Ghrelin Stimulates Myocyte Development

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Key Words

Ghrelin • Myogenesis • MyoD • Myosin heavy chain

Abstract

Ghrelin, a gastric peptide hormone, may regulate mesenchymal cell development. Here we show that ghrelin promotes myogenesis in vitro in the mouse myoblast cell line C2C12 cells. Cells expressing ghrelin demonstrated a significant increase in the differentiation of premyocytes into myocytes. The mean myogenic index in cells stably expressing ghrelin increased significantly relative to control cells ($42 \pm 2\%$ vs. $16 \pm 1\%$, $p < 0.05$). Western blotting showed that expression of MHC protein was elevated in cells expressing ghrelin compared to control cells. MyoD expression increased after treatment of C2C12 cells with exogenous ghrelin (10^{-7} M). The stimulatory effect of ghrelin on myogenesis was abolished by either replacement of the third amino acid serine with alanine or deletion of its first nine amino acids. This study demonstrated that ghrelin is a potent stimulator for myogenesis.

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Introduction

Ghrelin, a 28 amino acid peptide, is secreted by gastric oxyntic glands and circulates in blood [1]. Whereas ghrelin was originally reported to stimulate growth hormone release [1-3], the wide distribution of its receptor, growth hormone secretagogue receptor 1a (GHSR1a), has suggested a potentially broader array of actions. Expression of ghrelin and its related receptor in the kidney indicates that ghrelin may play an important role in the regulation of renal function [4]. In rats, ghrelin has been found to stimulate food intake [2, 5], to induce adiposity [6], and to increase body weight [2, 5, 6], suggesting a possible role in regulation of feeding behavior and energy metabolism. In the gastrointestinal system, ghrelin has been recently reported to regulate secretion of gastric acid [7, 8], gastric motility [7, 9], and pancreatic protein output [10].

Several studies have shown that ghrelin may play a role in the regulation of cell proliferation and differentiation. Dependent on the cell type, ghrelin either stimulates or inhibits cell proliferation. Mitotic effects of ghrelin have been demonstrated in preosteoblasts [11, 12], neuronal precursors [13-15], preadipocytes [16, 17],

cardiomyocytes [18] and the rat GH3 pituitary cell line [19]. In cell lines derived from carcinomas including prostate [20], thyroid [21], mammary gland [22] and lung [23], ghrelin acts to inhibit proliferation. In vitro studies suggest that ghrelin induces the differentiation of several cells including osteoblasts [11, 12], adipocytes [16, 17] and neurons [13-15]. Investigators [24] have reported that ghrelin stimulates proliferating myoblast cells to differentiate and fuse into multinucleated myotubes. Since osteoblast, adipocyte and myocytes are all derived from a common precursor cell-the mesenchymal stem cell, these reports suggest that ghrelin may play a role in the modulation of mesenchymal cell differentiation.

The object of this study was to determine whether ghrelin promotes myogenesis and to examine its structure-function relationship. We report here that in the C2C12 cells (1) expression of ghrelin stimulates the differentiation of premyocytes into the multinucleated myotubes; (2) ghrelin upregulates the expression of the early marker, MyoD transcript factor, and the late marker, myosin heavy chain (MHC), of muscle cell differentiation; (3) replacement of the third amino acid serine of ghrelin with alanine or deletion of its first nine amino acids abolishes the stimulatory effect of ghrelin on myogenesis.

Materials and Methods

Stable cell transfection

Mouse myoblast cell line C2C12 cells were maintained in DMEM containing 15% fetal calf serum, 1% penicillin-streptomycin, 1% sodium pyruvate, and 1% glutamine. Cells (1.5×10^6) were plated, and cDNA constructs were transfected into cells by Lipofectamine (Invitrogen, Carlsbad, CA). Cells expressing ghrelin constructs were selected with 800 μ g/ml neomycin. Stable cell lines were maintained with 400 μ g/ml neomycin. C2C12 cells stably transfected with pcDNA3.1 were also established and used as the control. Successful transfection and transcription were confirmed by RT-PCR. PCR products using primers derived from the ghrelin gene was shown in Figure 1. Expression of ghrelin mRNA was observed in cells transfected with full length ghrelin-pGhrelin, but not in native C2C12 or cells transfected with pcDNA3.1 empty vector.

