Research Article

Acinetobacter baumannii Lipopolysaccharide Influences Adipokine Expression in 3T3-L1 Adipocytes

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Received 19 January 2017; Revised 21 May 2017; Accepted 6 June 2017; Published 5 July 2017

Acinetobacter baumannii is one of the most important nosocomial opportunistic pathogen worldwide. In addition, obesity has been associated with an increased risk of nosocomial infection, suggesting that there may be an association between A. baumannii and white adipose tissue. However, the effects of A. baumannii on adipocytes have not been well studied at the molecular level. Here, we investigated the potential role of A. baumannii-derived lipopolysaccharides (LPS) as signaling molecules that affect adipocyte functionality. We tested the effect of increasing concentrations of A. baumannii-derived LPS (10, 100, or 1000 ng/mL) on the 3T3-L1 adipocyte cell line. Exposure to LPS was found to increase the expression of several adipokines (e.g., MIP-2, MCP-1, TNF-α, IL-6, lipocalin-2, and FABP4) in 3T3-L1 adipocytes and significantly reduced the expression of leptin and adiponectin. The effects of A. baumannii-derived LPS on MIP-2 expression were similar in comparison with that of LPS prepared from Pseudomonas aeruginosa and Escherichia coli in our cell culture-based system. This study suggests that A. baumannii-derived LPS functions as a signaling molecule that impacts the inflammatory function of white adipose tissue on the level of gene expression.

1. Introduction

Acinetobacter baumannii is a widespread gram-negative bacterium that primarily causes pulmonary, urinary tract, bloodstream, and surgical wound infections [1]. These bacteria easily and rapidly develop resistance to multiple antibiotics, and the presence of bacteremia in infected patients is associated with a poor prognosis [2]. Thus, A. baumannii is one of the most challenging pathogens for healthcare institutions worldwide [1–3]. Furthermore, A. baumannii is thought to possess only limited number of virulence factors and does not produce any known cytotoxins, suggesting that the pathogenetic mechanism of A. baumannii is poorly understood [4–6]. The opportunistic pathogen has lipopolysaccharides (LPS) in the outer membrane and may play an important role in the pathogenesis [4, 6].

Obesity is defined as excess adipose tissue. Adipose tissue function as key endocrine organs by releasing numerous proinflammatory and anti-inflammatory cytokines, as well as families of growth factors [7–9]. Thus, adipose tissue may represent an important site of inflammation. Recent studies have indicated that obesity is associated with an increased risk of nosocomial infections, such as a surgical wound, pulmonary, and urinary tract infections, and might contribute to an increase in future cases of sepsis [10, 11]. Furthermore, a more recent study focused on A. baumannii demonstrated that an altered nutritional status, resulting from hyperalimentation and obesity, could promote A. baumannii infection by reducing physiologic defense mechanisms [12]. These evidences suggest that A. baumannii and white adipose tissue may have a reciprocal relationship, and A. baumannii infection in obese patients may be associated with an increased risk of future sepsis.

The ability of LPS from several bacterial strains (i.e., Escherichia coli, Neisseria meningitidis, and Salmonella enterica serovar Minnesota) to induce systemic inflammation in response to a low-dose bacterial challenge has been well recognized [13–16]. In particular, A. baumannii-derived LPS...
(A. b-LPS) were reported to stimulate inflammatory signaling (e.g., interleukin-8, IL-8, and tumor necrosis factor, TNF-α) via human Toll-like receptors in human monocytic THP-1 cells [5]. These lines of evidence have raised the possibility that A. baumannii may have an influence on the physiological functionality of adipocytes, particularly in the synthesis and secretion of adipokines through LPS as a signaling molecule. Although we have previously demonstrated that A. b-derived LPS can influence the expression of several immunomodulatory genes in human neutrophils [17], the possible effects of A. b-LPS on adipocytes remain unknown.

