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The constitutive activity of the ghrelin receptor attenuates apoptosis via a protein kinase C-dependent pathway

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ABSTRACT

The ghrelin receptor (GHS-R1a) displays a high level of constitutive signaling through a phospholipase C/protein kinase C-dependent pathway. Therefore, we have investigated the role of agonist-dependent and agonist-independent signaling of GHS-R1a in apoptosis using the seabream GHS-R1a stably expressed in human embryonic kidney 293 cells (HEK-sbGHS-R1a cells). Cadmium-induced activation of caspase-3 was significantly attenuated in HEK-sbGHS-R1a cells compared to wild-type HEK293 cells, while the apoptotic responses to the protein kinase C inhibitor staurosporine were similar. GHS-R1a ligands had no effect on caspase-3 activation or on cell proliferation. Concentrations of the inverse agonist [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]-substance P sufficient to inhibit constitutive inositol phosphate generation did not enhance caspase-3 activity, suggesting a possible role of phosphatidylcholine-specific phospholipase C in the anti-apoptotic activity of GHS-R1a. In conclusion, our data suggests that the constitutive activity of sbGHS-R1a may be sufficient alone to attenuate apoptosis via a protein kinase C-dependent pathway.
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1. Introduction

Ghrelin is a 28-amino acid peptide with an acylated octanoic acid at Ser3 which is essential for activation of the ghrelin receptor (Kojima et al., 1999). Ghrelin, and other growth hormone secretagogues such as GHRP-6, stimulate a Gq/11-protein coupled receptor (Howard et al., 1996) now known as the ghrelin receptor or GHS-R1a (Davenport et al., 2005). In addition to GHS-R1a, there is wide spread distribution of a splice variant comprising the first five predicted transmembrane regions of GHS-R1a, known as GHS-R1b (Howard et al., 1996). The truncated ghrelin receptor polypeptide (GHS-R1b) does not bind ghrelin but acts as a dominant-negative for the expression of GHS-R1a at the cell surface and therefore has a marked effect on the constitutive (agonist-independent) signaling properties of GHS-R1a when co-transfected into human embryonic kidney (HEK) 293 cells (Chu et al., 2007; Leung et al., 2007). The ghrelin receptor is unusual in displaying extremely high levels of constitutive activity (Holst et al., 2003), and it has been proposed that a lack of constitutive activity is responsible for the altered growth status in persons expressing novel variants of the *GHSR* gene (Holst and Schwartz, 2006).

There is much conflicting information in the literature concerning the role of ghrelin as a proliferative agent, in part because not all ghrelin-stimulated responses are mediated through GHS-R1a (van der Lely et al., 2004). For example, in myocardium there are at least three functional receptors for ghrelin: GHS-R1a, GHS-Ru (unknown) and CD36 which is a glycoprotein type B scavenger receptor (Cao et al., 2006). But if we focus on cellular responses which are attributable to ghrelin stimulation of GHS-R1a (i.e. assays where nonacylated ghrelin was inactive and/or GHS-R1a was identified by protein or mRNA expression), we see that ghrelin stimulates proliferation which can be inhibited by [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]-substance P (SPa) or D-Lys(3)-GHRP-6 in rat and human primary cell cultures (Andreis et al., 2003; Li et al., 2007; Sato et al., 2006; Zhang et al., 2005) and in cell lines (Jeffery et al., 2002; Kim et al., 2005; Nanzer et al., 2004). And, while ghrelin either has no effect on apoptosis (Andreis et al., 2003) or enhances apoptotic deletion (Belloni et al., 2004), the predominant effect appears to be the ability to inhibit apoptosis, as shown in adipocytes (Kim et al., 2004), pancreatic cells (Andreis et al., 2003) and in rat hypothalamic cell cultures (Chung et al., 2007). In addition, GHRP-6 also inhibits apoptosis in rat central nervous system (Delgado-Rubín de Célix et al., 2006; Frago et al., 2002).

Since the discovery of GHS-R1a and GHS-R1b, researchers have attempted to correlate the relative expression of these proteins (at the mRNA level) with the state of malignancy of human tumors. For example, (i) somatotroph adenomas had significantly higher GHS-R1b mRNA copy number compared to normal pituitary (Korbonits et al., 2001), (ii) prostate cancer cell lines express GHS-R1a and

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GHS-R1b, whereas normal prostate only has GHS-R1a (Jeffery et al., 2002), (iii) 50% of human endocrine pancreatic tumors express GHS-R1b and 50% of these also contain GHS-R1a, with the majority of these tumors expressing GHS-R1a also expressed ghrelin (such co-expression was not seen in normal endocrine pancreas) (Volante et al., 2002), (iv) human pancreatic adenocarcinoma cell lines express both GHS-R1a and GHS-R1b (Duxbury et al., 2003), (v) in contrast to normal lung, a human bronchial neuroendocrine tumor expresses ghrelin, GHS-R1a and GHS-R1b (Arnaldi et al., 2003), and (vi) the ratio of ghrelin to GHS-R1a increases in highly differentiated testicular tumors (Gaytan et al., 2004). Whether or not the GHS-R1b polypeptide functions as a dominant-negative mutant of GHS-R1a under physiological conditions remains to be determined, but it has been proposed to form a heterodimer complex with neurotensin receptor 1 and thus function as a receptor for neuromedin U in non-small cell lung cancer cells (Takahashi et al., 2006). Thus, the presence of GHS-R1a and/or GHS-R1b may contribute to the tumor potential of cells.

