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Direct Peripheral Effects of Ghrelin Include Suppression of Adiponectin Expression

Abstract

The stomach-derived peptide, ghrelin, has recently been discovered as an important regulator of energy homeostasis. Central nervous system pathways involving stimulation of hypothalamic neuropeptides play a prominent role in mediating ghrelin's orexigenic effects. However, potential direct peripheral effects remain poorly understood. Using a brown adipocyte model, we tested ghrelin-mediated influences on adipose tissue. Chronic ghrelin stimulation of differentiating adipocytes did not affect the pattern or extent of fat accumulation. Furthermore, insulin-induced glucose uptake as a hallmark of adipocyte function was not altered by ghrelin pre-treatment. However, acute ghrelin treatment resulted in a significant time-dependent increase in p44/42 mitogen-activated protein kinase phosphorylation. There was no stimulation of phosphatidylinositol 3-kinase, JAK/STAT, or stress kinase signaling pathways. Furthermore, ghrelin did not significantly alter gene expression of the thermogenic uncoupling protein-1. By contrast, expression of the novel adipokine adiponectin, which has been implicated in the pathogenesis of insulin resistance and obesity, was strongly impaired. This inhibition occurred acutely, and was sustained for several hours. In summary, our data provide evidence for selective effects of ghrelin on adipocyte signaling and function and thus propose a role for adipose tissue as a novel mediator of ghrelin's effects on energy balance and glucose homeostasis.

Key words
Ghrelin · Brown Adipocyte · MAP Kinase · Adiponectin · Obesity · Insulin Resistance

Introduction

Ghrelin, a 28-amino-acid acylated peptide synthesized in the stomach [1], has recently been discovered as an important regulator of feeding and energy homeostasis [2]. Circulating ghrelin levels are increased during weight loss, fasting, and hypoglycemia [3], and acutely decreased in obesity, and after food intake and glucose ingestion [4 – 6]. Studies in rodents and humans have shown ghrelin treatment to result in increased food intake and weight gain [7 – 10]. Moreover, human studies have shown a preprandial surge in ghrelin levels, implicating ghrelin in meal initiation [11]. These data suggest an orexigenic role of this factor in the central nervous system regulation of body weight involving stimulation of orexigenic neuropeptides such as NPY and AGRP in the hypothalamus [12 – 14]. However, whether direct effects of ghrelin on peripheral tissues contribute to the regulation of bodyweight and energy homeostasis is unknown. Here, we demonstrate differential direct effects of ghrelin on adipocyte signaling, metabolism, and adipokine expression using a well-characterized brown-adipocyte model [15 – 21].

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Materials and Methods

Materials

The phosphospecific antibody against p44/p42 MAP kinase was from Cell Signaling Technology, Inc.; Beverly, MA, USA. Synthetic ghrelin was a generous gift from Eli Lilli and Co., Indianapolis, Indiana, USA, where it was generated as described previously [22]. Glucose uptake assays were performed with 2-deoxy-[^{3}H]glucose from NEN Life Technologies (Dreieich, Germany). Primers for expression analysis were ordered from Biometra (Goettingen, Germany) and TIB Molbiol (Berlin, Germany). Unless stated otherwise, all other chemicals were purchased from Sigma (Deisenhofen, Germany).

Cell culture

Cells used in all experiments were SV40T-immortalized brown adipocytes generated as previously described [15,17]. Pre-adipocytes were cultured to confluence in Dulbecco's modified Eagle's medium (Life Technologies, Paisley, Scotland), supplemented with 4.5 g/l glucose, 20 nM insulin, 1 nM T3, 20% fetal bovine serum (Sigma-Aldrich Co.; St. Louis, MO, USA), and penicillin/streptomycin (BioWhittaker, Vervier, Belgium) ("differentiation medium"). Upon attaining confluence, 500 µM isobutylmethylxanthine, 250 µM indomethacine, and 2 µg/ml dexamethasone were added to the differentiation medium to induce cell differentiation. After 24 h, cells were returned to differentiation medium and cultured for 4 to 5 more days. Cells used for protein assays were starved for 24 to 48 h in serum-free medium prior to carrying out the experiments. No starvation was performed prior to total RNA isolation.

