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Journal of Cellular Biochemistry 106:626–632 (2009)

Journal of Cellular Biochemistry

Ghrelin Inhibits Early Osteogenic Differentiation of C3H10T1/2 Cells by Suppressing Runx2 Expression and Enhancing PPARγ and C/EBPα Expression

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ABSTRACT

Ghrelin is a 28-residue peptide identified in the stomach as an endogenous ligand of the growth hormone secretagogue receptor that is expressed in a variety of peripheral tissues, as well as in the brain. In previous studies, ghrelin has been shown to stimulate both adipogenic differentiation from preadipocytes and osteogenic differentiation from preosteoblasts or primary osteoblasts. This study was undertaken to investigate the direct effect of ghrelin on the lineage allocation of mesenchymal stem cells (MSCs). We identified ghrelin receptor mRNA in C3H10T1/2 cells, and we found the levels of this mRNA to be attenuated during osteogenic differentiation. Treatment of cells with ghrelin resulted in both proliferation and inhibition of caspase-3 activity. In addition, ghrelin decreased serum deprivation-induced bax protein expression and release of cytochrome *c* from the mitochondria, whereas it increased bcl-2 protein expression. Moreover, ghrelin inhibited early osteogenic differentiation, as shown by alkaline phosphatase activity and staining, and inhibited osteoblast-specific genes expression by altering Runx2, PPARγ, and C/EBPα protein expression. J. Cell. Biochem. 106: 626–632, 2009.

KEY WORDS: GHRELIN; MESENCHYMAL STEM CELLS; DIFFERENTIATION; Runx2; PPARγ

Ghrelin, a 28-residue acid peptide secreted from human and rat stomach, stimulates the release of growth hormone (GH) from the pituitary gland [Kojima et al., 1999]. In addition, ghrelin has various important physiological effects, such as inducing adiposity and increasing food intake [Asakawa et al., 2001]. Expression of the ghrelin receptor, a receptor for the GH secretagogue, has been detected in a wide range of tissues [Gnanapavan et al., 2002]. We have reported the expression of ghrelin receptors in preadipocytes [Kim et al., 2004] and various osteoblastic cell lines [Kim et al., 2005]. Furthermore, ghrelin enhances the osteoblastic differentiation of preoseteoblasts [Kim et al., 2005; Maccarinelli et al., 2005] or primary osteoblasts [Fukushima et al., 2005], as well as the adipocyte differentiation of preadipocytes [Choi et al., 2003; Kim et al., 2004]. Differentiation of mesenchymal progenitor cells into osteoblast or adipocyte lineages is regulated reciprocally. The developmental process by which mesenchymal progenitor cells become specific cell types can be divided into two stages: commitment and terminal differentiation [Weintraub et al., 1991]. Our previous results suggest that ghrelin affects terminal differentiation after commitment to a specific lineage. However, there is no data about the effect of ghrelin on the lineage allocation of mesenchymal progenitor cells. Apparently, pluripotent bone marrow mesenchymal progenitor cells can differentiate into osteoblasts or adipocytes based on the influence of several transcription factors. These differentiation "switches" control the fate of mesenchymal progenitor cells as they enter the bone or fat lineage [Nuttall and Gimble, 2004]. As a rule, the transcription factors Runx2, osterix, and β-catenin regulate osteoblast differentiation, and the C/EBP family and PPARγ regulate adipocyte differentiation. Runx2 is essential for the differentiation of mesenchymal progenitor cells into preosteoblasts, and it inhibits their differentiation into adipocytes and chondrocytes. In addition, β-catenin and osterix direct preosteoblasts to become immature

Abbreviations used: PPARγ, peroxisome proliferator activated receptor gamma; C/EBPα, CCAAT/enhancer binding protein alpha.

Grant sponsor: Seoul National University Hospital; Grant number: #04-2003-010-0.

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Received 3 April 2008; Accepted 2 December 2008 • DOI 10.1002/jcb.22042 • 2009 Wiley-Liss, Inc.
Published online 21 January 2009 in Wiley InterScience (www.interscience.wiley.com).

osteoblasts [Komori, 2006]. This study was undertaken to investigate the effect of ghrelin on lineage allocation to osteoblasts or adipocytes from uncommitted mesenchymal progenitor cells and to determine the expression of transcription factors involved in lineage allocation. We also examined various other biological properties of ghrelin, including its role in proliferation and in preventing apoptosis in mesenchymal progenitor cells.

MATERIALS AND METHODS

MATERIALS

Mouse ghrelin was purchased from Phoenix Pharmaceuticals (Belmont, CA). Polyclonal primary antibodies against PPARγ (E8), C/EBPα, bax, bcl-2, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit primary antibody against cytochrome *c* was from Cell Signaling (Beverly, MA), and HRP-conjugated anti-phospho anti-goat IgG, rabbit IgG and mouse IgG were from Biosource (Camarillo, CA).

