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**Pseudomonas aeruginosa** Quorum-Sensing Molecule Homoserine Lactone Modulates Inflammatory Signaling through PERK and eIF2α

Mark A. Grabiner, Zhu Fu, Tara Wu, Kevin C. Barry, Christian Schwarzer, and Terry E. Machen

*Pseudomonas aeruginosa* secrete N-(3-oxododecanoyl)-homoserine lactone (HSL-C12) as a quorum-sensing molecule to regulate bacterial gene expression. Because HSL-C12 is membrane permeant, multiple cell types in *P. aeruginosa*-infected airways may be exposed to HSL-C12, especially adjacent to biofilms where local (HSL-C12) may be high. Previous reports showed that HSL-C12 causes both pro- and anti-inflammatory effects. To characterize HSL-C12’s pro- and anti-inflammatory effects in host cells, we measured protein synthesis, NF-κB activation, and KC (mouse IL-8) and IL-6 mRNA and protein secretion in wild-type mouse embryonic fibroblasts (MEF). To test the role of the endoplasmic reticulum stress inducer, PERK we compared these responses in PERK−/− and PERK-corrected PERK−/− MEF. During 4-h treatments of wild-type MEF, HSL-C12 potentially activated NF-κB p65 by preventing the synthesis of IkB and increased transcription of KC and IL-6 genes (quantitative PCR). HSL-C12 also inhibited secretion of KC and/or IL-6 into the media (ELISA) both in control conditions and also during stimulation by TNF-α. HSL-C12 also inhibited PERK (as shown by increased phosphorylation of eIF2α) and inhibited protein synthesis (as measured by incorporation of [35S]methionine by MEF).Comparisons of PERK−/− and PERK-corrected MEF showed that HSL-C12’s effects were explained in part by activation of PERK- phosphorylation of eIF2α— inhibition of protein synthesis — reduced IkBα production— activation of NF-κB— increased transcription of the KC gene but reduced translation and secretion of KC. HSL-C12 may be an important modulator of early (up to 4 h) inflammatory signaling in *P. aeruginosa* infections. **The Journal of Immunology**, 2014, 193: 1459–1467.

*Pseudomonas aeruginosa* are Gram-negative bacteria that form biofilms in the airways of patients with cystic fibrosis (CF) (1). *P. aeruginosa* coordinate the production of biofilms and virulence factors using the small molecule N-(3-oxododecanoyl)-homoserine lactone (HSL-C12) as a lipid-soluble, diffusible, quorum-sensing molecule (2–4). HSL-C12 has multiple effects on mammalian cells, including inducing apoptosis and activating release of Ca2+ from endoplasmic reticulum (ER) stores (5–10). HSL-C12 has also been reported to affect inflammatory signaling, although some reports indicate an activation of proinflammatory signaling, whereas others indicate a suppression of inflammatory signaling (11–17).

The goal of this study was to elucidate HSL-C12’s role in inflammatory signaling and discover associated effector molecules. To accomplish this, we used mouse embryonic fibroblasts (MEF). Fibroblasts are expected to be exposed to the membrane-permeant HSL-C12 in *P. aeruginosa* biofilm-infected lungs. In addition, MEF are a genetically tractable system with many knockout lines available. We measured expression and secretion of KC (mouse equivalent of human IL-8) and IL-6, because these are important cytokines mediating epithelial immunity produced in response to NF-κB signaling. TNF-α and IL-1β were used as activators of the NF-κB proinflammatory signaling pathway. We show in the current study that both TNF-α and IL-1β cause increases in KC gene transcription and KC secretion. HSL-C12 increased KC gene transcription but did not increase KC secretion, even in the presence of TNF-α or IL-1β. This uncoupling of KC gene transcription from KC secretion could have resulted from an inhibition of protein synthesis resulting from HSL-C12-induced release of Ca2+ from the ER (9, 10, 18), resulting in decreased Ca2+ in the ER, activation of ER stress, and consequent inhibition of protein synthesis (19). We therefore explored the role of ER stress in the responses of MEF to HSL-C12.

We tested specifically the role of protein kinase RNA–like ER kinase (PERK), a transducer of ER stress, in HSL-C12–mediated translation inhibition. PERK, a membrane protein localized to the ER, is one of four kinases known to phosphorylate the eukaryotic translation elongation factor (eukaryotic initiation factor 2α [eIF2α]) (20). PERK becomes activated when BiP chaperone proteins, which usually inhibit PERK, release from binding to PERK and are sequestered to the ER lumen due to a buildup of unfolded proteins (21). PERK is also activated by reductions in Ca2+ in the ER. When PERK becomes active, it phosphorylates the translation elongation factor elF2α on serine 51 (S51 in human), which causes selective inhibition of protein synthesis and induces only certain chaperones and ER stress response proteins to be translated (22). Previous studies have shown that HSL-C12 increases phosphorylation of elF2α in MEF (23). We therefore tested whether HSL-C12 inhibited KC secretion through its effects to activate PERK by comparing protein synthesis, NF-κB translation elongation factor (MTT; MEF) and PERK-corrected MEF showed that HSL-C12’s effects were explained in part by activation of PERK- phosphorylation of eIF2α— inhibition of protein synthesis — reduced IkBα production— activation of NF-κB— increased transcription of the KC gene but reduced translation and secretion of KC. HSL-C12 may be an important modulator of early (up to 4 h) inflammatory signaling in *P. aeruginosa* infections. **The Journal of Immunology**, 2014, 193: 1459–1467.

