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Ghrelin and obestatin modulate early atherogenic processes on cells: enhancement of monocyte adhesion and oxidized low-density lipoprotein binding

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Abstract

Emerging evidence indicates the potential involvement of ghrelin, an endogenous ligand for the growth hormone secretagogue receptor, in low-grade inflammatory diseases such as obesity and atherosclerosis. The goal of the present study was to use cell culture models to investigate the influences of ghrelin and obestatin in processes participating in atherogenesis. We studied monocyte adhesion, monocyte chemoattractant protein–1, and adhesion molecule expression on endothelial cells as well as binding of oxidized low-density lipoprotein (LDL) and acetylated LDL to macrophages. Ghrelin treatment increased adhesion of calcein-labeled THP-1 monocytes to EA.hy 926 endothelial cells. Simultaneously, ghrelin increased the expression of intercellular adhesion molecule–1 measured by quantitative reverse transcriptase polymerase chain reaction. Tumor necrosis factor–α stimulation together with ghrelin treatment decreased both monocyte adhesion and vascular cell adhesion molecule–1 and monocyte chemoattractant protein–1 expression and, together with obestatin treatment, decreased vascular cell adhesion molecule–1 expression. Finally, ghrelin and obestatin increased binding of oxidized LDL to thioglycollate-elicited mouse peritoneal macrophages. No changes were observed in the uptake of acetylated LDL by mouse J774.A1 macrophages after exposure to ghrelin or obestatin. In conclusion, we found 3 lines of in vitro evidence supporting proatherogenic properties of ghrelin in the early stages of the disease. However, in the presence of tumor necrosis factor–α stimulation, opposite effects of ghrelin were observed, suggesting that ghrelin may also have an anti-inflammatory role in the presence of increased inflammation, for example, during the more progressed phases of atherogenesis.

1. Introduction

Ghrelin, an octanyolated 28-amino-acid–long peptide hormone, is an endogenous ligand for a growth hormone secretagogue receptor (GHS-R) [1] that is widely distributed in the body, including cardiovascular system [2,3]. Obestatin is a newly discovered 23-amino-acid–long amidated peptide identified by bioinformatic approach [4]. Both ghrelin and obestatin are posttranslationally cleavaged from the same polypeptide precursor preproghrelin in stomach [4]. Ghrelin has a modulatory role in inflammation by reducing proinflammatory cytokines, and there is also strong evidence suggesting that ghrelin plays a role in chronic inflammatory diseases [5,6]. Low-grade system inflammation plays an important role in cardiovascular diseases [7]. Indeed, in our previous study, plasma ghrelin levels were positively related to early atherosclerosis measured by carotid artery intima media thickness [8].

The adhesion of monocytes to vascular endothelial cells, accumulation of cholesterol esters and oxidized low-density lipoprotein (Ox-LDL) into macrophages, and formation of

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lipid-loaded foam cells are the early steps in the progression of atherosclerosis and have been demonstrated to be modulated by various circulating factors such as plasma cytokines and adipokines [7]. Monocyte recruitment and adhesion to endothelial cells are additionally induced by various chemotactic cytokines and monocyte chemoattractant proteins (MCPs) such as MCP-1 [9]. Increased expression of cell adhesion molecules facilitates adherence of monocytes to the endothelium and transmigration into the vessel wall [10]. The roles of ghrelin and obestatin in these early atherosclerotic processes modulated by inflammatory markers have not been fully established. Previously, ghrelin has been shown to decrease monocyte adhesion onto tumor necrosis factor (TNF) α–treated human umbilical vein endothelial cells (HUVECs) [11]. In addition, ghrelin was found to inhibit TNF-α–induced nuclear factor–κB activation [11], which has been demonstrated to increase the transcription of genes encoding chemotactic cytokines and adhesion molecules [12]. Furthermore, in another study, ghrelin has been found to increase both intercellular adhesion molecule–1 (ICAM-1) and vascular cell adhesion molecule–1 (VCAM-1) protein expression on nonstimulated HUVECs but not to affect monocyte adhesion in these cells [13].

The goal of the present study was to investigate in detail the in vitro effects of ghrelin and newly discovered obestatin on the early key events of atherosclerotic processes such as monocyte adhesion to endothelial cells, binding of Ox-LDL, and uptake of acetylated LDL (Ac-LDL) on macrophages.

2. Materials and methods

2.1. Materials

Human and mouse ghrelin and obestatin were purchased from Phoenix Pharmaceutical (Belmont, CA). Human TNF-α was from Bender MedSystems (Vienna, Austria), and calcein-AM was from Molecular Probes (Eugene, OR). Cell culture reagents were purchased from Sigma-Aldrich Chemie (Steinheim, Germany) except fetal bovine serum (FBS), which was from PromoCell (Heidelberg, Germany). Difco Fluid Thioglycollate medium was from Difco Laboratories/BD Diagnostic Systems (Sparks, MD).

