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Ghrelin stimulates proliferation and differentiation and inhibits apoptosis in osteoblastic MC3T3-E1 cells

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Abstract

Ghrelin is a 28-amino-acid peptide identified in the stomach as an endogenous ligand of the growth hormone secretagogue receptor (GHS-R) that strongly stimulates the release of growth hormone at the hypothalamus and pituitary level. Although GHS-Rs are expressed in a variety of peripheral tissues, little is known about its effect on bone independent of GH/IGF-1 axis. This study was undertaken to investigate whether ghrelin exerts a direct effect on osteoblasts. We identified mRNA and protein expression of GHS-R in primary osteoblasts as well as a number of osteoblastic cell lines, including MC3T3-E1, ROS 17/2.8, UMR-106, MG63, and SaOS2 cells. Treatment of ghrelin (10^{-11} to 10^{-7} M) to MC3T3-E1 cells showed dose-dependent stimulation of proliferation, which was abrogated by treatment with [D-Lys]-GHRP-6 (10^{-3} M), a selective antagonist of the ghrelin receptor. Ghrelin activated ERK1/2 MAPK and pretreatment with MAPK kinase inhibitors, PD98059 attenuated the ghrelin-induced cell proliferation. Ghrelin also inhibited TNFα-induced apoptosis and suppressed caspase-3 activation that occurs in response to TNFα as well as during in vitro differentiation process. Moreover, ghrelin treatment enhanced in vitro osteoblast differentiation as evidenced by matrix mineralization, alkaline phosphatase activity, and osteoblast-specific gene expression. These results suggest that ghrelin promotes proliferation and differentiation and inhibits apoptosis of osteoblasts.

Keywords: Ghrelin; Osteoblast; Proliferation; MAPK; Apoptosis; Differentiation

Introduction

Ghrelin is a 28-amino-acid peptide that has recently been discovered in human and rat stomach [12,19,26]. Ghrelin strongly stimulates the release of growth hormone [1,2,6,44] and is a natural ligand of the growth hormone secretagogue receptor (GHS-R), which belongs to a seven transmembrane receptor family [26]. The most abundant tissue source of ghrelin appears to be the stomach, where it is synthesized and secreted from a distinct endocrine cell type found in the submucosal layer [13]. It is similarly secreted from small and large intestine as well [39]. Ghrelin is also detected in normal adult plasma at concentrations of 100–120 fmol/ml [26]. These observations indicate that, unlike most digestive peptides, ghrelin is not limited to the gastrointestinal tract but is secreted into gastric blood vessels and circulates throughout the body. Expression of the ghrelin receptor,

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GHS-R, has been also identified in a wide range of tissues [18] including human brain [27], kidney [32], and prostate [22]. The expression of both ghrelin and ghrelin receptor is widely distributed in the human cardiovascular system as well [24].

Although the most prominent effect of ghrelin is their ability to release GH both in vivo and in vitro, the widespread distribution of their receptors suggests that ghrelin may have some peripheral actions. This notion was supported by recent studies that synthetic GHS-R ligand has protective effects against ischemic dysfunction of heart. Hexarelin, a 2-methyl D-Trp derivative of GH releasing peptide-6 (GHRP-6) (His-Trp-Ala-Trp-D-Phe-Lys-NH2), has been shown to improve cardiac function after experimental myocardial infarction [15,45] and protect against post-ischemic dysfunction of heart in rat models [14,28,38]. In vitro, ghrelin and hexarelin stimulate H9c2 cardiomyocytes proliferation [37].

