Introduction

Growth hormone secretagogues (GHS) are a family of predominantly synthetic molecules that have been developed through so-called reversed pharmacology, i.e. initial finding of an orphan receptor without an identified ligand. Ghrelin was subsequently identified as the natural ligand in 1999 by Kojima and co-workers. It is a 28 amino acid long peptide carrying a unique posttranslational modification (n-octanoic acid) in the third amino acid (figure 1). Rather than stimulating the cyclic AMP pathway as do the growth hormone releasing hormones, GHS act on the GHSR1a and induce intracellular calcium release, in turn triggering the GH synthesis in the hypophysis (figure 1). Because of the reversed pharmacology strategy exogenous GHS possess a high degree of structural variety and little homology with ghrelin (figure 1), and may be potentially used for doping purpose, as a substitute for, or in combination with growth hormone itself. The identification of GHS in biological fluids has thus far not been addressed and a priori presents serious difficulties due to the structural variety of the molecules, their complex nature, and the concentration at which they might be found in biological fluids. As such, a screening procedure should be based on the common feature of all molecules; their capacity to interact with the receptor.
Here we describe a strategy based on displacement of a labelled ligand bound to the receptor by non-labelled GHS present in a biological fluid. The loss of labelled material from the receptor is indicative of the presence of a secretagogue whereas its identification would require a more classical approach based on chromatographic purification and mass spectrometric analysis. Preliminary data on the stable recombinant production of growth hormone secretagogue receptor (GHSR1α), the characterisation of several synthetic GHS, the synthesis of the endogenous ligand (ghrelin), radiolabelling of this molecule, and binding/competition experiments under optimal are presented.

**Materials and Methods**

**Reagents** – Human ghrelin and desoctanoyl-ghrelin were synthesised through a standard solid-phase protocol at the Department of Experimental and Health Sciences, Pompeu Fabra University, Barcelona, Spain. Truncated variant, ghrelin AA1-14 and ghrelin AA1-10 were purchased from Peptides International, Inc (Louisville, Kentucky, USA), and Hexarelin, GHRP-6 and MK-0677 were a generous gift from Dr. G. Muccioli (Department of Anatomy, Pharmacology and Forensic medicine of University of Turin, Italy). Radioligand, $^{125}$I-ghrelin was labelled as described and also purchased from Amersham (Buckinghamshire, United Kingdom). Filters GF/C were acquired from Whatman International Ltd. (Brentford, Middlesex, England). Fluo-3 and Fluo-4 probes
and Pluronic-127 were purchased from Invitrogen (Carlsbad, USA). For cell culture: Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), penicillin, streptomycin, glutamine and G418 were purchased from Invitrogen (Carlsbad, USA), 100 mm dishes from Beckton Dickinson (San Diego, California) and multi-well plates from Nunc (Wiesbaden, Germany).

**Cell culture** – HEK 293 cells (human embryonic kidney), expressing the GHS-R1a were a kind gift from Dr. R. Smith (Baylor College of Medicine, Houston, USA) through Dr. F. Casanueva (University of Santiago de Compostela, Spain), while wild type HEK 293 cells were from Dr. C. Fillat (CRG, Barcelona Spain). Briefly, both cell lines were cultured up to 70-80% confluence and subjected to G418 selection (800 µg/ml).

**Membrane preparation** – Cell membranes were extracted as described by Guerlavais et al. Briefly, cells were scrapped from the plate with 500 µl of homogenization buffer. Cells were disrupted (3* freeze and thaw cycles) and centrifuged at 10,000g (20 min, 4ºC). The membrane pellet was resuspended, aliquoted and stored at -80 ºC. Total protein concentration was determined by the Bradford method.

**Verification of the presence of the GHSR1a** – Measurements of intracellular calcium concentrations were done using three different techniques: fluorescence spectroscopy, flow cytometry and microscopy. The protocol for fluorescence spectroscopy in plated cells was derived from Johansson et al. Approximately 30,000 cells per well were seeded in 96-well poly-D-lysine coated plates for 16h. The plates were washed with HBSS buffer containing 2.5 mM probenecid, pH 7.4. Then 100 µl Fluo-4 AM solution (4 µM) was added and left for 1 h at 37 ºC. Cells were then washed twice with the loading buffer and 100 µl buffer was added per well. [Ca²⁺] was measured in a Gemini XPS fluorescent spectrophotometer (Molecular Devices, Toronto, Canada) at λ<sub>ex</sub> 488 and λ<sub>em</sub> 538 nm.