Here, we examined the effects of A. b-derived LPS on adipokine expression in 3T3-L1 adipocytes in comparison with LPS isolated from other gram-negative bacteria, including pathogenic strains. Our results indicate that A. b-derived LPS affect adipocyte function, in particular, the upregulation of the chemotactant and the inflammatory cytokines and the alteration of adipokine expression. These results support the hypothesis that an A. baumannii infection in obese patients may be associated with an increased risk of future sepsis.

2. Methods

2.1. Reagents. Mouse 3T3-L1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Dulbecco’s modified Eagle’s medium (DMEM) with high glucose (11965-092), 1 mM sodium pyruvate, and 100 U/mL penicillin-100 μg/mL streptomycin were from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Fetal bovine serum (FBS) was obtained from Nissui Seiyaku (Tokyo, Japan). LPS from E. coli O111:B4 and LPS from E. coli O55:B5, 3-isobutyl-1-methylxanthine (IBMX), insulin, and dexamethasone were obtained from Sigma-Aldrich (St. Louis, MO). Mouse CXCL2/MIP-2 DuoSet ELISA was obtained from R&D Systems (Minneapolis, MN). All the reagents used were of the highest grade available from commercial sources.

2.2. Bacterial Strains. A. baumannii ATCC19606 and P. aeruginosa PAO1 were used as reference strains. The multidrug-resistant P. aeruginosa (MDR) strain and multidrug-resistant A. baumannii (MDRA) strain were clinical isolates from the Teikyo University hospital. These bacteria were grown for 16 h at 37°C on Luria-Bertani (LB) agar (Wako Pure Chemical Industries). A single colony of the bacteria on the LB agar was used for the large-scale culture of the bacteria in LB broth (Wako Pure Chemical Industries). After incubating with shaking at 37°C, the bacteria in the exponential phase was harvested by centrifugation, froze in liquid nitrogen, and stored at −20°C until use. In total, 50 L of the bacterial liquid culture was required for 5 g of the bacteria pellet. We contracted a company (Wako Pure Chemical Industries) to extract and purify LPS from these gram-negative bacteria using phenol and ultracentrifugation [17].

2.3. Cell Culture and Treatment. Unless otherwise specified, cell culture and further analyses were conducted at 37°C in an atmosphere comprising of 5% CO₂ and 95% air. 3T3-L1 cells were maintained as subconfluent cultures in DMEM, supplemented with 25 mM glucose, 10% FBS, 1 mM sodium pyruvate, and 100 U/mL penicillin-100 μg/mL streptomycin (medium A). These cells were seeded at a density of 4 × 10⁵ cells/well in 35 mm dishes and cultured for 3 days with 10 μg/mL insulin, 0.25 μg/mL dexamethasone, and 0.5 mM IBMX in medium A. The cells were further incubated for 5 days in medium A. The medium was changed every 2 days, as described previously [7]. The differentiated adipocytes thus prepared were characterized on day 8 by assessing the intracytoplasmic accumulation of lipid droplets; more than 80% of these adipocytes were stained positive with Oil red O [18]. These differentiated 3T3-L1 adipocytes were incubated for 0, 1, 24, 48, or 72 h in 35 mm dishes containing medium A with 100 ng/mL LPS. These cells were used for measuring adipokine expression levels, immunoblot analyses, and real-time quantitative PCR analyses, as described below.

2.4. RNA Preparation and Complementary DNA Synthesis. 3T3-L1 cells were treated with LPS for 1 h at 37°C. As control, saline (the solvent for the LPS) adjusted to a concentration of 0.1% was used. Total RNA of 3T3-L1 cells was extracted using the RNeasy mini kit (Qiagen, Dusseldorf, Germany). An Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) was used to determine the quantity and quality of the total RNA samples. Total RNA was reverse-transcribed to cDNA following the manufacturer’s instructions using the SuperScript® V10™ cDNA Synthesis Kit (Life Technologies). Briefly, 2 μg of total RNA was incubated with 4 μL of the 5 × V10 reaction mix and 2 μL of the SuperScript enzyme mix in a 20 μL reaction volume at 25°C for 10 min, followed by further incubation at 42°C for 120 min. The reaction was terminated by heating to 85°C for 5 min. The cDNA product was diluted to a final volume of 40 μL with deionized water and used for real-time quantitative PCR analysis.

