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Original Research

Ghrelin inhibits oxLDL-induced inflammation in RAW264.7 mouse macrophages through down-regulation of LOX-1 expression via NF-κB signaling pathway

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Abstract: Oxidized low-density lipoprotein (oxLDL) is one of the many causes of the initiation and progression of atherosclerosis, which can subsequently promote the uptake of oxLDL by macrophages and lead to inflammation in the blood vessels. In the present study, we evaluated the protective effects of ghrelin on oxLDL-induced RAW264.7 mouse macrophages. Ghrelin was able to inhibit the release of several pro-inflammatory cytokines including tumor necrosis factor (TNF)-α and interleukin (IL)-6. In addition, ghrelin also inhibited the expression of Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in oxLDL treated macrophages. Furthermore, we demonstrated that ghrelin could inhibit the expression of p-IκBα, and the inhibitory effects could be blocked by BAY 117082. Taken together, ghrelin possesses anti-inflammatory effects on oxLDL-induced inflammation in macrophages, suggesting that it can prevent or treat atherosclerosis, and deserves to be further studied and developed to be potent drug for treating atherosclerosis.

Key words: Ghrelin, atherosclerosis, inflammation, oxLDL, LOX-1.

Introduction

Atherosclerosis is a major threat to human health and life quality, which can cause cardiovascular diseases through passive accumulation of cholesterol in the artery wall. The accumulation of oxidized low-density lipoprotein (oxLDL) is one of the many causes of the initiation and progression of atherosclerosis, which can subsequently promote the uptake of oxLDL by macrophages and lead to foam cell formation (1, 2). Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is one of the well-known receptors responsible of the uptake of oxLDL. To date, LOX-1 has been found to be expressed on the membrane of many cell types, including endothelial cells (3), macrophages (4) and vascular smooth muscle cells (5). A number of evidences also suggest that LOX-1 is also involved in the process of atherosclerosis (6, 7).

Chronic inflammation is observed throughout atherosclerosis, and this chronic inflammatory process starts at the early stage of atherosclerosis (8). The oxidative modification of circulating LDLs is an important early process of atherosclerosis. The oxLDL particles bind to LOX-1 on macrophages leading to secretion of pro-inflammatory cytokines and chemokines, such as TNF-α and IL-6 (9). These pro-inflammatory molecules, in turn, activate and stimulate the expression of LOX-1 in macrophages, further enhancing the take-up of oxLDL (10).

Ghrelin is a polypeptide secreted by X/A-like enteroendocrine cells from the stomach (11). To date, this polypeptide is found to be able to modulate many physiological activities, including food intake, energy balance, growth hormone release and long-term regulation of body weight in humans (12). Modern pharmacological researches show that ghrelin known as the circulating orexigen exerts antagonistic activities on the leptin-induced reduction in food intake via activation of the hypothalamic neuropeptide Y-Y1 pathway (11, 12). Further study indicates that the effects of ghrelin are mainly medicated through one of G protein-coupled receptor named growth hormone secretagogue receptor (GHS-R) (13). The wide distribution of GHS-R in the tissues suggests that ghrelin possesses multifunctional effects on the process of human health. Recent evidences also demonstrate that ghrelin shows immunomodulative effects on human monocytes and T cells (14). The main purpose of our present study is to evaluate the effects of ghrelin on oxLDL-induced inflammation in RAW264.7 mouse macrophages and the underlying mechanisms.

Materials and Methods

The experimental protocols in the current study were approved by the Ethics Review Committee of the Laboratory Centre of General Hospital of Tianjin Medical University.