CELL CULTURE

Murine embryonic mesenchymal stem cells (MSCs) C3H10T1/2 (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 with 10% fetal bovine serum (FBS) and antibiotics at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

REVERSE TRANSCRIPTION PCR

First-strand cDNA was synthesized from 1 μg of total RNA using a Reverse Transcription System kit (Promega, Madison, WI). PCR was performed using 100 ng of cDNA, 20 pmol of each primer (synthesized by Bioneer Corp., Chungwon, Korea), 2.5 mM of dNTPs, 1 mM of $MgCl_2$, and 1 U of Taq polymerase in a 50 μl reaction volume containing 1× Taq polymerase buffer using a Perkin-Elmer Gene Amp PCR System 9600. The forward and reverse primer sequences used to amplify ghrelin receptor, osteoblast, and adipocyte genes are listed in Table I.

QUANTITATIVE REAL TIME PCR MEASUREMENTS OF GENE EXPRESSION

The mRNA expressions of adipocyte genes were determined by quantitative real-time PCR (SYBR® Green I). Reactions were performed in 20 μl with ~5 μl (10 ng/μl) cDNA, 10 μl 2× SYBR I Mix, and 0.8 μl primers. Cycling conditions used were 95°C for 30 s, 56°C for 30 s, and 95°C for 30 s for 40 cycles. Real-time PCR was performed in an ABI PRISM 7900 Sequence detection system (Applied Biosystems, Foster City, CA). All PCR reactions were performed in duplicate and the expression levels were normalized to β-actin signal in the same reaction.

ADENOVIRUS INFECTION

Adenoviral vectors expressing a wild-type (Ad-PPARγ or a dominant-negative form of PPARγ (Ad-dnPPARγ) were used for gene delivery. For adenovirus-mediated gene transfer, confluent C3H10T1/2 cells were exposed to Ad-PPARγ, Ad-dnPPARγ, or adenovirus expressing GFP alone (Ad-GFP). The preparation and infection of PPARγ and control GFP adenoviruses were performed as described previously [Fu et al., 2002].

BROMODEOXYURIDINE (BRDU) INCORPORATION ASSAY

Cell proliferation was assessed using the BrdU incorporation ELISA kit (Roche Applied Science, Basel, Switzerland) according to the manufacturers' instructions. Briefly, C3H10T1/2 cells were seeded onto a 96-well microplate at 1×10^3 cells/well. Cells were incubated in the presence of ghrelin with various doses for 24 h. Cells were incubated with BrdU labeling solution for 2 h at 37°C. After removal of the labeling solution, cells were fixed and denatured and incubated for 90 min with anti-BrdU antibody conjugate, which was subsequently removed by rinsing three times. Finally, cells were incubated in a substrate solution for 30 min at room temperature and proliferation was assessed by colorimetric detection.

CASPASE-3 ACTIVITY ASSAY

Cells were washed in phosphate buffer saline and analyzed for caspase-3 activity by using a caspase-3 colorimetric assay kit (R&D

TABLE I. Sequences of PCR Primers Used to Amplify Each of the Genes in RT-PCR

Gene	Primer	Sequence (5′–3′)	PCR product length (bp)
GHS-R1a	Forward	GAGCCTAACGTCACGCTGGA	240
	Reverse	TAGAGGTTGGTGGTGCG	
Runx2	Forward	TTTAGGGCGCATTCCTCATC	102
	Reverse	TGTCCTTGTGGATTAAAAGGA	
Osterix	Forward	CTGGGGAAAGGAGGCACAAAGAAG	474
	Reverse	GGGTTAAGGGGAGCAAAGTCAGAT	
ALP	Forward	TCCTGACCAAAAACCTCAAAGG	101
	Reverse	TGCTTCATGCAGAGCCTGC	
Col I	Forward	GCGAAGGCAACAGTCGCT	101
	Reverse	CTTGGTGGTTTTGTATTCGATGAC	
LPL	Forward	AAGGTCAGAGCCAAGAGAAGCA	98
	Reverse	CCAGAAAAGTGAATCTTGACTTGGT	
Adipsin	Forward	GCTATCCCAGAATGCCTCGTT	71
	Reverse	CCACTTCTTTGTCCTCGTATTGC	
aP2	Forward	GTCACCATCCGGTCAGAGAGTAC	86
	Reverse	TCGTCTGCGGTGATTTCATC	
GAPDH	Forward	GGTCGGTGTGAACGGATTTG	320
	Reverse	GTGAGCCCCAGCCTTCTCCAT	

ALP, alkaline phosphatase; Col I, collagen type I; LPL, lipoprotein lipase.