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activation and KC gene transcription (mRNA production), and KC secretion by PERK−/− MEF and PERK-corrected PERK−/− MEF.

Materials and Methods

Reagents

Unless otherwise specified, reagents and chemicals were obtained from Sigma-Aldrich. HSL-C12 (Cayman Chemical, Ann Arbor, MI, and Sigma-Aldrich) was dissolved in DMSO as 50 μM or 100 μM stocks, and freeze-thaw cycles were limited. HSL-C12 from different suppliers displayed different activities, and therefore 50 μM or 100 μM doses were used accordingly. TNF-α and IL-1β (both R&D Systems, Minneapolis, MN) were used at 10 or 20 ng/ml from 10 or 20 μg/ml stock solutions in water. The Ca2+-ATPase blocker thapsigargin (24) was prepared as a 1 mM stock in DMSO and used at 1 μM.

Cell culture of MEF

Wild-type (WT) MEF were obtained from C. Li (University of Louisville). PERK−/− and corresponding PERK-corrected PERK−/− MEF cell lines were obtained from R. Kaufman (SanfordBurnham Medical Research Institute). MEF were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin. All cells were passed at 1:5–1:15 dilutions, and the remaining cell suspension was seeded directly onto a 24-, 12-, or 6-well tissue culture plate (BD Falcon, Bedford, MA).

ELISA and quantitative PCR for KC and IL-6

Three replicates for each of the following cells, WT, PERK−/−, and PERK-corrected PERK−/− MEF, were grown to confluence on 24-well plates, and experimental cells were treated for 4 h with HSL-C12 (50 μM), TNF-α (10–20 ng/ml), IL-1β (10 ng/ml), or HSL-C12 in combination with TNF-α or IL-1β. The cell culture medium was removed, the cells were washed with PBS, and samples were taken using TRizol reagent (Life Technologies, Grand Island, NY). ELISAs were performed using R&D Systems Duo Set kit (R&D Systems). Capture Abs were incubated on Nunc MaxiSorp 96-well plates in 0.1 M sodium phosphate buffer (pH 8.0) overnight at 4°C, and then the plates were blocked with Dulbecco’s PBS with 1% BSA for 4 h at 4°C. A total of 50 μl culture media per well was taken from the MEFs and applied to the plate overnight at 4°C, followed by five washes with PBS with 0.1% Tween 20 (PBST) and biotinylated capture Abs in Dulbecco’s PBS with 1% BSA for 2 h at room temperature. Five more PBST washes were performed, followed by 30 min of streptavidin-HRP at room temperature. After five more PBST washes, wells were developed for 10 min at room temperature in the dark with 1 mg/ml α-phenylenediamine in 0.05 M sodium phosphate/0.05 M citrate buffer (pH 5.0) and stopped with 3 M HCl. Absorbance was read in a spectrophotometer at 490 nm.

Apoptosis-related cell death of MEF that occurred during HSL-C12 treatments (25) could have altered measurements of KC by ELISA. We attempted to measure the magnitude of this effect by normalizing to [protein] adherent to the cell culture plate after washing. Samples were taken from organic phase from TRizol preparation and dot blotted onto filter paper. Filter paper was then stained with Coomassie blue and then destained with 10% methanol 10% acetic acid. Filter paper was scanned and quantified using ImageJ. KC concentrations obtained through ELISA were divided by [protein] acquired through dot blot. This normalization did not appreciably alter measurements of KC secretion in response to any of the treatments. KC secretion has therefore been reported in ng/ml for studies to determine effects on NF-κB activation, as described previously (26). This vector contained the luciferase gene driven by four tandem copies of the NF-κB consensus sequence and was stored in 10 mM Tris with 20% glycerol at −80°C. The virus was added to MEF at a multiplicity of infection of 100, and they were returned to the incubator for 24 h. Cells were then washed to remove viruses and left to grow for another day. Previous experiments showed that the adenovirus elicits expression in ≥75% of the cells (26). WT MEF in media were left untreated or exposed to HSL-C12 (50 μM), TNF-α (10 ng/ml), or HSL plus TNF-α for 4 h. Cells were then washed and processed with the luciferase assay system by using Reporter Lysis Buffer (Promega, Madison, WI) to measure NF-κB-mediated transcriptional induction according to the manufacturer’s protocol. Measurements of luciferase activity (relative light units) were performed in triplicate for each sample and normalized to the protein concentration (Bradford assay). Averages were then expressed relative to the average control value in the epithelial cells, which was set equal to 1.0.

Western blotting

MEF were grown in 6-well plates to confluence and treated for up to 4 h with HSL-C12 or 1 h with 1 μM thapsigargin and then lysed in M-PER mammalian protein extraction reagent (Pierce, Rockford, IL) containing 5 μg/ml leupeptin, 5 μg/ml pepstatin, 1 mM PMSF, and 50 mM calyculin A. Protein sample concentrations were determined with Bradford reagent (Bio-Rad, Hercules, CA). Immunoblot analysis was performed by first separating protein (10–50 μg/lane) on NadodSO4-PAGE and then transferring it to nitrocellulose membranes. Individual gels with identical loading were run side by side when multiple primary Abs were used. Membranes were blocked (5% nonfat dried milk) in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20 for 1 h and then incubated with specific Abs overnight. Primary Abs (diluted 1:1000 in blocking buffer) for phospho-S51-eI-F2 (A-21), eI-F2 (51-285), and PERK-β activation, as described previously (25), were obtained from R. Kaufman (Sanford Burnham Medical Research Institute). MEF were grown on 24-well plates, and PERK-β corrected PERK−/− MEF were plated on a clear-bottom, white 96-well plate (Nunc, Penfield, NY) in 100 μl medium/well and grown to confluence. Cells were then treated with increasing doses of HSL-C12 in Ringer’s solution. After treatment, 100 μl reagent mix was added to each well. The plate was incubated at room temperature for 1 h on a shaker, and the end-point luminescence was measured in a plate-reader luminometer (LmaxII 384; Molecular Devices, Sunnyvale, CA). Data were background (blank) subtracted and averaged.

