Research Article
Octanoylated Ghrelin Inhibits the Activation of the Palmitic Acid-Induced TLR4/NF-κB Signaling Pathway in THP-1 Macrophages

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Received 7 October 2012; Accepted 24 October 2012

Academic Editors: J.-B. Corcuff and C. Fürienn

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To investigate the effect of acylated ghrelin on the activation of TLR4/NF-κB signaling pathway induced by palmitic acid in human monocyte-derived (THP-1) macrophages, THP-1 macrophages were cultured for 12 h by palmitic acid with various concentrations. The THP-1 macrophages were pretreated by acylated ghrelin at different doses for 4 h before cultivated by palmitic acid (200 μmol/L) for 12 h. We observed the level of TLR4, NF-κB p65 phosphorylation in THP-1 macrophages and TNF-α, IL-1β in culture supernatant. TLR4 mRNA was measured by real-time PCR. TLR4 protein and NF-κB p65 phosphorylation was measured by western blotting. The expression of TNF-α and IL-1β was detected by ELISA. Compared to the THP-1 macrophages without palmitic acid, the level of the pervious substances increased after treatment by palmitic acid in a dose-dependent fashion (P<0.05). Compared to the THP-1 macrophages with palmitic acid (200 μmol/L), the level of the pervious substances decreased after preadministration by acylated ghrelin in a dose-dependent fashion. So, we make a conclusion that acylated ghrelin can regulate the activation of TLR4/NF-κB signaling pathway and inhibit the release of inflammatory cytokines in THP-1 macrophages which are stimulated by palmitic acid in a dose-dependent fashion.

1. Introduction

Obesity is a chronic disease that is associated with low-grade inflammation [1–3]. Obesity can induce changes in metabolism and gene expression in adipocytes, thereby enhancing lipolysis and releasing proinflammatory free fatty acids (FFAs) and cytokines (such as monocyte chemotactic protein-1 (MCP-1) and tumor necrosis factor-alpha (TNF-α)), which are able to recruit and activate macrophages to produce high levels of proinflammatory mediators (such as TNF-α, interleukin-1 beta (IL-1β), and resistin). These proinflammatory factors can promote an insulin-resistant state in adipocytes and form a positive feedback loop. The establishment of this positive feedback pathway can further amplify the inflammatory response and insulin resistance [4]. Obesity is related to metabolic inflammation, which is not only the basic pathophysiological mechanism for insulin resistance but is also critical in the occurrence of obesity-related vascular complications. Obesity has been shown to be associated with the activation of the innate immunity pathway and inflammatory response, as well as an impaired insulin signaling pathway and insulin resistance [5–10].

Toll-like receptor 4 (TLR4) could serve as a bridge that links innate immunity, lipid metabolism, and insulin resistance [11, 12]. Patients with overnutrition and obesity often have elevated levels of FFAs in their circulatory system because large quantities of saturated fatty acids are released by lipolysis from hypertrophied adipocytes due to the macrophages’ activities. These fatty acids serve as a natural endogenous ligand of TLR4, thereby activating downstream targets, such as the proinflammatory transcription factor nuclear factor-kappa B (NF-κB), to mediate an inflammatory response and insulin resistance. In addition, TLR4 can also activate the transformation from monocytes...
to macrophages, thereby forming foam cells that lead to the formation of atherosclerotic plaques. In these previously mentioned processes, the NF-κB pathway plays an important role in the TLR4-mediated regulation of immunity [4, 9, 11–15].

Palmitic acid, also known as alkyl [16] acid, is a saturated fatty acid. Palmitic acid acts as a natural dietary ligand for the activation of TLR4 signal transduction, which ultimately leads to the activation of NF-κB and activator protein-1 in macrophages and adipocytes and promotes the release of proinflammatory cytokines and chemokines. Ghrelin is a gastrointestinal peptide hormone that is secreted by gastric X/A-like endocrine cells. Octanoylated ghrelin is the activated form of ghrelin, which can bind with its endogenous ligand, the growth hormone secretagogue receptor (GHSR), and has multiple biological functions, including potential therapeutic effects on the cardiovascular system. In particular, ghrelin has been shown to have significant anti-inflammatory effects in both animal experiments and in vitro studies [17–22]. Therefore, we explored the effects of octanoylated ghrelin on the activation of the TLR4 signaling pathway and the secretion of inflammatory factors from palmitic acid-induced human monocyte-derived (THP-1) macrophages.

