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CRF Type 2 Receptors Mediate the Metabolic Effects of Ghrelin in C2C12 cells

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Abstract

Objective—Ghrelin is known to regulate appetite control and cellular metabolism. The Corticotropin-Releasing Factor (CRF) family is also known to regulate energy balance. In this study, we investigated the links between ghrelin and the CRF family in C2C12 cells, a mouse myoblast cell line.

Design and methods—C2C12 cells were treated with ghrelin in the presence or absence of CRF receptor antagonists and then subjected to different metabolic analyses.

Results—Ghrelin enhanced glucose uptake by C2C12 cells, induced GLUT4 translocation to the cell surface and decreased RBP4 expression. A CRF-R2 selective antagonist, anti-sauvagine-30, blocked ghrelin-induced glucose uptake, Ghrelin upregulated CRF-R2 but not CRF-R1 levels. Moreover, ghrelin-treated C2C12 cells displayed a cAMP and pERK activation in response to Ucn3, a CRF-R2 specific ligand, but not in response to CRF or stressin, CRF-R1 specific ligands. Ghrelin also induced UCP2 and UCP3 expression, which were blocked by anti-sauvagine-30. Ghrelin did not induce fatty acids uptake by C2C12 cells or ACC expression. Even though C2C12 cells clearly exhibited responses to ghrelin, the known ghrelin receptor, GHSR1a, was not detectable in C2C12 cells.

Conclusion—Our results suggest that, ghrelin plays a role in regulating muscle glucose and, raise the possibility that suppression of the CRF-R2 pathway might provide benefits in high ghrelin states.

Keywords

Ghrelin; CRF; metabolism; glucose

Introduction

Ghrelin is a gut-brain peptide known to have a role in growth hormone regulation and in appetite control [1]. Two major forms of ghrelin are found in plasma: an acylated ghrelin

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Disclosure Summary

W.W.V. and E.G. have nothing to disclose.

with an O-*n*-octanoylated serine in position 3 and a non-acylated form, known as des-acyl ghrelin (2). The acylation is considered necessary for ghrelin actions via the growth hormone secretagogue receptor type 1a (GHSR1a) [1, 2]. In addition to appetite control regulation, ghrelin takes part in many other physiological processes, including control of metabolism in a variety of cell types, including muscle [3–8].

The effects of ghrelin on muscle metabolism have been demonstrated in several species. In rats, four days of ghrelin injection reduced the triglyceride content in gastrocnemius muscle but had no effect on triglyceride levels of soleus muscle [3, 4]. The soleus muscle of these rats, however, displayed enhanced AKT signaling and glucose transporter-4 (GLUT4) mRNA expression [4]. In addition, the ghrelin-injected rats exhibited higher blood glucose levels compared to control animals [4]. In human muscle, administration of exogenous ghrelin caused insulin resistance and stimulated lipolysis [9].

In addition to ghrelin, many other factors regulate food intake and energy balance in skeletal muscle. Of interest for this work is the Corticotropin-Releasing Factor (CRF) family of peptides [10]. CRF family peptides signal through two related receptors, CRF-R1 [11] and CRF-R2 [12, 13]. The CRF-R2 isoform has three functional splice variants in human (α , β , and γ) and two in mice and rat (α and β) that are produced by the use of alternate 5' exons [14–17]. Previous studies have shown that CRF-R2 is highly expressed in skeletal muscle [13, 18].

A link between ghrelin and the CRF family has been suggested by several studies; conflicting results have also been published. One study demonstrated that the administration of ghrelin stimulated food intake by increasing meal size in both WT and CRF-R2 null mice [19]. However, another study in rats showed that the effect of ghrelin on gastric motility was blocked by either the local administration of a CRF-R2 antagonist [20] or by microinjection of the CRF receptor antagonist astressin into the PVN [21]. Furthermore, ghrelin was shown to stimulate CRF expression and secretion both *in vitro* [22] and *in vivo* [23]. Intraperitoneal injection of ghrelin was found to upregulate hypothalamic CRF mRNA levels and also elevates circulating corticosterone levels [24]. Finally, administration of ghrelin to pregnant female mice led to reduced exploratory behavior and elevated CRF and ghrelin levels in their pups [25].