Caspar 37 assay

Caspar 37/47 assay was measured by cell-based homogeneous luminescent assays (Caspar-Glo; Promega, Madison, WI), in which a specific substrate that contains the tetrapeptide DEVD was cleaved by the activated cellular caspases to release aminoluciferin, which reacts with luciferase, resulting in the production of light. PERK−/− and PERK-corrected PERK−/− MEF were plated on a clear-bottom, white 96-well plate (Nunc, Penfield, NY) in 100 μl medium/well and grown to confluence. Cells were treated with increasing doses of HSL-C12 in Ringer’s solution after treatment, 100 μl reagent mix was added to each well. The plate was incubated at room temperature for 1 h on a shaker, and the end-point luminescence was measured in a plate-reader luminometer (LmaxII 384; Molecular Devices, Sunnyvale, CA). Data were background (blank) subtracted and averaged.

4S uptake into protein

WT MEF were grown in 24-well plates to confluence (Fig. 4). Wells were pretreated for 3 h with either 50 μM HSL-C12 or DMSO (control). At 3 h postinfection, medium was removed and incubated with 25 μM/ml [35S] methionine (Perkin Elmer, Waltham, MA) in RPMI 1640 medium without methionine supplemented with 10% serum, 2 mM l-glutamine, and 50 μM HSL-C12 or DMSO. Cells were labeled for 1 h, washed three times with cold PBS, and then lysed with radiolabeled preincubation assay buffer supplemented with 2 mM NaVO4, 1 mM PMSF, 25 μM NaF, and 1× Roche protease inhibitor mixture (no EDTA) (pH 7.2) for 10 min at 4°C. Total protein levels were measured by bicinchoninic acid assay, and equal amounts of protein were mixed with SDS sample buffer (40% glycerol, 8% SDS, 2% 2-ME, 40 mM EDTA, 0.05% bromophenol blue, and 250 mM Tris-HCl (pH 6.8)), boiled for 5 min, and then separated by SDS-PAGE. The gels were stained with Coomassie blue to show equal protein loading, dried, and exposed to a phosphor screen and visualized using a Typhoon Trio imager (GE Healthcare).

Statistics

Significance was tested using the two-tailed Student’s t test with equal or unequal distribution, as mentioned in the figure legends. The p values were
C12 activation of Cl−

Similar concentration dependence has been observed for HSL-C12, with a threshold of 1–10 μM, studies showing that HSL-C12 activated apoptosis in MEF. These concentrations were based on previous secretion measurements in response to TNF-α.

The secretion of KC was less than that stimulated by TNF-α. It is therefore possible that TNF-α caused increased transcription of KC mRNA (Fig. 1A) but a decrease in the secretion of KC (Fig. 1B). Similar exposure of MEF to TNF-α plus HSL-C12 caused increased KC gene transcription that was similar to that elicited by TNF-α-treated MEF (Fig. 1A), but secretion of KC was less than that stimulated by TNF-α and approximately equal to that of untreated, control MEF (Fig. 1B). Similar results were obtained when IL-6 mRNA transcription and secretion were measured in response to TNF-α and HSL-C12 (Fig. 2). These data showed that TNF-α caused predictable increases in transcription of KC and IL-6 mRNA and secretion of KC and IL-6. In contrast, C12-HSL, both on its own and in the presence of TNF-α, caused decreases in transcription of KC or IL-6 mRNA, but secretion of KC or IL-6 was either unaffected or inhibited.

HSL-C12 inhibits protein synthesis

The apparently contradictory effects of HSL-C12 (alone and in combination with TNF-α) to stimulate KC/IL-6 gene transcription but inhibit KC/IL-6 secretion could have resulted from an inhibition of secretion of the cytokines or from an inhibition of synthesis of these two proteins. Inhibition of protein synthesis could also explain the discrepancy between measured levels of mRNA induced in response to HSL-C12 (Figs. 1, 2) and levels of NF-κB–regulated luciferase induced by HSL-C12 (Fig. 3). The NF-κB–regulated luciferase method involves transfecting cells with a plasmid that expresses luciferase driven by a NF-κB–regulated promoter. When NF-κB signaling becomes activated, transcription of the luciferase gene also becomes activated, and luciferase protein is produced by the cells, which are then assayed after 4 h of incubation. If HSL-C12 were inhibiting protein synthesis, this could prevent increases in luciferase expression, as observed in this study (Fig. 3) and in previous experiments (23).

