INFECTION AND IMMUNITY, Dec. 2006, p. 7029–7031 Vol. 74, No. 12
0019-9567/06/$08.00+0 doi:10.1128/IAI.00038-06

Mouse and Human Cell Activation by *N*-Dodecanoyl-DL-Homoserine Lactone, a *Chromobacterium violaceum* Autoinducer▽

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Received 9 January 2006/Returned for modification 5 April 2006/Accepted 5 September 2006

Chromobacterium violaceum **produces autoinducers, including homoserine lactones (HSLs), for genetic regulation. Among the seven HSLs derived from *C. violaceum* we evaluated, only C_{12}-HSL stimulated the production of inflammatory cytokines in mammalian monocytic cell lines through the activation of the NF-κB signaling pathway besides their quorum-sensing role, like 3-oxo-C_{12}-HSL from *Pseudomonas aeruginosa*.**

Many bacteria sense their population density through a sophisticated cell-to-cell communication mechanism, and gene expression or their own growth rate is altered so that they can adapt to the surrounding environment. This type of gene regulation is known as quorum sensing (1, 19). The quorum-sensing system depends on diffusible signal molecules, termed autoinducers, which enable bacteria to monitor their own population density. This system controls some kinds of bacterial behavior, such as bioluminescence (8), swarming (5), biofilm formation (4), and the production of virulence determinants (6, 11).

The quorum-sensing system is considered to play a role in the pathogenesis of chronic respiratory infections due to *Pseudomonas aeruginosa* in patients with a compromised lower respiratory tract or a compromised systemic defense mechanism. In *P. aeruginosa*, two acyl-homoserine lactone-based systems, the *las* and *rhl* systems, have been described (10, 12, 14). Apart from its quorum-sensing function, the *P. aeruginosa N*-3-oxododecanoyl-homoserine lactone (3-oxo-C_{12}-HSL) autoinducer has been suggested to modulate the immune responses of the infected host. In this context, it was demonstrated that 3-oxo-C_{12}-HSL induces the production of interleukin-8 (IL-8), cyclooxygenase 2, and prostaglandin E2 in human fibroblasts and that it accelerates apoptosis in mammalian macrophages and neutrophils (15, 16, 18).

Chromobacterium violaceum, a gram-negative rod commonly found in soil and water, is an opportunistic pathogen, like *P. aeruginosa* (2, 3, 13). Although this bacterium was reported to produce several kinds of acyl-homoserine lactones (AHLs), including C_4-, C_6-, C_7-, C_8-, C_{10}-, C_{12}-, and C_{14}-HSL, the involvement of HSLs in inflammatory host responses has not been evaluated (9, 17).

In this study, we examined the immunostimulatory function of *C. violaceum*-derived HSLs bearing a different carbon chain moiety and demonstrated that only C_{12}-HSL exerted biological activities in mammalian cells similar to those exerted by the autoinducer of *P. aeruginosa*, 3-oxo-C_{12}-HSL.

To examine the immunostimulatory function of *C. violaceum*, mouse macrophage-like RAW264.7 cells (kindly provided by K. Kawasaki and M. Nishijima, National Institute of Infectious Disease, Tokyo, Japan) were cultured in the presence of *N*-acyl HSLs bearing a different carbon chain moiety, i.e., C_4-, C_6-, C_7-, C_8-, C_{10}-, C_{12}-, and C_{14}-HSL derived from *C. violaceum* (Fluka, Buchs SG, Switzerland) (Fig. 1), dissolved in dimethyl sulfoxide, at 37°C for 6 or 24 h in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. The concentration of tumor necrosis factor alpha (TNF-α), a representative of cytokines yielded by activated macrophages, was determined in the supernatants by enzyme-linked immunosorbent assay (ELISA). As shown in Fig. 2A, only C_{12}-HSL stimulated TNF-α production by RAW264.7 cells compared with all other groups, but no other HSLs induced TNF-α production, regardless of the incubation period, compared with medium alone. Similar results were achieved with RAW264.7 cells stimulated with the acyl-HSLs at an equal molar concentration (100 μM) (data not shown). Production of IL-1β by RAW264.7 cells was significantly higher when cells were treated with C_{12}-HSL than with C_4-HSL or medium alone (Fig. 2B). Similar results were achieved regarding IL-8 production by THP-1 cells, a human monocytic leukemia cell line (obtained from the Health Science Research Resources Bank, Osaka, Japan) maintained in RPMI 1640 medium with 10% heat-inactivated fetal calf serum and preincubated with 0.1 μM 22-oxyacalcitriol, an analogue of 1_α,25-dihydroxy-vitamin D_3 (Chugai Pharmaceutical, Tokyo, Japan) for 72 h before stimulation with AHLs (Fig. 2C). These results suggest that only C_{12}-HSL of *C. violaceum* exerts immunostimulatory effects on mouse and human monocytic cells.