Differentiation of C2C12 cells

To induce cell proliferation, cells were grown to confluence and culture medium was switched to differentiation medium containing DMEM supplemented with 2% fetal calf serum, 1% penicillin-streptomycin, 1% sodium pyruvate, and 1% glutamine. Cells were observed for 7-10 days for morphological changes. Differentiated myocytes (myotubes) were identified with hematoxylin staining by their distinct morphology and multiple nuclei. As a morphological parameter

Fig. 1. Stable expression of ghrelin in C2C12 cells: Total RNA was extracted from stable cell lines transfected with pcDNA3.1 or pcDNA3.1 containing ghrelin cDNAs (pGhrelin). RT-PCR was performed using ghrelin sense and antisense primers as indicated in the text. Expression of ghrelin mRNA was detected in cells transfected with pGhrelin. No endogenous ghrelin mRNA was detected in either native C2C12 cells or cells transfected with pcDNA3.1 control vector. Sequence analysis demonstrated that the signal detected matches the published sequence of ghrelin cDNA.

of muscle differentiation, the myogenic index was defined as the number of nuclei in cells containing three or more nuclei divided by total number of nuclei in hematoxylin stained cells. Results were expressed as mean $\pm$ SEM. Statistical significance of $P < 0.05$ was established using student t test or ANOVA.

Western blot analysis

Cultured cells were washed with cold PBS and harvested at the times indicated. Cells were lysed in radioimmunoprecipitation assay buffer (1x PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). Protease inhibitor cocktail (Roche Diagnostics) and 1 mM phenylmethylsulfonyl fluoride were added to 10ml of radioimmunoprecipitation assay buffer before lysing cells. Total protein was determined with a bicinchoninic acid assay (Pierce Chemical, Rockford, IL). Then, 50 μ g of total protein was loaded onto a 7.5–10% denaturing gel. Proteins were transferred to polyvinylidene difluoride membrane for analysis. MHC and MyoD protein were detected using specific antibodies.

Construction of ghrelin mutants and establishment of stable cell lines expressing ghrelin mutants

Two ghrelin mutants, ala-ghrelin and ghrelin(10-28), were constructed using site directed mutagenesis (Stratagene, La Jolla, CA). In the ala-ghrelin mutant, the third amino acid serine was replaced with alanine. For the ghrelin (10-28) mutant, the first nine amino acids were deleted. C2C12 cell lines stably transfected with pcDNA3.1, pGhrelin, pAla-ghrelin, or pGhrelin(10-28) were established and maintained in medium containing 400 μ g/ml neomycin as described above.

Fig. 2. Stimulation of myogenesis: C2C12 cells transfected with pcDNA3.1 or pGhrelin were assayed for differentiation into myocytes as indicated by the appearance of the myotubes (A, B) and expression of myosin heavy chain (C) and MyoD (D).

RT-PCR

Total RNA was isolated from cultured C2C12 cells by using RNeasy mini kit (QIAGEN). Single strand cDNA synthesis was performed as follows: 30 μ l of reverse transcription mixture contained 1 μ g of DNase I pretreated total RNA, 0.75 μ g of oligo d(T) primer, 6 μ l of 5x RT buffer, 10 mM dithiothreitol, 0.5 mM deoxynucleotides, 50 U of RNase inhibitor, and 240 U of reverse transcriptase (Invitrogen). The RT reaction was carried out at 40°C for 70min followed by heat inactivation at 95°C for 3min. PCR primers used for ghrelin and β -actin mRNA detection were deduced from published sequences.

Results

Stimulation of myogenesis

C2C12 cells, a mouse skeletal muscle satellite derived cell line, is commonly used to study cellular and molecular mechanisms of muscle differentiation. When cultured in differentiation medium, C2C12 myoblasts exit cell cycle, differentiate and fuse into multinucleated myotubes. To examine the effect of ghrelin on the differentiation of myoblasts, cells were transfected with control pcDNA3.1 or pGhrelin and assayed for differentiation into myocytes as indicated by the appearance of myotubes. Cells expressing ghrelin demonstrated increased myogenesis as indicated by an increase in myogenic index (Figure 2A, B).