To test for potential HSL-C12–induced inhibition of protein synthesis, we used a [35S]methionine metabolic labeling approach to measure global translation levels in MEF after treatment with HSL-C12 (Fig. 4). During the final hour of HSL-C12 or DMSO 4-h treatments on WT MEF, the media were replaced with media in which the only source of methionine was [35S]methionine. Any new proteins synthesized during this time would incorporate radioactive methionine, and the level of incorporation would be directly proportional to the amount of translation occurring in those cells. When protein samples were taken, run on SDS-PAGE gel, and exposed on a phosphor screen, WT MEF samples treated with only DMSO contained radiolabeled proteins over a wide range of m.w. (Fig. 4A). In contrast, MEF treated with HSL-C12 showed very little 35S labeling, indicating that HSL-C12 was inhibiting protein synthesis. Coomassie labeling of the SDS-PAGE gel revealed equal protein loading between lanes (Fig. 4B), indicating that the difference in radiolabeling in response to HSL-C12 was not caused by differential loading. These results suggested that the HSL-C12–induced decoupling of increased KC gene transcription with no increases in KC secretion in MEF may have resulted at least in part from a global reduction in protein synthesis.

ER stress transducer PERK plays a role in HSL-C12–mediated inhibition of protein translation

To assay the role of PERK in HSL-C12–mediated inhibition of translation, we compared HSL-C12–induced responses in PERK−/− and corresponding PERK-corrected PERK−/− (termed PERK-corrected from here on) MEF cell lines. Western blot analysis indicated that PERK levels were similar in the WT and PERK-corrected MEF, and PERK was absent in PERK−/− MEF (Fig. 5A, 5B). Western blots of PERK from WT and PERK-corrected MEF displayed similar gel shifts when treated with HSL-C12, consistent with phosphorylation and activation of PERK during treatment with HSL-C12 (Fig. 5A).

We tested the functional PERK activity of these cell lines by activating ER stress using the common ER stress inducer thapsigargin, which, like HSL-C12, causes release of Ca2+ from the ER (9, 10). As measured in Western blots, thapsigargin caused only a small increase in eIF2α phosphorylation in PERK−/− cells, whereas PERK-corrected cells displayed much more phosphorylation (Fig. 5C, 5D).

**RESULTS**

**HSL-C12 increases proinflammatory cytokine gene transcription but reduces cytokine secretion**

We used 50–100 μM HSL-C12 for studies testing inflammatory responses in MEF. These concentrations were based on previous studies showing that HSL-C12 activated apoptosis in MEF with a threshold of 1–10 μM and maximal effects at 50–100 μM (25). Similar concentration dependence has been observed for HSL-C12 activation of Cl− secretion (9) and apoptosis (10) by airway epithelial cells.

To determine HSL-C12’s effects on inflammatory signaling in MEF, we measured both transcription of the proinflammatory gene KC (mouse equivalent of IL-8) into mRNA using quantitative PCR and secretion of KC protein product into the cell medium using ELISA. MEF were treated for 4 h with HSL-C12 (50 μM), TNF-α (10 ng/ml), or a combination of the two. Results have been summarized in Fig. 1. Compared with untreated MEF, TNF-α caused expected increases in KC gene transcription (Fig. 1A) and KC secretion (Fig. 1B). In contrast, treatment with HSL-C12 caused increased transcription of KC mRNA (Fig. 1A) but a decrease in secretion of KC (Fig. 1B). Similarly, exposure of MEF to TNF-α plus HSL-C12 caused increased KC gene transcription that was similar to that elicited by TNF-α-treated MEF (Fig. 1A), but secretion of KC was less than that stimulated by TNF-α and approximately equal to that of untreated, control MEF (Fig. 1B). Similar results were obtained when IL-6 mRNA transcription and secretion were measured in response to TNF-α and HSL-C12 (Fig. 2). These data showed that TNF-α caused predictable increases in transcription of KC and IL-6 mRNA and secretion of KC and IL-6. In contrast, C12-HSL, both on its own and in the presence of TNF-α, caused decreases in transcription of KC or IL-6 mRNA, but secretion of KC or IL-6 was either unaffected or inhibited.

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**FIGURE 1.** HSL-C12 and TNF-α stimulate KC gene transcription, but C12-HSL inhibits KC secretion. WT MEF were rinsed with fresh media and then left untreated or treated with TNF-α (10 ng/ml), HSL-C12 (50 μM), or TNF-α + HSL-C12 for 4 h. Samples were taken from cell media at t = 4 h, and KC contents were assayed by ELISA. At the end of the experiment, RNA was isolated, and cDNA was formed for quantitative PCR assay. (A) Quantitative PCR data for KC mRNA are given as RQ score normalized to RPS17 cDNA. Averages displayed with minimum and maximum. TNF-α and HSL-C12 each increased production of KC mRNA, and treatment with TNF-α + HSL-C12 caused an even larger increase than during treatment with either agonist alone. *Comparison with control. (B) KC secretion (in ng/ml) for the same treatments. TNF-α increased KC secretion, but HSL-C12 decreased KC secretion. Averages ± SE. n = 3 biological replicates for all conditions. *Comparison with control; #comparison with TNF-α.
A total of 50 μM HSL-C12 also increased phosphorylation of eIF2α to high levels in PERK-corrected cells but much less in PERK−/− cells, particularly during the first 1–2 h of treatment (Fig. 6A, 6B). A summary of eIF2α from three experiments using 100 μM HSL-C12 is shown in Fig. 6C. The higher dose of HSL-C12 resulted in an even greater difference in the response of PERK+/+ and PERK−/− cells. HSL-C12 also increased phosphorylation of eIF2α in WT MEF (data not shown). These results indicated that HSL-C12 would induce a more pronounced inhibition of protein synthesis in PERK-corrected cells compared with PERK−/− cells. Measurements of protein synthesis using 35S labeling showed that when cells were treated with HSL-C12 plus TNF-α, there was greater inhibition of protein synthesis in PERK-corrected than PERK−/− cells (Fig. 7). Similar PERK-dependent inhibition of 35S labeling was obtained during treatment with HSL-C12 alone (data not shown). Together these results indicated that a portion of HSL-C12’s inhibitory effects on protein synthesis resulted from activation of PERK and the phosphorylation of eIF2α.