Systems, Minneapolis, MN) according to the manufacturers' instructions. Briefly, cells were lysed in 50 μl of cell lysis buffer provided in the kit. Protein concentrations of the lysates were determined by using the Bradford protein assay. Equal amounts of lysates were incubated with the caspase-3 substrate, 200 μM DEVD-pNA, at 37°C for 2 h. Absorbances of the samples were read every 60 min in a Spectramax 250 (Molecular Dynamics) microplate reader at 405 nm. Caspase-3 activity was proportional to the optical density at 405 nm.

WESTERN BLOT ANALYSIS

Cells were lysed in 50 mM Tris (pH 7.5), 150 mM NaCl, and 1% Triton X-100 supplemented with a protease inhibitor mixture (Sigma, St. Louis, MO; used at a dilution of 1:100). Lysates were fractionated by SDS–PAGE and transferred to a Hybond ECL nitrocellulose membrane (Amersham, Piscataway, NJ). For the detection of bax, bcl-2, and cytochrome *c*, cells were fractionated into mitochondria and cytosol using the Mitochondria Fractionation Kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. The membrane was first blocked with 5% milk for 1 h and then incubated with primary and secondary antibodies for 24 and 1 h, respectively. Signal was developed using an enhanced chemiluminescence kit (Amersham).

HISTOCHEMICAL ANALYSIS

For alkaline phosphatase (ALP) staining, cells were fixed in citrate-buffered acetone (60%) for 30 s at room temperature. After washing with deionized water, cells were incubated at room temperature for 30 min in an aqueous solution of 0.6 mg/ml fast blue RR salt and 0.1 mg/ml naphthol ASMX phosphate (Sigma). For Oil Red O staining, cells were fixed with 10% buffered neutral formalin for 5 min at room temperature and rinsed in distilled water. A working stock solution of Oil Red O was prepared from 1% (w/v) Oil Red O in 99% isopropanol, and diluted to 0.3% (v/v) with distilled water. Cultures were stained with Oil Red O working solution for 30 min with gentle rocking, and then rinsed with distilled water.

ALP ASSAY

To assess ALP activity, cells were washed three times with ice-cold Tris-buffered saline (TBS), pH 7.4, and scraped immediately after addition of 0.5 ml of ice-cold 50 mM TBS. The lysates were then sonicated for 20 s at 4°C. Enzyme activity assays were performed in assay buffer (10 mM MgCl2 and 0.1 M alkaline buffer, pH 10.3) containing 10 mM *p*-nitrophenylphosphate as a substrate. The reaction was stopped by addition of 0.3 N NaOH, and absorbance was read at OD405. Relative ALP activity is defined as mmol of *p*-nitrophenol phosphate hydrolyzed per min per mg of total protein.

STATISTICAL ANALYSIS

All data are presented as mean ± SD. The data were analyzed by one-way ANOVA. When the ANOVA indicated a significant difference between two groups, the difference was subsequently evaluated by the Student–Newman–Keuls multiple comparison test. A *P*-value <0.05 was considered significant for all statistical analyses.

RESULTS

EXPRESSION OF GHRELIN RECEPTOR IN C3H10T1/2 CELLS

To study the expression of ghrelin receptor (GH secretagogue receptor 1a; GHS-R1a) in C3H10T1/2 cells, ghrelin receptor mRNA was amplified by RT-PCR (Fig. 1A). 3T3L-1 cells were used as a positive control to verify detection of the target band of 240 bp. The level of ghrelin receptor mRNA increased during adipogenic differentiation of C3H10T1/2 cells, and it decreased during osteogenic differentiation (Fig. 1B).

EFFECT OF GHRELIN ON SURVIVAL OF C3H10T1/2 CELLS

We investigated the effect of ghrelin on the proliferation of C3H10T1/2 cells using BrdU uptake. Ghrelin significantly increased the proliferation of C3H10T1/2 cells at 10^{-13} and 10^{-11} M (Fig. 2A). We also examined whether ghrelin exerts an anti-apoptotic effect on C3H10T1/2 cells. Ghrelin decreased caspase-3 activity significantly at concentrations between 10^{-13} and 10^{-7} M compared to control (Fig. 2B). This anti-apoptotic effect of ghrelin was comparable to that of insulin-like growth factor-I (IGF-I). Moreover, treatment with ghrelin decreased serum deprivation-induced bax protein expression, whereas it increased bcl-2 protein expression in the