Seabream GHS-R1a shares 60% amino acid identity with mammalian GHSRs and has higher affinity for GHRP-6 than for human ghrelin (Chan and Cheng, 2004). Therefore, having established a cell line stably expressing seabream GHS-R1a (HEK-sbGHS-R1a) (Chan and Cheng, 2004), we chose this system to investigate the role of GHS-R1a and GHS-R1b in regulating apoptosis and proliferation. As we have shown previously that GHS-R1b behaves as a dominant-negative mutant of GHS-R1a and can attenuate the constitutive activity of GHS-R1a (Leung et al., 2007), and that D-Lys(3)-GHRP-6 can inhibit constitutive activation of ERK1/2 in HEK-sbGHS-R1a cells (Chan et al., 2004), we have used these HEK-sbGHS-R1a cells to study the role of agonist-dependent and agonist-independent signaling through sbGHS-R1a in the regulation of apoptosis and proliferation. In order to determine the role of sbGHS-R1a signaling through the Gq/phospholipase C/protein kinase C pathway, we have compared the effect of apoptosis inducers predicted to be protein kinase C-dependent (staurosporine, Ozawa et al. (1999)) and protein kinase C-independent (cadmium, Hamada et al. (1996)).

Here we show that the constitutive activity of sbGHS-R1a, when stably expressed in HEK293 cells, appears to attenuate apoptosis via a protein kinase C-dependent pathway but does not affect proliferation of these cells.

2. Materials and methods

2.1. Reagents

GHRP-6 and [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]-substance P were obtained from Phoenix Pharmaceutical Inc. (Beijing, China). D-Lys(3)-GHRP-6 was obtained from Bachem AG (Bubendorf, Switzerland). Unless specified, all other compounds were supplied by Biomol (Plymouth Meeting, PA), Invitrogen (Carlsbad, CA) or Sigma Chemical Co. (St. Louis, MO).

2.2. Cell culture and transfection

HEK293 cells were purchased from American Type Culture Collection (Manassas, VA) and were maintained in DMEM containing 100 i.u./ml penicillin, 100 μg/ml streptomycin and 10% (v/v) foetal bovine serum, and were incubated in a humidified atmosphere of 5% CO_2/95% air at 37 °C. A clonally selected HEK293 cell line stably expressing sbGHS-R1a was developed as previously described (Chan and Cheng, 2004) and was maintained in DMEM containing 500 μg/ml G418. Cells were transfected at 80% confluency in culture plates using LipofectAMINE 2000 liposome reagent and Opti-mem I reduced serum medium (Invitrogen, Carlsbad, CA) for 5 h, according to the manufacturer's instructions, and all cells were assayed 48 h post-transfection.

2.3. Preparation of epitope-tagged sbGHS-R1a and sbGHS-R1b

The HA-sbGHS-R1a and HA-sbGHS-R1b were constructed by PCR amplification from seabream pituitary cDNA. Gene specific primers were designed flanking the entire coding regions of sbGHS-R1a and sbGHS-R1b respectively, with the appropriate restriction enzyme cutting sites (Kpn I and Xba I) engineered at the 5′ ends of the primers. A HA tag was introduced to the N-terminal of the receptors by incorporating the HA sequence to the forward primers. The primers for HA-sbGHS-R1a were sbGHS-R1a-start-HA (a forward primer having the sequence: GAG GTA CCG AAA TGT ATC CAT ATG ATG TTC CAG ATT ATG CTC CCT CTT GGC CAA ATC TC) and sbGHS-R1a-stop (a reverse primer having the sequence: CAC ATC TAG ATT AGA AGC TGA TTG TGG A). The primers for HA-sbGHS-R1b were sbGHS-R1a-start-HA (the same as for sbGHS-R1a) and sbGHS-R1b-stop (a reverse primer having the sequence: CAC ATC TAG ACT ACA TGG ATA AAG TTA TG). PCR was carried out in a 50 μl final volume containing 50 mM KCl, 10 mM Tris–HCl at pH 9, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.02% bovine serum albumin (BSA), 200 μM dNTP, 0.2 pmol primers, 2.5U *Taq* polymerase, and 1 μl cDNA. The reaction was performed with annealing temperature of 50 °C for 25 cycles. The PCR product was size separated on an agarose gel, gene-cleaned, unidirectionally subcloned into the eukaryotic expression vector pcDNA3.1(+)/Zeo, and all modifications were confirmed by DNA sequencing.