Western blotting

Cell lysis, protein isolation, and protein content determination were performed after stimulation with ghrelin essentially as previously described [15]. Subsequent to separation by SDS-PAGE, protein was transferred to nitrocellulose membranes (Schleicher and Schuell Inc.; Keane, NH, USA). After blocking membranes overnight with 10 mM Tris, 150 mM NaCl, 0.05% Tween (pH 7.2), and 3% BSA, membranes were incubated for a period of 1 – 2 h with the respective antibodies. Visualization of protein bands was achieved employing chemiluminescence (Roche Molecular Biochemicals; Mannheim, Germany) and enhanced chemiluminescence films (Amersham Pharmacia Biotech; Freiburg, Germany).

Glucose uptake assay

Cells were assayed for glucose uptake essentially as described [15,18]. After a serum-free starvation of 48 h and pre-treatment with ADA (2 U/ml), fully differentiated monolayers of brown adipocytes were incubated with insulin (100 nM) and/or ghrelin (1 µM) for the indicated times. After the stimulation period, cells were exposed to 50 µl of 2-deoxy-[^{3}H]glucose (0.5 µCi/ml final concentration) for 4 min, washed in ice-cold PBS three times, and lysed in 0.1% SDS. The radioactivity incorporated was determined using liquid scintillation counting.

Oil red O staining

Dishes were washed twice with phosphate-buffered saline and fixed with 10% buffered formalin for at least 1 h at room temperature. Cells were then stained for 2 h at room temperature with a filtered oil red O solution (0.5 g of oil red O in 100 ml of isopropanol) and washed twice with water afterwards.

Gene expression analysis

Reverse transcription (RT) followed by polymerase chain reaction (PCR) was performed in order to analyze UCP-1 and adiponectin mRNA expression with hypoxanthine guanine phosphoribosyl transferase (HPRT) analyzed as a housekeeping gene control. Briefly, total RNA isolation from stimulated brown adipocytes was performed using the TRIzol reagent (Invitrogen; Karlsruhe, Germany) followed by cleanup using the RNeasy kit (Qiagen; Hilden, Germany). RNA quality was tested by RNA visualization on an agarose gel and photometry analysis. Up to 2 µg total RNA was reverse-transcribed using Superscript II (Invitrogen; Karlsruhe, Germany) and an oligo $p(DT)_{15}$ primer (Roche Molecular Biochemicals; Mannheim, Germany) in the presence of RNase inhibitor (Roche Molecular Biochemicals; Mannheim, Germany) in a 20 µl reaction. 1 µl of each RT reaction was amplified in a 25 µl PCR containing 2.5 mM $MgCl_2$, 250 nM of each primer, and 1X QuantiTect SYBR Green PCR-Mix (Qiagen; Hilden, Germany). PCR was performed on a GeneAmp 5700 Sequence Detection System (Applied Biosystems; Foster City, CA, USA). The following primers were used: UCP-1 (acc. no. M21222 + M21244) 5'-ATG GTG AAC CCG ACA ACT TCC GAA GTG-3' (sense) and 5'-GTA CTG GAA GCC TGG CCT TCA CCT TGG-3' (antisense); Adiponectin (acc. no. AF304466) 5'-AAG GAC AAG GCC GTT CTC T-3' (sense) and 5'-TAT GGG TAG TTG CAG TCA GTT GG-3' (antisense); HPRT (acc. no. NM_013556) 5'-GTT GGA TAC AGG CCA GAC TTT GT-3' (sense) and 5'-CAC AGG ACT AGA ACA CCT GC-3' (antisense). PCR for all targets was performed as follows: initial denaturation at 95 °C for 900 s, 40 cycles with 95 °C for 20 s, 55 °C for 20 s, 72 °C for 20 s. Specific amplification was confirmed by producing melting curve profiles (cooling the samples to 65 °C for 10 s and heating to 95 °C in steps of 0.2 °C/s with continuous measurement of fluorescence). Optimized relative quantification was performed by using the Relative Expression Software Tool (REST) [23].

Statistical analysis

Sigma Plot software (SPSS Science; Chicago, IL, USA) was used for statistical analysis of all data. Results are presented as the mean ± SEM. Student's *t*-test for unpaired variables was used for determination of statistical significance. Values of $p < 0.05$ were considered significant, those < 0.01 highly significant.

Results

Ghrelin does not alter adipocyte differentiation

Chronic stimulation of differentiating cells with 1 µM ghrelin did not affect the increasing extent of fat accumulation per tissue culture plate over the differentiation period as assessed by staining cells with the fat-specific oil red O (Fig. **1**). This stain indicates the amount of stored intracellular triglycerides by red color intensity. Furthermore, the microscopic pattern of multilocular fat droplets typical of fully differentiated brown adipocytes was unchanged in ghrelin-treated cells (data not shown).

