Ghrelin Inhibits Proinflammatory Responses and Nuclear Factor-κB Activation in Human Endothelial Cells

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Background—Ghrelin is a novel growth hormone–releasing peptide that has been shown to improve cachexia in heart failure and cancer and to ameliorate the hemodynamic and metabolic disturbances in septic shock. Because cytokine-induced inflammation is critical in these pathological states and because the growth hormone secretagogue receptor has been identified in blood vessels, we examined whether ghrelin inhibits proinflammatory responses in human endothelial cells in vitro and after administration of endotoxin to rats in vivo.

Methods and Results—Human umbilical vein endothelial cells (HUVECs) were treated with or without tumor necrosis factor-α (TNF-α), and induction of proinflammatory cytokines and mononuclear cell adhesion were determined. Ghrelin (0.1 to 1000 ng/mL) inhibited both basal and TNF-α–induced cytokine release and mononuclear cell binding. Intravenous administration of ghrelin also inhibited endotoxin-induced proinflammatory cytokine production in rats in vivo. Ghrelin inhibited H2O2–induced cytokine release in HUVECs, suggesting that the peptide blocks redox-mediated cellular signaling. Moreover, ghrelin inhibited basal and TNF-α–induced activation of nuclear factor-κB. Des-acyl ghrelin had no effect on TNF-α–induced cytokine production in HUVECs, suggesting that the antiinflammatory effects of ghrelin require interaction with endothelial growth hormone secretagogue receptors.

Conclusions—Ghrelin inhibits proinflammatory cytokine production, mononuclear cell binding, and nuclear factor-κB activation in human endothelial cells in vitro and endotoxin-induced cytokine production in vivo. These novel antiinflammatory actions of ghrelin suggest that the peptide could play a modulatory role in atherosclerosis, especially in obese patients, in whom ghrelin levels are reduced. (Circulation. 2004;109:2221-2226.)

Key Words: hormones, peptide • inflammation • endotoxins • monocytes • nuclear factor-κB

Ghrelin is a newly discovered hormone, produced primarily in the stomach, and has been identified as the endogenous ligand for the growth hormone secretagogue receptor (GHSR).1 Recently, ghrelin and its receptors were detected in cardiovascular tissues,2,3 indicating that the peptide may play a role in cardiovascular regulation. Stimulation of GHSR has been shown to prevent cardiac damage after ischemia/reperfusion in hypophysectomized rats.4 Moreover, administration of ghrelin improved left ventricular function and attenuated the development of cardiac cachexia in a heart failure model.5 Furthermore, ghrelin improved mortality and corrected the hemodynamic and metabolic abnormalities associated with endotoxic shock in rats.6

Proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), are upregulated in patients with congestive heart failure, particularly those with cardiac cachexia, and have been implicated in the pathophysiology of this disease.7,8 TNF-α and other proinflammatory cytokines have also been shown to participate in the pathogenesis of atherosclerosis and septic shock, both of which entail vascular inflammation.9 On the basis of the aforementioned observations, we hypothesized that ghrelin may have significant antiinflammatory effects in the vasculature. In the present study, we examined the effects of ghrelin on inflammatory responses in cultured human umbilical vein endothelial cells (HUVECs) and in vivo after endotoxin administration. Our findings provide the first evidence that ghrelin acts as an antiinflammatory peptide in the cardiovascular system.

Methods

Cell Culture

Cultured HUVECs were prepared by collagenase treatment of freshly obtained human umbilical veins. Cells were cultured and
characterized as previously described \(^{10}\) and used in experiments at passage 2.

**Interleukin-8, TNF-α, and Monocyte Chemotactic Protein-1 Enzyme-Linked Immunosorbent Assays**

Cytokines were measured by enzyme-linked immunosorbent assay (ELISA) as described previously.\(^ {11}\)

**Adhesion of U937 Mononuclear Cells to HUVEC Monolayers**

To evaluate monocyte adhesion, HUVEC monolayers were incubated in M199 containing 0.25% FBS with human TNF-α (10 ng/mL) for 24 hours in the absence or presence of ghrelin (100 ng/mL). The media were removed, and U937 cells labeled with the fluorescent probe bis-carboxyethyl-carboxyfluorescein acetoxymethyl ester (BCECF-AM; Molecular Probes) were added to wells (6×10^4 per well) and incubated for 10 minutes at 37°C. The wells were washed and filled with fresh media; the plates were sealed, inverted, and spun at 100g for 5 minutes to remove nonadherent U937 cells. Adherence of labeled U937 cells was determined in a plate reader. After the results were normalized to percentage of control values, data were expressed as mean±SEM.

