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Ghrelin enhances the proliferating effect of thyroid stimulating hormone in FRTL-5 thyroid cells☆

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Abstract

Ghrelin regulates cell proliferation through the growth hormone secretagogue receptor (GHS-R). We confirmed the expression of GHS-R in FRTL-5 thyroid cells and investigated the effects of ghrelin in thyrocytes using FRTL-5 cells. Ghrelin increased intracellular calcium levels but not intracellular cyclic AMP levels. Ghrelin activated Erk within 2 min, then activated Akt and STAT3. Erk phosphorylation was inhibited by the calcium inhibitor cyclopiazonic acid (CPA). Ghrelin alone did not stimulate FRTL-5 cell proliferation but enhanced the effects of thyroid stimulating hormone (TSH). Pretreatment with TSH potentiates the growth effects of ghrelin in thyroid cells, and p66Shc, a growth factor receptor adaptor protein, might mediate these synergistic effects. Ghrelin phosphorylated TSH-induced p66Shc, which was inhibited by CPA. Ghrelin did not affect the proliferation of ARO cells, which showed no increased expression of p66Shc after TSH treatment. Thus, ghrelin-induced intracellular calcium signaling enhanced the TSH-induced proliferation of thyrocytes, possibly mediated by the p66Shc pathway.

Keywords: Ghrelin; Thyroid; Proliferation; Erk; Calcium; p66Shc

1. Introduction

Ghrelin is a 28 amino acid hormone with multiple functions that acts through the growth hormone secretagogue receptor (GHS-R) (Kojima et al., 1999; Arvat et al., 2001; Broglio et al., 2001; Caminos et al., 2002). It is predominantly produced by the stomach but is also produced in normal thyroid C cells (Raghay et al., 2006). Moreover, Gnanapavan et al. (2002) showed that GHS-R type 1a mRNA is expressed in the thyroid, together with GHS-R1b. Several studies have shown the expression of ghrelin in thyroid and medullary thyroid carcinomas (Kanamoto et al., 2001; Volante et al., 2003; Morpurgo et al., 2005; Raghay et al., 2006) but not in benign thyroid diseases (Zhang et al., 2006) including thyroid adenomas. Ghrelin could play a role in the proliferation of thyrocytes or in the development of thyroid tumors. However, the effects of ghrelin on the proliferation of thyrocytes remain unclear.

Ghrelin activates phospholipase C, protein kinase C and intracellular calcium mobilization after binding to the GHS-R in HeLa-T4 cells (Cunha and Mayo, 2002). Intracellular calcium mobilization is also an important signal in thyrocytes (Ginsberg et al., 1997; Schofl et al., 1997). Therefore, it is conceivable that ghrelin may be involved in the regulation of function of thyrocytes via the calcium signal activated by GHS-R. Therefore, we aimed to investigate the signal pathways and proliferative effects of ghrelin using normal rat FRTL-5 thyroid cells.

Abbreviations: GHS-R, growth hormone secretagogue receptor; CPA, cyclopiazonic acid; EGTA, ethylene glycol bis(β-aminoethylether)tetraacetic acid; pErk, phosphorylation of Erk; GHRP, growth hormone-related peptide.

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2. Materials and methods

2.1. Tissue and cell culture

A fresh subclone of FRTL-5 rat thyroid cells was obtained from the Interthyr Research Foundation (Dr. Leonard D. Kohn, Ohio Univ., Athens, OH). The doubling time of these cells is 36 ± 6 h when cultured in the presence of TSH, but they do not proliferate in the absence of TSH. Cells were grown in 6H5 medium, consisting of Coon's modified F-12 medium supplemented with 5% calf serum, 1 mM nonessential amino acids and a mixture of six hormones: bovine TSH (10 U/L), insulin (10 mg/L), hydrocortisone (0.4 mg/L), human transferrin (5 mg/L), glycyl-L-histidyl-L-lysine acetate (10 μg/L) and somatostatin (10 μg/L). Before the experiments and after the cells were about 80% confluent, the cells were grown for 5 d in 5H5 medium depleted of TSH. In some experiments, the cells were treated in 3H0.5 medium (depleted of TSH, insulin and somatostatin) for 12 h before treatment. Human anaplastic thyroid carcinoma (ARO) cell lines were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin and 50 μg/mL streptomycin (Invitrogen) at 37 °C under 5% CO_2 in air.

The cells were plated in 100 mm dishes for reverse transcriptase-polymerase chain reaction (RT-PCR) amplification, in 6-well plates for immunoprecipitation and Western blotting, in 24-well plates for cAMP assay and in 96-well plates for the proliferation study (MTT assay).

Normal human thyroid tissues were obtained from surgical operations. Tissue samples of rat thyroid or pituitary glands were obtained from male Sprague–Dawley rats (120–130 g) fed with normal chow. All experiments were conducted in accordance with the guidelines proposed in The Declaration of Helsinki (http://www.wma.net) involving humans and the use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the Clinical Research Institute and the Institutional Review Board at Seoul National University Hospital.

2.2. Materials

Rabbit polyclonal anti-total Shc antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA), and anti-rabbit secondary antibodies from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-phosphotyrosine antibodies, 4G10, were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Bovine TSH, growth hormone-related peptide (GHRP), IGF-I and insulin were obtained from Sigma–Aldrich (St. Louis, MO, USA). cAMP RIA kits were obtained from Incstar (Minneapolis, MN, USA). All other reagents were obtained from Sigma–Aldrich unless otherwise stated.