2.5. Real-Time Quantitative PCR Analysis. Real-time quantitative PCR was performed to determine gene expression levels of target genes in mouse 3T3-L1 cells using ABI7300 real-time PCR System (Applied Biosystems; Foster City, CA). Real-time PCR reactions were performed in 10 μL reaction volumes containing the following components: 1 μL of the cDNA solution, 5 μL of the SYBR® Green PCR Master Mix (Applied Biosystems; Foster City, CA), 300 nM of each primer (real-time PCR primer set for adipose (mouse); Hokkaido System Science Co., Ltd.), and deionized water. The sequence of the forward primer for macrophage inflammatory protein-2 (MIP-2) was 5′-GCTGTCCCTCAACGGAA GAA-3′, and that of the reverse primer was 5′-CAGGATCG ATCCAGGGTTTCC-3′. The sequence of the forward primer for lipocalin-2 was 5′-CACACACGGACTACAAACAG-3′, and that of the reverse primer was 5′-CCTTCAGGTCAAGG GACAGC-3′. The sequence of the forward primer for TLR4 was 5′-CTCTGGGAGGGACATCTTC-3′, and that of the reverse primer was 5′-TCAGGTCCAAAGTGCGTTT-3′. The sequence of the forward primer for the endothelial nitric oxide synthase (eNOS) sequence was 5′-GGTTGCAAGGCT GCCAATT-3′, and that of the reverse primer was 5′-
TAACTACCACAGCCGAGGA-3’. The cDNA amplification conditions were 95°C for 2 min, 40 cycles at 95°C for 15 sec, 60 sec at the annealing temperature 60°C. The target gene expression levels were normalized to the internal control (β-actin). Relative expression levels were calculated using the formula $2^{-\delta \Delta Ct}$, where $\delta \Delta Ct = \Delta Ct\text{(target gene)} - \Delta Ct\text{(β-actin)}$; $\Delta Ct\text{(target gene)} = Ct\text{(target gene)} - Ct\text{(β-actin)}$ control condition and Ct is the cycle at which the threshold is crossed. RT-PCR analysis was performed in triplicate on a mean of at least three separate experiments.

2.6. MIP-2 Measurement. After treating the adipocytes with LPS, the culture medium was collected and centrifuged at 10,000g for 10 min at 4°C, and the cells were solubilized with 1.0 mL of 0.1 N NaOH to determine the protein concentration using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). The amounts of MIP-2, the rodent equivalent of human IL-8, secreted in the supernatant were determined using a sandwich ELISA kit, with recombinant mouse MIP-2 as the standard, according to the manufacturer’s instructions. Corrections to MIP-2 concentrations were made according to the protein content of the cells cultured in each well. The minimal detectable MIP-2 level in this assay was 15.6 pg/mL.

2.7. Statistical Analysis. The data was expressed as means ± SD. The program Prism 6 (GraphPad Software, Inc., San Diego, CA, USA) was used to perform the statistical analysis. Comparisons between groups were performed by one-way analysis of variance (ANOVA) and Dunnett posttest. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Exposure to A. b-LPS Increases the Expression of Chemoattractant and Inflammatory Cytokines in 3T3-L1 Adipocytes. To determine the effect of A. b-LPS on adipocyte functionality, on day 8, 3T3-L1 adipocytes were incubated for 1 h in the presence or absence of 100 ng/mL A. b-LPS. The expression levels of the chemoattractant (e.g., MIP-2 and monocyte chemotactic protein 1, MCP-1) and inflammatory cytokines (e.g., TNF-α and IL-6) increased at the mRNA level in adipocytes treated with A. b-LPS compared to the control cells without LPS treatment (Figure 1). Treatment of the adipocytes with 100 ng/mL A. b-LPS produced a 33-fold induction of MIP-2 and TNF-α, a 49-fold induction of MCP-1, and a 15-fold induction of IL-6. Treatment of the adipocytes with E. coli O111:B4 (O111-LPS) exhibited the same effect on inflammatory cytokine expression. Conversely, mRNA expression levels of TLR4 and eNOS were not affected by both A. b-LPS and O111-LPS.

