Abstract. N-3-oxododecanoyl homoserine lactone (3-oxo-C12-HSL), a quorum-sensing signal molecule produced by Pseudomonas aeruginosa (P. aeruginosa), is involved in the expression of bacterial virulence factors and in the modulation of host immune responses by directly disrupting nuclear factor-kB (NF-kB) signaling and inducing cell apoptosis. The unfolded protein response (UPR) triggered by endoplasmic reticulum (ER) stress may suppress inflammatory responses in the later phase by blocking NF-kB activation. It was recently demonstrated that 3-oxo-C12-HSL may induce UPR in human aortic endothelial cells (HAECs). Therefore, 3-oxo-C12-HSL may also inhibit NF-kB activation and suppress inflammatory responses by activating UPR. However, the possible underlying mechanism has not been fully elucidated. Accordingly, we investigated the effects of 3-oxo-C12-HSL on cellular viability, UPR activation, lipopolysaccharide (LPS)-induced NF-kB activation and inflammatory response in the RAW264.7 mouse macrophage cell line. Treatment with 6.25 µM 3-oxo-C12-HSL was not found to affect the viability of RAW264.7 cells. However, pretreating RAW264.7 cells with 6.25 µM 3-oxo-C12-HSL effectively triggered UPR and increased the expression of UPR target genes, such as CCAAT/enhancer-binding protein β (C/EBP β) and CCAAT/enhancer-binding protein-homologous protein (CHOP). The expression of C/EBP β and CHOP was found to be inversely correlated with LPS-induced NF-kB activation. 3-Oxo-C12-HSL pretreatment was also shown to inhibit LPS-stimulated proinflammatory cytokine production. Hence, 3-oxo-C12-HSL may attenuate LPS-induced inflammation via UPR-mediated NF-kB inhibition without affecting cell viability. This may be another mechanism through which P. aeruginosa evades the host immune system and maintains a persistent infection.

Introduction

N-3-oxododecanoyl homoserine lactone (3-oxo-C12-HSL), a quorum-sensing signal molecule produced by the opportunistic pathogen Pseudomonas aeruginosa (P. aeruginosa), plays an important role in the regulation of bacterial virulence genes, as well as in the modulation of inflammatory responses (1-3). 3-Oxo-C12-HSL directly disrupts lipopolysaccharide (LPS)-induced nuclear factor-kB (NF-kB) signaling and represses NF-kB-responsive genes that encode inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-12 in macrophages (2,4). 3-Oxo-C12-HSL also induces cellular apoptosis in macrophages/lymphocytes and alleviates host immune responses (5). These data indicate that 3-oxo-C12-HSL plays a critical role in the inhibition of inflammation.

The unfolded protein response (UPR) is an adaptive cellular response that protects cells against the stress of unfolded proteins in the endoplasmic reticulum (ER) (6). Recent studies reported that UPR triggers NF-kB activation in the early phase (7) and suppresses inflammatory responses to stimuli in the later phase through NF-kB activity inhibition (8-12). CCAAT/enhancer-binding protein (C/EBP) is a family of transcription factors that possess a highly conserved, basic-leucine zipper (bZIP) domain, which is required for DNA binding and dimerization (13). C/EBP β, one of the 6 C/EBP family members, may be triggered by ER stress and interact with transcription factors, including NF-kB (14). Several studies confirmed that C/EBP β induced by UPR depresses NF-kB phosphorylation (12,15,16). CCAAT/enhancer-binding protein-homologous protein (CHOP) is a transcription factor that stably forms heterodimers with C/EBP family members through their bZIP domain and is upregulated by the activation of UPR (17). Recent studies demonstrated that UPR-mediated...
induction of CHOP reduces inflammatory cytokine secretion and NF-κB activation (18,19). A recent study also demonstrated that 3-oxo-C12-HSL may induce the expression of UPR genes, such as C/EBP β and CHOP, in human aortic endothelial cells (HAECs) (20). These data strongly indicate that 3-oxo-C12-HSL may inhibit NF-κB activity through the induction of UPR.