2.3. cAMP measurement

Cyclic AMP assays in FRTL-5 cells were performed in NaCl-free Hanks balanced salt solution (HBSS) containing 20 mmol/L HEPES (pH 7.4), 1% bovine serum albumin, 0.5 mmol/L 3-isobutyl-1-methylxanthine and 222 mmol/L sucrose to make it hypotonic. Aliquots of 1 nM ghrelin or 10 U/L TSH, dissolved in 300 μL incubation media, were incubated with cells plated in 24-well plates for 2 h at 37 °C; supernatants were aspirated and stored at −20 °C, and cAMP released into the medium was measured by radioimmunoassay (RIA) as described (Kim et al., 1996).

2.4. RNA and RT-PCR

To confirm the expression of GHS-R in normal thyroid cells and tissues, we performed RT-PCR using FRTL-5 cells and rat normal pituitary tissues as positive controls. In addition, to detect the presence of GHS-R in thyroid cancers, we used ARO cells and human normal thyroid tissues as controls. For FRTL-5 and ARO cells, when the cells reached 70% confluence, total RNA was extracted from the cell pellets using the Trizol method (Invitrogen) according to the manufacturer's instructions. For normal pituitary and thyroid tissues, we also extracted total RNA from the tissue using homogenizer model PRO 200 (Pro Scientific Inc., Oxford, CT, USA). One microgram of total RNA from each sample was used to obtain cDNAs using MMLV Reverse Transcriptase (Invitrogen). For PCR, the following primers were used for the total rat GHS-R (512 bp): 5′-CTCAGGCAACCTGCTCA-3′ (sense) and 5′-TACCGGTCTTCTGCCTC-3′ (antisense), and for human GHS-R (130 bp): 5′-CTCCAAGCATCCTCCCTA-3′ (sense) and 5′-CAGCTCTCACTGACGAA-3′ (antisense). The cycle consisted of 5 min at 65 °C, 2 min at 37 °C, 50 min at 37 °C, followed by 15 min at 70 °C on a PTC-200 thermal cycler (MJ Research, Ramsay, MN, USA). A PCR premix reagent set (AccuPower, Bioneer, Seoul, Korea) was used for PCR. The specimens were then electrophoresed on 1% agarose gels.

2.5. Western blot analysis and immunoprecipitation

The cells were cultured in six-well culture plates. After each indicated treatment, the collected cells were lysed in a lysis buffer (20 mM Tris–HCl pH 7.4, 2 mM EGTA, 2 mM EDTA, 25 mM beta-glycerophosphate, 1% Triton X 100 and 500 μL of 10% glycerol and protease inhibitors; Sigma–Aldrich). The supernatant from the cell lysates was centrifuged at 14,000 rpm for 15 min and the supernatant containing total protein was collected. Protein concentrations were measured using the BCA (bicinchoninic acid) assay (Smith et al., 1985). Aliquots of 15 μg of protein were boiled for 5 min in 4× loading buffer (500 mM Tris–HCl pH 6.8, 10% SDS, 10% glycerol, 20 mM DTT and 0.1% bromophenol blue) and then electrophoresed with a prestained protein marker (Fermentas, San Francisco, CA, USA) on an 8% SDS–PAGE gel. The protein was transferred to a nitrocellulose membrane (Whatman, Schleicher and Schuell, Dassel, Germany) for 1 h in transfer buffer (250 mM Tris, 1.92 M glycine and 20% methanol). The membrane was blocked for 30 min in blocking solution (5% bovine serum albumin; BSA). This was followed by incubation of the membrane in primary anti-Erk (extracellular signal-regulated kinase), anti-phospho-Erk, anti-STAT3 (signal transducer and activator of transcription 3), anti-phospho-STAT3, anti-Akt, anti-phospho-Akt (Cell Signaling, Beverly, MA, USA), and anti-Shc antibody solutions at 4 °C overnight in 1% BSA. After washing in TBS-T (Tris-buffered saline containing 0.1% (w/v) Tween 20), the membrane was incubated with an anti-rabbit secondary antibody at room temperature for 1 h. After washing, an enhanced chemiluminescence (ECL) solution (GE Healthcare, Buckinghamshire, UK) was layered onto the membrane and incubated for 5 min. The membrane was then exposed to X-ray film and developed using a chemiluminescent detection system (FPM 1300, Fuji, Mortsem, Belgium).

For immunoprecipitation, the specimens were mixed with 1 mg of protein and anti-phosphotyrosine antibody (4G10) and then rotated at 4 °C overnight. Thereafter, 50 μL of G sepharose was added, and the mix was rotated for 2 h at 4 °C. Centrifugation at 14,000 rpm for 5 min and washing with IP buffer was repeated three times. The specimens were then electrophoresed with a prestained protein ladder (Fermentas) on an 8% SDS–PAGE gel. The same specimens were immunoblotted with the anti-Shc antibody.

2.6. MTT [thiazolyl blue tetrazolium bromide] assay

Cell growth was assessed by MTT assay. The FRTL-5 cells were seeded into each well of 96-well culture plates and then cultured in 5H5 medium depleted of TSH for 5 d and then 3H0.5 medium overnight, followed by 3H0.5 medium (negative control) and 1 nM ghrelin, 10 nM GHRP, 100 ng/mL IGF-1 or 10 μM CPA for 5–10 min with or without 1 U/L TSH (24 h pretreatment). ARO cells were also treated with various concentration of ghrelin with or without TSH pretreatment. After incubation, 150 μL of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution (2 mg/mL in phosphate-buffered saline) was added to each well, and the plates were then incubated for 4 h at 37 °C. After removing the supernatant and shaking with 50 μL of dimethyl sulfoxide (Jersey Lab Supply, Livingston, NJ, USA) for 30 min, absorbance was measured at 460 nm. All experiments were repeated at least three times.

3. Results

3.1. GHS-R expression in thyroid cells and tissues

We confirmed the expression of GHS-R in rat thyroid tissues as well as in rat pituitary tissues (positive control). GHS-R was