The fact that both ghrelin and the CRF family exert metabolic effects on muscle cells, combined with evidence from studies demonstrating that CRF receptors mediate some ghrelin actions, led us to investigate the possible regulatory and functional links between the metabolic effects of ghrelin and the CRF/Ucn systems in muscle cells. Specifically, we explored the effects of ghrelin on CRF/Ucn receptor expression, activity and signaling pathways and investigated whether ghrelin effects are dependent upon CRF/Ucn signaling. In this study, we demonstrate that ghrelin upregulates CRF receptor expression and signaling in C2C12 cells. We further provide evidence for ghrelin effects on C2C12 metabolism. Finally, we show that the ghrelin-induced metabolic changes in C2C12 cells can be blocked by selective CRF receptor antagonist.

Materials and Methods

Reagents

Ghrelin, CRF, stressin, Ucn3 and anti-sauvagine-30 were synthesized and generously provided by Dr. Jean Rivier (Salk Institute). Antalarmin was a gift of Dr. G. Chrousos. Acetyl-CoA carboxylase, phospho-ERK and total-ERK antibodies as well as the HRP linked secondary antibody were purchased from Cell Signaling (Danvers, MA). Anti-actin antibody was purchased from AbCam (Cambridge, MA). GLUT4 antibody was purchased from Millipore (Temecula, Ca), this antibody was raised to the C-terminus (amino acids 498–510) of mouse GLUT-4. RNA extraction kit was purchased from QIAgen (Valencia, CA). High capacity cDNA synthesis kit was purchased from Applied Biosystems (Carlsbad, CA). LightCycler 480 SYBER Green Master mix for real time PCR was purchased from Roche (Mannheim, Germany). Hotmaster taq DNA polymerase was purchased from 5-prime (Gaithersburg, MD). The mounting solution, containing DAPI, Vectastain, was purchased from Vector laboratories (Burlingame, CA). Alexa488-conjugated anti-rabbit secondary antibody was purchased from Invitrogen (Carlsbad, CA). Oil red O, Oleic acid and palmitic acid were purchased from Sigma (St. Louis, MO).

Cells

C2C12 cells, a mouse myoblast cell line (obtained from ATCC), were grown in DMEM (Invitrogen, Carlsbad, CA) with 10% fetal calf serum (FBS, Hyclone, Logan, Utah) at 37°C under 5% CO₂. For ghrelin treatments, cells were plated in 12 well Costar plates, allowed to recover for 24 h, then ghrelin or vehicle was added to the medium for the indicated durations. When treated with CRF antagonists, the cells were plated in 12 well plates, allowed to recover for 24h, at which time ghrelin or vehicle was added for 24 hours. Then, antalarmin, a CRF-R1 specific antagonist or anti-sauvagine-30, a CRF-R2 specific antagonist, was added for an additional 48 hours. The medium with both ghrelin and the inhibitors was refreshed every 24 hours until the end of the incubation. At the end of the incubations, cells were harvested and evaluated as described below.

C2C12 membrane enrichment and GLUT4 Western blot analysis

C2C12 membrane fractions were prepared as described previously [11]. Briefly, C2C12 cells were treated with 100 nM ghrelin or vehicle. After 72 hours, cells were washed with HDB (Hepes dissociation buffer) and detached by incubation with 0.5 mM EDTA in HDB for 15 min at ambient temperature. The cells were washed twice more with HDB and homogenized in 5% sucrose. The homogenates were centrifuged at 600 × g for 5 min, after which the supernatants were removed and centrifuged at 40,000 × g for 20 min. The resulting membrane fractions were resuspended and protein concentrations were measured and adjusted to 1–4 mg/ml in 10% sucrose. Western blot analysis was performed as described below.

Glucose uptake by C2C12 cells

Glucose uptake by C2C12 cells was measured as described previously [18]. Briefly, cells were incubated with low glucose, serum-free DMEM containing 0.1% BSA for 2h. Cells

were then washed with Hank's balanced saline solution (HBSS) and incubated with the same buffer for an additional 2h. Insulin was added at 10nM concentration, and incubation was continued for an additional 30 min, after which a mixture of [³H]-deoxyglucose (0.2mCi/ml) and nonradioactive 2-deoxyglucose (final concentration, 0.1mM) was added to the cells for additional 5 min. Cells were rapidly washed 4 times with ice-cold PBS and incubated with 1M NaOH for 30 minutes. An aliquot for protein determination was taken before neutralizing the samples with 1M HCl. The extracts were counted for radioactivity in EcoLume scintillation fluid using a beta-counter.