2.2. Preparation of IRDye 800–labeled Ox-LDL

Human LDL (d = 1.019-1.063 g/mL) was isolated from normolipemic EDTA plasma by sequential ultracentrifugation [14] using Beckman Ti 50.2 rotor (Beckman Coulter Inc., Fullerton, CA) at 218 000g at +15°C at least for 20.5 hours. Low-density lipoprotein was removed from the top by aspiration and oxidized as previously described [15] with 5 μmol/L copper sulfate at +37°C for 24 hours. The degree of the modification was verified by picrylsulfonic acid (TNBS) method [16] and testing monoclonal anti–Ox-LDL antibody binding (EO6 binding [17]). Copper Ox-LDL was labeled with the IR800 dye using IRDye 800CW labeling kit (LI-COR Biosciences, Lincoln, NE) according to the manufacturer's instruction in a molar ratio of 2 to 1. Labeled Ox-LDL was dialyzed against 3 changes of phosphate-buffered saline (PBS) with 0.27 mmol/L EDTA.

2.3. Preparation of ^{3}H-CE–labeled Ac-LDL

Isolated human LDL was stored at −20°C in density solution (d = 1.063 g/mL). For acetylation, LDL was first dialyzed into LDL buffer (150 mmol/L NaCl and 1 mmol/L EDTA [pH 7.4]) and acetylated as described previously [18] with mild modification. Typical preparation was performed as follows: 2 mL of saturated solution of sodium acetate was added gently to 10 mg of LDL (5 mg/mL) with gentle stirring in ice bath. Acetic anhydride was added in small aliquots (2.5 μL at a time) during 1 hour. The total number of microliters of added acetic anhydride was 2 times the number of milligrams of used LDL. Thereafter, the mixture was gently stirred for another 1 hour and dialyzed against LDL buffer. The LDL modification was tested by TNBS reactivity by the mild modification of the method of Habeeb [16]: 12.5 μg of native and modified LDL was diluted to 0.2 mg/mL with water. Thereafter, 125 μL of 4% $NaHCO_3$ and 12.5 μL of 0.1% TNBS were added; and the mixture was incubated at 37°C for 1 hour. Fifty microliters of 1 N HCl and 25 μL of 10% sodium dodecyl sulphate were added, and the reaction was incubated at room temperature for 15 minutes; thereafter, the absorbance was read at 340 nm. The results are given as percentage of masked amino groups and were calculated as follows: 100% − (absorbance of modified LDL/absorbance of native LDL). The typical preparation of Ac-LDL contained approximately 76% of modified groups. The Ac-LDL was labeled with ^{3}H-cholesterol oleate. Briefly, 40 μCi of ^{3}H-cholesterol oleate in dimethyl sulfoxide was added per 1 mg of LDL; and the mixture was diluted with LDL buffer to make the total concentration of LDL to 1 mg/mL. Dimethyl sulfoxide concentration in preparation was 10% of total volume. The labeling was performed at least for 2 hours at +40°C in water bath, and unbound label was removed by extensive dialysis against LDL buffer.

2.4. Cell culture

Human monocytic cell line THP-1 (ATCC, Rockville, MD) was grown in RPMI 1640 supplemented with 2 mmol/L L-glutamine, 10 mmol/L HEPES, 1 mmol/L Na-pyruvate, 0.05 mmol/L β-mercaptoethanol, and 4.5 g/L glucose. Human endothelial cell line EA.hy 926 was grown in Dulbecco modified Eagle medium (DMEM) with addition of 10 mmol/L hypoxanthine-aminopterin-thymidine and 10 mmol/L HEPES. Mouse-elicited macrophages and mouse monocyte/macrophages J774.A1 (ATCC) were grown in DMEM supplemented with 2 mmol/L L-glutamine, 1 mmol/L Na-pyruvate, and 4.5 g/L glucose. All growth media were supplemented with 100 U/mL penicillin,

0.1 mg/mL streptomycin, and 10% FBS. Cell cultures were maintained in humidified incubators at 37°C in an atmosphere of 5% CO_2. The cells were counted using 0.4% (wt/vol) trypan blue.