**Western Blotting**

Nuclear protein was prepared as described previously.\(^ {12}\) Proteins were separated on 7.5% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes. Immunoreactive proteins were detected by incubating the blots with diluted p65 rabbit polyclonal antibody at 4°C overnight, followed by horseradish peroxidase–conjugated secondary antibody (Santa Cruz Biotechnology) and visualization with an enhanced chemiluminescence system (NEN). The resulting images were analyzed with NIH Image software.

**Adenovirus Transfection and Luciferase Assay**

A recombinant adenoviral vector expressing the luciferase reporter gene driven by nuclear factor-kB (NF-kB) transcripational activation (Ad.NFkBBlue) was generously provided by Dr Paul McCray through the Gene Transfer Vector Core Facility at the University of Iowa.\(^ {13}\) Confluent HUVECs were incubated with 100 MOI Ad.NFkBLuc in serum-free M199 for 3 hours. The medium was replaced with fresh medium containing 2% FBS, and the incubation was continued for an additional 24 hours. Thereafter, cells were incubated with or without 100 ng/mL ghrelin for 1 hour and then treated with 0.1 ng/mL TNF-α for 24 hours. Subsequently, luciferase activity was determined with the use of a kit (Promega) according to the manufacturer’s protocol. All measurements of luciferase activity (relative light units) were normalized to the protein concentration.

**Dichlorodihydrofluorescein Diacetate Fluorescence**

HUVECs were grown to confluence in 48-well Costar plates and exposed to 100 ng/mL ghrelin in M199 medium with 0.25% serum for 6 hours at 37°C. The oxidant-sensitive probe dichlorodihydrofluorescein diacetate (H₂DCFDA, 50 μM/mL; Molecular Probes) was added, and the cells were incubated at 37°C for 30 minutes. As H₂DCFDA enters cells, intracellular esterase activity removes the fluorochrome group; the resulting H₂DCF leaves the cell more slowly, effectively increasing the intracellular concentration of the probe. After 30 minutes of incubation, H₂O₂ (0 to 50 μM/mL) was added to the wells, and the plate was placed in a Fluostar Optima microplate fluorometer (BMG Laboratories). Relative fluorescence (excitation 485 nm; emission 538 nm) was determined every 4 minutes over a 2-hour period at 37°C.

**MTS Assay for Cell Viability**

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay with the use of a test kit (Promega) according to the manufacturer’s instructions.\(^ {14}\) For H₂O₂-induced cytotoxicity evaluation, HUVECs were exposed to 0 to 200 μM H₂O₂ for 30 to 120 minutes, and then the cells were washed and reincubated with fresh H₂O₂-free medium. Subsequently, the MTS viability assay was performed 24 hours after exposure to H₂O₂.

**Experimental Animals**

Sprague-Dawley rats were purchased from Peking University Animal Center (Beijing, China) and randomly divided into 3 treatment groups: injection of normal saline; injection of endotoxin (lipopolysaccharide [LPS]) (10 mg/kg); and injection of LPS plus simultaneous injection of rat ghrelin (10 nmol/kg). All injections were performed through the tail vein. After 4 hours, the rats were killed, and plasma was collected. Interleukin-8 (IL-8), TNF-α, and monocyte chemotactic protein-1 (MCP-1) levels in plasma were measured by ELISA. The experimental protocol was in accordance with institutional guidelines and was approved by the Institutional Animal Care and Use Committee of Peking University.

**Statistical Analysis**

Results are expressed as mean±SEM. The data were analyzed by ANOVA followed by Bonferroni t testing. Probability values <0.05 were considered statistically significant.