RNA extraction and RT-PCR analysis

Total RNA from C2C12 cells was extracted using RNeasy mini columns, according to the manufacturer's guidelines. RNA was converted into cDNA with the High-Capacity cDNA kit. Gene expression was quantified by qRT-PCR using SYBR green chemistry on a Roche Lightcycler 480 platform). Relative expression levels (RRct) were calculated by normalizing to hypoxanthine guanine phosphoribosyl transferase (HPRT). Primers (supplementary Table S1) were designed using the primer3 website (<http://frodo.wi.mit.edu/primer3>). The same cDNAs were also used for semi-quantitative PCR amplification of GHSR1a and β -actin.

Immunofluorescence

C2C12 cells were plated on polylysine coated cover slips in 12 well Costar plates. The cells were incubated in DMEM with 10% FBS for 24 hours after which ghrelin or vehicle was added for 72 hours. The cells were washed once with PBS and fixed with 4% paraformaldehyde for 20 minutes at room temperature and washed again with PBS. The cells were incubated in blocking solution (2% normal goat serum and 0.2% Triton x100 in PBS) and incubated overnight at 4°C with an anti-GLUT4 primary antibody (dilution 1:100 in blocking solution). The next day, cells were washed 3 times with PBS and incubated at room temperature for 1 hour with an Alexa488-conjugated anti-rabbit secondary antibody (dilution 1:500 in blocking solution). Cells were washed 3 times with PBS and mounted on slides with Vectastain mounting solution containing DAPI. Staining was visualized using Zeiss LSM 710 Laser Scanning Confocal Microscope.

cAMP Measurements

C2C12 cells were plated in DMEM with 10% FBS into 24-well Costar tissue culture dishes and allowed to recover for 24 h. Cells were treated with vehicle or ghrelin for 72 hours. The medium was changed to DMEM with 0.1% FBS at least 2 h before treatments. The cells were preincubated for 30 min with 0.1 mM 3-isobutyl-1-methylxanthine and then exposed to stressin, CRF or Ucn3 for 30 min at 37 C. Intracellular cAMP was measured from triplicate wells using an RIA kit (Biomedical Technologies, Stoughton, MA).

ERK activation

C2C12 cells were plated in DMEM with 10% FBS into 24-well Costar tissue culture dishes and allowed to recover anotherfor 24 h. Cells were treated with vehicle or ghrelin for 72 hours. The cells were equilibrated in DMEM with 24% FBS during the last 24 hours before

they were exposed to CRF or Ucn3 for 5 min at 37 °C. Cells were harvested immediately in 100 µl of sample treatment buffer [50 mM Tris.HCl, pH 6.8, 100 mM DTT, 2% (wt/vol) SDS, 0.1% (wt/vol) bromophenol blue, 10% (wt/vol) glycerol]. The samples were boiled for 5 min then analyzed using Western Blot technique as described in the next section.

Western blot analysis

Total C2C12 cells proteins were extracted, and Laemmli buffer (125 mM Tris, pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, and 2% s-mercaptoethanol) was added. The samples were then separated on a 12% acrylamide gel, followed by their transfer to a nitrocellulose membrane. After blocking with 10% skimmed milk, the membranes were incubated with the primary antibodies overnight at 4°C (dilution for GLUT4- 1:1000, for pERK and total ERK-1:5000 in 5% milk in TBS-T), then with the secondary antibodies for 1 h at room temperature (dilution for secondary 1:5000 in 5% milk in TBS-T). The immunoreactive bands were detected by ECL (Amersham, Buckinghamshire, England).

Statistical analysis

For statistical comparisons, replicate experiments were averaged and analyzed by the two way ANOVA test and considered statistically different when $P < 0.05$. All numerical data shown in the figures are from representative experiments expressed as the means $\pm$ S.E.M of replicates.

Results

The effects of ghrelin on muscle metabolism have been previously demonstrated [3–8]. We examined if ghrelin has similar effects on C2C12 cells. Ghrelin treatment dose-dependently increased the uptake of glucose into C2C12 cells (Fig. 1A). To determine if the CRF/Ucn peptide family participates in this action of ghrelin, we measured glucose uptake in C2C12 cells treated with ghrelin in the presence or absence of CRF receptor isoform-selective antagonists. Antalarmin, a CRF-R1 specific antagonist, when added concomitantly with ghrelin during the last 24h of incubation, had no effect on the ghrelin-induced rise in glucose uptake (Fig. 1B). By contrast, the addition of anti-sauvagine-30, a CRF-R2 specific antagonist, blocked the ghrelin-induced glucose uptake into C2C12 cells (Fig. 1C). These results suggested that ghrelin effects on glucose uptake by C2C12 cells are linked to CRF-R2 activation.