2. Materials and Methods

2.1. Cell Culture and Treatment. The THP-1 monocyctic cell line (Shanghai Institute of Biological Sciences) was used in this study. THP-1 monocyes were cultured in complete culture medium containing 100 nM phorbol-12-myristate-13-acetate (Sigma, USA) for 72 hours to induce the differentiation of THP-1 macrophages. After the medium was discarded, the macrophages were cultured in serum-free medium for 12 hours. Two separate experiments were then performed. In the first experiment, THP-1 macrophages were incubated with different concentrations (0 μmol/L, 100 μmol/L, 200 μmol/L, and 500 μmol/L) of palmitic acid and 10 ng/mL lipopolysaccharide (LPS) for 12 hours. The second experiment included the following five groups: a control group (medium containing fetal bovine serum); a 200 μmol/L palmitic acid-incubated group; a 1 ng/mL octanoylated ghrelin (AnaSpec) + 200 μmol/L palmitic acid intervention group; a 10 ng/mL octanoylated ghrelin + 200 μmol/L palmitic acid intervention group; a 100 ng/mL octanoylated ghrelin + 200 μmol/L palmitic acid intervention group. Different concentrations of octanoylated ghrelin were added to THP-1 macrophages in each of the groups, and the cells were incubated for 4 hours. Palmitic acid (200 μmol/L) was then added followed by 12 hours of incubation. The cells from all of the groups were collected for protein and mRNA extractions. The cell culture supernatants were also collected and stored in at –70°C prior to TNF-α and IL-1β analyses.

2.2. RT-PCR. Total RNA was extracted from the THP-1 cells using TRizol after the intervention. A UV protein and nucleic acid analyzer was used to determine the RNA concentration and purity, which was based on an OD260/OD280 ratio between 1.8 and 2.0. The 18S gene was used as an internal reference gene. The primers for human TLR4 included the sense primer 5′-AAGCGGAAAGTGATTGTG-3′ and the antisense primer 5′-CTGAGCAAGGTCTTCTCCAC-3′. The primers for the internal reference gene (18S) included the sense primer 5′-AGTCCCTGCCCCCTTTGTAACA-3′ and the antisense primer 5′-CGATCCAGGGCCTACTA-3′. A One Step SYBR PrimeScript RT-PCR Kit II (Takara) system was used according to the manufacturer’s instructions. The amplification and melting curves were used to calculate the Ct value of the TLR4 gene and the difference between the Ct values (ΔCt) of the TLR4 and 18S genes. In addition, the equation 2^−ΔΔCt was used to determine the relative amount of TLR4 mRNA.

2.3. Western Blotting. After the THP-1 macrophages were collected, they were washed twice with PBS. The cells were then lysed in a radioimmunoprecipitation assay buffer and phenylmethylene sulfonyl fluoride mixture at a 98:2 ratio to extract the proteins. The protein levels in each group were adjusted to the same level based on a bicinchoninic acid protein assay kit. For each group, 40 μg of the protein sample was mixed with sodium dodecyl sulfate (SDS) gel-loading buffer (20% w/v) and was boiled at 98°C for five minutes. β-actin was used as an internal reference. Following SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and protein transfer to polyvinylidene difluoride (PVDF) membranes, the membranes were blocked in a TBST (Tris-buffered saline/TWEEN-20) solution containing 10% nonfat dry milk for 1 hour. The primary antibodies for all of the proteins were proportionally diluted into working solutions with antibody diluents. The PVDF membranes were incubated in the primary antibody solution overnight at 4°C. Subsequently, the primary antibody solution was discarded. The membranes were rinsed three times in TBST for 15 minutes on a shaking table. The secondary antibody working solutions were prepared using the secondary antibody diluted in 5% nonfat milk at a ratio of 1:3000. The PVDF membranes
were incubated in the secondary antibody solution at room temperature for 1 hour and were subsequently rinsed three times (15 minutes each rinse). Enhanced chemiluminescence reagents were used for staining. The films were scanned thereafter and stored using Image Lab software. Image J software was used to determine the gray value of each protein band. The gray value ratios of the TLR4 and phosphorylated NF-κB bands over the internal reference β-actin band were used to quantify the expression of TLR4 and phosphorylated NF-κB.