2.5. Monocyte adhesion

To evaluate monocyte adhesion, human EA.hy 926 endothelial cells were grown overnight in serum-free DMEM (10 mmol/L hypoxanthine-aminopterin- thymidine, penicillin-streptomycin, Na pyruvate) in 96-well plate and exposed to 10 and 100 ng/mL of ghrelin (2.96 and 29.6 nmol/L)) and obestatin (3.93 and 39.3 nmol/L)) for 4 hours for basal adhesion studies and for 1 hour before incubation with TNF-α (10 and 100 ng/mL) for 4 hours. The medium was removed, and fluorescently labeled (calcein-AM, Molecular Probes, Eugene, OR) THP-1 monocytes (125 000/well) were added to wells and incubated for 30 minutes at 37°C. The wells were inverted and spun at 200*g*. After the medium was added into the wells, fluorescence of the plate was measured in a plate reader. The results were normalized to control values.

2.6. Mouse-elicited macrophage isolation

The mice used in this study were C57BL/6 mice from the Experimental Animal Core Facility barrier of the University of Oulu, Finland. Mice were maintained in collective cages in an appropriate room with controlled temperature with a 12-hour light cycle, and mice were allowed access to food and water ad libitum. The study was approved by the Animal Ethics Committee of University of Oulu.

Elicited mouse macrophages were harvested from male mice by peritoneal lavage with PBS 3 days after an intraperitoneal injection of 2 mL of thioglycollate medium (3%). The macrophages were plated for 96-well plates at a density of 150 000 cells per well in DMEM media containing 10% FBS for binding assay or 6-well plates at a density of 4×10^6 cells per well for the demonstration of scavenger receptors RNA expression. Nonadherent cells were removed by washing the cells 3 hours after plating before overnight incubation at 37°C.

2.7. Binding of Ox-LDL

Elicited mouse macrophages were harvested and plated for 96-well plates at a density of 150 000 cells per well in DMEM media containing 10% FBS. After overnight culture at +37°C, the cells were washed with PBS and exposed to peptides in serum-free medium for 1 hour at +37°C. The plates were placed on ice for 20 minutes before incubation with IR800 dye–labeled (Licor Biosciences, Lincoln, NE) Ox-LDL (15 μg/mL) at +4°C for 3 hours. The specificity of the IRDye-labeled Ox-LDL binding was assessed with the excess (30×) of unlabeled Ox-LDL that blocked the binding by 85%. After washes with PBS for 3 times, the plate was allowed to dry and scanned with Odyssey IR scanner using 800-nm laser (Licor Biosciences).

2.8. Uptake of Ac-LDL

J774.A1 cells were plated for 96-well culture plates at a density of 50 000 cells per well in DMEM media containing 10% FBS. After 2 days culture, cells were washed twice with PBS and preincubated with 100 ng/mL of ghrelin (29.6 nmol/L) or obestatin (39.3 nmol/L) in serum-free DMEM for 1 hour before tritium-labeled Ac-LDL was added to the cells for 3 hours at +37°C. Cells were washed 2 times with PBS before 100 μL of 0.1 mol/L NaOH was added to the wells and kept in plate shaker to lyse the cells. Twenty-five microliters of cell lysate and 200 μL of Optiphase Supermix (PerkinElmer, Turku, Finland) were added to 96-well sample plate (1450-401, PerkinElmer), and radioactivity counts were obtained with a β-counter (Microbeta, PerkinElmer). The protein concentrations of the cell lysates were determined using protein assay (DC Protein Assay; Bio-Rad, Hercules, CA). Acetylated LDL uptake was calculated as counts per second in cell per microgram of cell protein.

Table 1
Nucleotide primers used in qRT-PCR

Gene	Accession no.	Primer
GAPDH (human)	P04406	Forward: 5′ GAGTCAACGGATTTGGTCGT 3′ Reverse: 5′ GACAAGCTTCCCGTTCTCAG 3′
ICAM-1 (human)	X06990	Forward: 5′ CAGAGGTTGAACCCCACAGT 3′ Reverse: 5′ CCTCTGGCTTCGTCAGAATC 3′
VCAM-1 (human)	BC085003	Forward: 5′ TAAAATGCCTGGGAAGATGG 3′ Reverse: 5′ GGTGCTGCAAGTCAATGAGA 3′
MCP-1 (human)	NM_002982	Forward: 5′ CCCCAGTCACCTGCTGTTAT 3′ Reverse: 5′ TGGAATCCTGAACCCACTTC 3′
β-Actin (mouse)	NM_007393	Forward: 5′ AGAGCTACGAGCTGCCTGAC 3′ Reverse: 5′ AGCACTGTGTTGGCGTACAG 3′
CD36 (mouse)	NM_007643	Forward: 5′ GGATCTGAAATCGACCTTAAAG 3′ Reverse: 5′ TAGCTGGCTTGACCAATATGTT 3′
SR-A (mouse)	AF203781	Forward: 5′ GGATCTGAAATCGACCTTAAAG 3′ Reverse: 5′ GTTGCTTTGCTGTAGATTCACGG 3′