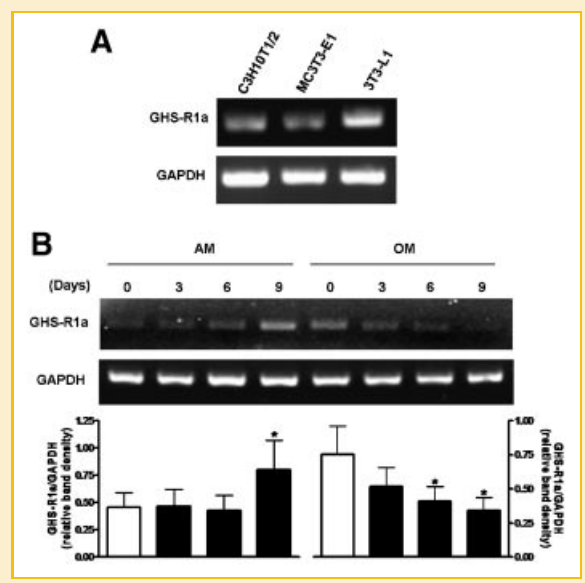

Fig. 1. Expression of ghrelin receptor in C3H10T1/2 cells. A: RT-PCR analysis of the ghrelin receptor (GHS-R1a). Messenger RNA coding for the ghrelin receptor was detected by PCR amplification of cDNA from reverse-transcribed total RNA prepared from C3H10T1/2 as well as MC3T3-E1, murine preosteoblasts. Mouse preadipocytes, 3T3L-1, were used as a positive control for ghrelin receptor expression. B: Change in ghrelin receptor mRNA levels during osteogenic and adipogenic differentiation. Cells were cultured in DMEM/F12 containing 10% FBS and induced to undergo osteoblastic differentiation with osteogenic media (OM; 50 μg/ml ascorbic acid and 10 mM of β-glycerophosphate) or induced to undergo adipogenic differentiation with adipogenic media (AM; 10 μg/ml insulin, 0.1 mM indomethacin, and 0.1 μM dexamethasone). The amplification of GAPDH was used as an internal control for cDNA quality and PCR efficiency. All results are representative of three independent experiments. Each bar represents the mean ± SD. $^{*}P<0.05$ versus control.

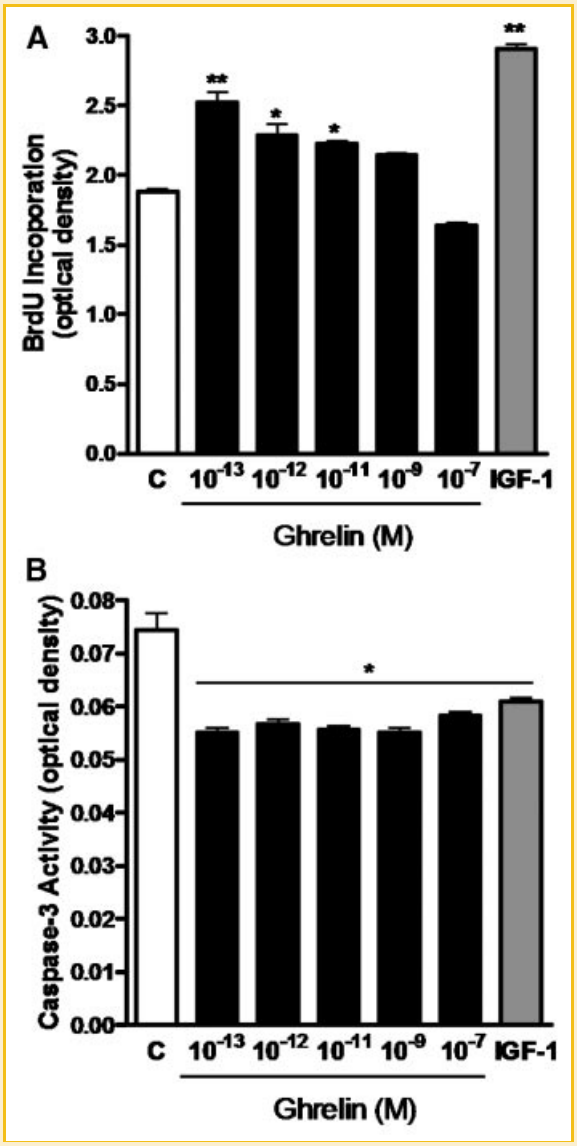

Fig. 2. Effect of ghrelin on the survival of C3H10T1/2 cells. A: Cells (2×10^3 cells/well) were plated and cultured for 24 h in the absence or presence of different concentrations of ghrelin (10^{-13}–10^{-7} M). BrdU labeling solution was added to cells, which were harvested 2 h later, and cell proliferation was quantified by a colorimetric assay. IGF-I (100 ng/ml) was used as a positive control. B: Cells (1×10^4 cells/well) were plated and cultured for 24 h in DMEM/F12 without FBS in the absence or presence of different concentrations of ghrelin (10^{-13}–10^{-7} M). Cell lysates were incubated with 200 μM DEVD-pNA for 3 h and analyzed in the colorimetric caspase substrate assay. IGF-I (100 ng/ml) was used as a positive control for anti-apoptotic effect. The results are representative of three independent experiments. Each bar represents the mean ± SD. $^{**}P < 0.01$ versus control, $^{*}P < 0.05$ versus control.

mitochondrial fraction (Fig. 3). In addition, ghrelin inhibited serum deprivation-induced release of cytochrome *c* from the mitochondria into the cytosol (Fig. 3).