3.2. Effect of A. b-LPS on Adipocyte Expression in 3T3-L1 Adipocytes. The expression of a large number of cytokines, such as lipocalin-2, FABP4, leptin, and adiponectin, are markedly induced during the differentiation of preadipocytes into adipocytes [19, 20]. Clinical and experimental studies indicate an important role of lipocalin-2, FABP4, and leptin...
as inflammatory adipokines associated with obesity and related complications [20–22]. Unlike several other adipokines, adiponectin has been associated with insulin-sensitizing and anti-inflammatory properties [20, 23]. Therefore, we next evaluated the effect of *A. baumannii*-LPS on the expression of these adipokines in 3T3-L1 adipocytes by incubating cells for 1 h and 24 h in the absence or presence of *A. baumannii*-LPS. After 24 h treatment with *A. baumannii*-LPS, a significant increase in lipocalin-2 and FABP4 was observed, whereas mRNA levels of leptin and adiponectin reduced in 3T3-L1 adipocytes (Figure 2). Furthermore, the expression of lipocalin-2, leptin, and adiponectin was not affected by *A. baumannii*-LPS for at least 1 h (data not shown).

### 3.3. MIP-2 mRNA Was Upregulated by *A. baumannii*-LPS in 3T3-L1 Adipocytes

IL-8 is the human equivalent of rodent MIP-2 and a potential chemoattractant for neutrophil infiltration into adipose tissue secreted by adipocytes in models of obesity [24–26]. Therefore, we focused on the alteration of MIP-2 expression by *A. baumannii*-derived LPS (*A. baumannii*-LPS) and stimulated differentiated adipocytes with various concentrations of *A. baumannii*-LPS (10, 100, or 1000 ng/mL) for 1 h. The level of MIP-2 mRNA in adipocytes was upregulated by *A. baumannii*-LPS in a dose-dependent manner, with a maximum increase (125-fold) at 1000 ng/mL, compared to the levels exhibited by the untreated cells (Figure 3(a)). Furthermore, the time course of MIP-2 expression induced by *A. baumannii*-LPS was compared with the LPS of O111-LPS. MIP-2 expression was maximum at the end of the 1 h time point and subsequently decreased. After 1 h, *A. baumannii*-LPS induced a 60-fold upregulation of MIP-2 expression, but at 3 h and 6 h, the expression level had decreased to 30 and 6.3-fold, respectively (Figure 3(b)). MIP-2 expression in 3T3-L1 adipocytes treated with 1000 ng/mL O111-LPS was upregulated by 125-fold, similar to that exhibited by *A. baumannii*-LPS (Figure 3(a)). Under these conditions, the LPS used for stimulation had no effect on cytotoxicity, as assessed by both Oil-Red-O staining (Figure 3(c)) and a WST-8 cell proliferation assay (data not shown).