Chemicals and reagents

Rat ghrelin with the same peptide sequence as of mouse ghrelin was purchased from AnaSpec, San Jose, CA, USA. Unless otherwise stated, other chemicals and reagents in the current study were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

The murine macrophage cell line RAW 264.7 was obtained from the Institute of Biochemistry and Cell Biology (Shanghai Institute for Biological Science, the Chinese

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Academy of Science, Shanghai, China) and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies, CA, USA) supplemented with 10% fetal bovine serum and 1% of penicillin and streptomycin (Gibco, Life technologies). The cells were incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

Lipoprotein preparation

Total LDL was isolated from the plasma of normal healthy volunteers using discontinuous ultracentrifugation as described previously (15). The extracted LDL was then diluted to a concentration of 1 mg/ml with PBS and dialysed thrice to remove residual EDTA. OxLDL was preduced by incubating LDL with CuSO4 solution (0.5 μM, freshly prepared) in PBS for 24 h in a shaking water bath with the temperature at 37 °C. The degree of oxidation of oxLDL was examined in an agarose gel with increased mobility using native LDL as control. Protein concentration was assessed using a commercial BCA protein assay kit (Pierce Chemical Company, Rockford, IL, USA). The produced oxLDL was endotoxin free, tested by endotoxin enzyme-linked immunosorbent assay (ELISA) kit (Lonza, Swiss).

Cell viability assay

Cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. In brief, RAW 264.7 cells were plated in 96-well plates with a density of 3×10^4 cells/ml and cultured for 12 h. The cells were then incubated with different concentrations of oxLDL (25, 50, 100, and 200 μg/ml), ghrelin (1, 10, and 100 nM) with or without oxLDL (50 μg/ml). After treatment for 48 h, 30 μl of MTT (5 mg/ml) solution was added into each well and further incubated for 4 h. After replacing the culture medium with 100 μl dimethyl sulfoxide (DMSO), the optical density (OD) was measured at a wavelength of 570 nm by using a microplate reader (TECAN, Austria).

Quantitative real-time polymerase chain reaction (qPCR)

After treatment with ghrelin, the cells were collected in centrifuge tubes, and the total RNA was extracted with TRIzol® reagent (Life Technologies, NY, USA), following the manufacturer's instruction. For each sample, 1 μg of RNA was used for the reverse transcription reaction using Oligo dT (18T) (Omega, NY, USA). The cDNA products were amplified for *LOX-1*, *TNF-α*, and *IL-6* gene expression via qPCR using specific primers as shown in Table 1. PCR was carried out in triplicate with SYBR Green PCR Master Mix by using a 7900HT qPCR system thermal cycler (Applied Biosystems, CA). In this study, 18s mRNA was used as internal control for each sample. Results were obtained from three independent experiments. The Ct values for each sample were normalized to 18s mRNA.

Measurement of TNF-α and IL-6 levels

After treatment with ghrelin and oxLDL, the supernatants of each well were collected. The concentrations of inflammatory cytokines TNF-α and IL-6 in the supernatants were determined using ELISA according to the manufacturer's instructions.

Western blot assay

After treatment, protein samples were extracted using RIPA solution (Sigma, USA), and the concentrations of protein samples were examined by BCA assay kits according to the manufacturers' instruction. Protein samples were separated on 10% v/v sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA). The membranes were then immunoblotted with primary anti-LOX-1 (1:1000, Abcam, China), anti-IκBα, anti-p-IκBα (1:1000, Santa Cruz Biotechnology, China) or anti-β-actin (1: 3000, Cell Signaling Technology, USA) for over night at 4°C. The membranes were then incubated with respective secondary antibodies for 1h at room temperature. Finally, protein were visualized by enhanced chemiluminescence substrate (Thermo, USA).

Neutralized assay

The cells were pre-treated with phosphate-buffered saline (PBS), IgG or anti-LOX-1 antibody (5 μg/ml) for 1 h, and then treated with ghrelin and oxLDL as previous stated. The supernatants were also collected for the determination of TNF-α and IL-6 levels using ELISA assay as previous stated.

Blocking assay

The cells were pre-treated with PBS, DMSO or BAY11-7082 (5 μM) for 1 h, and then treated with ghrelin and oxLDL as previous stated. The supernatants were also collected for the determination of TNF-α and IL-6 levels using ELISA assay as previous stated.