Previous studies have established that urocortin effects in muscle are mediated by CRF-R2 and that Ucn2 is the major peptide of the CRF family expressed in this tissue [18]. We therefore examined whether C2C12 express Ucn2 and either or both of the two CRF receptor isoforms. Using semi-quantitative PCR, we observed that C2C12 cells express Ucn2, CRF-R1 and CRF-R2 (Fig. 2A). Next, we investigated whether chronic treatment of C2C12 cells with ghrelin is associated with changes in the expression of Ucn2, CRF-R1 or CRF-R2. Real-time PCR analysis revealed that ghrelin had no effect on the level of Ucn2 (Fig. 2B) or CRF-R1 expression (Fig. 2C–D). Ghrelin, however, caused a time- and dose-dependent upregulation of CRF-R2 mRNA levels (Fig. 2E–F). We further examined the effect of des-acyl ghrelin, the other isoform of ghrelin on CRF receptors expression. Des-

acyl ghrelin significantly increased the expression levels of CRF-R1 (fig. 2G–H) as well as CRF-R2 (fig. 2I–J) in a time- and dose-dependent manner. This data suggest that both ghrelin and des-ghrelin might be using the same pathway and effect similarly on C2C12 cells glucose uptake.

The prolonged treatment with ghrelin has been reported to induce differentiation of C2C12 myoblasts to myotubes [26]. Moreover, it has been shown that differentiation of C2C12 to myotubes is associated with CRF-R2 up-regulation [27]. Consistent with these reports, we show that a 72 hour, but not a 24 hour, exposure of C2C12 cells to ghrelin caused a time- and dose-dependent downregulation of the negative regulator of myogenesis, Id2 (Supp. Fig. S1A–B). In addition, ghrelin exposure led to a minor induction of MyoD, a myogenic determination factors (Supp. fig. S1C–D). These data suggest that the upregulation in CRF-R2 expression in differentiated C2C12 cells is a mechanism that facilitates ghrelin's actions and promotes glucose metabolism. To determine if ghrelin-induced changes in CRF-R2 expression alters signaling via this receptor isoform, we measured the effects of ghrelin pre-treatment on cAMP accumulation and pERK induction in response to Ucn3, a CRF-R2 selective agonist as well as in response to CRF-R1 specific agonists, stressin and CRF. Ghrelin by itself did not produce a statistically significant effect on cAMP levels of C2C12 cells (Fig. 3A–C). The CRF-R1 selective agonists, stressin and CRF, also failed to modify cAMP accumulation in ghrelin-treated or untreated C2C12 cells (Fig. 3A–B). By contrast, Ucn3, a CRF-R2-selective peptide, had a small effect on basal cAMP levels in vehicle-treated cells and dose-dependently stimulated cAMP accumulation in ghrelin-treated C2C12 cells (Fig. 3C). In addition, Ghrelin by itself did not cause ERK activation in C2C12 cells (Fig. 3D–E). CRF also failed to stimulate ERK phosphorylation in both ghrelin-treated and untreated C2C12 cells (Fig. 3D). However, Ucn3 dose-dependently stimulated ERK phosphorylation in ghrelin-treated C2C12 cells (Fig. 3E). Taken together, these results suggest that ghrelin can influence CRF-R2 mediated responses of C2C12.

One of the key players in glucose uptake is GLUT4, a glucose transport protein. We examined the effect of ghrelin on GLUT4 expression and found no effect (Fig 4A). The main regulation on GLUT4 activity is the translocation of GLUT4 from the ER to the cell surface. Therefore, we examined the possibility that ghrelin regulates GLUT4 translocation to the cell membrane. Indeed, we found that ghrelin induced GLUT4 translocation to the cell surface, as determined by immunofluorescence (Fig 4B) and Western blot analysis of membranes extracted from ghrelin-treated C2C12 cells (Fig. 4C). These experiments demonstrated that GLUT4 signal was enriched in membranes isolated from ghrelin-treated cells compared to membranes from vehicle-treated cells (Fig. 4C).

