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Unacylated ghrelin is not a functional antagonist but a full agonist of the type 1a growth hormone secretagogue receptor (GHS-R)

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Abstract

Recent findings demonstrate that the effects of ghrelin can be abrogated by co-administered unacylated ghrelin (UAG). Since the general consensus is that UAG does not interact with the type 1a growth hormone secretagogue receptor (GHS-R), a possible mechanism of action for this antagonistic effect is via another receptor. However, functional antagonism of the GHS-R by UAG has not been explored extensively. In this study we used human GHS-R and aequorin expressing CHO-K1 cells to measure $[Ca^{2+}]_i$ following treatment with UAG. UAG at up to 10^{-5} M did not antagonize ghrelin induced $[Ca^{2+}]_i$. However, UAG was found to be a full agonist of the GHS-R with an EC_{50} of between 1.6 and 2 μM using this *in vitro* system. Correspondingly, UAG displaced radio-labeled ghrelin from the GHS-R with an IC_{50} of 13 μM. In addition, GHS-R antagonists were found to block UAG induced $[Ca^{2+}]_i$ with approximately similar potency to their effect on ghrelin activation of the GHS-R, suggesting a similar mode of action. These findings demonstrate in a defined system that UAG does not antagonize activation of the GHS-R by ghrelin. But our findings also emphasize the importance of assessing the concentration of UAG used in both *in vitro* and *in vivo* experimental systems that are aimed at examining GHS-R independent effects. Where local concentrations of UAG may reach the high nanomolar to micromolar range, assignment of GHS-R independent effects should be made with caution.

Keywords: Ghrelin; GHS-R; Antagonism

1. Introduction

Ghrelin was discovered through its ability to activate the type 1a growth hormone secretagogue receptor (GHS-R) and stimulate growth hormone release *in vivo* (Smith et al., 2005; van der Lely et al., 2004). An evolutionarily conserved feature of ghrelin is the acylation of its third residue, usually with *n*-octanoic and, less commonly, with *n*-decanoic acid (Hosoda et al., 2003). Kojima et al. (1999) were the first to describe the requirement that ghrelin be acylated on its third serine residue for activation of the GHS-R in the nanomolar range, with an EC_{50} for increased $[Ca^{2+}]_i$ of 2.5×10^{-9} M. In the circulation, ghrelin also occurs as a unacylated isoform (UAG) at 10–50 times the concentration of acylated ghrelin (Kojima and Kangawa, 2005, and our unpublished observations).

Of great interest to us has been the finding that in humans co-administration of UAG can antagonize the metabolic effects of ghrelin *in vivo*. Ghrelin administration causes hyperglycemia, hypoinsulinemia, increased circulating free fatty acids and worsening insulin sensitivity, but these effects are reversed or prevented by co-administration with UAG (Broglio et al., 2004; Gauna et al., 2004). These effects seem to be specific to ghrelin's metabolic activity since UAG has no impact on GH, PRL or ACTH secretion (Broglio et al., 2004). This suggested a direct action on the endocrine pancreas, and perhaps on hepatic glucose production. In relation to these *in vivo* findings, we have shown that UAG not only suppresses glucose output, but also blocks ghrelin induced glucose release by primary hepatocytes (Gauna et al., 2005). In support of these findings, a recent report demonstrated the antagonistic effect in fish where ghrelin's orexigenic effects were blocked by administration of UAG. Furthermore, this effect appears to occur both centrally and peripherally (Matsuda et al., 2006). There are now many

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reports of direct biological activity of UAG *in vitro* that suggest a receptor mediated cellular response, perhaps via a specific receptor that is not GHS-R (Baldanzi et al., 2002; Cassoni et al., 2004; Chen et al., 2005; Gauna et al., 2006; Muccioli et al., 2004; Nanzer et al., 2004; Thompson et al., 2003; Toshinai et al., 2006). Despite these findings, the current consensus appears to be that UAG is inactive as an agonist of the GHS-R. However, the possibility remains that UAG is somehow able to block the ghrelin response by antagonizing the GHS-R. Therefore, we have explored in more detail, in a defined *in vitro* system, the ability of UAG to antagonize activation of the GHS-R by ghrelin, the potency of UAG at the GHS-R, and the effects of GHS-R antagonists.

2. Materials and methods

2.1. Peptides

Human UAG was obtained from NeoMPS (Strasbourg, France) and Thera Technologies (Montreal, Canada). Human ghrelin, [D-Lys^3]GHRP-6, somatostatin28, obestatin and glucagon were obtained from NeoMPS. The ghrelin analog BIM28163, a potent antagonist of the GHS-R, was kindly provided by IPSEN Pharmaceuticals (Milford, MA). All peptides had been assessed for purity and integrity by high performance liquid chromatography and mass spectrometry.