3-oxo-C12-HSL may inhibit the expression levels of TNF-α and monocyte chemoattractant protein-1 (MCP-1) by directly impairing the regulation of NF-κB functions when stimulating bone marrow-derived macrophages and peripheral blood mononuclear cells together with LPS for ≤3 h (2). However, whether pretreatment with 3-oxo-C12-HSL is able to induce UPR and whether this UPR can inhibit LPS-induced inflammation in macrophages has yet to be determined. In the present study, we investigated the effects of 3-oxo-C12-HSL-triggered UPR on LPS-induced NF-κB activation and inflammation in RAW264.7 cells.

Materials and methods

Reagents. *P. aeruginosa* quorum-sensing molecule 3-oxo-C12-HSL and LPS from *Escherichia coli* were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. RAW264.7 cells (China Center for Type Culture Collection, Wuhan, China) were cultured in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (Hyclone, Logan, UT, USA). The cultured cells were incubated with serum-free basal medium for 12 h and then washed twice prior to the challenge. For the challenge experiments, the cells were exposed to various concentrations of 3-oxo-C12-HSL (6.25-100 µM). At indicated times following incubation, the cultures were centrifuged at 450 x g for 10 min at 4°C. The cultured supernatants and the cells were collected and stored at -80°C for further experiments.

Cell viability assay. The cells (5x10^5 cells/well) were cultured in a flat-bottom 96-well microtiter plate containing 100 µl of serum-free basal medium with various concentrations of 3-oxo-C12-HSL (21). The cell viability was determined using the WST-1 cell proliferation and cytotoxicity assay kit (Beyotime, Haimen, China) according to the manufacturer's instructions. Absorbance was measured using a microplate reader (BioTek, Winooski, VT, USA) at 450 nm and the reference wavelength was set at 630 nm. The percentage cytotoxicity was calculated in comparison with that of the untreated control cells.

Apoptosis assay. Cell apoptosis was analyzed by flow cytometry using the Annexin V-fluorescein isothiocyanate (FITC) apoptosis kit (Keygen Biotech, Nanjing, China). The RAW264.7 cells (1x10^5 cells/ml) were incubated with different concentrations of 3-oxo-C12-HSL for 9 h. After the challenge, the cells were suspended and labeled with propidium iodide and Annexin V-FITC for 15 min at 25°C in the dark. The cells were observed on FACSAria (BD Biosciences, San Diego, CA, USA) and the data were analyzed using the FACSDiva Cytometer software (BD Biosciences). The cells that stained positive for Annexin V were counted as apoptotic.

RNA extraction, quantitative reverse transcription-polymerase chain reaction (qRT-PCR). TRIzol reagent (Invitrogen Life Technologies) was used to extract total RNA. The primers used for qPCR are as follows: GAPDH, forward 5'-TGCACTACCACTGCTTAG-3' and reverse 5'-GATGCAGGATGATGGTC-3'; C/EBP β, forward 5'-ACCAGGTTCGGGACGTTGA-3' and reverse 5'-GTTGGCTAGTCCTGGCTTCAA-3'; CHOP, forward 5'-CTGGAAACCTGTATGAGGAT-3' and reverse 5'-CAGGGTCAAGGATGGAAGT-3'. RT-PCR was performed using the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) and qPCR analysis was performed with SYBR-Green Realtime PCR master mix-plus (Toyobo) according to the manufacturer's instructions.

Western blot analysis. Following the challenge and removal of the culture medium, the cells were lysed in radioimmunoprecipitation assay buffer (ProMob, Changsha, China) and then boiled for 5 min. Aliquots of cell extracts were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (Bio-Rad, Richmond, CA, USA) and blotted with anti-β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-C/EBP β (BioLegend, San Diego, CA, USA), anti-CHOP (Santa Cruz Biotechnology, Inc.) and anti-phospho-NF-κB p65 pSer536 antibody (Cell Signaling Technology, Inc., Danvers, MA, USA).

Enzyme-linked immunosorbent assay (ELISA). The cytokine levels of MCP-1 and TNF-α were measured by ELISA using mouse MCP-1 and TNF-α kits (R&D Systems, Minneapolis, MN, USA), as previously described (22). Cell supernatant was added to the wells, which were coated with antibodies against MCP-1 and TNF-α. After washing twice with washing buffer, peroxidase-conjugated avidin, biotinylated antibodies and chromogenic substrate were added to each well. The absorbance was read at 450 nm in an ELISA plate reader.