Recent studies indicate that synthetic GHSs are also involved in the regulation of bone growth and metabolism as one of their peripheral effects. GHRPs have been reported to increase growth velocity in children with short stature/GH deficiency [31]. Short-term treatment of obese men with the non-peptidyl GHS, MK-677, increased circulating biochemical markers of bone formation and bone resorption [43], and GHS treatment increased bone mineral content in rodents [42]. However, given that growth hormone/IGF-1 systems are important regulators of bone growth and metabolism [5,16], whether GHSs exert direct peripheral effects on osteoblasts independent of GH/IGF-1 system is yet to be established. This study was undertaken to investigate the direct peripheral effects of ghrelin on osteoblasts. We have demonstrated that primary osteoblasts as well as osteoblastic cell lines of various species express ghrelin receptor. We have also provided evidence that ghrelin treatment directly stimulates proliferation and differentiation, whereas it inhibits apoptosis in mouse osteoblastic MC3T3-E1 cells.

Materials and methods

Materials

Rat ghrelin, GHRP-6, and D-Lys3-GHRP-6 were purchased from Phoenix Pharmaceuticals (Belmont, CA). Rabbit anti-GHS-R1a antibody was purchased from Alpha Diagnostic International Inc (San Antonio, TX). TNFα was obtained from R&D system (Minneapolis, MN), and cycloheximide (CHX) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) were from Sigma-Aldrich (St. Louis, MO). PD 98059 and U0126 were from Calbiochem (Darmstadt, Germany). SuperScript One-Step RT-PCR with Platinum *Taq* system was purchased from Invitrogen (Carlsbad, CA). Fluorogenic caspase substrates Ac-DEVD-AMC were obtained from Calbiochem-Novabiochem (San Diego, CA). Rabbit polyclonal anti-caspase-3 antibody was from BD Biosciences (San Diego, CA), and goat polyclonal anti-actin, rabbit polyclonal anti-phospho-ERK1/2, and anti-ERK1/2 antibodies were from Cell Signaling (Beverly, MA), and anti-phopho HRP-conjugated anti-goat IgG, rabbit IgG, and mouse IgG were from Biosource (Camarillo, CA).

Cell culture

Mouse MC3T3-E1 (RIKEN cell bank, Tsukuba, Japan) osteoblastic cells were derived from spontaneously immortalized calvaria cells and represent immature osteogenic cells. Four osteosarcoma cell lines from rat (ROS 17/2.8 and UMR-106) and human (MG63 and SaOS2) exhibit features of mature osteoblasts. All the experiments were performed using cells at passage 9–12. MC3T3-E1, ROS 17/2.8, UMR-106, and MG63 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 with 10% fetal bovine serum (FBS) and SaOS2 cells in McCoy's 5A medium with 15% fetal bovine serum and antibiotics at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Normal mouse or rat osteoblasts were isolated from 21-day fetal mouse or rat calvariae using a well-characterized technique essentially as described previously [17].

Construction of GHS-R expression plasmid and transient transfection

Full length cDNA of rat GHS-R was obtained by RT-PCR using total RNA isolated from rat brain. The sense and antisense primers were 5′-ATGTGGAACGCGACCCC-CAGCGA-3′, and 5′-ACCCCCAATTGTTTCCAGACC-CAT-3′, respectively [26]. The amplified cDNA was ligated into pcDNA3 vector and used for transient transfection experiment.

HeLa cells were maintained in DMEM supplemented with glutamine, penicillin, streptomycin, and 10% FBS. Purified rat GHS-R plasmid was introduced into the cells (70–80% confluent) using LipofectAMINE (Life Technologies Inc.) according to manufacturer's recommendations. Forty-eight hours after transfection, the cell lysates were subjected to Western blot analysis.

Reverse transcription PCR

For the detection of GHS-R expression, ~2 μg of total RNA isolated from osteoblastic cells was used for RT-PCR analysis using Superscript™ One-step RT-PCR with Platinum^R Taq system (Invitrogen). The forward and reverse primer sequences for mouse GHS-R1a were 5′-GAGCC-TAACGTCACGCTGGA-3′ and 5′-TAGAGGTTGGT-GGTGCG-3′; for rat, 5′-TTCTGCCTCACTGTGCTCTAC-AGT-3′ and 5′-GGACACCAGGTTGCAGTACTGG-3′; and for human, 5′-CCTCGCTCAGGGACCAGAACCA-3′