EFFECT OF GHRELIN ON EARLY OSTEOGENIC DIFFERENTIATION OF C3H10T1/2 CELLS

To investigate the effect of ghrelin on the early osteogenic differentiation program, C3H10T1/2 cells were cultured for 7 day

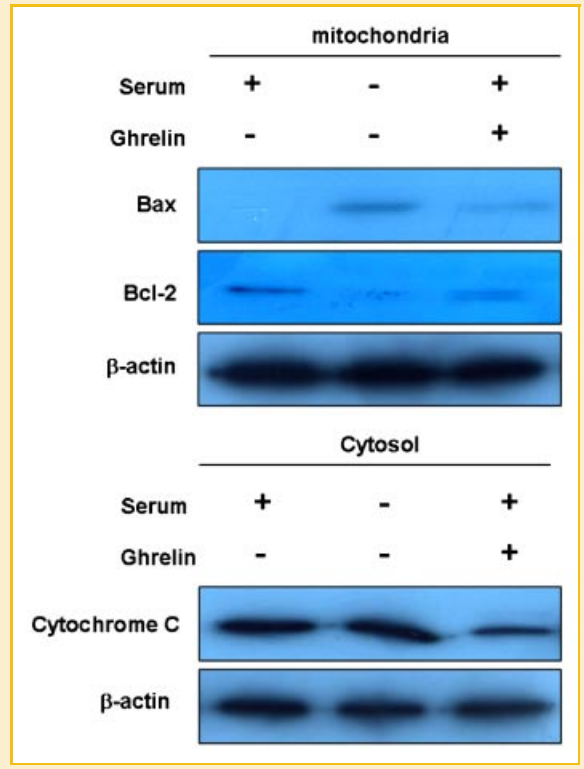

Fig. 3. Anti-apoptotic effect of ghrelin on C3H10T1/2 cells through a mitochondria pathway. Cells (1×10^6 cells/well) were plated and cultured for 24 h in DMEM/F12 without FBS in the absence or presence of ghrelin (10^{-11} M). Bax and bcl-2 protein levels in the mitochondria fraction and cytochrome *c* protein levels in the cytosolic fraction were assessed by Western blot. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in DMEM/F12 medium with 10% FBS supplemented with 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate. Treatment with ghrelin for 7 day reduced ALP staining and activity in a dose dependent manner with a significant response at 10^{-7} M of ghrelin (Fig. 4A). Consistent with this, ghrelin also decreased mRNA levels of osterix, ALP, and collagen type 1 (Fig. 4B). Taken together, these results indicate that ghrelin inhibits early osteogenic differentiation of C3H10T1/2 cells.

THE INHIBITORY EFFECT OF GHRELIN ON EARLY OSTEOGENIC DIFFERENTIATION THROUGH SUPPRESSION OF RUNX2 EXPRESSION AND INDUCTION OF PPARγ AND C/EBPα

To understand how ghrelin inhibits early osteogenic differentiation from C3H10T1/2 cells, we examined the effects of ghrelin treatment on the expression of Runx2, PPARγ, and C/EBPα. These are all key transcription factors that act as molecular switches between the osteogenic and adipogenic pathways in MSCs. Cells were cultured for 3, 6, and 9 day in DMEM/F12 medium with 10% FBS supplemented with either ghrelin or vehicle alone. PPARγ, and C/EBPα protein expression significantly increased in the ghrelin-treated cells compared to the vehicle-treated cells (Fig. 5A). Moreover, incubating C3H10T1/2 cells in osteogenic differentiation media containing 10^{-7} M ghrelin for 7 day enhanced PPARγ, and