**HSL-C12’s effects on KC gene transcription and secretion in MEFs are PERK dependent**

The data to this point suggested that HSL-C12 caused a PERK-dependent block in host mRNA-to-protein translation that could have inhibited the production of KC. Therefore, we explored PERK’s role in KC responses to TNF-α and IL-1β (a more potent activator than TNF-α of the NF-κB pathway in MEF) in combination with HSL-C12. Results from these experiments have been summarized in Fig. 8. Similar to WT MEF (Fig. 1), PERK−/− and PERK-corrected MEF both responded to treatment with TNF-α or IL-1β with increases in KC gene transcription (Fig. 8A, 8C) and KC secretion (Fig. 8B, 8D).

In PERK-corrected cells, HSL-C12 elicited modest increases in KC gene transcription (Fig. 8A, 8C, but KC secretion decreased markedly (i.e., compared with treatment with TNF-α or IL-1β alone; Fig. 8B, 8D). These results were similar to those obtained in WT MEF (Figs. 1, 2). Results were different in PERK−/− MEF, as follows: HSL-C12 caused only small changes in either KC gene transcription (Fig. 8A, 8C) or KC secretion (Fig. 8B, 8D). In the presence of either TNF-α or IL-1β, HSL-C12 had only small effects on KC gene transcription (i.e., compared with TNF-α or IL-1β alone; Fig. 8A, 8C), and KC secretion was either not affected or was modestly inhibited (i.e., compared with TNF-α or IL-1β alone; Fig. 8B, 8D). These data were consistent with HSL-C12 stimulating PERK-dependent increases in KC gene expression while inhibiting KC secretion. These PERK-dependent effects of HSL-C12 were particularly evident when HSL-C12 was added in combination with TNF-α or IL-1β: in PERK-corrected MEF treated with either TNF-α or IL-1β, HSL-C12 caused large increases in KC gene transcription but inhibitions of KC secretion, whereas in PERK−/− MEF treated with either TNF-α or IL-1β, HSL-C12 caused only small stimulations of KC gene transcription, and KC secretion remained elevated.

**HSL-C12 activates NF-κB: PERK dependence**

The ability of HSL-C12 to stimulate KC gene transcription on its own and to synergize with TNF-α or IL-1β in stimulating KC gene expression in PERK-corrected (but not PERK−/−) MEF (Fig. 8) increased KC gene transcription (Fig. 8A, 8C), but KC secretion decreased markedly (i.e., compared with treatment with TNF-α or IL-1β alone; Fig. 8B, 8D). Therefore, we explored the activity of HSL-C12 on NF-κB activity.

**FIGURE 2.** Effects of HSL-C12 and TNF-α on IL-6 gene transcription and IL-6 secretion. WT MEF were treated with TNF-α (10 ng/ml), HSL-C12 (50 μM), or TNF-α + HSL-C12 for 4 h, and ELISA and quantitative PCR were performed as in Fig. 1. (A) Quantitative PCR data for IL-6 mRNA are given as RQ score normalized to RPS17 cDNA. Averages displayed with minimum and maximum. *Comparison with control; †comparison with TNF-α. (B) IL-6 secretion (in ng/ml) for conditions shown. Averages ± SE, n = 3 biological replicates for all conditions. *p comparison with control; †comparison with TNF-α.

**FIGURE 3.** HSL-C12 reduces activation of NF-κB luciferase. TNF-α (10 ng/ml) stimulated NF-κB luciferase activity. Compared with control or TNF-α–treated MEF, HSL-C12 (50 μM) decreased NF-κB luciferase activity. Data are averages (±SD) of three biological replicates, each performed in triplicate. *Comparison with control; †comparison with TNF-α.

**FIGURE 4.** HSL-C12 inhibits protein synthesis. (A) [35S]methionine radiolabeling of bulk protein from WT MEF treated with 50 μM HSL-C12 or DMSO (mock treated) and then imaged with a phosphor screen. HSL-C12 treatments were for 4 h total with radiolabeling performed during final hour. (B) Coomassie-stained SDS-PAGE gel from same experiment. Results are representative of two experiments.
may be explained by the inhibition of protein synthesis. IκB, the repressor of NF-κB, is a high turnover protein (27). Treatment with an activator of the NF-κB pathway (e.g., TNF-α or IL-1β) causes phosphorylation and increased degradation of IκBα (27). It has been reported that activators of PERK, including UV light, can also lead to NF-κB signaling by inhibiting protein synthesis, thereby decreasing the available pool of IκBα (28). We measured IκBα and p65 (a subunit of NF-κB) levels and found that over the course of a 4-h treatment HSL-C12 caused IκBα levels to decrease more in PERK-corrected than PERK<sup>−/−</sup> cells, particularly during the first 2 h of treatment (Fig. 9A). Levels of p65 were similar between the two lines, so the ratio of p65 to IκBα, and therefore potential NF-κB activity, was greater in PERK-corrected cells during the first 2 h of treatment (Fig. 9B). These results indicated that PERK was responsible for a major part of the effects of HSL-C12 to potentially activate NF-κB (Fig. 9) and thereby increase KC gene transcription (Fig. 8A, 8C). By inhibiting protein synthesis (Figs. 4, 7), PERK may have prevented the production and secretion of KC (Fig. 8B, 8D), while synergistically increasing KC gene transcription when cotreated with IL-1β (Fig. 8A, 8C) by causing reduced resynthesis of the NF-κB repressor, IκB.