Expression of early and late markers of skeletal muscle differentiation

To confirm the observation that ghrelin acts to enhance the differentiation of C2C12 myoblast cells, we

investigated the ability of ghrelin to induce the expression of MyoD, an early marker, and MHC, a late marker of skeletal muscle differentiation. As shown in Figure 2C, expression of MHC was significantly enhanced in cells transfected with pGhrelin relative to control cells. When native C2C12 cells were treated with ghrelin (10^{-7} M) for 24 hours, the expression of MyoD protein was significantly increased compared to controls (Figure 2D).

Minimal structural requirements for ghrelin's activity in myogenesis

To elucidate the structural requirements for ghrelin in the modulation of myocyte differentiation, we generated two ghrelin mutants: ala-ghrelin and ghrelin (10-28). These constructs are illustrated in Figure 3A. The stable expression of these constructs was confirmed by RT-PCR. We used a sense primer derived from the T7 promoter of pcDNA3.1 and an antisense primer derived from the C-terminus of ghrelin gene to demonstrate transfection of the pGhrelin construct. mRNA containing the sequence of pGhrelin was demonstrated only in cells transfected with pGhrelin, pAla-ghrelin, or pGhrelin(10-28), but not in native C2C12 cells or cells transfected with pcDNA3.1 (Figure 3B). Sequencing analysis confirmed the sequences of these mutants. When using a sense primer designed from the first 9 amino acid residues of ghrelin and a ghrelin anti-sense primer, only C2C12 cells transfected with pGhrelin expressed ghrelin mRNA, while no ghrelin mRNA was detected in native C2C12 cells, cells transfected with pAla-ghrelin, cells transfected with pGhrelin (10-28) or cells transfected

Fig. 3. Establishment of stable cell lines expressing ghrelin mutants: (Top) Structure of two ghrelin mutants: Ala-ghrelin and ghrelin (10-28). (Bottom) Studies used a sense primer derived from the T7 promoter of pcDNA3.1 and an antisense primer derived from the C-terminus of ghrelin gene to demonstrate the successful transfection of the pGhrelin construct. mRNA containing the sequence of pGhrelin was demonstrated only in cells transfected with pGhrelin, pAla-ghrelin, or pGhrelin(10-28), but not in native 3T3-L1 cells or cells transfected with pcDNA3.1 (A). Sequencing analysis confirmed the sequences of these mutants. When using a sense primer designed from the first 9 amino acid residues of ghrelin and a ghrelin anti-sense primer, only C2C12 cells transfected with pGhrelin expressed ghrelin mRNA, while no ghrelin mRNA was detected in native C2C12 cells, cells transfected with pAla-ghrelin, cells transfected with pGhrelin(10-28) or cells transfected with pcDNA3.1 (B).

with pcDNA3.1 (Figure 3C).

As showed in Figure 4, cells expressing ghrelin demonstrated a significant increase in the myogenic index compared to cells stably transfected with pcDNA3.1 vector ($44 \pm 3\%$ vs. $17 \pm 2\%$, $p < 0.05$), indicating an increment in myogenesis. Replacement of the third amino acid serine residue of ghrelin by alanine eliminated ghrelin-mediated effects on myogenesis ($18 \pm 3\%$). No increase in myogenesis was observed when the first nine amino acids of ghrelin were deleted ($15 \pm 1\%$).

To further confirm that acylation of the third amino acid serine residue of ghrelin is required for its stimulatory effect on myogenesis, we next examined the effect of exogenous ghrelin and des-acyl ghrelin. Consistent with the above results of ghrelin mutation, des-acyl ghrelin demonstrated no effect on myogenesis at a dose of 10^{-7} M with a myogenesis index similar to control cells ($98 \pm 5\%$ of the control, $n=6$). In the paralleled experiments, cells treated with exogenous ghrelin (10^{-7} M) showed a significant increase in the myogenesis index ($160 \pm 6\%$ of the control, $n=6$).