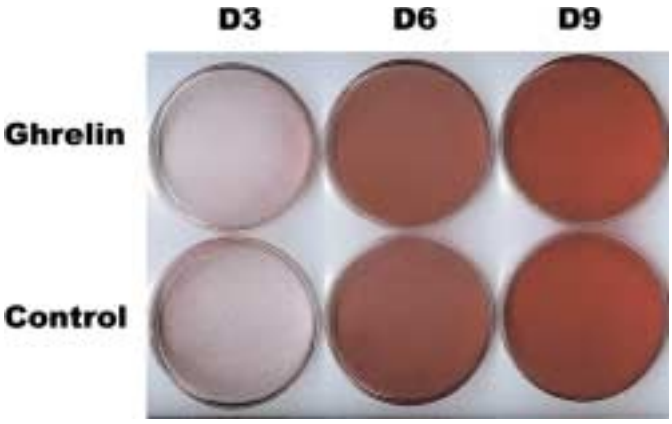

Fig. **1** Ghrelin does not affect adipocyte differentiation. Oil red O-stained cells at the indicated days (D) of differentiation (after the end of the induction period).

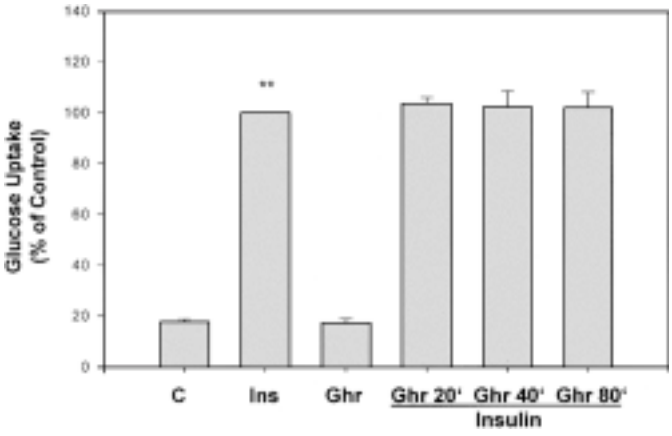

Fig. **2** Insulin-stimulated glucose uptake is unaltered by ghrelin pretreatment. Differentiated adipocytes were treated with insulin (Ins) (100 nM) for 30 min or ghrelin (Ghr) (1 μM) alone or in combination for the indicated periods of time (ghrelin prior to insulin). ** $p < 0.01$ comparing non-treated control cells (C) to cells treated with insulin alone. A bar graph analysis of 4 independent experiments is shown.

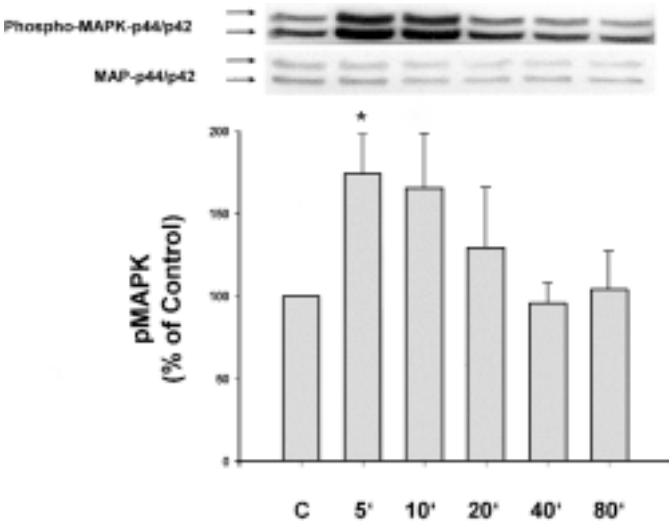

Fig. **3** Ghrelin induces MAP kinase activation. Differentiated cells were treated with ghrelin (1 μM) for the indicated periods of time and immunblots of cell lysates were prepared using a phosphospecific p44/p42 MAP kinase antibody. * $p < 0.05$ comparing non-stimulated control (C) to ghrelin-treated cells. A representative blot and a bar graph analysis of three independent experiments are shown. As loading control for total protein amounts a representative immunoblot using a p44/p42 MAP kinase antibody is shown below the phosphospecific blot.

Ghrelin does not affect insulin-induced glucose uptake

Insulin-induced glucose uptake is a hallmark of adipocyte function, and a ghrelin-insulin axis may play a role in the control of energy homeostasis. Insulin stimulation for 30 min resulted in a highly significant approximately five-fold increase in glucose uptake in fully differentiated brown adipocytes as compared with non-treated cells (Fig. **2**). Ghrelin stimulation alone did not have any effect on basal glucose uptake. Furthermore, pre-treatment for periods between 20 and 80 min did not alter the insulin-induced positive effect (Fig. **2**).