We also tested whether the ER stress pathway that is based on IRE1α affected the HSL-C12–mediated transcriptional regulation of KC in IRE1α<sup>−/−</sup> and IRE1α<sup>+/+</sup> MEF cells. Data shown in Supplemental Fig. 1 indicate that C12-mediated activation of KC transcription was not dependent on IRE1α because increased KC mRNA levels were observed upon C12 treatment in IRE1α<sup>−/−</sup> MEF cells.

**Role for PERK in HSL-C12–triggered apoptosis?**

Given the apparent role for PERK in HSL-C12 inflammatory phenotypes, we also tested whether PERK might play a role in other HSL-C12–triggered responses. PERK and other members of the unfolded protein response are known to produce proapoptotic signals under certain conditions (29–31), and 50 μM HSL-C12 increased caspase 3/7 activity in WT MEF (25). In the present experiments, HSL-C12 caused equivalent concentration-dependent activation of caspase 3/7 in PERK-corrected and PERK<sup>−/−</sup> MEF (Fig. 10). HSL-C12 has also been reported to cause depolarization of mitochondrial membrane potential (10), but PERK-corrected and PERK<sup>−/−</sup> cells showed similar HSL-C12–induced rates of depolarization of mitochondrial membrane potential as measured using the dye JC-1 (data not shown). Together, these results indicated that HSL-C12 affected KC signaling and apoptosis through different pathways (Fig. 11).

**Discussion**

Results from this study showed that, depending on the assay, HSL-C12 had both proinflammatory and anti-inflammatory effects on WT MEF, but these responses appeared to be mediated at least in part through a common molecular mechanism. HSL-C12 caused increased transcription of KC and IL-6 genes, but the expected increase in KC and IL-6 secretion did not occur—secretion of these two proinflammatory cytokines was either reduced (KC) or remained unaffected (IL-6). Because HSL-C12 also increased the phosphorylation of eIF-2α and inhibited protein synthesis, it seems likely that at least part of the inhibitory effect of HSL-C12 on proinflammatory cytokine secretion in the face of increases in gene transcription resulted from HSL-C12 inhibiting synthesis of these cytokines. It also seems likely that similar effects contributed to HSL-C12–triggered increases in TNF-α or IL-1β–stimulated KC or IL-6 gene expression but decreases in TNF-α–stimulated secretion of KC or IL-6. These apparently contradictory effects of HSL-C12 to elicit both proinflammatory (increased gene transcription) and anti-inflammatory (reduced activation of NF-κB, reduced secretion of KC and IL-6) or IL-6–stimulated KC or IL-6 gene expression but decreases in TNF-α–stimulated secretion of KC or IL-6. These apparently contradictory effects of HSL-C12 to elicit both proinflammatory (increased gene transcription) and anti-inflammatory (reduced activation of NF-κB, reduced secretion of KC and IL-6) may explain some of the apparent contradictions surrounding previously reported proinflammatory (11, 12, 15) versus anti-inflammatory (16, 23) effects of HSL-C12 on host cells.

Many of HSL-C12’s effects to alter inflammatory signaling and responses appeared to be mediated largely through HSL-C12–induced activation of PERK. This conclusion is based on the following observations: 1) HSL-C12 caused larger increases in eIF-2α phosphorylation, greater inhibition of protein synthesis, and larger degradation of IκBα in PERK-corrected MEF compared with PERK<sup>−/−</sup> MEF. 2) Compared with treatments with IL-1β or TNF-α.
alone, treatments with IL-1β or TNF-α plus HSL-C12 caused larger increases in KC gene transcription and less KC secretion in PERK-corrected MEF compared with PERK^{−/−} MEF. These data are consistent with the interpretation that HSL-C12 acts through PERK and eIF-2α to inhibit protein synthesis, leading to reduced production of the high turnover protein IκBα, increased activation of NF-κB, and increased KC gene transcription but decreased KC synthesis and secretion, particularly in the presence of the proinflammatory mediators IL-1β and TNF-α.

A flow chart summarizing these conclusions about the effects of HSL-C12 is presented in Fig. 11. HSL-C12 activates PERK and the subsequent phosphorylation of eIF-2α to inhibit protein synthesis, leading to reduced production of the high turnover protein IκBα, increased activation of NF-κB, and increased KC gene transcription but decreased KC synthesis and secretion, particularly in the presence of the proinflammatory mediators IL-1β and TNF-α.