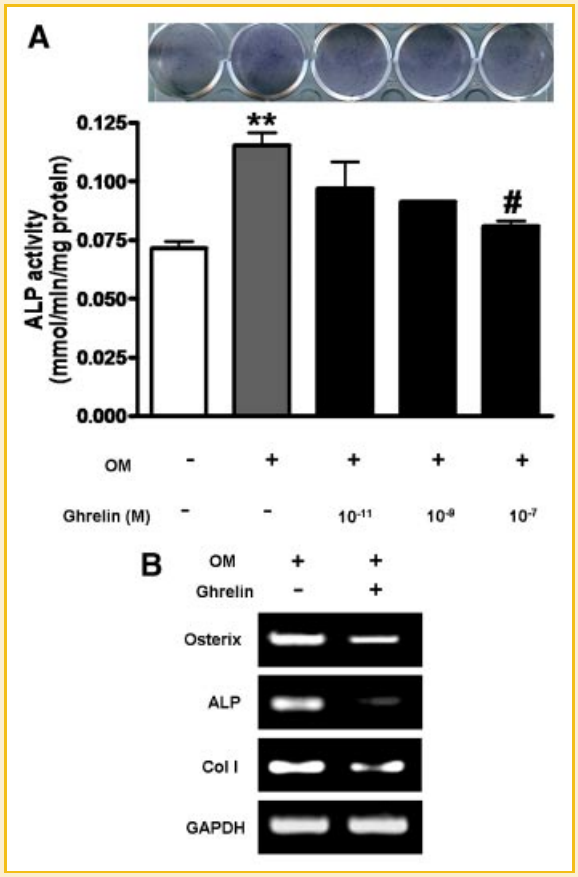

Fig. 4. The inhibitory effect of ghrein on early osteogenic differentiation of C3H10T1/2 cells. A: The effect of ghrelin on the ALP staining and activity in C3H10T1/2 cells. Cells were seeded at 70–80% density in a 6-well plate and grown for 7 day in DMEM/F12 containing osteogenic media (OM) in the presence of varying concentrations of ghrelin or vehicle. ALP activity was determined and normalized to protein content. ALP staining and activity were evaluated as described in Materials and Methods Section. The results are representative of three independent experiments. Each bar represents the mean ± SD. $^{**}P < 0.01$ versus control, $^{\#}P < 0.05$ versus OM. B: Effect of ghrelin on mRNA expression of early osteogenic genes. Cells were seeded at 70–80% density in a 6-well plate and grown for 7 day in DMEM/F12 containing osteogenic media in the presence of ghrelin (10^{-7} M) or vehicle. Total RNA was extracted from the C3H10T1/2 cells and RT-PCR analysis on osterix, ALP, and collagen type 1 (Col I) mRNA was performed as described in Materials and Methods Section.

C/EBPα protein expression, but suppressed Runx2 protein expression (Fig. 5B). These results suggest that ghrelin inhibits early osteogenic differentiation in C3H10T1/2 cells by altering the expression levels of proteins related to lineage allocation.

EFFECT OF GHRELIN ON ADIPOGENIC DIFFERENTIATION OF C3H10T1/2 CELLS

Cells were cultured for 7 day in DMEM/F12 medium with 10% FBS supplemented with 10 μg/ml insulin, 0.1 mM indomethacin, and 0.1 μM dexamethasone. During the culture period, both the vehicle-treated cells and the ghrelin-treated cells grew in multilayers and accumulated lipid droplets in their cytoplasm (data not shown). This effect was visible following Oil Red O staining (data not shown).

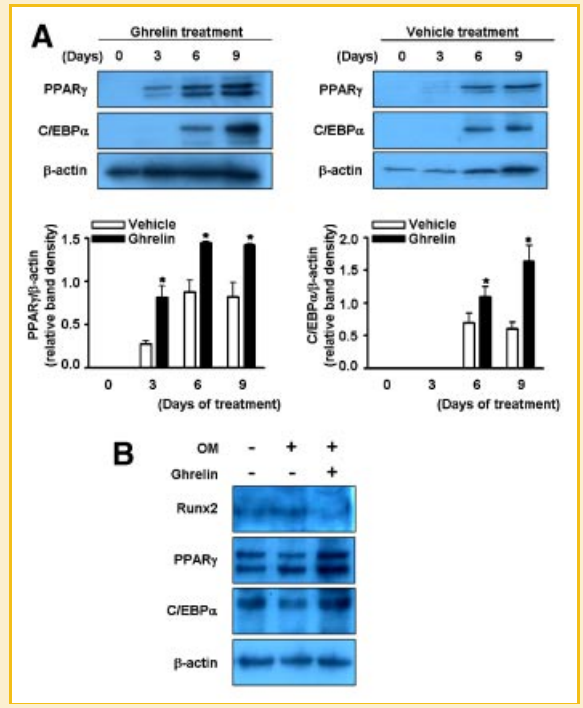

Fig. 5. Effect of ghrelin on Runx2, PPARγ, and C/EBPα protein expression during osteogenic differentiation. A: Cells were incubated for 3, 6, and 9 day in DMEM/F12 containing FBS with either ghrelin (10^{-7} M) or vehicle. PPARγ and C/EBPα protein expression was determined by Western blot analysis. All the results are representative of three independent experiments. Each bar represents the mean ± SD. $^{*}P < 0.05$ versus vehicle. B: After C3H10T1/2 cells were incubated for 7 day in osteogenic media (OM) with ghrelin (10^{-7} M), Runx2, PPARγ, and C/EBPα protein expression was determined by Western blot analysis. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Quantitative real-time PCR analysis was performed to examine potential differences at the molecular level. Consistent with cytochemical data, mRNA levels of adipocyte genes such as adipsin, lipoprotein lipase (LPL) and aP2 were comparable to the levels in vehicle-treated cells (Fig. 6). Because ghrelin did not enhance adipogenic differentiation in C3H10T1/2 cells even though it induced expression of PPARγ and C/EBPα, we investigated the effect of ghrelin on the expression of adipocyte genes in cells with elevated PPARγ expression. We found that the mRNA expression of adipsin, LPL, and aP2 increased with elevated PPARγ expression, but not following ghrelin treatment (Fig. 7).