**Other Reagents**

Human and rat ghrelin and des-acyl ghrelin were obtained from Phoenix Pharmaceuticals, Inc (purity >95%). TNF-α was purchased from R&D Systems. Endotoxin (Escherichia coli LPS) was purchased from Difco Laboratories. All other reagents were purchased from Sigma.

**Results**

**Ghrelin Inhibits TNF-α–Induced IL-8 and MCP-1 Production in HUVECs**

To investigate whether ghrelin has antiinflammatory effects in human endothelial cells, we first pretreated HUVECs with vehicle or ghrelin for 1 hour and then exposed the cells to TNF-α for 24 hours. Media were then collected and assayed for IL-8 and MCP-1 by ELISA. TNF-α produced potent, dose-dependent increases in the production of IL-8 (Figure 1A) and MCP-1 (not shown) in HUVECs. Ghrelin inhibited TNF-α–induced IL-8 release in a concentration-dependent manner, with significant inhibition observed at a concentration of ghrelin as low as 10 ng/mL under these conditions (Figure 1B). Ghrelin likewise inhibited TNF-α–induced MCP-1 release (8.7±0.3 ng per well [control], 24.6±0.3 ng per well [1 ng/mL TNF-α], 13.0±0.6 ng per well [TNF-α+1000 ng/mL ghrelin; P<0.05 versus TNF-α alone]; n=3 per group). Ghrelin also dose-dependently inhibited basal IL-8 and MCP-1 release in the absence of stimulation with TNF-α (data not shown).

When HUVECs were preincubated with ghrelin for 6 hours, both basal (Figure 2A) and TNF-α–induced (Figure 2B) IL-8 production were inhibited by very low concentrations of the peptide (0.1 to 1 ng/mL). In contrast, when HUVECs were exposed concomitantly to ghrelin and TNF-α (ie, no precubulation with ghrelin), the magnitude of inhibition was less, suggesting that the antiinflammatory effects of ghrelin are time dependent (Figure 2C). However, des-acyl ghrelin, at concentrations up to 1000 ng/mL, did not inhibit TNF-α–induced IL-8 release (Figure 1B). These findings suggest that n-octanoylation (serine-3) of ghrelin is critical for its antiinflammatory effects in human endothelial cells.
Ghrelin Inhibits U937 Cell Adhesion in HUVECs

Mononuclear cell adhesion plays a crucial role in vascular inflammation and atherosclerosis and is stimulated by chemotactic cytokines. Therefore, we investigated whether ghrelin inhibits mononuclear cell adhesion to endothelial cells. Stimulation with 1 ng/mL TNF-α/H9251 produced an approximately 6-fold increase in adhesion of U937 cells to HUVEC monolayers compared with baseline. Treatment with ghrelin inhibited TNF-α-stimulated U937 cell binding by approximately 60% (Figure 3). Ghrelin also inhibited basal mononuclear cell binding in the absence of TNF-α. Thus, our findings indicate that ghrelin potently inhibits production of chemotactic cytokines and adhesion of mononuclear cells to HUVECs, suggesting that the peptide attenuates vascular inflammation.

Ghrelin Inhibits Cytokine Release in a Rat Model of Endotoxic Shock

To determine whether ghrelin exhibits antiinflammatory effects in vivo, we injected rats with vehicle (saline) or endotoxin (LPS, 10 mg/kg) with or without ghrelin (10 nmol/kg). After 4 hours, we determined the plasma levels of the proinflammatory cytokines TNF-α, IL-8, and MCP-1. As expected, treatment with LPS induced a dramatic increase in cytokine production (Table). Coadministration of ghrelin markedly attenuated the plasma levels of each of these cytokines during treatment with LPS.