Statistical analysis

All data were expressed as the mean ± standard deviation (SD) from three indepedent tests. One-way ANOVA followed by *Dunnett tests* was performed for statistical analyses by using GraphPad PRISM software version 5.0 (GraphPad Software, USA), and $p < 0.05$ was considered statistically significant.

Results

Ghrelin showed no cytotoxicity in oxLDL-treated RAW 264.7 cells

As shown in Figure 1A, oxLDL treatment at 25 and 50 μg/ml did not show any significant cell toxicity in RAW264.7 cells. However, the numbers of RAW264.7

Table 1. The sequences of primers used for real-time quantitative PCR.

Gene	Forward	Reverse
LOX-1	5'-GAGCTGCAAACTTTTCAGG-3'	5'-GTCTTTCATGCAGCAACAG- 3'
TNF-α	5'-GGCTGCCCCGACTACGT-3'	5'-TTTCTCCTGGTATGAGATAGCAAATC-3'
IL-6	5'-GGGAAATCGTGGAAATGAGAAA-3'	5'-AAGTGCATCATCGTTGTTCATACA-3'
18s	5'-GCAATTATTCCCCATGAACG-3'	5'-GGCCTCACTAAACCATCCAA-3'

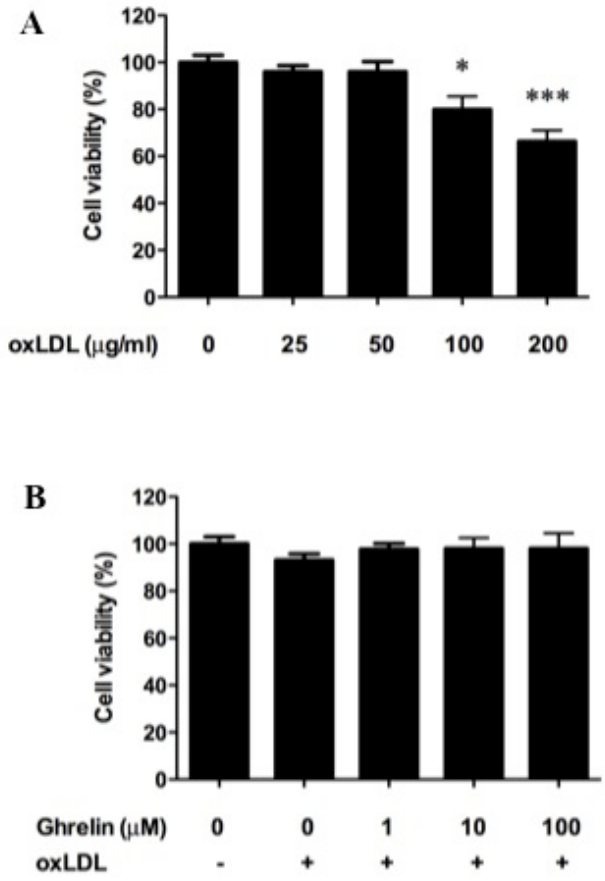

Figure 1. Effects of ghrelin on cell viability of mouse macrophage RAW 264.7 cells. A, RAW264.7 cells were exposed to oxLDL (25, 50, 100, and 200 microg/ml) for 48 h. B, RAW264.7 cells were incubated with ghrelin (1, 10, and 100 nM) with or without oxLDL (50 μg/ml) for 48 h. Data are the means (SD; error bars) from three independent experiments. * $P < 0.05$, *** $P < 0.001$ versus vehicle control.

cells were remarkably decreased after treatment with oxLDL at 100 and 200 μg/ml, with inhibition rate of 20% and 31%, respectively. Therefore, in our following experiments, we chose 50 μg/ml as the working dose for oxLDL. However, as shown in Figure 1B, the cell viability of oxLDL-treated RAW 264.7 cells were not affected by ghrelin treatment with 1, 10 and 100 nM for 48 h.