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The NF-κB inhibitor (28), will be inhibited. As IκBα levels fall, NF-κB will be released to enter the nucleus and induce transcription of proinflammatory gene products such as KC. (HSL-C12 causes p65 to migrate from the cytosol to the nucleus within 30–60 min in the airway epithelial cell line JME [C. Schwarzer and T. Machen, unpublished observations].) However, because protein synthesis has been inhibited, new synthesis and secretion of KC will be inhibited, even in the presence of ligands like TNF-α and IL-1β that normally induce inflammatory secretion. Of course, this proposal requires that p65 that enters the nucleus will be the phosphorylated, active version that serves as the potent proinflammatory transcription factor, and it has been argued that p65 that enters the nucleus of HSL-C12–treated cells remains unphosphorylated (29). Further experiments testing the mechanism by which HSL-C12 activates proinflammatory gene tran-

**FIGURE 6.** HSL-C12 increases phosphorylation of eIF-2α. (A) PERK^{−/−} and PERK-corrected MEF were treated with 50 μM HSL-C12 for 0, 1, 2, 3, or 4 h. Protein samples were taken and run at equal concentrations on SDS-PAGE gel; Western blots were performed using anti-phosphoS51-eIF-2α and anti-eIF-2α Abs. Results typical of three similar experiments. (B) Results from (A) were quantified and displayed as the ratio of phosphoS51-eIF-2α to bulk eIF-2α. (C) Average induction (±SEs) of eIF-2α phosphorylation of WT, PERK^{−/−}, and PERK-corrected MEF in response to treatment with 100 μM HSL-C12 for 1 h from two further experiments. *Comparison PERK^{−/−} with PERK-corrected, n = 3 experiments.

**FIGURE 7.** HSL-C12’s inhibition of protein synthesis is PERK dependent. (A) [35S]methionine radiolabeling of bulk protein from PERK^{−/−} and PERK-corrected MEF untreated or treated with 100 μM HSL-C12 + 10 ng/ml TNF-α imaged with a phosphor screen. MEF were treated with HSL-C12 + TNF-α for 4 h, with radiolabeling performed during final hour. (B) Coomassie-stained SDS-PAGE gel from same experiment. (C) Graph of phosphor images from (A) was quantified and normalized to Coomassie image from (B). Normalized [35S]S incorporation was calculated from the entire lane for PERK-corrected and PERK^{−/−} MEF, untreated or treated with HSL-C12 + TNF-α. Results typical of two similar experiments.
scription will help clarify the specific roles of PERK, eI-F2α, and NF-κB p65 in responses to HSL-C12.

Although the present data indicate that HSL-C12–triggered effects on inflammatory signaling and responses in MEF were mediated largely through activation of PERK, it is also clear that HSL-C12 had effects that were mediated through other effector pathways. PERK was not required for HSL-C12 to activate caspase 3/7, indicating that PERK was not involved in mediating HSL-C12–triggered apoptosis. Instead, HSL-C12–triggered apoptosis (10, 25) seems to require activation of another ER stress pathway mediated by IRE1α and XBP-1 (31). In addition, HSL-C12 elicited a less than maximal but still detectable increase in eI-F2α phosphorylation, inhibition of protein synthesis, and degradation of IκB in PERK2/2 MEF. All of these effects most likely resulted from HSL-C12 activating other effector pathways besides PERK-eI-F2α. One potential PERK-independent molecule that could become activated by HSL-C12 is the eI-F2α kinase GCN2, which is activated by metabolic stress through the buildup of unloaded tRNAs (31). HSL-C12’s effects on the mitochondria (10, 14) may lead to metabolic stress and subsequent GCN2 activation. Previous work showed that HSL-C12 activates both p-38 and c-Jun in MEF (31), so these signaling pathways may also be involved in HSL-C12–triggered responses. The newly identified, selective inhibitors of the anti-inflammatory and proapoptotic effects of HSL-C12 on MEF (triazolo[4,3-a] quinolones) should help in testing for alternative signaling pathways affected by HSL-C12 (23).

Although it has not been fully established that HSL-C12 is an important P. aeruginosa virulence factor in vivo, the present data indicate that HSL-C12 is an important virulence factor in vitro.

**FIGURE 8.** HSL-C12 effects on KC gene expression and secretion are PERK dependent. PERK−/− and PERK-corrected MEF were rinsed with fresh media and then treated for 4 h with TNF-α (20 ng/ml), HSL-C12 (100 μM), or TNF-α + HSL-C12 (A and B) or with IL-1β (10 nM), HSL-C12 (100 μM), or IL-1β + HSL-C12 (C and D). Samples were taken from the media after 4 h for measurement of KC secretion (in ng/ml medium) by ELISA (A and C). Cells were then treated with TRIzol and cDNA was prepared for quantitative PCR analysis, where results are given as RQ score normalized to RPS17 cDNA (B and D). Averages ± SE; n = 3 biological replicates for all conditions, except KC secretion by PERK−/− MEF, where n = 2. For experiments on PERK-corrected MEF: *comparisons with control; #comparison with TNF-α (A and B) or IL-1β (C and D). For experiments on PERK−/− MEF: *comparison with control; #comparison with TNF-α (A and B) or IL-1β (C and D).

**FIGURE 9.** HSL-C12-induced reduction in IκBα is PERK dependent. PERK−/− and PERK-corrected MEF were treated with 100 μM HSL-C12. (A) Protein samples were taken from cells at times shown and run at equal concentrations on SDS-PAGE gel; Western blots were performed using anti-IκBα and anti-p65 Abs. (B) Results from (A) quantified and displayed as ratio p65 to bulk IκBα. High ratios indicate high potential for NF-κB p65-driven transcription. Results typical of two experiments.