Statistical analysis. Representative data from triple independent experiments are expressed as means ± SD. One-way ANOVA was performed to determine statistical significance among multiple groups and the Student's t-test was used to compare differences between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Determination of a proper concentration of 3-oxo-C12-HSL in RAW264.7 cells. At a proper concentration, 3-oxo-C12-HSL may activate UPR without affecting cell viability, as previously described (11). 3-Oxo-C12-HSL was shown to induce cell apoptosis and death in macrophage/lymphocytes (5). Thus, the cytotoxic effects of different concentrations of 3-oxo-C12-HSL were investigated in order to determine a proper concentration that elicits an appropriate UPR. The RAW264.7 cells exhibited a significantly reduced viability following treatment with 100 µM 3-oxo-C12-HSL (Fig. 1A). Cell viability decreased from 33.4% at 1 h to 2.25% at 24 h following 3-oxo-C12-HSL addition (Fig. 1A). By contrast, 6.25 µM 3-oxo-C12-HSL did
The mRNA expression of molecular chaperones in the UPR pathway. Kim et al. (20) confirmed that 50 μM 3-oxo-C12-HSL effectively induces UPR and activates UPR target genes, such as C/EBP β and CHOP in HAECs. Therefore, the mRNA expression of representative target genes of UPR activation, such as C/EBP β and CHOP, was analyzed using qRT-PCR to determine whether 6.25 μM 3-oxo-C12-HSL was able to efficiently activate these genes (Fig. 2A and B). C/EBP β mRNA was found to be increased at 3 h and reached a peak at 12 h (Fig. 2A). Similarly, CHOP mRNA expression was also increased after 3-oxo-C12-HSL stimulation at time points between 3 and 12 h (Fig. 2B). These results suggested that 6.25 μM 3-oxo-C12-HSL may effectively stimulate the expression of UPR target genes in RAW264.7 cells. The C/EBP β [liver-enriched transcriptional activator protein (LAP) isoform] and CHOP proteins are activated by UPR and may suppress the activation of NF-κB in the later phase (15,18). Thus, we also investigated the protein expression pattern of UPR following incubation with 3-oxo-C12-HSL. Enhanced expression of the C/EBP β (LAP isoform) and CHOP proteins was observed following stimulation with 3-oxo-C12-HSL. The protein level of C/EBP β (LAP isoform) increased from 9 to 12 h (Fig. 2C). The level of CHOP was also significantly increased after 9 h, reaching a peak at 12 h (Fig. 2D). These results indicated that 3-oxo-C12-HSL pretreatment may effectively activate UPR-mediated C/EBP β and CHOP expression. Although the levels of C/EBP β (LAP isoform) and CHOP at 12 h were marginally higher compared to those at 9 h, no statistically significant difference was observed (P>0.05). Therefore, the time point for analyzing the abrogation of LPS-induced inflammation in RAW264.7 cells would be the point at which the UPR target genes were effectively activated following pretreatment with 3-oxo-C12-HSL. In the present study, 9 h was selected as the appropriate time point based on the aforementioned results.

3-oxo-C12-HSL pretreatment suppresses LPS-induced NF-κB activation and inflammatory cytokine production. To further verify whether 3-oxo-C12-HSL pretreatment suppresses LPS-induced NF-κB activation, we observed the LPS-induced NF-κB phosphorylation in cells pretreated with 3-oxo-C12-HSL and untreated cells. Following stimulation with LPS for 12 h, NF-κB phosphorylation was significantly decreased in the group pretreated with 3-oxo-C12-HSL for 9 h. Furthermore, the expres-

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**Figure 1.** Effects of N-3-oxododecanoyl homoserine lactone (3-oxo-C12-HSL) on RAW264.7 cell viability. (A) RAW264.7 cells were treated with 6.25 or 100 μM 3-oxo-C12-HSL for 1-24 h. Cell viability was measured with the WST-1 assay. (B) Following incubation with increasing concentrations of 3-oxo-C12-HSL for 12 h, cell viability was measured with the WST-1 assay (*P<0.05 compared to the 0 and 6.25 μM 3-oxo-C12-HSL-treated groups). (C) The RAW264.7 cells were treated with 0, 6.25, 50 or 100 μM 3-oxo-C12-HSL for 9 h. The cells were stained with propidium iodide and fluorescein isothiocyanate (FITC)-conjugated Annexin V and the percentage of Annexin V-positive cells was measured by flow cytometry.
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sion of C/EBP β (LAP isoform) and CHOP was significantly negatively correlated with NF-kB phosphorylation (Fig. 3A).