Ghrelin inhibited inflammatory effects in oxLDL-induced RAW264.7 Cells

Chronic inflammation is observed throughout atherosclerosis, and this chronic inflammatory process starts at the early stage of atherosclerosis. As shown in Figure 2A and 2B, exposure of oxLDL induced markedly upregulation of inflammatory responses in terms of mRNA expression levels of both TNF-α and IL-6 by qPCR analysis. However, ghrelin treatment could significantly decreased oxLDL induced mRNA expression levels of TNF-α and IL-6 in a dose-dependant manner. This agreed with the observed suppression of TNF-α and IL-6 protein expression, as shown in Figure 2C and 2D.

Ghrelin suppressed oxLDL-induced LOX-1 expression in RAW264.7 cells

LOX-1 is one of the scavenger receptors for regulating the uptake of the oxidative LDL in macrophages.

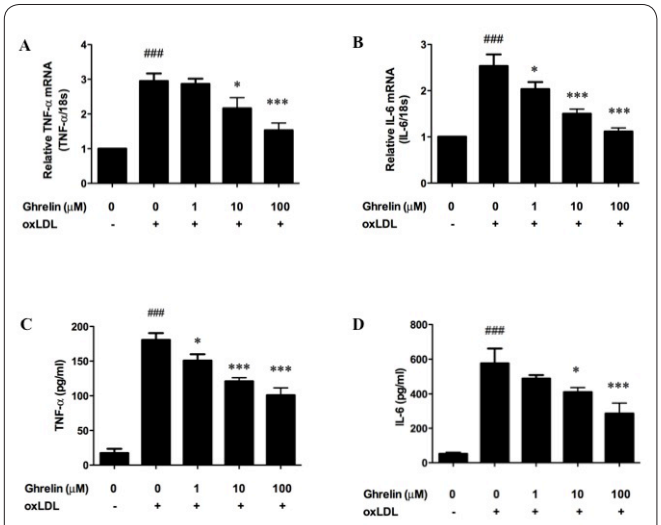

Figure 2. Anti-inflammatory effects of ghrelin on oxLDL-induced inflammation in RAW 264.7 cells. The effects of ghrelin on the mRNA levels of TNF-α (A) and IL-6 (B) in oxLDL-induced inflammation were evaluated by qPCR. The effects of ghrelin on the protein levels of TNF-α (C) and IL-6 (D) in oxLDL-induced inflammation were evaluated by ELISA. Data are the means (SD; error bars) from three independent experiments. ### $P < 0.001$ versus vehicle control; * $P < 0.05$, *** $P < 0.001$ versus oxLDL only treatment group.

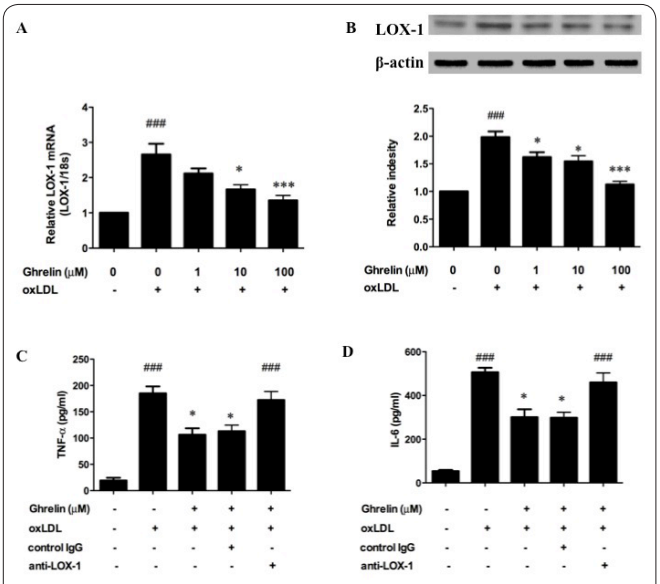

Figure 3. Ghrelin suppressed oxLDL-induced LOX-1 expression in RAW264.7 cells. A, The mRNA levels of LOX-1 were detected by qRT-PCR after ghrelin treatment in oxLDL-induced RAW264.7 cells. B, the protein levels of LOX-1 were detected by western blot after ghrelin treatment in oxLDL-induced RAW264.7 cells. The effects of LOX-1 antibody on the inhibitory activity of ghrelin of TNF-α (C) and IL-6 (D) expression in oxLDL-induced inflammation were evaluated by ELISA. Data are the means (SD; error bars) from three independent experiments. ### $P < 0.001$ versus vehicle control; * $P < 0.05$, *** $P < 0.001$ versus oxLDL only treatment group.