To further examine the effects of ghrelin on the metabolic profile of C2C12 cells, we tested several parameters. Retinol binding protein 4 (RBP4), is an adipokine shown to be linked to reduced insulin sensitivity and to diabetes. Treating C2C12 cells with ghrelin caused a downregulation of RBP4 mRNA levels in a time- and dose-dependent manner (Fig. 5A–B). Consistent with the effects on glucose uptake, the change in RBP4 expression by ghrelin was blocked by anti-sauvagine-30 (Fig. 5C). Anti-sauvagine-30 by itself had no effect on RBP4 expression (data not shown).

We further found that ghrelin time- and dose-dependently increased the expression levels of UCP2 (fig. 6A, C) and UCP3 (fig. 6B, D), two genes that play an important role in controlling energy homeostasis in cells. These UCP2 and UCP3 responses to ghrelin were blocked by anti-sauvagine-30 (Fig. 6E–F). Anti-sauvagine-30 alone had no effect on UCP2 and UCP3 expression (data not shown). By contrast to these results, ghrelin had no effect on fatty acid uptake in C2C12 cells (fig. 7A), nor did it alter acetyl-CoA carboxylase (ACC) levels, an enzyme that catalyzes a pivotal step in fatty acid synthesis pathway (fig. 7B).

The actions of ghrelin are largely mediated via GHSR1a [1, 2]. We therefore examined if C2C12 cells express GHSR-1a, and if ghrelin treatment alters its expression. Previous studies have provided conflicting data about GHSR1a expression in C2C12 [26, 28]. In agreement with one of these studies ([26]), we did not detect GHSR1a transcripts in either ghrelin-treated or untreated C2C12 cells, as determined by quantitative real-time PCR (Fig. 8A–B). By contrast, GHSR1a mRNA expression in mouse pituitaries was readily detectable (Fig. 8A–B). These results suggest that as yet an unidentified receptor must mediate the actions of ghrelin in C2C12 cells.

Discussion

In this study, we have demonstrated that ghrelin is able to induce changes in vital cellular functions of C2C12 cells, such as glucose uptake. By analyzing the actions of ghrelin on C2C12 cells, we have uncovered a functional link between CRF-R2 signaling and ghrelin actions in these cells. Our data demonstrate that anti-sauvagine-30, a CRF-R2 selective antagonist blocks the action of ghrelin on glucose uptake by C2C12 cells. We show that ghrelin upregulates CRFR2 levels in these cells without affecting CRF-R1. Consistently, ghrelin-treated cells respond to Ucn3, a CRF-R2 selective peptide, with an increase in cAMP accumulation and ERK activation whereas CRF-R1 selective peptides, stressin and CRF, have no effect on either cAMP or ERK activation in ghrelin-treated or untreated cells. These observations suggest that ghrelin has an important role in regulating C2C12 functions and that excessive ghrelin signaling could interfere with normal metabolic functions of these cells. Our findings also provide additional support for a link between ghrelin and the CRF system.

The prolonged treatment with ghrelin has been reported to induce differentiation of C2C12 myoblasts to myotubes [26]. Moreover, it has been shown that, whereas C2C12 myoblasts express CRF-R1 but not CRF-R2, differentiation of C2C12 to myotubes is associated with CRFR2 up-regulation [27]. Consistent with these reports, we show that CRF-R2 expression is upregulated in ghrelin-treated cells and that ghrelin treatment of C2C12 cells is associated with metabolic effects. These data suggest that the upregulation in CRF-R2 expression in differentiated C2C12 cells is a mechanism that facilitates ghrelin's actions and promotes glucose metabolism. In addition, our findings that ghrelin decreases the expression of Id2, a negative regulator of myogenesis and induces the expression of MyoD, a myogenic determination factor raise the possibility that CRF-R2 mediates the differentiation effects of ghrelin on C2C12 cells by promoting glucose metabolism. Our data further suggest that glucose metabolism stimulation by CRF-R2 is necessary for facilitating ghrelin action on C2C12 cells differentiation. However, the minor effect we observed on the differentiation

markers expression, suggest that ghrelin by itself is not sufficient for C2C12 differentiation and other means, such as reduced serum concentrations in the culture medium are necessary for fully C2C12 differentiation to happen. Our data showing that upregulation of CRF-R2 is also associated with RBP4 downregulation is also consistent with a previous report demonstrating that RBP4 expression is significantly lower in skeletal muscle isolated from CRF-R2 null mice compared to WT [27]. These observations, combined with our data showing that a CRF-R2 antagonist can block the effect of ghrelin on RBP4 expression in C2C12 cells, provide further support to the notion that the CRF receptor system mediates the metabolic effects of ghrelin in C2C12 cells. Such a link is not unique, as similar associations between ghrelin and the CRF family have been documented both in the central nervous system and the periphery [19–25].