2.4. Measurement of sbGHS-R1a and sbGHS-R1b mRNA

Quantitative real time PCR was performed to quantify the expression of sbGHS-R1a and sbGHS-R1b, essentially as described previously for human ghrelin receptors (Leung et al., 2007). Oligonucleotide primers were: 5′-GTGGGAATGAATGGGACTGG-3′ as the forward primer for both sbGHS-R1a and sbGHS-R1b, and 5′-GCCAACACCACCACCACCAAC-3′ for the sbGHS-R1a reverse primer, and 5′-GGATAAAGTTATGCGTGTGCG-3′ for the sbGHS-R1b reverse primer. PCR reactions were performed in duplicate in three independent experiments using the ABI Prism 7700 Sequence Detector System (Applied Biosystems, Foster City, CA) in a total volume of 25 μl containing Sybr green PCR buffer (Applied Biosystems, UK), $MgCl_2$, dNTP blend, AmpliTaq Gold, AmpErase UNG primers, water and diluted cDNA template. Negative controls contained template without performing reverse transcription. The PCR conditions were: 50 °C for 2 min and denaturing at 95 °C for 10 min, followed by 40 cycles at 94 °C for 45 s and 64 °C for 45 s. Absolute quantification of transcripts was performed against standard curves obtained by amplification of serially diluted solutions of plasmid clones containing sbGHS-R1a and sbGHS-R1b sequences as templates. Levels of mRNA expression were normalized against total RNA (Whelan et al., 2003).

2.5. Caspase-3 assay

For the comparison of caspase-3 activity in HEK-sbGHS-R1a and HEK-sbGHS-R1b cells, cells were seeded in 6-well culture plates at 8×10^5 cells per well, and maintained in DMEM plus 10% fetal bovine serum. After 24 h, the cells were transfected and apoptosis inducers added 48 h post-transfection. After 8 h incubation of triplicate wells with apoptosis inducer or control solution, the medium was removed and cells were washed and harvested in PBS. Cell pellets were lysed (lysis buffer: 50 mM Hepes, 1 mM DTT, 0.1 mM EDTA, 0.1% CHAPS, pH 7.4), centrifuged at $10{,}000 \times g$ for 10 min at 4 °C, and lysates assayed for caspase-3 activity. Activated caspase-3 cleaves a specific caspase-3 I substrate (Calbiochem, San Diego, CA) to release *p*-nitroaniline which was detected by absorbance at 405 nm in a 96-well plate ELISA reader (Universal Microplate Reader Elx800, Bio-Tek Instruments Inc., Winooski, VT). The protein content of the cell lysates was determined using a Micro BCA™ Protein Assay Kit (Pierce, Rockford, IL) and caspase-3 specific activity was then determined.

To compare the effect of GHS-R1a ligands, cells were seeded as above. Cells were incubated with DMEM or SPa (1 μM) or D-Lys(3)-GHRP-6 (100 μM) for 30 min before DMEM or GHRP-6 (100 μM), then caspase-3 activity was measured in duplicate wells after 24 h incubation with DMEM (control), 10 μM staurosporine (stau), or 25 μM cadmium.

2.6. DNA fragmentation

Cells were seeded in 6-well culture plates at 8×10^5 cells per well. The next day, cells were incubated for 8, 16 or 24 h with apoptosis inducer or control (0.1% DMSO) solution, then harvested with the addition of lysis buffer (Promega, Madison, IN). Genomic DNA was isolated using Wizard SV Genomic DNA Purification System (Promega, Madison, IN). DNA (2 μg) was electrophoresed on a 1.5% agarose gel and the pattern of DNA fragmentation was photographed with a ChemiDoc XRS (Bio-Rad, Hercules, CA) after staining with GelRed (Biotium Inc., Hayward, CA).

2.7. Cell viability assay

Cells were seeded in 96-well culture plates, pretreated with poly-D-lysine (10 μg/ml), at 10^4 cells per well, then maintained and transfected as described for the caspase-3 assay. The conversion of 2-(4′,5′-dimethyl-2′-thiazolyl)-3-(4′-sulfophenyl) (MTS) solution to a colored formazan product in metabolically active cells was determined, in duplicate, by incubation for 4 h in a CO_2 incubator, and the absorbance at 570 nm was recorded with a 96-well plate ELISA reader (Universal Microplate Reader Elx800, Bio-Tek Instruments Inc., Winooski, VT).

2.8. Cell proliferation assay

Cells were seeded in 12-well culture plates, pretreated with poly-D-lysine (10 μg/ml), at 10^5 cells per well, and cultured in DMEM plus 10% fetal bovine

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