Ghrelin induces MAP kinase activation

To investigate adipocyte signaling, serum-starved mature adipocytes were stimulated with ghrelin for time periods from 5 to 80 min. There was no significant stimulation of JAK/STAT-, p38 stress kinase-, protein kinase A (PKA)-, and phosphatidylinositol 3-kinase (PI 3-Kinase) signaling pathways as assessed by using phosphospecific antibodies against STAT3, p38 MAP kinase, CREB, and Akt (data not shown). However, acute stimulation by ghrelin (1 μM) significantly increased p44/p42 MAP kinase phosphorylation by almost 2-fold as compared to basal levels without altering the amount of total protein (Fig. **3**). This effect was time-dependent and maximal after 5 min (Fig. **3**). Furthermore, ghrelin-induced increases could be observed at ghrelin concentrations as low as 0.1 nM (data not shown).

Ghrelin inhibits adiponectin gene expression

Given the prominent role of MAP kinase in regulation of gene transcription, we finally tested potential ghrelin effects on the thermogenic brown fat-specific UCP-1, leptin, and on adiponectin, a newly discovered adipokine involved in the pathogenesis of obesity and insulin resistance. Ghrelin stimulation between 30 min and 4 h did not significantly affect basal UCP-1 mRNA expression in differentiated brown adipocytes (Fig. **4**). There was a trend towards an acute 30% increase in UCP-1 mRNA levels after 30 min of ghrelin stimulation (Fig. **4**). Furthermore, ghrelin did not have any effect on leptin gene expression (data not shown). Interestingly however, basal adiponectin mRNA expression was decreased by approximately 50% in ghrelin-treated cells (Fig. **4**). This inhibition already occurred after 30 min and lasted for up to 4 h (Fig. **4**).

Discussion

Here, we demonstrate direct effects of the newly discovered stomach-derived hormone ghrelin on adipocyte signaling and adipokine expression. Evidence is rapidly accumulating that indicates a pivotal role for ghrelin, not only in the regulation of growth hormone secretion but also in the central nervous system control of energy homeostasis [1, 2, 24 – 27]. It has been demonstrated that ghrelin acts on hypothalamic neurons that regulate