Inhibition of TNF-α–Induced NF-κB Activation by Ghrelin

NF-κB activation plays a key role in the production of chemotactic cytokines and in adhesion molecule expression,15 and both TNF-α and LPS are potent activators of NF-κB. To test whether ghrelin inhibits TNF-α–induced NF-κB activation in HUVECs, 2 complementary methods were used. First, we performed Western blotting to examine NF-κB nuclear translocation. Cells were preincubated with vehicle or 100 ng/mL ghrelin for 1 hour, and then 0.1 to 1 ng/mL TNF-α was added for 20 minutes. Nuclear extracts were prepared and tested for p65 protein levels by Western blotting. Figure 4, A and B, shows that TNF-α induced nuclear translocation of p65 protein, which was markedly attenuated by ghrelin. Second, we transfected HUVECs with
Effects of Ghrelin on H$_2$O$_2$-Induced IL-8 Release and Oxidative Stress in HUVECs

TNF-α–induced IL-8 release from endothelial cells can be blocked by antioxidant compounds. Moreover, reactive oxygen species (ROS) such as H$_2$O$_2$ can activate inflammatory signaling pathways, including NF-κB, in vascular cells. We therefore examined whether ghrelin inhibits IL-8 release evoked by H$_2$O$_2$ in endothelial cells. We first identified concentrations of H$_2$O$_2$ that would not induce cytotoxicity in HUVECs. Incubation with up to 50 μmol/L H$_2$O$_2$ for up to 2 hours produced no measurable cytotoxicity as determined by MTS assay after 24 hours (vehicle=0.97±0.10 absorbance units versus H$_2$O$_2$=0.93±0.15 absorbance units; n=4; P>0.05). Under these same conditions, H$_2$O$_2$ (50 μmol/L) produced an approximately 2.5-fold increase in IL-8 release from HUVECs, which was inhibited by treatment with ghrelin (Figure 5A). To determine whether ghrelin could inhibit H$_2$O$_2$-induced IL-8 release by scavenging intracellular ROS, we examined fluorescence of H$_2$DCF, an oxidant-sensitive probe. A 2-hour exposure to H$_2$O$_2$ resulted in H$_2$DCF fluorescence in HUVECs, which was unaffected by ghrelin (Figure 5B). Ghrelin also did not affect the rate of increase of H$_2$DCF fluorescence after application of H$_2$O$_2$, even at low concentrations of H$_2$O$_2$ (Figure 5C). These findings suggest that the mechanism(s) by which ghrelin inhibited H$_2$O$_2$–induced IL-8 release in HUVECs was neither by directly scavenging intracellular H$_2$O$_2$ nor by upregulating intracellular H$_2$O$_2$-scavenging mechanisms, but rather by interfering with redox signaling to inhibit cytokine release.

Discussion

Ghrelin, a recently discovered 28–amino acid peptide, has received considerable attention for its effects on food intake and adiposity, in addition to its growth hormone–releasing properties. Putative receptors for ghrelin have been identified in blood vessels and endothelial cells, suggesting that the peptide could play a modulatory role in cardiovascular function. In the present study, we show for the first time that ghrelin inhibits basal and TNF-α–induced chemotactic cytokine production and mononuclear cell adhesion in human vascular endothelial cells. These effects were seen at concen-
trations of ghrelin that fall within the physiological range.\textsuperscript{19} We also demonstrate that intravenous treatment with ghrelin inhibits cytokine release induced by systemic administration of endotoxin in vivo. These antiinflammatory effects could help to explain the beneficial consequences of ghrelin administration in myocardial reperfusion injury, cardiac cachexia, and septic shock. Furthermore, we demonstrate that ghrelin inhibits NF-κB activation in endothelial cells, suggesting a potential mechanism for the observed antiinflammatory effects.

Induction of chemotactic cytokines such as IL-8 and MCP-1 is thought to play a key role in monocyte recruitment and adhesion to endothelial cells in atherosclerosis.\textsuperscript{20} Interestingly, obesity has been associated with elevated levels of proinflammatory cytokines, which are thought to contribute to increased cardiovascular morbidity in these patients.\textsuperscript{21} The mechanisms responsible for enhanced proinflammatory cytokine production in obesity remain to be elucidated. Recent studies suggest that plasma levels of ghrelin are reduced in obese patients.\textsuperscript{22} Thus, our findings raise the possibility that reduction in endogenous ghrelin could potentially contribute to the proinflammatory state and the increased incidence of atherosclerosis in obese patients.