DISCUSSION

In the present study, we detected expression of the ghrelin receptor in C3H10T1/2 cells. We also showed that ghrelin inhibits early osteogenic differentiation of cells by affecting the expression of key transcription factors related to lineage allocation.

We previously reported expression of the ghrelin receptor in osteoblastic cell lines and 3T3L-1 cells [Kim et al., 2004, 2005]. Our previous and current results indicate that ghrelin receptors are expressed throughout osteoblast and adipocyte lineages from MSCs, suggesting that ghrelin plays a role in cells of mesenchymal origin.

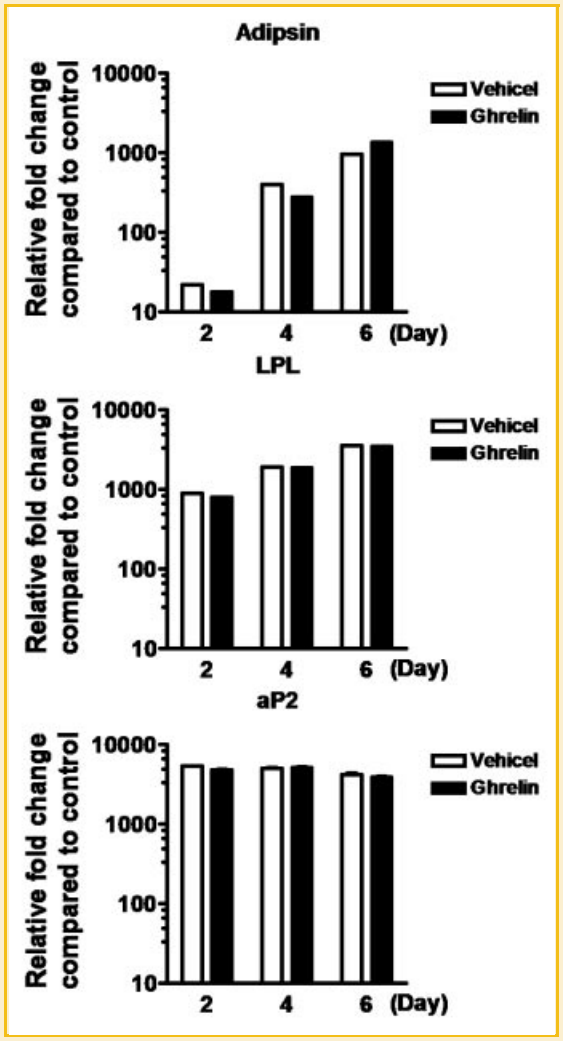

Fig. 6. Effect of ghrelin on adipogenic differentiation of C3H10T1/2 cells. Cells were seeded at 70–80% density in a 6-well plate and grown for 7 day in DMEM/F12 containing adipogenic media in the presence of ghrelin (10^{-7} M) or vehicle. Total RNA was extracted from C3H10T1/2 cells and quantitative real-time PCR analysis of adipsin, lipoprotein lipase (LPL) and aP2 was performed as described in Materials and Methods Section.

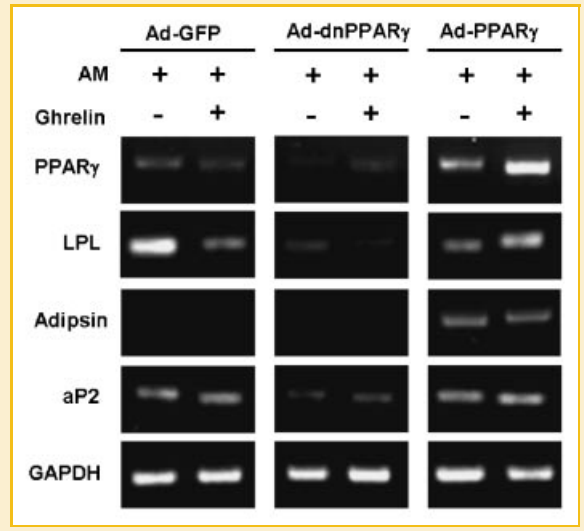

Fig. 7. Effect of ghrelin on expression of adipocyte genes due to modulation of PPARγ expression in C3H10T1/2 cells. In the adenovirus experiments, at 2 day post-confluence, cells were exposed to maintenance medium containing either adenoviral storage buffer or 2×10^9 PFU of adenovirus per well (MOI ~50 PFU/cell) for 12 h with agitation before washing with PBS. Fluorescence microscopy of living cells showed infection rates >95% for the Ad-GFP (control), Ad-PPARγ, and Ad-dnPPARγ mutant viruses (dominant-negative PPARγ. C3H10T1/2 cells infected with Ad-GFP, Ad-PPARγ, or Ad-dnPPARγ adenoviruses were incubated for 8 day in adipogenic media in the presence or absence of ghrelin (10^{-7} M). Total RNA was extracted from C3H10T1/2 cells, and RT-PCR analysis of adipogenic genes was performed as described in Materials and Methods Section.