Incubation of RAW264.7 cells with oxLDL resulted in significantly upregulation of LOX-1 expression at both mRNA and protein levels, which was markedly inhibited by co-treatment with ghrelin (1 – 100 nM) in a concentration dependent manner (Figure 3A and 3B). To further confirm whether ghrelin mediate anti-inflammatory effects in oxLDL-induced inflammation via LOX-1 receptor, we used an anti-LOX-1 antibody for the neu-

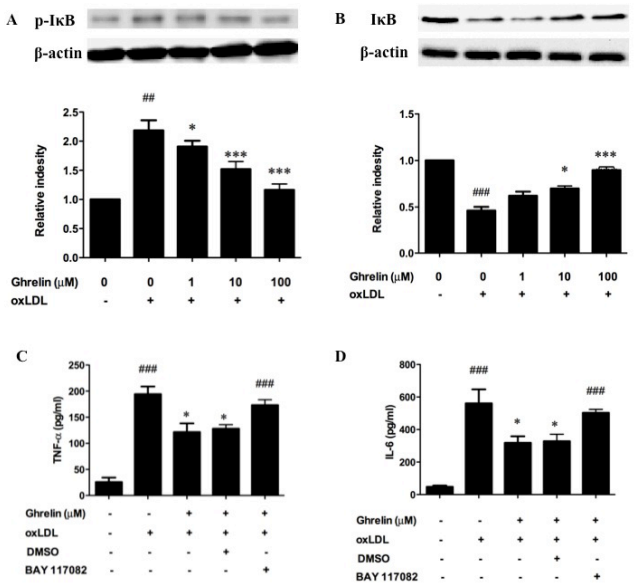

Figure 4. Ghrelin exerted its effects through NF-κB signaling pathway in oxLDL-treated RAW264.7 macrophages. The protein levels of IκB phosphorylation (A) and degradation (B) were detected by western blot after ghrelin treatment in oxLDL-induced RAW264.7 cells. The effects of BAY 117082 on the inhibitory activity of ghrelin of TNF-α (C) and IL-6 (D) expression in oxLDL-induced inflammation were evaluated by ELISA. Data are the means (SD; error bars) from three independent experiments. ### $P < 0.01$, ### $P < 0.001$ versus vehicle control; * $P < 0.05$, *** $P < 0.001$ versus oxLDL only treatment group.

tralized experiments. Our results showed that pretreatment with anti-LOX-1 antibody could almost recover the inhibitory effects of ghrelin-induced downregulation of TNF-α and IL-6 expression in oxLDL treated macrophages (Figure 3C and 3D).

Ghrelin exerted its effects through NF-κB signaling pathway in oxLDL-treated RAW264.7 macrophages

To determine the intracellular mechanism regarding with the anti-inflammatory activity of ghrelin, we explored the role of transcription factor NF-κB in the regulatory process. In our current study, cytoplasmic protein of IκBα was determined by western blot assay. As shown in Figure 4A, challenging with oxLDL significantly increased IκBα phosphorylation and degradation in RAW264.7 cells. While, ghrelin (1 – 100 nM) suppressed the increased expression of IκBα phosphorylation (Figure 4A) and degradation (Figure 4B). In addition, BAY 117082, a NF-κB pathway blocker, could markedly recover the inhibitory effects by ghrelin exposure in oxLDL-induced TNF-α (Figure 4C) and IL-6 (Figure 4D) expression.