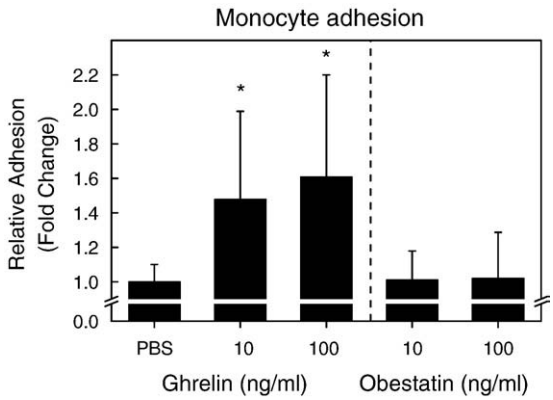

Fig. 1. Effect of ghrelin and obestatin on adhesion of THP-1 human monocytes on EA.hy 926 human endothelial cells. Ghrelin increases monocyte adhesion dose dependently. Endothelial cells were incubated with ghrelin or obestatin for 4 hours at +37°C before monocyte adhesion. Peptide concentrations of 10 and 100 ng/mL equal 2.96 and 29.6 nmol/L of ghrelin and 3.93 and 39.3 nmol/L of obestatin. Statistically significant differences in adhesion compared with PBS control in the panel. *$P < .001$; Mann-Whitney U test. Data from 5 different experiments are presented as mean fold changes ± SD to PBS control.

2.9. Quantitative reverse transcriptase polymerase chain reaction

EA.hy 926 cells were exposed to human ghrelin (100 ng/mL = 29.6 nmol/L) or obestatin (100 ng/mL = 39.3 nmol/L) in the presence and absence of TNF-α (10 ng/mL) in serum-free media for 1 hour (ICAM-1 and VCAM-1) or 3 hours (MCP-1). The messenger RNA (mRNA) expression of adhesion molecules VCAM-1, ICAM-1, and MCP-1 was determined in EA.hy 926 cells; and the mRNA expression of the scavenger receptors CD36 and SR-A was assessed in elicited mouse macrophages treated with ghrelin or obestatin (100 ng/mL). RNA was extracted with RNeasy Mini Kit (Qiagen, Valencia, CA). Half microgram of RNA was used to prepare complementary DNA (cDNA) with First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). One microliter of cDNA samples was used for the quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis (iQ SYBR Green I Supermix, Bio-Rad) in the iCycler Thermal Cycler (Bio-Rad). The qRT-PCR conditions were as follows: 95°C for 3 minutes followed by 40 cycles of 95°C for 10 seconds for denaturation, 60°C for 10 seconds for annealing, and 72°C for 10 seconds for extension. The cDNA levels of mouse SR-A and CD36 genes were normalized to the β-actin gene; and human ICAM-1, VCAM-1, and MCP-1, to GAPDH. Primer sequences are presented in Table 1. iCycler iQ optical system software (version 2.0, Bio-Rad) was used for analysis.

2.10. Statistical analysis

Data analyses were performed with the software package SPSS for Windows (16.0; SPSS, Chicago, IL). The results for different variables are presented as mean ± SD. To compare the treatments, 1-way analysis of variance, or Wilcoxon-Mann-Whitney test if the variables were not normally distributed, was used. P value < .05 (2-sided) was regarded as statistically significant.

3. Results

3.1. Effects of ghrelin and obestatin on monocyte adhesion to endothelial cells

Monocyte adhesion to endothelial cells is a key early step in the development of atherosclerosis [7]; therefore, we first investigated the effects of ghrelin and obestatin on THP-1 monocyte adhesion to EA.hy 926 endothelial cells. Fig. 1 shows that ghrelin increased monocyte adhesion to endothelial cells by 48% and 61% when incubated with 10 ng/mL (2.96 nmol/L) and 100 ng/mL (29.6 nmol/L) of ghrelin for 4 hours. Obestatin did not influence the adhesion (Fig. 1).

3.2. Effects of ghrelin and obestatin on adhesion molecules and MCP-1 without TNF-α stimulation

To assess the possible mechanism behind the monocyte adhesion, we investigated the effect of ghrelin and obestatin on adhesion molecules and MCP-1 expressions in EA.hy 926 cells by qRT-PCR. The cells were incubated with peptides to measure ICAM-1, VCAM-1, and MCP-1 expressions. Ghrelin increased ICAM-1 expression in endothelial cells (Fig. 2), a result consistent with our monocyte adhesion experiment (Fig. 1). Ghrelin did not alter MCP-1 expression (data not shown). Obestatin treatment did not change either ICAM-1 (Fig. 2) or