Following the cytokine analysis, we examined the activation of NF-κB, a key signaling molecule involved in inflammatory immune responses, using RAW/kB cells. These are stably transformed RAW264.7 cells that express luciferase in an NF-κB-dependent manner (7). RAW/kB cells were stimulated at 37°C for 6 h with C_4-, C_6-, C_7-, C_8-, C_{10}-, C_{12}-, and C_{14}-HSLs,

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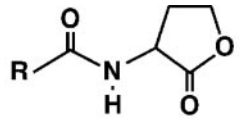
▽ Published ahead of print on 18 September 2006.



HSLs (*C. violaceum*)

R	Chemical name	Abbreviation
CH3CH2CH2	N-Butyryl-DL-HSL	BHL (C_4-HSL)
CH3(CH2)4	N-Hexanoyl-DL-HSL	HHL (C_6-HSL)
CH3(CH2)5	N-Heptanoyl-DL-HSL	HPHL (C_7-HSL)
CH3(CH2)6	N-Octanoyl-DL-HSL	OHL (C_8-HSL)
CH3(CH2)8	N-Decanoyl-DL-HSL	DHL (C_{10}-HSL)
CH3(CH2)10	N-Dodecanoyl-DL-HSL	dDHL (C_{12}-HSL)
CH3(CH2)12	N-Tetradecanoyl-DL-HSL	tDHL (C_{14}-HSL)

FIG. 1. Chemical structure of autoinducers derived from *C. violaceum*.

and luciferase activity was measured (Fig. 3). Incubation with C_{12}-HSL significantly increased luciferase expression, whereas incubation with other HSLs did not influence the reporter gene expression. Similar results were achieved with RAW/kB cells stimulated with the acyl-HSLs at an equal molar concentration (100 μM) (data not shown).

In this study, C_{12}-HSL derived from *C. violaceum* stimulated the production of TNF-α and IL-1β in mouse RAW264.7 cells. It also induced the activation of NF-κB. Moreover, it induced the production of IL-8 in human THP-1 cells. These findings showed that C_{12}-HSL induced the production of cytokines in mammalian monocytic cells. In this regard, several studies have demonstrated that 3-oxo-C_{12}-HSL derived from *P. aerugi-*