Since LPS-induced MCP-1 and TNF-α production involves NF-kB phosphorylation (23,24), the effects of 3-oxo-C12-HSL on LPS-induced cytokine production were then investigated in RAW264.7 cells. LPS-induced MCP-1 and TNF-α production was significantly decreased in cells pretreated with 3-oxo-C12-HSL compared to that in untreated cells (Fig. 3B and C). These results indicated that pretreatment with 6.25 µM 3-oxo-C12-HSL significantly inhibits LPS-induced inflammatory responses in RAW264.7 cells.

Therefore, 3-oxo-C12-HSL may inhibit NF-kB phosphorylation and cytokine production in RAW264.7 cells through the activation of UPR, particularly the expression of UPR target genes, such as C/EBP β and CHOP.

Discussion

P. aeruginosa is an opportunist pathogen that causes persistent infection in patients with cystic fibrosis, mechanical ventilation, HIV infection and various malignancies (25). It was previously confirmed that macrophage dysfunction contributes to the susceptibility to infection and the persistence of P. aeruginosa in experimental models (1,3). 3-Oxo-C12-HSL, a vital quorum-sensing signal molecule produced by P. aeruginosa, regulates the expression of P. aeruginosa extracellular virulence factors and induces the incompetence of macrophages by impairing the regulation of NF-kB function and accelerating macrophage apoptosis (2,5,26,27). Incubation with 12-50 µM 3-oxo-C12-HSL was shown to directly induce the apoptosis of bone marrow-derived macrophages (5). Incubation with 25 µM 3-oxo-C12-HSL may also directly disrupt NF-kB activation (2). Those results indicated that 3-oxo-C12-HSL at concentrations >12 µM may directly inhibit macrophage activation.

UPR induced by a subcytotoxic dose of subtilase cytotoxin was shown to prevent LPS-associated inflammatory responses through a UPR-dependent inhibition of NF-kB activation, without affecting RAW264.7 cell viability (11). A recent investigation also reported that 3-oxo-C12-HSL effectively activated UPR in HAECs (20). Therefore, it is important to determine whether pre-incubation with a subcytotoxic dose of 3-oxo-C12-HSL may induce UPR and inhibit NF-kB activation in RAW264.7 cells, as this may be another mechanism through which 3-oxo-C12-HSL inhibits the function of macrophages and maintains the persistent infection of P. aeruginosa. The present data demonstrated that 6.25 µM 3-oxo-C12-HSL may effectively induce the expression of UPR-responsive genes and proteins without affecting cell viability. UPR induced by

Figure 2. Expression of molecular chaperones in the unfolded protein response pathway. RAW264.7 cells were treated with 6.25 µM 3-oxododecanoyl homoserine lactone (3-oxo-C12-HSL) for 0, 3, 9 and 12 h prior to gene expression analysis. The relative quantitative expression of (A) CCAAT/enhancer-binding protein (C/EBPβ) and (B) CCAAT/enhancer-binding protein-homologous protein (CHOP) at different time points were determined using quantitative reverse transcription-polymerase chain reaction analysis (P<0.05 compared to untreated controls). Subsequently, the RAW264.7 cells were incubated with 6.25 µM 3-oxo-C12-HSL for the indicated times and the level of (C) C/EBP β [liver-enriched activator protein (LAP)] and (D) CHOP were determined by western blot analysis and semi-quantified by densitometry (P<0.05 compared to untreated controls; P>0.05 compared to each other).