Discussion

The major finding of the present study is that ghrelin stimulates myogenesis in C2C12 myoblast cells. This conclusion is supported by two distinct observations: (1)

Fig. 4. Effect of ghrelin mutation on myogenesis: C2C12 cells stably transfected with pcDNA3.1 (control), pcDNA3.1 containing full length ghrelin (pGhrelin), Ala-ghrelin or Ghrelin (10-28) were induced to differentiate for 7 days (A). Myogenic index expressed as mean $\pm$ SEM (B). * indicates $P < 0.05$.

expression of ghrelin enhances myogenesis as demonstrated by increase in the myogenic index and in expression of MHC and Myo-D, two common markers of myocyte differentiation; (2) the stimulatory effect of ghrelin on myotube differentiation is abolished by replacement of the third amino acid serine of ghrelin with alanine or by deletion of the first nine amino acids of ghrelin.

Since its discovery in 1999, ghrelin has been the focus of studies ranging from neuroendocrine control mechanisms, food intake and energy metabolism, cardiovascular function, immunologic functions and growth and development. While genetic knockout studies demonstrate that absence of ghrelin does not prevent normal fetal growth and development [25], pharmacological and developmental studies have demonstrated the importance of ghrelin in the proliferation and differentiation of a variety of cell types [11-19]. Ghrelin is present in the fetus where its tissue distribution differs from the adult [26], suggesting a potential role of this hormone during growth and development. The abundance of ghrelin in the fetal endocrine pancreas suggests that ghrelin may regulate β -cell development [27]. This concept is supported by a report demonstrating the expansion of ghrelin-producing cells at the expense of β -cells in mice lacking the homeodomain protein Nkx2.2 [28]. The significance of ghrelin in growth and development is further demonstrated by studies showing that ghrelin regulates the proliferation and differentiation of a variety of cell types derived from endodermic, mesodermic and ectodermic tissues [11-23].

Our laboratory has reported the actions of ghrelin on the lineage differentiation of mesenchymal stem cells. We have previously reported that ghrelin inhibits the differentiation of adipocytes [16]. Since myocytes and adipocytes are derived from a common progenitor cell, and muscle and adipose tissue development often has a reciprocal relationship, the finding that ghrelin stimulates myocyte differentiation supports a potential role of this hormone in lineage determination of mesenchymal stem cells. This concept is consistent with a recent study in which ghrelin has been shown to increase the differentiation of myoblast cells [24].

The ghrelin active core has been shown to include a motif surrounding the third amino acid serine [1, 29, 30]. Octanoyl modification of this third amino acid serine is critical for binding and activation of the growth hormone secretagogue receptor 1a (GHSR1a) [1, 29, 30]. The

current study shows that replacement of the third amino acid serine with alanine renders ghrelin inactive in the stimulation of myocyte differentiation. In addition, deletion of the first amino acids of ghrelin also abolishes its function in the regulation of myocyte differentiation. These findings suggest that the first nine amino acids, and specifically the third amino acid serine, are required for stimulation of myogenesis. This observation is in contrast to the study of Filegheddu et al [24] in which acylation of the third amino acid serine was not required for regulation of myoblast cell proliferation and differentiation. Since the classical ghrelin receptor GHSR1a is not present in C2C12 cells [24], our study and others [16, 18] may also suggest the existence of an unknown subtype of ghrelin receptor. Whether this novel subtype of ghrelin receptor is an alternative receptor for ghrelin or a des-acyl ghrelin receptor proposed by Filegheddu et al [24] requires further investigation. Until this novel ghrelin receptor subtype is cloned, the possibility of an alternative ghrelin receptor or putative des-acyl ghrelin receptor remains speculative. Indirect evidences have been reported to support the existence of both these receptors. For example, radiolabeled peptidyl GHS was shown to bind to a more diverse array of human tissues than did ghrelin or non-peptidyl GHS [31]. In addition, studies have reported discrepancies between actions of neutral, competitive antagonists of ghrelin receptor in vitro compared with their effects on energy homeostasis in vivo [32]. These results are often interpreted as evidences supporting the existence of an alternative receptor for ghrelin. A small but growing body of literature suggests that des-acyl ghrelin is functionally active [33]. Because des-acyl ghrelin does not bind to and activate the classical ghrelin receptor GHSR1, nor does it exert any endocrine effect [33], a putative des-acyl ghrelin receptor is proposed. However, most of the data suggesting actions of des-acyl ghrelin come from the pharmacological or in vitro studies and these observations may reflect the cross-reaction with heterologous receptors of superphysiologic dose of des-acyl ghrelin.

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