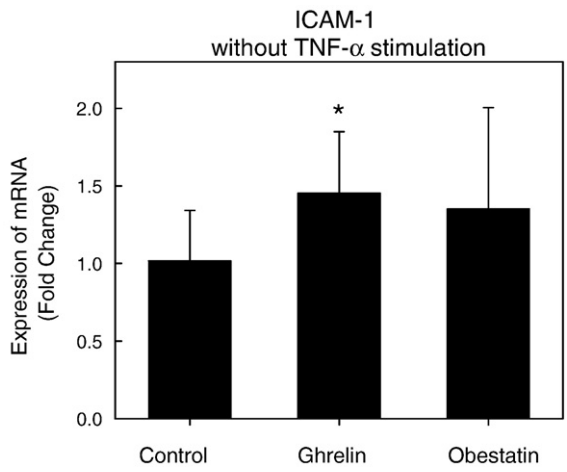

Fig. 2. The effects of ghrelin and obestatin on ICAM-1 mRNA expression in cultured EA.hy 926 endothelial cells without TNF-α stimulation. Ghrelin statistically significantly increased ICAM-1 expression. EA.hy 926 cells were treated with ghrelin (100 ng/mL = 29.6 nmol/L) or obestatin (100 ng/mL = 39.3 nmol/L). Messenger RNA levels were demonstrated by qRT-PCR. Statistically significant differences in expressions compared with PBS control in the panel. *$P < .05$; Mann-Whitney U test. Data from 3 different experiments (n = 9) are presented as mean fold changes ± SD to control.

MCP-1 expression (data not shown). The level of VCAM-1 expression was too low to be assessed without TNF-α stimulation as it has been earlier described in literature [19].

3.3. Effects of ghrelin and obestatin on monocyte adhesion to endothelial cells stimulated with TNF-α

Low-grade inflammation is known to be associated with obesity [20] and also with the initiation and progression of vascular atherosclerosis [7]. Therefore, to mimic an inflammatory stage, we then stimulated the cells with a proinflammatory cytokine TNF-α after ghrelin and obestatin treatment. Tumor necrosis factor–α (10 ng/mL) treatment alone was able to increase monocyte adhesion by 47% compared with PBS control (Fig. 3). However, when endothelial cells were pretreated with ghrelin (10 or 100 ng/mL), significant decreases (25% and 27%) in monocyte adhesion to TNF-α–stimulated endothelial cells were observed (Fig. 3). Obestatin pretreatment did not change monocyte adhesion to TNF-α–stimulated endothelial cells (Fig. 3).

3.4. Effects of ghrelin and obestatin on ICAM-1, VCAM-1, and MCP-1 expressions on endothelial cells stimulated with TNF-α

To determine the mechanisms behind the TNF-α–stimulated monocyte adhesion, we again examined the adhesion molecule and MCP-1 expressions in EA.hy 926 cells by qRT-PCR. When the endothelial cells were treated with TNF-α (10 ng/mL) alone, the expression of adhesion molecule ICAM-1 was increased by 35-fold, VCAM-1 by 1500-fold, and MCP-1 by 58-fold compared with PBS control. When the cells were incubated with ghrelin together with TNF-α (10 ng/mL), no significant changes in the ICAM-1 expression (data not shown) but reductions in VCAM-1 (Fig. 4A) and MCP-1 expressions (Fig. 4B) were observed compared with TNF-α treatment alone. Obestatin treatment together with TNF-α also decreased VCAM-1

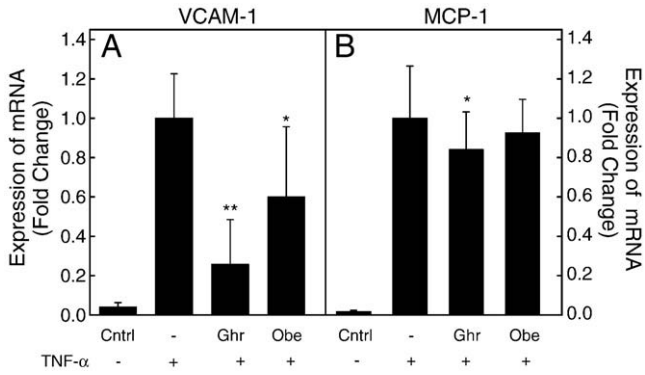

Fig. 4. The effects of ghrelin and obestatin on VCAM-1 and MCP-1 mRNA expressions in EA.hy 926 endothelial cells stimulated with TNF-α. A, Ghrelin and obestatin decreased VCAM-1 expression in the presence of TNF-α. B, Ghrelin decreased MCP-1 expression in the presence of TNF-α. EA.hy 926 cells were treated with ghrelin (100 ng/mL = 29.6 nmol/L) or obestatin (100 ng/mL = 39.3 nmol/L) together with TNF-α (10 ng/mL). Messenger RNA levels were demonstrated by qRT-PCR. A and B, Statistically significant differences in expressions compared with TNF-α. $^{**}P < .001$ (1-way analysis of variance) and $^{*}P < .05$ (Mann-Whitney *U* test). Data from 3 different experiments (n = 9) are presented as mean fold changes ± SD to control. Cntrl indicates PBS control; Ghr, ghrelin; Obe, obestatin.