### 3.4. Effect of *A. baumannii*-LPS on MIP-2 Secretion from 3T3-L1 Adipocytes

We next explored whether MIP-2 secretion increased in 3T3-L1 adipocytes stimulated with *A. baumannii*-LPS. The differentiated adipocytes were incubated for 72 h with or without *A. baumannii*-LPS (300, 1000, or 3000 ng/mL). A small amount of MIP-2 was secreted into the culture medium, with the basal level of 27.2 pg/mg of cellular MIP-2 protein. Moreover, MIP-2 secretion from 3T3-L1 adipocytes treated with 3000 ng/mL *A. baumannii*-LPS increased by 4.4-fold. Furthermore, incubation with 3000 ng/mL O111-LPS also increased MIP-2 expression by 4.4-fold (Figure 4(a)). Next, the time course of MIP-2 secretion by *A. baumannii*-LPS was compared with that of the untreated cells. At 24 h, MIP-2 secretion exhibited a 1.8-fold increase following treatment with 1000 ng/mL *A. baumannii*-LPS, and at both 48 h and 72 h, MIP-2 secretion was upregulated by 2.5-fold (Figure 4(b)). Together, these results indicate that *A. baumannii*-LPS and O111-LPS can enhance MIP-2 expression at the mRNA level, leading to MIP-2 secretion from the stimulated adipocytes.
3.5. Adipokine Expression following Stimulation with LPS Isolated from Nosocomial Opportunistic Pathogens. Finally, we tested the effect of six types of LPS isolated from *A. baumannii*, MDRA, *E. coli* O55:B5, *E. coli* O111:B4, *P. aeruginosa* PAO1, and MDRP on MIP-2 expression in 3T3-L1 adipocytes (Figures 5(a) and 5(b)). At the mRNA level, *A. baumannii*-LPS treatment for 1 h resulted in a 62-fold upregulation of MIP-2 expression compared with the control cells that did not receive LPS treatment. Stimulation with LPS derived from the other bacteria also significantly increased MIP-2 expression (68-fold for MDRA, 47-fold for *E. coli* O55:B5, 48-fold for *E. coli* O111:B4, 77-fold for PAO1, and 60-fold for MDRP; Figure 5(a)). ELISA studies revealed that the LPS prepared from these bacterial strains also increased the level of MIP-2 secretion from 3T3-L1 adipocytes by 4.4-fold for *A. baumannii*, 4.1-fold for MDRA, 4.6-fold for *E. coli* O55:B5, 4.4-fold for *E. coli* O111:B4, 4.2-fold for PAO1, and 4.1-fold for MDRP under the LPS treatment for 48 h (Figure 5(b)). A comparison of the induced MIP-2 expression between the six LPSs revealed no significant differences. Under these conditions, the LPS prepared from these bacteria exhibited no effect on cytotoxicity, as assessed by the WST-8 assay described above (data not shown).

4. Discussion

In the present study, we found that *A. baumannii*-LPS mediates the upregulation of chemokine expression in adipocytes, including MIP-2, MCP-1, TNF-α, and IL-6, at the mRNA level in 3T3-L1 adipocytes. Furthermore, *A. baumannii*-LPS reduces the expression of leptin and adiponectin in 3T3-L1 adipocytes. A previous report indicated that an altered nutritional status due to obesity could promote an *A. baumannii* infection by reducing physiologic defense mechanisms [12]. Thus, taken together with our results, these findings imply that *A. baumannii*-LPS may act as a direct signaling molecule that impacts the inflammatory function of adipose tissue.

Adipose tissue is an important source of TNF-α and other cytokines [19]. In adipose tissue, the adipocytes are surrounded by immune cells; every gram of adipose tissue contains 1-2 million adipocytes and 4-6 million stromal-vascular cells, of which more than half are leukocytes [27].
In addition, a number of studies demonstrate that neutrophil infiltration into adipose tissue precedes macrophage infiltration during a classical immune response [28, 29]. In this study, we demonstrated that A. b-LPS induce the upregulation of MIP-2, a strong chemoattractant of neutrophils, and MCP-1 in 3T3-L1 adipocytes (Figures 1 and 3). Furthermore,
we have previously demonstrated that A. b-LPS can influence the expression of several proinflammatory and immunomodulatory genes in human neutrophils [17]. Thus, we speculate that when the neutrophils and macrophages infiltrate into obese adipose tissue induced by the chemoattractant, the cells are also an important source of inflammatory cytokines. These inflammatory cytokines from adipose tissue may evoke systemic inflammation and a cytokine storm.