2.2. Aequoscreen assay for ghrelin and UAG activity

Aequoscreen cells were kindly provided by Euroscreen s.a. (Gosselies, Belgium). These cells (CHO-A5) are stably transfected with a pIRES-puro (Clontech, Mountain View, CA) construct containing mitochondrially targeted apoaequorin which allows luminometric determination of $[Ca]_i$. An identical cell line was also provided that had also been stably transfected with a human GHS-R1a expression construct with a *Neo* cassette (CHO-A5-GHSR). These cell lines were maintained in HAM F12 containing 10% fetal calf serum, 2.5 μg/mL amphotericin, 100 IU/mL penicillin, 100 μg/mL streptomycin, 5 μg/mL puromycin. The GHS-R expressing cells were maintained in the same medium but with the addition of 400 μg/mL G418. On the day of the assay the cells were resuspended in BSA assay buffer (DMEM/HAM's F12, with HEPES, without phenol red, 0.1% BSA, 2.5 μg/mL amphotericin, 100 IU/mL penicillin, 100 μg/mL streptomycin) at 5×10^6 cells/mL, and then coelenterazine h (Sigma, St. Louis, MO) was added to a final concentration of 5 μM. Cells were incubated at room temperature for 4 h and kept in suspension by gentle rotation. Cells were then diluted with BSA assay buffer to 5×10^5 cells/mL, and 100 μL was injected into wells of a black 96-well plate containing 100 μL of various concentrations and combinations of ghrelin, UAG and other peptides. Luminescence was integrated for 15 s using a Victor2 1420 multilabel counter (Perkin-Elmer, Wellesley, MA, USA), a short enough time that it is very unlikely that modification of the UAG could occur during the assay. After collection of data, the residual response of the cells was measured by permeabilizing their membranes with 100 μL of 1% Triton X-100. Data were calculated as the fractional response to agonist relative to the total response of the cells to agonist and Triton X-100 (fractional response $= x(x+y)^{-1}$, where x = agonist response and y = residual response). In experiments with antagonists we have normalized the data as percentage of maximal response in the absence of antagonist. UAG was able to saturate the response of the aequoscreen cells in the presence of an EC_{75} concentration of ghrelin (Fig. 1A). Therefore, we have set the maximal response of UAG at that of ghrelin in the correlation function used for regression analysis. Non-linear regression analysis was performed using Graphpad Prism version 3 (San Diego, CA).

2.3. Radioligand binding studies

Membranes were prepared from CHO-A5-GHSR cells using a protocol from Euroscreen. Briefly, cells at 80% confluence in monolayer culture in 75 cm^2 flasks, were scraped into PBS, then pelleted at $1500 \times g$ for 3 min. The cell pellet was then resuspended in buffer A (15 mM Tris–HCl (pH 7.4), 2 mM $MgCl_2$, 0.3 mM EDTA, 1 mM EGTA) at 4 °C and homogenized in a glass/Teflon homogenizer. The crude membrane fraction was collected by two consecutive centrifugation steps at $40{,}000 \times g$ for 25 min separated by a washing step in buffer A. Membrane pellets were resuspended in buffer B (75 mM Tris–HCl (pH 7.5), 12.5 mM $MgCl_2$, 0.3 mM EDTA, 1 mM EGTA, 250 mM sucrose) and stored at −80 °C until use. Protein content was measured using the Biorad protein assay (Biorad, Hercules, CA).

Competitive binding dose curves for ghrelin and UAG were then run using 75 μg of membrane protein and 50,000 cpm (0.1 nM) of [His[^{125}I]]-ghrelin (NEX388, Perkin-Elmer, Boston, MA) in binding buffer (25 mM HEPES pH7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$) in a total volume of 100 μL. Binding was carried out at 21 °C for 60 min, then membranes were collected by centrifugation, washed with ice-cold binding buffer, and radioactivity was counted. Samples were run in duplicate. Non-linear regression analysis was performed using Graphpad Prism.

3. Results

The main reason for examining UAG modulation of GHS-R activity was to elucidate a mechanism for our finding that this

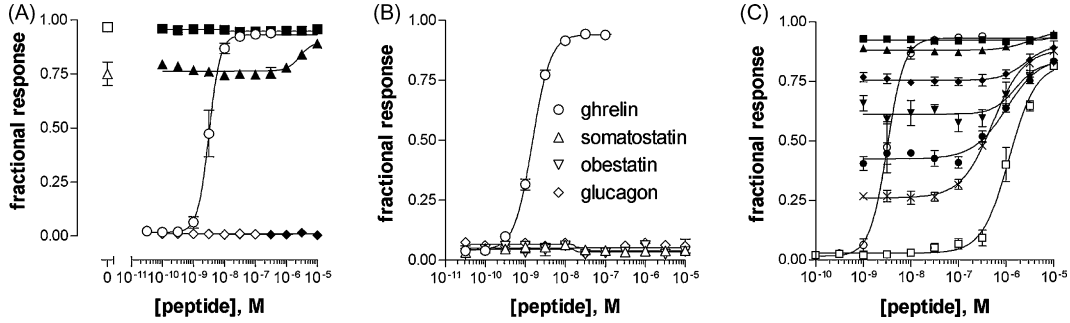

Fig. 1. (A) UAG does not antagonise ghrelin's action on the GHS-R. Open circles represent the $[Ca^{2+}]_i$ response of CHO-A5-GHS-R cells to a dose curve of ghrelin. The cells' response to 2.5 nM (triangles) and 5 nM ghrelin (squares), in the absence (open symbols) and presence (closed symbols) of dose curves of UAG are superimposed on the ghrelin dose response curve. UAG further stimulates the effects of 2.5 nM ghrelin, approaching the maximal response obtained with ghrelin alone (fractional response of 0.93). Neither ghrelin (open diamonds) nor UAG (closed diamonds) activate aequorin cells that lack the GHS-R. (B) Peptide ligands (somatostatin28, obestatin and glucagon) of similar molecular weight to UAG do not activate the GHS-R in aequoscreen cells in the 10^{-11} to 10^{-5} M range. A ghrelin dose response was run concurrently as a positive control. (C) UAG (open squares), activates the GHS-R with an EC50 in the low micromolar range, but does not antagonize activation of the GHS-R by ghrelin in a range of concentrations corresponding to 25–100% of maximal response (closed symbols represent UAG dose response curves run in increasing concentrations of ghrelin from 0.75 to 9 nM). The dose response for ghrelin is shown for comparison (open circles). Error bars represent ±S.E.M.