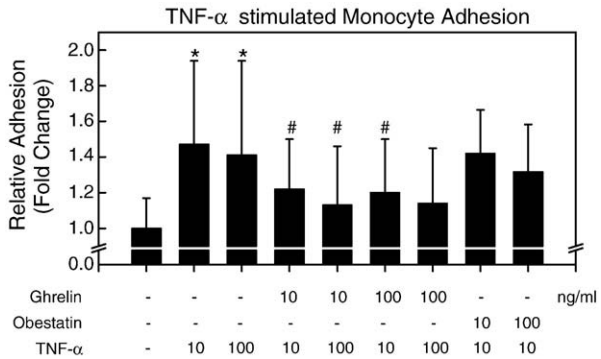

Fig. 3. Effect of ghrelin and obestatin on adhesion of THP-1 human monocytes on EA.hy 926 human endothelial cells stimulated with TNF-α. Ghrelin statistically significantly decreases monocyte adhesion on endothelial cells. The endothelial cells were preincubated with ghrelin or obestatin for 1 hour at +37°C before TNF-α stimulation for 4 hours and performing monocyte adhesion experiment. Peptide concentrations of 10 and 100 ng/mL equal 2.96 and 29.6 nmol/L of ghrelin and 3.93 and 39.3 nmol/L of obestatin. Statistically significant differences in adhesion compared with PBS control in the panel, $^{*}P < .001$, and with TNF-α; $^{\#}P < .05$; Mann-Whitney *U* test. Data from at least 4 different experiments are presented as mean fold changes ± SD to PBS control.

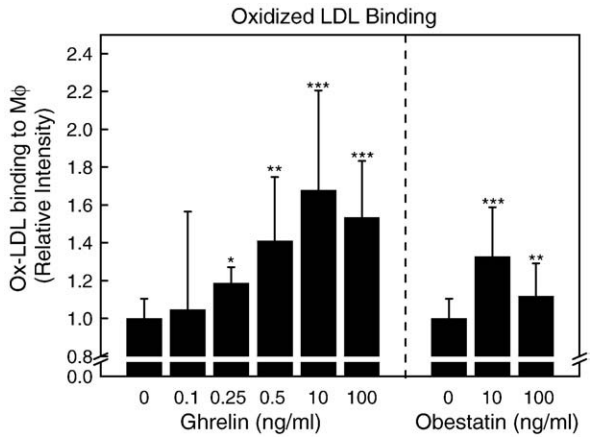

Fig. 5. Ghrelin and obestatin increase the binding of Ox-LDL to thioglycollate-elicited mouse peritoneal macrophages. Ghrelin increased the Ox-LDL binding to macrophages dose dependently. The macrophages were preincubated with peptides for 1 hour at 37°C before the binding of IR800 dye–labeled Ox-LDL at +4°C for 2 hours. The intensities were measured using Odyssey scanner. Peptide concentrations of 10 and 100 ng/mL equal 2.96 and 29.6 nmol/L of ghrelin and 3.93 and 39.3 nmol/L of obestatin. Statistically significant differences in binding compared with control in the panel. $^{***}P < .0001$, $^{**}P < .005$, and $^{*}P < .05$; Mann-Whitney *U* test. Data from 3 different experiments (n = 18) are presented as mean fold changes ± SD to control. Mϕ indicates macrophage; IR, infrared.

expression (Fig. 4A) but did not alter ICAM-1 (data not shown) and MCP-1 expressions (Fig. 4B) compared with TNF-α alone.

3.5. The effects of ghrelin and obestatin on Ox-LDL binding to mouse-elicited macrophages

Formation of foam cells from monocyte-derived macrophages by intake of Ox-LDL is an essential step in atherogenesis [7]. Therefore, we also wanted to explore the influences of ghrelin and obestatin on Ox-LDL binding to mouse-elicited peritoneal macrophages. Fig. 5 shows that ghrelin dose dependently increased the binding of Ox-LDL on mouse macrophages. In addition, obestatin treatment increased binding of Ox-LDL to the macrophages (Fig. 5). However, no changes were seen in CD36 or SR-A mRNA expression by ghrelin or obestatin treatment on the mouse peritoneal macrophages (data not shown).

3.6. The effects of ghrelin and obestatin on uptake of Ac-LDL on J774A.1 mouse macrophages

The uptake of modified LDL (other than Ox-LDL) occurs via various scavenger receptors, and SR-A has been found to be responsible for most (~80%) of the macrophage uptake of Ac-LDL [21]. Ghrelin and obestatin did not influence the uptake of Ac-LDL by macrophages (data not shown).