Discussion

The main aim of the present study was to investigate whether ghrelin could protect RAW264.7 macrophages against oxLDL-induced inflammation. In addition, the underlying mechanisms of this anti-inflammatory effect were also explored. The results showed that ghrelin was able to inhibit the release of several pro-inflammatory cytokines including TNF-α and IL-6. In addition, ghrelin also inhibited the expression of Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in oxLDL treated macrophages. Furthermore, we also demonstrated that ghrelin could also inhibit the phosphorylation of IκBα, and the inhibitory effects could be blocked by BAY 117082.

LDL is sometimes referred to as “bad” cholesterol, which is normally found in tissues such as the intima of arteries. After modification, LDL can bind to the proteoglycan matrix, which is believed to be an early event during atherogenesis according to the “response to retention” hypothesis (16). Generally, native LDLs are internalized by macrophages and cause a small portion of foam cell formation. However, the oxidative LDL induces up-regulation of its uptake by macrophages via scavenger receptors including LOX-1, cluster of differentiation (CD) 36, and scavenger receptor (17). Uptake of oxLDL subsequently leads to intracellular cholesteral accumulation and foam cells formation, and induces inflammatory microenvironment, thereby promoting atherogenesis. In the present study, we demonstrated that LOX-1 expression was significantly increased after oxLDL stimulation, and ghrelin could remarkably reverse the up-regulatory effects.

Chronic inflammation is observed throughout atherosclerosis, and this chronic inflammatory process starts at the early stage of atherosclerosis (8). The oxidative modification of circulating LDLs is an important early process of atherosclerosis. The oxLDL particles bind to LOX-1 on macrophages leading to secretion of pro-inflammatory cytokines and chemokines, such as TNF-α and IL-6 (9). These pro-inflammatory molecules, in turn, activate and stimulate the expression of LOX-1 in macrophages, further enhancing the take-up of oxLDL (10). In the present study, oxLDL stimulation significantly up-regulated the release of TNF-α and IL-6, which could significantly reversed by ghrelin treatment. These data suggested that ghrelin could be an effective agent for treating atherogenesis through the anti-inflammatory pathway. In our current results (Figure 2), we noticed that ghrelin at 1 μM showed different effects on transcription (mRNA levels) and translation (protein levels). These might were due to the lower rate of mRNA transcription compared to protein translation in mammalian cells.

The NF-κB signaling pathway plays a crucial role in the ability of cells to integrate external molecules and induce the appropriate responses. As a downstream factor of LOX-1, NF-κB also involved in several inflammatory diseases including atherosclerosis. Activation of NF-κB signals were found in the whole precess of atherosclerosis beginning from plaque formation to its destabilization and rupture (18, 19). In the current study, we demonstrated the phosphorylation of IκBα were remarkably increased by oxLDL treatment (Figure 4A), indicating the NF-κB pathway was activated in oxLDL-stimulated macrophages. The data also showed that Ghrelin herein dose-dependantly inhibit the up-regulation of p-IκBα in oxLDL-treated RAW 264.7 cells. To further elucidate the important role of activation of NF-κB signaling, BAY 117082, a NF-κB pathway blocker, was used to suppress the activation of NF-κB pathway in oxLDL-stimulated macrophages. As shown in Figure 4C and 4D, pre-treatment with BAY 117082 significantly recovered the inhibitory effects on pro-inflammatory cytokines (TNF-α and IL-6) release caused by ghrelin

treatment. All these data suggested that NF-κB signaling pathway played a crucial role in the anti-inflammatory activities of ghrelin in oxLDL-induced inflammation.

In conclusion, our present study suggested that treatment with ghrelin significantly attenuated oxLDL-induced proinflammatory cytokines TNF-α and IL-6 expression in macrophages. In addition, ghrelin treatment also remarkably decreased IκB phosphorylation in oxLDL-stimulated macrophages. Moreover, ghrelin treatment significantly inhibited the expression of LOX-1 in oxLDL-activated RAW cells, and the inhibitory effects could be challenged by anti-LOX-1 antibody. Our findings pointed to a novel mechanism for the anti-atherosclerotic effect of ghrelin, indicating that ghrelin might be a potent candidate for treating human atherosclerosis.

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