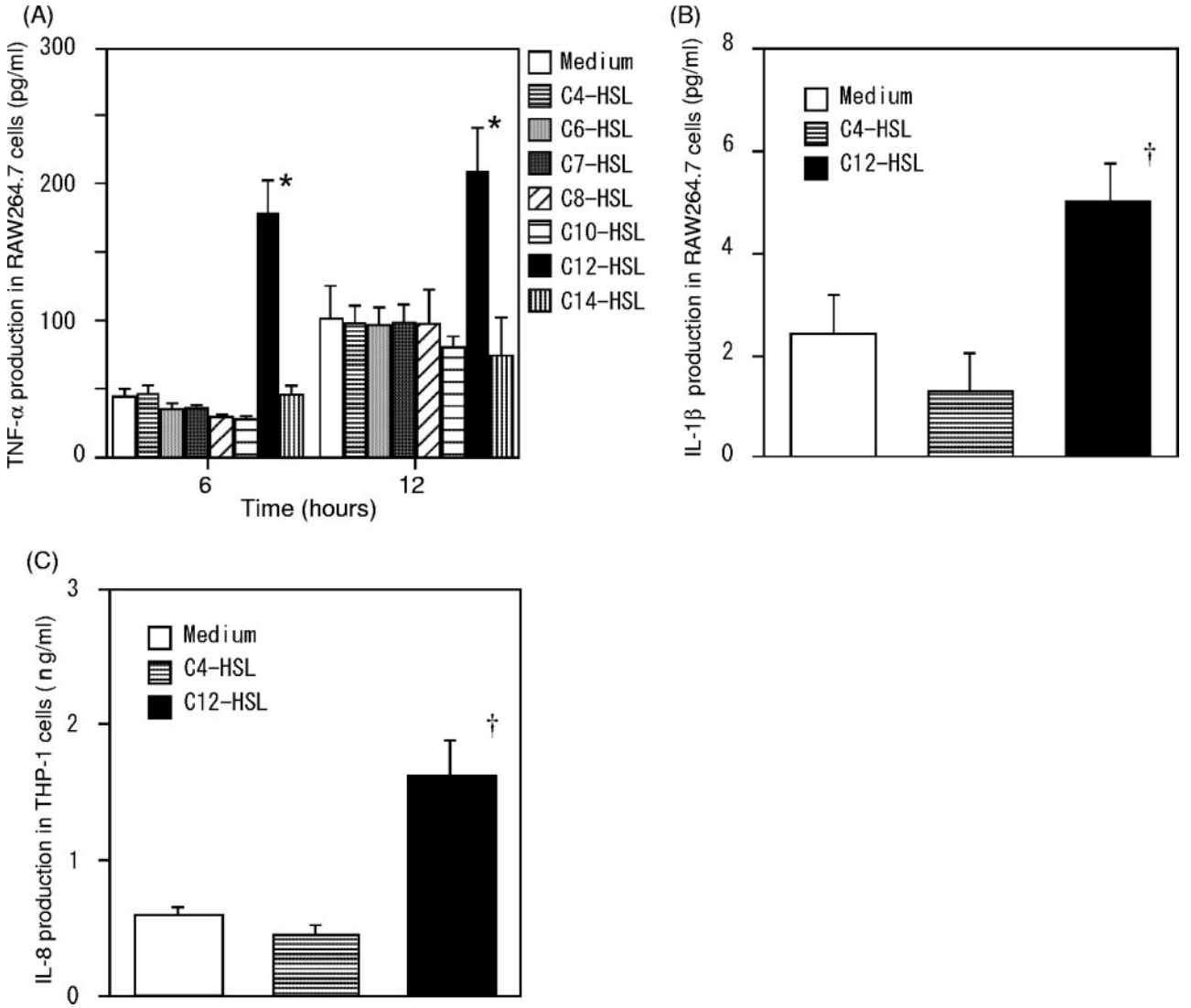


FIG. 2. C_{12}-HSL derived from *C. violaceum* induces TNF-α and IL-1β production by RAW264.7 cells and IL-8 production by THP-1 cells. (A) RAW264.7 cells (5×10^5 cells/500 μl of cell culture medium in a 24-well plate) (Corning) were stimulated with 10 μg/ml AHLs. After stimulation at 37°C for 6 or 24 h, the culture supernatant was collected, and the concentration of TNF-α was measured using an ELISA kit (Biosource). (B) The experiment was similar to that described in panel A, but only C_4-HSL and C_{12}-HSL were used, and IL-1β in the supernatants was measured. (C) THP-1 cells (1×10^5 cells/200 μl of cell culture medium in a 96-well plate) (Falcon) were cultured in the presence of C_4-HSL and C_{12}-HSL as described in panel B, and the level of IL-8 in the supernatant was determined using an ELISA kit (BD Pharmingen). In all panels, results represent the means ± standard errors ($n = 3$ to ~4 per data point); cells cultured in medium alone served as the control. A two-tailed Student *t* test was used for statistical comparison. *, $P < 0.001$ compared with all other groups; †, $P < 0.05$ compared with medium alone or C_4-HSL.

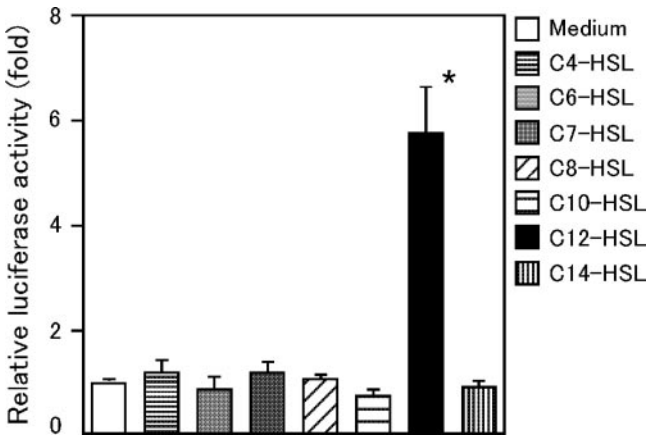


FIG. 3. C_{12}-HSL derived from *C. violaceum* activates NF-κB. RAW/kB cells (4×10^4 cells/100 μl of cell culture medium in a 96-well plate) (Corning) were stimulated with 10 μg/ml AHLs or 1 μg/ml lipopolysaccharide (positive control) (data not shown). After stimulation at 37°C for 6 h, the cells were lysed in 25 μl of 5× cell lysis reagent (Promega Corp.), and then luciferase activity was measured using 5 μl of the lysate and 25 μl of luciferase assay substrate (Promega Corp.). Luminescence was quantified with a luminometer (Berthold Japan, Tokyo, Japan). Luciferase activity was normalized to the activity in the cells cultured without AHLs (medium alone) and presented as relative induction (*n*-fold). Results represent the means ± standard errors ($n = 3$ per data point). A two-tailed Student *t* test was used for statistical comparison. *, $P < 0.001$ compared with all other groups.

nosa exerts biological activities in mammalian cells. Taken together, these findings suggest that the carbon chain moiety of HSLs is important for the recognition of the autoinducer by the host cells. On the other hand, C_8 acyl chain (octane), C_8-OH (1-octanol), C_{12} acyl chain (dodecan), and C_{12}-OH (1-dodecanol) (all chemicals were purchased from Sigma Aldrich) failed to activate NF-κB in the RAW/kB cells (data not shown). These results indicate that the acyl moiety alone is not sufficient to activate macrophages and that the whole structure of acyl-HSL is necessary for it to exert a biological activity. Furthermore, it is possible to infer that autoinducers derived from various bacteria are likely to exert biological activities in host cells. Moreover, by means of these autoinducers pathogenic microorganisms might alter the surrounding environment to survive and infect host tissues.

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Editor: J. L. Flynn