4. Discussion

The first major finding of the present study was that ghrelin increased monocyte adhesion to endothelial cells simultaneously with increased ICAM-1 mRNA expression. However, when endothelial cells were further stimulated with TNF-α after the ghrelin treatment, we observed decreased monocyte adhesion and also reduced mRNA expression of VCAM-1 adhesion molecule and MCP-1. Finally, ghrelin and obestatin treatments increased Ox-LDL binding to macrophages but did not change Ac-LDL uptake by macrophages. These data clearly demonstrate that ghrelin and obestatin are influencing the important key events in atherogenesis and thus also suggest that they may play a role in atherosclerosis.

Until now, the roles of ghrelin and obestatin in atherosclerosis have not been conclusively confirmed. Diseases with known high plasma ghrelin levels, such as Prader-Willi syndrome [22], have been linked to atherosclerosis in a few case reports [23,24]. In other diseases, such as cachexia in renal disease, plasma ghrelin levels have been reported to be increased; but the origin of atherosclerotic complications in these diseases is likely to be more complex [25]. Furthermore, plasma ghrelin levels have been shown to be increased in anorexia nervosa, a disease reported to be associated with premature atherosclerosis [26]; however, systemic reports are not available. In contrary, low ghrelin levels have been associated to obesity [27], a known risk factor for cardiovascular diseases [28]. We recently reported that low plasma ghrelin concentrations were associated with increased systolic and diastolic blood pressure, insulin resistance, and obesity in a large population-based cohort study (n = 1034) [29]. Thus, these data suggest that low ghrelin concentrations may be hemodynamically harmful and that ghrelin may modulate atherogenesis. Surprisingly, our further studies demonstrated a positive association between plasma ghrelin levels and the degree of subclinical atherosclerosis measured as intima media thickness of the carotid artery in the same population-based cohort among the male subjects [8]. Later, opposite findings on association between plasma ghrelin levels and subclinical atherosclerosis in different study populations have been published [30-32]. In experimental studies, ghrelin receptor (GHS-R) density has been shown to be increased in atherosclerotic vessels [33]; and ghrelin has been demonstrated to have antiapoptotic actions in cardiomyocytes and endothelial cells [11,34], suggesting also a beneficial role of ghrelin for human atherosclerosis. Based on these findings, it seems likely that ghrelin has modulatory functions on vascular system and atherogenesis; and therefore, our objective was to study the effect of ghrelin and obestatin on atherosclerosis in vitro by exploring cells participating in the early steps of atherogenesis.

We found 3 lines of in vitro evidence supporting the proatherogenic properties for ghrelin and, in the presence of TNF-α stimulus, 3 lines of evidence showing anti-inflammatory properties. The first line of our proatherogenic evidence showed that ghrelin increased monocyte adhesion to endothelial cells (EA.hy 926). In atherosclerosis, monocyte adhesion to endothelial cells is a key early step in the development of atherosclerotic lesions [35] and is known to be modulated by adhesion molecules, chemokines, proinflammatory cytokines such as TNF-α, adipokines, and also oxidized lipoproteins [36]. In the present article, we used human endothelial cell EA.hy 926 as a model of monocyte adhesion to aortic endothelium. The EA.hy 926 cell line has been generated by the fusion of HUVEC with human lung carcinoma cell line A549 [37]. These immortalized and well-characterized cells express several characteristics common to endothelial cells, such as von Willebrand factor, integrins, and also adhesion molecules (ICAM, VCAM, E-selectin) upon stimulation with TNF-α, thus providing a good model of large-vessel endothelium to study adhesion and proinflammatory responses [38]. In contrast to the monocyte adhesion data of our present study, ghrelin was not shown to influence monocyte adhesion to endothelial cells in a study using isolated human monocytes and HUVECs [13]; and in another study, ghrelin was shown to decrease human mononuclear cell (U937) adhesion to HUVEC endothelial cells [11]. All these studies were conducted at the basal level without any stimulation. The contradictory results between these studies may be due to differences in cell lines and conditions. Furthermore, the ghrelin concentrations used were lower [13] and higher [11] than in our study.

The second line of proatherogenic evidence demonstrated that ghrelin treatment also increased ICAM-1 adhesion molecule expression in the endothelial cells. Increased expression of cell adhesion molecules such as VCAM-1 and ICAM-1 has been associated with increased adherence of monocytes onto the endothelium and also increased transmigration of monocyte into the vessel wall [10]. Similar observations have been reported earlier on HUVEC cells in a study where ghrelin dose dependently increased both ICAM-1 and VCAM-1 expression at the protein level [13].