The CRFR2 pathway has been implicated as a physiological system that affects the known reciprocal relationships between psychological and physiological challenges and the metabolic syndrome [27]. One study showed that Ucn2 is highly expressed in skeletal muscle [18] and probably serves as the endogenous local ligand for skeletal muscle CRF-R2 to inhibit the interactions between insulin-signaling pathway components and insulin-induced glucose uptake, as demonstrated in cultured skeletal muscle cells, and in C2C12 myotubes [18]. These published studies and our data from C2C12 cells presented here are consistent with the possibility that CRF-R2 might be a downstream effector of ghrelin action and as such may be involved in mediating ghrelin effects on skeletal muscle metabolism. These observations further imply that CRF-R2 is a player in modulating metabolic effects of ghrelin in response to psychological and physiological challenges, an idea that is supported by the association between ghrelin and CRF family in the central nervous system [19, 22, 24, 25] as well as in peripheral tissues [20]. Our data demonstrate that the CRF family is regulating the effects of ghrelin not only on gut motility or on central nervous system functions, but also in muscle.

Consistent with a previous report using cardiomyocytes, we did not observe any effect of ghrelin on fatty acid metabolism or uptake [29]. Similar observations of ghrelin having effects on glucose but not fatty acid metabolism have been reported from *in vivo* studies. Mice treated with ghrelin showed increased glucose disposal as compared to control [30]. Another study detected lower glucose levels and higher sensitivity to insulin in mice lacking both ghrelin and leptin (ob/ob mice) [31]. This study further suggested that the increase in glucose sensitivity might be explained by a decrease in UCP2 expression [31]. Similarly, other studies have also demonstrated that chronic ghrelin infusion leads to a decrease in UCP2 mRNA levels in white adipose tissue [32], liver [3] and pancreas [31]. In addition, chronic i.p. ghrelin treatment to UCP2-null mice significantly increased body fat and body weight as compared to WT mice [32]. These results combined with our results imply that ghrelin plays an opposite role in adipose tissues and in muscle. While in adipocytes, ghrelin seems to enhance lipogenesis and fat storage [30, 32], in C2C12 cells, ghrelin has no effect on fatty acid metabolism and favors the glucose pathway by inducing UCP2 expression. The increase in UCP2 promotes fat oxidation and restricts lipogenesis [32]. Taken together, our results and those of previous studies demonstrate that the ghrelin plays a more important role in the regulation of cellular glucose metabolism rather than the fatty acid metabolic

pathway. Given that fatty acids inhibit C2C12 differentiation [33, 34], an intriguing possibility is that ghrelin's effects on C2C12 differentiation might be linked to ghrelin's actions to upregulate UCP2 expression and to reduce fatty acid content and lipogenesis.

To date, the only known receptor for ghrelin is GHSR1a. We did not detect any GHSR1a mRNA expression in C2C12 cells, consistent with one published study but in contrast to another study that reported the presence of GHSR1a in differentiated C2C12 cells [26, 28]. Filigheddu et al., did not find evidence for GHSR1a expression but reported that C2C12 cells express high affinity binding sites recognized by both ghrelin and des-acyl-ghrelin [26]. This is in line with our data showing that, despite the lack of detectable GHSR1a expression, ghrelin and des-acyl ghrelin exert similar actions on CRF-R2, but not CRF-R1, expression in C2C12 cells and act via overlapping pathways including pAkt activation (data not shown). These observations are consistent with the idea that a common receptor or pathway, other than GHSR1a, is involved in mediating the actions of ghrelin and des-acyl-ghrelin in C2C12 cells.