There is an in vitro study demonstrating a significant decrease in leptin mRNA in 3T3-L1 adipocytes stimulated by LPS [30], as similar to our results (Figure 2). On the other hand, there are in vivo studies demonstrating that the plasma level of leptin was elevated after the intraperitoneal injection of LPS into mice [31, 32]; these data seem to be inconsistent with the present interpretation in our cell-based system. In a different context, there are in vivo and in vitro evidences for the involvement of TNF-α in the induction of leptin by LPS [31]. A systemic inflammatory response induced by LPS may predominate over the LPS-dependent leptin regulation possibly occurring in the local area of inflammatory responses. Further studies are necessary to understand the complex relationship between the local cellular responses and the systemic responses of leptin to LPS.

A. baumannii has the propensity to accumulate mechanisms of antimicrobial resistance, leading to the rapid acquisition of pan-drug resistance. Because it is difficult to eradicate multidrug-resistant bacteria (e.g., MDRA and MDRP) at the site of infection, persistent infections caused by these gram-negative bacteria may develop following the chronic release of LPS. In our study, the LPS derived from MDRA and MDRP increased the expression of MIP-2 in 3T3-L1 adipocytes. Thus, our findings imply that the LPS derived from multidrug-resistant bacteria promote adipocyte inflammation.

Korneev et al. investigated that the structural variations in lipid A as an LPS structural component affect the ability of LPS from different bacteria to activate the innate immune response through TLR4 [33]. The ability of LPS isolated from A. baumannii 1053 (a clinical isolate from a patient with cystic fibrosis), to induce cytokine production (e.g., IL-6 and TNF), is similar to that of LPS isolated from E. coli O130 in murine bone marrow-derived macrophages [33]. In contrast, they demonstrated that the biological activity of LPS from the P. aeruginosa 2192 (a clinical isolate from a cystic fibrosis patient) did not induce measurable cytokine production [33]. The reasons for the difference in the effect of LPS are as follows: the impact of LPS steadily decreases with the number and the length of the acyl chains of lipid A. We previously demonstrated that gene expression levels in human neutrophils in response to LPS from drug-sensitive strains (A. baumannii or P. aeruginosa PAO-1) differ from that of LPS from their respective drug-resistant strains (MDRA and MDRP) [17]. Thus, we speculate that it is possible that these LPS subtypes contain different biochemical modifications and are structurally distinct. However, in the present study, the effects of A. b-LPS on the expression of MIP-2 were similar compared to those of LPS prepared from MDRA, MDRP, PAO1, and E. coli in our cell culture-based system (Figure 5); these results do not explain fully the development of antibiotic resistance or poor prognosis associated with A. baumannii infections as compared to other gram-negative bacteria. Further studies will be necessary to understand how even the smallest structural differences between very similar bacterial ligands may affect the activation of the inflammatory response in adipocytes. These studies may provide a mechanism to fine tune the inflammatory response of adipocytes and novel insight into the progression of obesity-associated systemic inflammation.

To our knowledge, this study is the first to investigate the biological activity of LPS derived from A. baumannii in adipocytes. A. b-LPS induces the upregulation of chemoattractants and inflammatory cytokines, including MIP-2, MCP-1, TNF-α, and IL-6, and also reduces the expression of leptin and adiponectin in 3T3-L1 adipocytes. These findings suggest that the LPS derived from A. baumannii may act as a direct signaling molecule that impacts the inflammatory function of adipose tissue and is associated with an increased risk of future septic events.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors’ Contributions

Yuka Unno designed and conducted the experiments, performed data analysis, and wrote the manuscript. Yoshinori Sato, Ryuichi Nakano, and Akiyo Nakano assisted in the experiments. Yoshinori Sato, Satoshi Nishida, Tsuneyuki Ubagai, and Yasuo Ono have supervised the exercise intervention. Yasuo Ono reviewed and edited the manuscript.

Acknowledgments

The authors are grateful to Ms. Chizuru Miyazaki (Teikyo University School of Medicine) for her excellent technical assistance. This work was supported by JSPS KAKENHI Grant nos. 25750048, 15K16233, and 24591490.

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