The third line of evidence showing proatherogenic properties of ghrelin was the demonstration that ghrelin dose dependently increased Ox-LDL binding to macrophages. Increase in Ox-LDL uptake by macrophages will lead to lipid accumulation and foam cell formation and eventually into fatty streak formation in the arterial wall [7]. Oxidized LDL and Ac-LDL but not native LDL are internalized through different scavenger receptors by macrophages [39]. Although ghrelin increased Ox-LDL binding, it did not have any effect on Ac-LDL uptake on macrophages. The differences in these binding and uptake results might be due to different pathways and mechanism behind the lipid uptake of these ligands. Acetylated LDL is taken up mostly (~80%) by SR-AI receptor [21], whereas Ox-LDL is taken up by macrophages (for up to 90%) through SR-A and CD36 scavenger receptors [40]. Previously, hexarelin, a synthetic growth hormone–releasing peptide binding to ghrelin receptor (GHS-R1a) and CD36, has been demonstrated to reduce lipid accumulation in human THP-1 macrophages [41]. The differences might be due to different binding properties of ghrelin and hexarelin to GHS-R and CD36 receptors [42].

In contrast to the proatherogenic properties of ghrelin listed above, the actions of ghrelin on TNF-α–treated cells were reversed. We observed that, when the cells were treated with ghrelin together with TNF-α, not only the monocyte adhesion but also VCAM-1 and MCP-1 expressions were decreased. Our results are in line with a previous study showing that ghrelin decreases MCP-1 expression and monocyte adhesion to HUVEC endothelial cells stimulated with TNF-α [11]. Similar findings in a very recent study demonstrated also that ghrelin inhibits nicotine-induced VCAM-1 expression in HUVEC cells [43]. Tumor necrosis factor–α, a proinflammatory cytokine, stimulates adhesion of monocytes to the surface of endothelial cells by enhancing the expression of adhesion molecules (ICAM-1 and VCAM-1) and stimulates infiltration of vascular wall by monocytes (MCP-1) and their transformation to macrophages [44]. Do our data suggest that the role of ghrelin may be different during inflammation? Several previous studies have already established that ghrelin exhibits anti-inflammatory properties. Ghrelin has been shown to inhibit expression and production of proinflammatory cytokines (eg, interleukin [IL]-1b, IL-2, IL-6, and TNF-α) through GHS-R in human peripheral blood mononuclear cells and T cells [45,46]. In addition, in vivo studies on rats have demonstrated down-regulation of proinflammatory cytokines TNF-α and IL-6 in sepsis [47]. These findings are analogous with our data suggesting anti-inflammatory modulatory function for ghrelin, and this may also apply to chronic inflammatory diseases such as atherosclerosis.

Obestatin is a 23–amino-acid amidated peptide arising from the same 117–amino-acid preprohormone as ghrelin [4]. Originally, obestatin was proposed as a ligand of GPR39, an orphan receptor in ghrelin receptor family, and was also thought to display effects opposite to ghrelin, such as decreasing food intake, decreasing body weight, and delaying gastric emptying, and to antagonize the actions of ghrelin when both peptides are coadministered [4]. However, several groups have been unable to confirm the properties of obestatin on GPR39 and its activation [48,49]. Several previous studies have already established the modulatory role of ghrelin in inflammation and atherosclerosis, but similar studies have not yet been carried out with obestatin. In the present study, we found that obestatin did not affect monocyte adhesion to endothelial cells and ICAM-1 and MCP-1 expression in endothelial cells with or without TNF-α treatment. Interestingly, obestatin decreased VCAM-1 expression in endothelial cells when stimulated together with TNF-α. In addition, obestatin increased Ox-LDL binding to macrophages. In the present in vitro study, the effects of obestatin on atherosclerosis using cell models seem to be similar or less effective than ghrelin.

In summary, we have demonstrated that ghrelin increased monocyte adhesion and ICAM-1 expression in endothelial cells and increased Ox-LDL binding to macrophages. However, the actions of ghrelin were reversed in the presence of TNF-α; ghrelin inhibited monocyte adhesion and VCAM-1 and MCP-1 expressions. In our studies, obestatin exhibited similar properties as ghrelin. These data suggest that ghrelin and obestatin may modulate the processes participating in atherogenesis but that the effect can vary depending on the inflammatory condition. Thus, these data propose ghrelin to be proatherogenic in the early stages of disease development. However, during the more progressed phases of atherogenesis and inflammation, for example, in the presence of increased TNF-α [7], ghrelin may exhibit a more antiatherogenic role. Further validation of the detailed properties of ghrelin and obestatin is warranted because ghrelin has already been suggested to be a tempting treatment for inflammatory conditions, obesity, and cardiovascular diseases.

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