High ghrelin levels are observed in many diseases including obesity [35], which often leads to the development of type 2 diabetes, as well as Prader-Willi syndrome [36–38] and anorexia nervosa [39, 40]. Exploring the actions of ghrelin on muscle development and function could provide a better understanding of the mechanisms of ghrelin actions affecting muscle metabolism, both in normal as well as in pathological conditions, and help in the development of new treatments for these various metabolic conditions. Our data provide a link between ghrelin and the CRF pathway in muscle metabolism and raise the possibility that suppression of the CRF pathway would be beneficial during high ghrelin states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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EG conceived, carried out experiments and analysed data. WWV conceived experiments. All authors were involved in writing the paper and had final approval of the submitted and published versions.

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‘What is already known about this subject’

- In human and rat muscle, administration of exogenous ghrelin affects glucose metabolism
- CRF-R2 is highly expressed in skeletal muscle and regulates its response to insulin
- A link between ghrelin and the CRF family has been suggested both in the central nervous system and in the periphery by several studies.

‘What this study adds’

- ghrelin plays a role in regulating muscle glucose, but not fatty acid metabolism
- CRF mediates ghrelin activity in muscle
- suppression of the CRF-R2 pathway might provide benefits in high ghrelin states

Fig. 1. Ghrelin regulation on glucose uptake can be blocked by CRF-R2 inhibitor
 Ghrelin increase H³-deoxy-glucose uptake into C2C12 cells (A). Antalarmin, a CRF-R1 specific antagonist, failed to block the ghrelin-induced glucose uptake (B), while anti-sauvagine 30 inhibited the uptake stimulated by ghrelin (C). The examples shown are representative of at least three experiments. * p<0.05 vs. control.

Fig. 2. Ghrelin effects on CRF family members expression levels

C2C12 cells express Ucn2, CRF-R1 and CRF-R2 (A). Incubating C2C12 cells with Ghrelin did not affect Ucn2 (B) and CRF-R1 expression (C and D), but increased CRF-R2 levels (E and F). Incubating C2C12 cells with des-acyl Ghrelin increased CRF-R1 (G and H) and CRF-R2 levels (I and J). The examples shown are representative of three experiments. * $p < 0.05$ vs. control

Fig. 3. Ghrelin effects on cAMP levels of C2C12 cells treated with CRF-R1 or CRFR2 selective ligands

Ucn3, but not stressin or CRF, stimulated cAMP accumulation in C2C12 cells pretreated with ghrelin (A–C). Ucn3, but not CRF, stimulated pERK activation in ghrelin pretreated C2C12 cells (D–E). Each panel shows the data from a representative of at least three experiments. * $p < 0.05$ vs. control.

Fig. 4. the effect of ghrelin on GLUT4

Ghrelin had no effect on GLUT4 mRNA expression levels in C2C12 cells (A); however, ghrelin stimulated GLUT4 translocation to the cell membrane as shown both by Immunofluorescence staining. Arrows indicate GLUT4 localization in the cytoplasm (Ba) and its translocation to the cell surface (Bb). In other experiments (C), western blot analysis with a specific antibody for GLUT4 was performed on membranes extracted from C2C12 either treated or not treated with ghrelin. The examples shown are representative of three experiments.

Fig. 5. RBP4 expression is regulated by ghrelin

Prolong exposure to Ghrelin down regulates RBP4 mRNA expression levels (A) in a dose dependent manner (B). Anti-sauvagine 30, a specific CRF-R2 inhibitor, blocked this effect of ghrelin on RBP4 mRNA levels (C). The examples shown are representative of at least three experiments. * $p < 0.05$ vs. control.

Fig. 6. UCP2 and UCP3 mRNA expression is regulated by ghrelin

Prolong exposure to Ghrelin increase both UCP2 and UCP3 mRNA expression levels (A–B) in a dose dependent manner (C–D). Anti-sauvagine 30, a specific CRF-R2 inhibitor, blocked this effect of ghrelin on both, UCP2 and UCP3 mRNA levels (E–F). The examples shown are representative of at least three experiments. * $p < 0.05$ vs. control.

Fig. 7. ghrelin does not regulate fatty acid uptake and fatty acid related protein expression
Ghrelin did not have an effect on fatty acid uptake into C2C12 (A) and did not modify the protein level of ACC (B). The examples shown are representative of at least three experiments.

Fig. 8. GHSR1a expression in C2C12 cells and mouse pituitaries

RNA was extracted from C2C12 cells treated with vehicle or increasing doses of ghrelin and real time PCR was performed using specific primers for GHSR1a. Expression was quantified relative to HPRT (A) and amplicon size was further verified on an agarose gel (B).