2.4. Measurement of IL-1β and TNF-α by ELISA. The cytokine measurements were performed according to the manufacturer’s instructions for the ELISA kit (BD Biosciences, USA).

2.5. Statistical Analysis. Statistical comparisons were conducted using a one-way analysis of variance (ANOVA) and least significant difference test.

3. Results
The effects of palmitic acid on TLR4, intracellular NF-κB p65 phosphorylation, and the TNF-α and IL-1β levels in THP-1 macrophages are shown in Figures 1–3.
In our study, THP-1 macrophages were incubated with different concentrations of palmitic acid (100 μmol/L, 200 μmol/L, and 500 μmol/L) and 10 ng/mL LPS for 12 hours. The RT-PCR results showed that the TLR4 mRNA expression increased with increasing palmitic acid concentrations. These results were all statistically significant ($P < 0.05$) relative to the control group (Figure 1). The western blot analyses showed that palmitic acid increased the TLR4 and phosphorylated NF-κB p65 protein levels in THP-1 macrophages in a dose-dependent manner. These differences were all statistically significant ($P < 0.05$) relative to the control group (Figure 2). The ELISA results showed that palmitic acid increased the secretion of IL-1β and TNF-α by THP-1 macrophages in a dose-dependent manner. These results were also statistically significant ($P < 0.05$) relative to the control group (Figure 3).

We incubated THP-1 macrophages in different concentrations of octanoylated ghrelin (0 nmol/L, 1 nmol/L, 10 nmol/L, and 100 nmol/L) for 4 hours. Palmitic acid (200 μmol/L) was subsequently added prior to additional 12 hours of incubation. The RT-PCR results showed that relative to the 200 μmol/L palmitic acid-incubated group, the addition of octanoylated ghrelin caused a decrease in the TLR4 mRNA expression in a dose-dependent manner ($P < 0.05$) (Figure 4). The western blot analyses showed that octanoylated ghrelin amendment led to decreases in TLR4 protein levels and NF-κB p65 phosphorylation in THP-1 macrophages in a dose-dependent manner ($P < 0.05$) relative to the 200 μmol/L palmitic acid-incubated group (Figure 5). The ELISA results showed that octanoylated ghrelin caused a decrease in the secretion of IL-1β and TNF-α by THP-1 macrophages in a dose-dependent manner ($P < 0.05$) relative to the 200 μmol/L palmitic acid-incubated group (Figure 6).

4. Discussion

We chose to incubate THP-1 macrophages with palmitic acid, which is a type of saturated fatty acid. The results showed that the concentrations of IL-1β and TNF-α in the supernatants of the cultured cells significantly increased with increasing palmitic acid concentrations. This observation suggested that palmitic acid could have a proinflammatory effect in a dose-dependent manner.
TLR4 is a member of the TLRs that have a natural pattern recognition. The function of TLR4 is to mediate transmembrane signaling transduction in which TLR4 could serve as a bridge that links innate immunity, lipid metabolism, insulin resistance, and vascular inflammation. TLR4 widely recognizes specific pathogen-associated molecular patterns, such as the LPS layer of gram-negative bacilli and FFAs that are secreted by adipocytes, and couples signal transduction pathways to activate innate immune cells and inflammatory cells, which results in a series of immune and inflammatory responses and leads to the synthesis and release of cytokines and inflammatory mediators. TLR4 induces a high level of expression of target genes with proinflammatory abilities by regulating the activity of signaling-dependent transcription factors (such as NF-κB). The NF-κB pathway plays an important role in TLR4-mediated immune regulation [23].

In TLR4-deficient mice, this vascular proinflammatory gene cannot be expressed, regardless of the extent of obesity, dyslipidemia, or high fat intake [24]. Increased plasma levels of palmitic acid can serve as the natural endogenous ligand of TLR4 that results in an abnormal TLR4 gene expression and TLR/NF-κB signal transduction and increases in the gene expression of the inflammatory cytokine IL-6 and SOD2 (mitochondrial superoxide dismutase), which could be one of the pathological mechanisms of insulin resistance [25]. Our study confirmed that palmitic acid could induce the activation of the TLR4/NF-κB signaling pathway in THP-1 macrophages in a dose-dependent manner, thereby establishing a cellular model of metabolic inflammation.