The endocrine activity of ghrelin is dependent on its acylation and subsequent interaction with GHSR-1a.\textsuperscript{1,3} However, it has been shown recently that both ghrelin and des-acyl ghrelin inhibit apoptosis in cardiomyocytes and endothelial cells,\textsuperscript{23} suggesting that ghrelin may also act through a novel, yet to be identified receptor, which is distinct from GHSR-1a. In the present study, the antiinflammatory effects of ghrelin in HUVECs appear to be dependent on the acylation of ghrelin, because des-acyl ghrelin did not inhibit TNF-α-induced cytokine release. These results suggest that, unlike the antiapoptotic effects, the antiinflammatory effects of ghrelin in endothelial cells are likely mediated through activation of GHSR-1a.

ROS such as H$_2$O$_2$ are thought to participate in TNF-α-induced IL-8 release by endothelial cells.\textsuperscript{16} Thus, to examine the potential mechanisms whereby ghrelin inhibits inflammatory responses in endothelial cells, we tested its capacity to inhibit cytokine release induced by H$_2$O$_2$. Treatment of HUVECs with H$_2$O$_2$ did, in fact, result in increased IL-8 release, which was effectively blocked by ghrelin. However, studies with H$_2$DCF in H$_2$O$_2$-treated cells indicate that ghrelin did not reduce the level of intracellular ROS. Taken together, these findings suggest that ghrelin does not upregulate endogenous cellular H$_2$O$_2$ removal pathways, nor does it act as an antioxidant per se (ie, it does not scavenge ROS), but it may instead interfere with redox signaling to inhibit cytokine release. It is conceivable that ghrelin could modulate redox signaling in HUVECs by stimulating the production of NO. Very recently, NO was reported to protect endothelial cells against H$_2$O$_2$-induced apoptosis by blocking transferrin receptor–mediated iron uptake.\textsuperscript{24} Moreover, a recent report suggests that ghrelin may induce the release of endogenous NO.\textsuperscript{25} However, treatment with N$^\circ$-nitro-L-arginine methyl ester, an inhibitor of NO synthase, did not block the inhibitory effects of ghrelin on TNF-α-induced IL-8 release (data not shown). This latter finding suggests that stimulation of NO release does not mediate inhibition by ghrelin of TNF-α–induced cytokine release in HUVECs.

NF-κB is a critical signaling molecule in TNF-α–induced inflammation and in responses produced by a variety of
stimuli that include growth factors, lymphokines, UV irradiation, pharmacological agents, and oxidant stress. Therefore, a potential mechanism whereby ghrelin could modulate inflammatory responses to both TNF-α and H₂O₂ is by blocking activation of the transcription factor NF-κB. In its inactive form, NF-κB is sequestered in the cytoplasm, bound by members of the I-κB family of inhibitor proteins. Phosphorylation of I-κB by an I-κB kinase complex exposes nuclear localization signals on the NF-κB subunits and induces translocation of the molecule to the nucleus. In the nucleus, NF-κB binds with consensus sequences of various genes, activating their transcription. Our observations that ghrelin attenuated TNF-α–induced nuclear translocation of NF-κB suggest a likely mechanism of the antiinflammatory effects of ghrelin. To our knowledge, this is the first study to report that ghrelin inhibits NF-κB activation. The molecular mechanisms leading to inhibition of NF-κB activation by ghrelin remain to be determined.

In summary, we demonstrate for the first time that ghrelin has potent antiinflammatory effects in human endothelial cells, likely mediated by inhibition of NF-κB activation. Ghrelin also inhibited endotoxin-induced systemic cytokine production in vivo. These findings may help to explain the beneficial effects of ghrelin administration in various pathological states associated with inflammation, including experimental models of heart failure and septic shock. Moreover, our findings suggest that a reduction in endogenous ghrelin could contribute to the increased incidence of atherosclerosis in patients with obesity. Consequently, activation of the ghrelin signaling pathway could represent a novel approach in the prevention and/or treatment of atherosclerosis.

Acknowledgments
This work was supported by National Institutes of Health grants HL-49264, HL-62984, and HL-070860 (Dr Weintraub), by the American Heart Association Grant-in-Aid Award (Dr Li). Finally, the authors appreciate the technical assistance of Papri Chatterjee in conducting the experimental protocols.

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_Circulation_. 2004;109:2221-2226; originally published online April 26, 2004;

doi: 10.1161/01.CIR.0000127956.43874.F2

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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