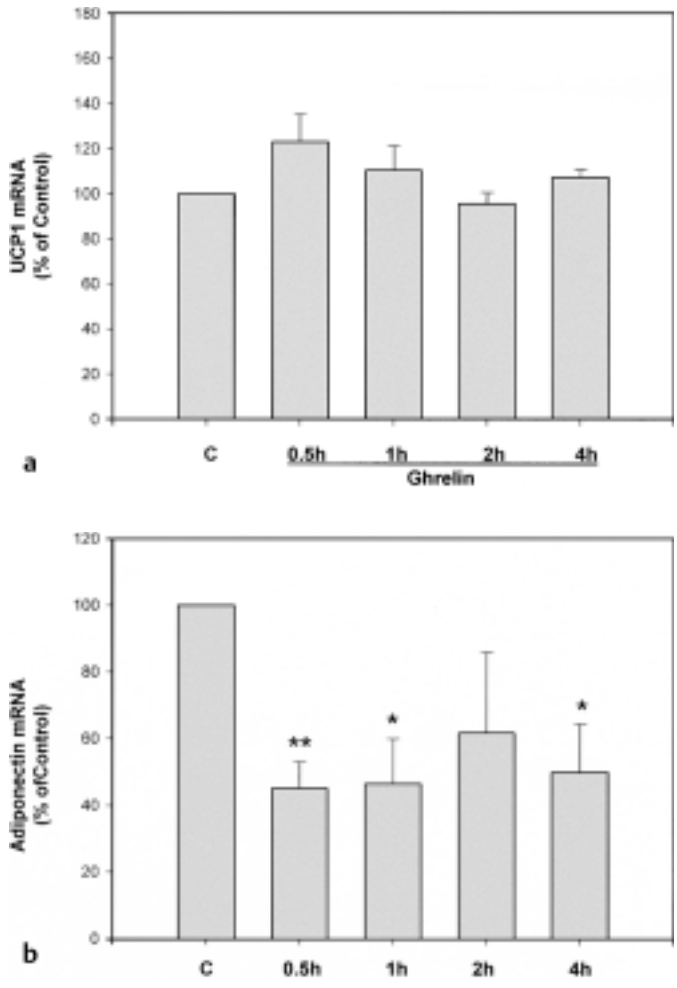

Fig. **4** Ghrelin inhibits adiponectin gene expression. Mature adipocytes were stimulated with ghrelin (1 μM) for the periods of time indicated and quantitative mRNA expression analysis of uncoupling protein-1 (UCP-1) (**a**) and adiponectin (**b**) was performed as described in **Materials and Methods**. $^{*}p < 0.05$ and $^{**}p < 0.01$ comparing non-stimulated (C) to ghrelin-treated cells. Bar graph analyses of 5 (**a**) and 3 (**b**) independent experiments are shown.

energy balance [28], and intracerebroventricular ghrelin injections result in significant increases in food intake in rats [10, 29]. However, peripheral actions of this stomach hormone – particularly on tissues that contribute to the control of energy homeostasis – are poorly understood, although the ghrelin receptor is expressed in adipocytes, for example [30]. Other studies have described stimulatory effects on pancreatic insulin secretion [31 – 33] and insulin signaling in hepatoma cells [34].

Our data presented here are the first results characterizing acute and chronic influences of ghrelin on adipocyte signaling and endocrine function. The data presented here expand the spectrum of biological activities of this novel hormone to adipose tissue and highlight the potential relevance of an adipose-gastric interplay for mediating ghrelin's anabolic effects.

First preliminary evidence suggestive of a potential role for ghrelin in influencing adipocyte biology has been derived from studies demonstrating expression of ghrelin and a putative ghrelin receptor subtype in fat [1, 30, 35]. We have found a selective activation of the MAP kinase-signaling pathway in our experiments. This is consistent with data from 3T3 L1 adipocytes [36] and hepatoma cells [34]. However, signaling elements mediating this activation and potential downstream effectors remain unknown to date.

We used a previously characterized brown adipocyte cell model in our studies [15 – 21, 37]. Brown adipocytes display a number of features that are similar to white adipocytes, in particular insulin sensitivity and leptin secretion. In addition, brown-adipose tissue contributes to energy homeostasis by its thermogenic capacity that is mediated by the mitochondrial protein UCP-1. We did not find a significant alteration in gene expression of this protein by ghrelin. This can be taken to suggest that ghrelin's effects on adipocytes may not involve thermogenesis, and may not be brown-fat-specific.

Our data do not support a role for ghrelin on adipocyte differentiation or insulin-activated endpoints such as glucose uptake. In view of reports on ghrelin effects on pancreatic [31 – 33] and liver cells [34], complex antipodal interactions between ghrelin and insulin action in insulin-sensitive tissue must be postulated.

However, this is the first study to demonstrate that ghrelin triggers a robust acute and sustained inhibition of adiponectin expression in differentiated adipocytes. Adiponectin is a novel adipokine that has been implicated in the pathogenesis of obesity and insulin resistance in rodents and humans [38]. Adiponectin levels are decreased in obese states and increased in individuals losing weight [38]. Furthermore, it has been demonstrated that administration of adiponectin improves insulin resistance *in vivo* in mice [39, 40], and that disruption of the adiponectin gene results in diet-induced insulin resistance [41]. These data indicate that adiponectin is not only an adipokine passively downregulated by insulin resistance, but it also actively influences insulin sensitivity. From a teleological point of view, our findings appear consistent with weight gain-inducing effects of ghrelin [10]. Furthermore, they demonstrate a potential physiological mechanism for earlier reports, showing that ghrelin receptor agonists can induce insulin resistance [42]. Moreover, they lend support to the hypothesis that adipose tissue may contribute to ghrelin's effects on energy homeostasis, which points to another level of complexity in the network of signaling pathways connecting peripheral tissues with the brain. In view of adiponectin's potential role in regulating functions of the vascular wall [43, 44], it is also tempting to speculate on a possible modulation of these processes by ghrelin. However, a general caveat of this study is its focus on gene expression level. Analyses of adiponectin protein levels in hyperghrelinemic states are underway, and represent a necessary extension of the findings presented here. Finally, as we have demonstrated previously, regulation of adiponectin is multifactorial, and most likely the result of a complex interplay of different hormones and cytokines [45, 46].

In summary, we demonstrate direct influences of ghrelin on adipocyte signaling and metabolism. By virtue of regulation of adipokine expression, this may assign a novel role for adipose tissue as an important relay station in mediating ghrelin's effects on energy homeostasis.

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