D C B A
3-oxo-C12-HSL was significantly inversely correlated with the LPS-induced inflammatory responses in RAW264.7 cells. 3-Oxo-C12-HSL at a concentration range of 10–100 µM was shown to induce cell death in bone marrow-derived macrophage, breast cancer, mast and epithelial cell lines (5,21,28). A concentration of 50 µM 3-oxo-C12-HSL was also found to induce the expression of C/EBP β and CHOP through UPR (20). In the present study, we observed that incubation with 12.5–100 µM 3-oxo-C12-HSL induced apoptosis and death in RAW264.7 cells. Moreover, 6.25 µM 3-oxo-C12-HSL did not affect cell viability but effectively activated UPR gene expression, increasing the mRNA expression levels of C/EBP β and CHOP. Thus, we selected 6.25 µM as the proper concentration to stimulate RAW264.7 cells. C/EBP β and CHOP protein levels were significantly elevated at 9 h following pretreatment with 3-oxo-C12-HSL. The levels of these proteins were higher at the time point of 12 h compared to those at 9 h. However, there was no significant difference between the two groups. Therefore, 9 h was selected as the appropriate pretreatment time in this experiment.

UPR was first confirmed to abrogate the induction of MCP-1 and inducible nitric oxide synthase by TNF-α (8). Other studies reported that UPR activation also suppresses cytokine or LPS-triggered activation of NF-κB and the subsequent production of proinflammatory cytokines (9–12). In a previous study, it was proven that the C/EBP β gene has an unfolded protein response element (UPRE) at its 3'-end. This UPRE was shown to be responsible for the upregulation of C/EBP β by ER stress (29). Another study confirmed that C/EBPs interact with the Rel domain of NF-κB via its bZIP domain (30). Prosch et al (31) further indicated that C/EBP α/β interacts with the NF-κB p65 subunit and inhibits the activation of NF-κB. The upregulated C/EBP β induced by UPR was confirmed to inhibit NF-κB phosphorylation (12,15). Although the exact underlying mechanism remains unclear, it is hypothesized that CHOP expression is upregulated by UPR and consequently reduces the phosphorylation of NF-κB and inflammatory cytokine production (16,18). In the present study, we demonstrated that the expression of C/EBP β and CHOP is significantly inversely correlated with the phosphorylation of NF-κB in RAW264.7 cells. In addition, pretreatment with 3-oxo-C12-HSL represses NF-κB-mediated MCP-1 and TNF-α production in these cells. All these findings indicated that C/EBP β and CHOP, which are upregulated by 3-oxo-C12-HSL-mediated UPR, inhibit LPS-induced NF-κB activation and inflammatory cytokine production.

Three major branches of UPR were generally triggered by ER stress to cause downstream transcriptional events: the double-stranded RNA-activated protein kinase-like ER kinase pathway, the inositol-requiring enzyme 1 (IRE1) pathway and the activating transcription factor 6 pathway (6). Although the role of stress response pathways in 3-oxo-C12-HSL-mediated cytotoxicity has not been fully elucidated, it was recently reported that 3-oxo-C12-HSL does not activate the splicing X-box binding protein 1 (XBP-1) function of IRE1 (32). However, another study indicated that the splicing XBP-1
function of IRE1 is not associated with the induction of C/EBP \( \beta \) (12). Thus, 3-oxo-C12-HSL may activate the UPR of ER stress through other branches in order to inhibit NF-\( \kappa \)B activation.

All three branches of UPR are involved in the regulation of inflammatory responses and different branches of UPR play major roles in different cell types (8-11). Therefore, the exact role of each UPR branch on 3-oxo-C12-HSL-induced UPR-mediated inhibition of NF-\( \kappa \)B requires further investigation.

In summary, this preliminary study indicated that 3-oxo-C12-HSL-mediated UPR in macrophages may inhibit LPS-induced inflammatory responses by disrupting NF-\( \kappa \)B activation. Although 3-oxo-C12-HSL was originally identified as a bacterial autotoxin for controlling virulence gene expression in \textit{P. aeruginosa} infection (27), it may also prevent LPS-mediated inflammatory responses by UPR, a mechanism possibly used by \textit{P. aeruginosa} to evade the host immune system and maintain persistent infection. Further investigations are required to determine the exact role of C/EBP \( \beta \) and CHOP or other branches of UPR in 3-oxo-C12-HSL-mediated NF-\( \kappa \)B inhibition through loss-of-function studies.

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