Ghrelin stimulated the proliferation of C3H10T1/2 cells and inhibited the serum deprivation-induced activation of caspase-3 at concentrations between 10^{-11} and 10^{-13} M. This is analogous to the findings that ghrelin increases adipoctye proliferation and reduces adipocyte apoptosis at lower dose of ghrelin (10^{-11} and 10^{-13}M) [Kim et al., 2004]. Moreover, ghrelin had an anti-apoptotic effect on C3H10T1/2 cells by acting through a mitochondria pathway. These results are in agreement with previous reports that ghrelin stimulates survival in a variety of cells [Murata et al., 2002; Pettersson et al., 2002]. These further suggest that ghrelin acts as a mitogen of uncommitted mesenchymal progenitor cells in physiologic concentrations.

Previous studies showed that ghrelin can stimulate osteoblastic differentiation from preosteoblasts and primary osteoblasts [Fukushima et al., 2005; Kim et al., 2005; Maccarinelli et al., 2005]. However, in this study, expression of the ghrelin receptor in C3H10T1/2 cells was reduced during osteogenic differentiation, and ghrelin inhibited early osteogenic differentiation from C3H10T1/2 cells. The reason for this discrepancy may be related to the effects of ghrelin on Runx2, PPARγ, and C/EBPα expression. Our results suggest that ghrelin suppresses Runx2 protein expression. Previous in vivo and in vitro data indicate that Runx2 triggers the expression of major bone matrix protein genes at an early stage of osteogenic differentiation, while maintaining osteoblastic cells in an immature stage [Liu et al., 2001]. This suggests that Runx2 may be involved in the differential effects of ghrelin on osteogenic differentiation at early and late stages. Ghrelin can strongly induce adiposity and stimulate adipocyte differentiation [Tschop et al., 2000; Choi et al., 2003]. Consistent with these results, in the present study, ghrelin induced PPARγ and C/EBPα protein expression. The mechanism by which ghrelin suppresses Runx2 is uncertain. In our previous study, PPARγ was shown to inhibit Runx2 expression and transcription from Runx2 promoters in MC3T3-E1 cells [Jeon et al., 2003]. We postulate that ghrelin suppresses Runx2 expression and thereby mediates PPARγ expression in C3H10T1/2 cells, although we did not elucidate the interaction between PPARγ and Runx2 in this study. Akune et al. [2004] reported that heterozygous PPARγ-deficient mice have high bone mass with increased osteoblastogenesis, which when compared to wild type is enhanced in bone marrow cell culture but not in differentiated osteoblast culture. This is in agreement with our findings, suggesting that PPARγ signaling may be involved in the early stages of osteogenic differentiation, but not in the late stages of differentiation.

In the present study, ghrelin also induced the expression of another adipocyte-specific transcription factor, C/EBPα in C3H10T1/2 cells. C/EBPα regulates the differentiation of preadipocytes to adipocytes and converts fibroblasts into adipocytes [Freytag et al., 1994]. Recent in vivo and in vitro studies suggest that ghrelin is involved in bone metabolism, but such effects have yet to be studied comprehensively. As leptin inhibits bone formation and thereby mediates the sympathetic nervous system via the hypothalamus [Takeda et al., 2002], ghrelin in physiologically opposite position of leptin in regards to food intake and energy expenditure may be anticipated to play a potential role in bone metabolism. When C3H10T1/2 cells were cultured with adipogenic media for 7 day, treatment with ghrelin did not result in additional adipogenic differentiation. Furthermore, when the expression levels of PPARγ were altered in these cells in the presence or absence of ghrelin, adipocyte gene expression was found to depend on PPARγ expression but not on ghrelin treatment. This suggests that adipogenic media (containing insulin, dexamethasone, and indomethacin) strongly induce PPARγ and C/EBPα expression, and that this effect is much stronger than that of ghrelin on the cells.

In summary, we have demonstrated novel actions of ghrelin in C3H10T1/2 cells, specifically its ability to stimulate proliferation and inhibit apoptosis and early osteogenic differentiation. Moreover, ghrelin inhibits the osteogenic commitment of MSCs by suppressing the protein expression of Runx2 and enhancing that of PPARγ and C/EBPα.

ACKNOWLEDGMENTS

This study was supported by a grant from Seoul National University Hospital (#04-2003-010-0).

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