystic fibrosis, where *P. aeruginosa* infection is common, the overall success of such therapy may depend on the identification of PPARγ agonists whose activity is not interfered with by the 3-oxo-C12-HSL produced by infecting *P. aeruginosa*. It is encouraging that Perez et al. showed in a mouse model of cystic fibrosis that troglitazone could reduce the inflammatory response to *P. aeruginosa* lung infection [25]. Since troglitazone has approximately 100-fold lower binding affinity for PPARγ than rosiglitazone [48], this suggests that the concentrations of 3-oxo-C12-HSL present in the lung during *P. aeruginosa* infection may not be sufficient to completely inhibit the activity of strong PPARγ agonists.

In conclusion, we have confirmed that 3-oxo-C12-HSL can bind to the PPARγ ligand binding domain, and that at nanomolar concentrations it can block binding of the synthetic PPARγ agonist rosiglitazone. These results imply that modulation of PPARγ activity by 3-oxo-C12-HSL produced by *P. aeruginosa* during infections could contribute to inflammation, particularly in cystic fibrosis patients where endogenous PPARγ levels are low. We speculate that successful use of PPARγ agonists to treat inflammation during *P. aeruginosa* lung infection may depend on identifying agonists that are able to overcome the inhibitory effects of 3-oxo-C12-HSL.

Acknowledgments

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