**FIGURE 10.** HSL-C12–induced apoptosis is PERK independent. PERK−/− and PERK-corrected MEF were treated with increasing doses of HSL-C12 for 4 h, and caspase 3/7 activation was measured. Data are averages ± SE (n = 3 biological replicates).
RESULTS indicate that HSL-C12’s effects on both inflammatory signaling and apoptosis might affect the course of *P. aeruginosa* lung infections. HSL-C12 secreted by *P. aeruginosa* would have the short-term effects of suppressing secretion of proinflammatory mediators (present work) and activating anti-inflammatory mediators (17). Consistent with this idea, we have found that Calu-3 airway epithelial cells secreted more IL-8 during exposure to planktonic *P. aeruginosa* compared with exposure to biofilm *P. aeruginosa*, which are expected to produce higher (HSL-C12) compared with planktonic *P. aeruginosa* (Supplemental Fig. 2). The apparent inhibitory effect of biofilm (i.e., compared with planktonic) *P. aeruginosa* on IL-8 secretion appeared to be mediated in part by HSL-C12 because the inhibitory effect of the biofilm PA01 was reduced by biofilm PA01 that were defective in expression of lasI, the enzyme responsible for producing HSL-C12 (Supplemental Fig. 2). HSL-C12 is also expected to create gaps in epithelia resulting from its proapoptotic effects (5–7, 10), allowing *P. aeruginosa* access to the basolateral membrane, an important factor for virulence (32, 33).

The roles of PERK in modulating host cell inflammatory responses to HSL-C12 (Fig. 11) and of IRE1α and XBP-1 in mediating the apoptosis response to HSL-C12 (30) suggest ties to phenotype observed in cells from CF patients. CF airway cells are characterized by increased ER volume and a chronically active unfolded protein response (34), perhaps indicating prolonged exposure to ER stress inducers like HSL-C12. However, these conclusions are mostly based on results from our short-term (up to 4 h) experiments, and CF patients may be chronically exposed to *P. aeruginosa* biofilms and HSL-C12. The experimental conditions applied in our current study did not allow us to monitor long-term effects of HSL-C12 because increased cell death dominated over and masked other cellular responses. Further studies of HSL-C12 effects on airway and other epithelia during exposures longer than the 4-h treatments and with lower doses used in this study are therefore warranted. Previous work on the intestinal cell line Caco-2 has shown that HSL-C12–triggered degradation of barrier function and tight junction structure is largely reversed during 24-h exposures (35, 36), and it will be important to test whether there is a similar reversal in inflammatory and apoptotic responses of airway epithelia during 24-h treatments with HSL-C12. Despite the uncertainty associated with making conclusions about chronic conditions in vivo based on results from short-term experiments in vitro, accumulating evidence indicates that HSL-C12 is an important virulence factor in *P. aeruginosa* infections not only because it regulates expression of key bacterial genes but also because it has direct, pathological effects on host cells.

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DISCLOSURES

The authors have no financial conflicts of interest.

REFERENCES


SUPPLEMENTAL FIGURES AND LEGENDS TO ACCOMPANY:

*Pseudomonas aeruginosa* Quorum-Sensing Molecule Homoserine Lactone Modulates

Inflammatory Signaling through PERK and eIF2α

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SUPPLEMENTAL FIGURE 1

Fig. S1. HSL-C12 effects on KC gene expression are not IRE-1α-dependent. IRE-1α−/− and IRE-1α-corrected MEF were rinsed with fresh media then treated for 4 hours with IL-1β (10 nM), HSL-C12 (100 µM) or IL-1β + HSL-C12. Cells were then treated with Trizol, and cDNA was prepared for qPCR analysis, where results are given as RQ score normalized to RPS17 cDNA. Averages +/- Std error; n = 3 biological replicates for all conditions. For experiments on IRE-1α-corrected MEF: * comparisons to control; # comparison to IL-1β. For experiments on IRE-1α−/− MEF, $ comparison to control, % comparison to IL-1β.
SUPPLEMENTAL FIGURE 2

![Secreted IL8 (ng/mL)]

**Fig. S2.** Compared to planktonic *P. aeruginosa*, biofilm *P. aeruginosa* inhibit IL8 secretion by Calu-3 airway epithelial cells; this inhibition is mediated in part by lasI, the enzyme responsible for production of HSL-C12 in *P. aeruginosa*. Calu-3 cells, an airway epithelial cell line, was grown to confluence on filters. *P. aeruginosa* strains PAO1 and PAO1lasI (missing lasI gene, which encodes the enzyme responsible for the last step of synthesis in HSL-C12) were grown in LB media (“planktonic”) or as biofilms on small, permeable filters on the surface of agar plates to permit growth as biofilms (“biofilm”) (Schwarzer et al, 2012. *Cell Micro* 14: 698-709). Planktonic PAO1 and biofilm PAO1 and PAO1lasI were resuspended in tissue culture MEM at $10^8$ cfu/ml. 100 µl of the suspensions were added to the apical surface of the Calu-3 monolayers, which were placed in the incubator for 4 hrs. Samples (100 µl) were taken from the basolateral surface of the epithelial monolayers at $t = 0$ and $t = 4$ hrs, and IL8 was assayed by ELISA. IL8 secreted during this time was largest during exposure to planktonic PAO1, less during exposure to biofilm PAO1 and intermediate during exposure to biofilm PAO1lasI. Data are averages +/- Std errors, n = 3 biological replicates, each measured in triplicate. * comparison to control; # comparison to PAO1 planktonic; $ comparison to PAO1 biofilm.