Ghrelin is the endogenous ligand of GHSR and is a 28-amino acid peptide. The two forms of ghrelin that are found in the body are n-octanoyl ghrelin, which is octanoylated...
with an n-octanoyl group on Ser3, and des-acyl ghrelin. The former plays important roles in biological activities. The octanoylated form is the ligand of GHSR-1 and is able to pass through the blood-brain barrier to bind with GHSR-1α in the central nervous system to exert its endocrine function. Ghrelin is mainly secreted by gastric X/A-like endocrine cells. A small amount of ghrelin secretion is also observed in the intestines, pancreas, kidneys, immune system, placenta, testis, pituitary, and hypothalamus. The receptor of ghrelin, GHSR, is a G protein-coupled receptor that exists as two types, that is, GHSR-1α and GHSR-1b. Most of the physiological effects of ghrelin are mediated by GHSR-1α. GHSR-1α mRNA expression mainly occurs in the hypothalamus and pituitary gland but also occurs in the peripheral tissues, such as the gastrointestinal tract, thyroid, pancreas, myocardium, and kidneys [26, 27]. After binding with its receptors, octanoylated ghrelin has many biological effects, including the promotion of the release of growth hormone, increasing food intake, the regulation of lipid metabolism, and the inhibition of inflammatory cytokine secretion. Ghrelin can relax blood vessels to lower blood pressure, reduce myocyte apoptosis, protect endothelial cells, improve weakened cardiac function, and inhibit the stress reaction of the endoplasmic reticulum; ghrelin also potentially has protective effects on the cardiovascular system [28, 29]. The levels of plasma ghrelin are negatively correlated with body weight, fasting insulin levels, and insulin resistance (HOMA-IR) and are positively correlated with the insulin sensitivity index (ISI). These relationships suggest that the reduced ghrelin levels in patients with obesity may lead to insulin resistance. However, the mechanism causing the negative correlation between plasma ghrelin and insulin resistance has not been elucidated [30, 31].

Ghrelin plays an important role in the inflammatory response, such as the inhibition of the release of inflammatory cytokines. A recent study [18] suggested that ghrelin showed strong anti-inflammatory effects by regulating the secretion of macrophage proinflammatory cytokines, such as IL-1β and TNF-α, and the anti-inflammatory cytokine IL-10 after LPS stimulation through the NF-κB and P38 mitogen-activated protein kinase (MAPK) signaling pathways. In lymphocytes and monocytes, ghrelin can also specifically inhibit the synthesis of leptin and LPS-induced proinflammatory cytokines, such as IL-1, IL-6, and TNF-α [19]. An in vitro experiment showed that ghrelin could inhibit the secretion of proinflammatory cytokines from human endothelial cells, the adhesion of monocytes, and the activity of NF-κB. The intravenous administration of ghrelin can also inhibit the in vivo synthesis of endotoxin-induced proinflammatory cytokines in rats [20]. Recently, an additional study showed that ghrelin could increase NF-κB activity in B-lymphocytes [32]. Our previous study showed that the plasma from obese patients could promote the release of inflammatory factors through activation of the TLR4/NF-κB pathway [33]. Newgard et al. [34] found that the ghrelin levels of obese patients were significantly lower than those of control individuals (P < 0.0001). Our study demonstrated that ghrelin could inhibit the release of inflammatory cytokines.
via the inhibition of palmitic acid-induced activation of the TLR4/NF-κB signaling pathway in THP-1 macrophages in a dose-dependent manner.

The activation of the TLR4/NF-κB signaling pathway can induce and aggravate insulin resistance, which leads to obesity and related metabolic disorders. Ghrelin can have an anti-inflammatory effect by inhibiting the TLR4/NF-κB pathway's activity. Therefore, The anti-inflammatory effect of ghrelin might be used to explore new drugs to prevent and therapy obesity, diabetes, and other metabolic inflammatory diseases.

**Conflict of Interests**
The authors declare that they have no conflict of interests.

**Acknowledgment**
This work was supported by grants from the Hunan Provincial Science and Technology Plan Foundation of China (no. 2011FJ3019).

**References**