For flow cytometry experiments wild-type and GHS-R1a transfected HEK293 cells were grown up-to 50-60 % confluence, collected in polypropylene tube, and centrifuged at 2000 rpm. The cell pellet was re-suspended in HBSS at cell concentrations of ~2x10⁶ cells/ml. Fluo-4 (4 µM) and Pluronic (0.08 µM) mixtures added to the cell suspension and incubated for 30 minutes at 37 ºC. Then, cells were centrifuged at 2000 rpm, and the pellet was re-suspended in cell loading medium at the desired cell concentration (10⁶ cells/ml). Analyses were performed with BD LSR Flow
Cytometer (Beckton Dickinson, San Diego, California). The instrument was then extensively flushed with 10% bleach and afterward cells were treated with different agonists at different concentrations. Baseline was determined for about 30 s, then cell aspiration was paused, and the agonist added to the sample. This injection time was between 6 and 8 seconds. Acquisition was then resumed, and changes in fluorescence versus time were recorded for approximately 8 minutes. Analysis of cytometer data was done using in-house developed software to localize and eliminate the gap corresponding to the injection time and rendering the mean value of all intensities in a given time interval.

For microscopy experiments HEK 293 cells (expressing GHS-R1a and wild-type) were seeded on circular coverslips two days before performing the experiment and grown as explained for other cell experiments. Then cells were incubated with Fluo-3 (containing in nM: 140 NaCl, 5 KCl, 1.2 CaCl2, 0.5 MgCl2, 5 Glucose, 10 HEPES, pH 7.4 and 300 mosmol/l) for 40 minutes at room temperature and the coverslips were mounted in a perfusion chamber sealed with grease to avoid leakage. Before initiating the experiments, the perfusion chamber was warmed-up continuously perfusing it with pre-heated isotonic solution, at 38 ºC for 5 minutes. Isotonic perfusion was stopped and cells were allowed contact with ghrelin for 10 minutes and then isotonic perfusion was activated again to wash ghrelin. Results were obtained using an Olympus IX70 inverted microscope (Hamburg, Germany) with a 20x objective (Olympus, Hamburg, Germany) supplied with excitation light (490nm), which was directed towards the cells in the field of view by a 595DR dichromatic mirror (Omega Optical, Battleboro, USA). Fluorescent images were collected with a digital CCD camera (Hamamatsu Photonics, Japan), using the AquaCosmos software program version 2.5.0.0 (Hamamatsu Photonics, Japan). Once the images were collected, the software analyzed the regions of interest (one individual cell each region). The image analysis was performed using AquaCosmos software.

**Binding Experiments** – Two different types of binding tests were done to determinate secretagogues affinity for receptor. For whole-cell binding assay confluent HEK GHS-R1a monolayer cells (about 70–80%, grown as explained above) were counted and then re-suspended in binding buffer containing DMEM, and 1% BSA (pH 7.2). About 5–6 x 10^5 were incubated with 100,000 cpm of ^125^I-ghrelin (Amersham) for 2 h at 4 ºC. After incubation, cells were pelleted and radioactivity was measured in a LKB 1260 gamma
counter (Wallac, Turku, Finland). Total binding values were obtained. Non-specific binding was determined in parallel incubations of non-transfected HEK 293 cells with $^{125}$I-Ghrelin, or parallel incubations of transfected HEK 293 cells with $^{125}$I-Ghrelin in which was added 1 µM of Ghrelin (final concentration). Values of non-specific binding were subtracted from total binding to yield specific binding values.

For membrane binding assays binding tests, saturation and competition, were carried out with $^{125}$I-Ghrelin as radioligand. To show binding-site specificity, different GHSs, natural (Ghrelin) and synthetic (hexarelin, GHRP-6, MK-0677), as well as truncated ghrelin peptides AA1-14 and AA1-10 were used as competitors in a displacement assay. Briefly, saturation assay was performed as follows: 0.71 µg of membrane protein (Human Ghrelin receptor – GHS-R1a from Perkin Elmer, Boston, Maryland, USA) was diluted in 200 µl incubation buffer (25 mM HEPES, pH 7.4, 5 mM MgCl$_2$, 1 mM CaCl$_2$, 2.5 mM EDTA and 0.4% BSA) together with different concentration of radioligand. Non-specific binding was calculated as binding in the presence of 1 µM unlabelled Ghrelin. The binding reaction was performed with mild shaking at 25 ºC and terminated after 40 minutes by rapid filtration over Whatman GF/C filters pre-soaked in 0.5% polyethyleneimine to minimize non-specific binding of the radioligand to the filters. Filters were rinsed four times with wash buffer (1 ml of ice-cold 50 mM Tris-HCl pH 7.4) and the bound radioactivity measured in an LKB 1260 gamma counter (Wallac, Turku, Finland).

Competition assay were conducted with 16 pM of $^{125}$I-Ghrelin and concentrations of the competitors ranged from 0.01 pM to 1 µM. The data are presented as a percentage of specific binding in the absence of unlabelled competitor. The final volume was 0.4 ml. Incubation, filtration and counting was equal to the saturation assay. Competition analyses were performed with ghrelin and various ghrelin analogous (MK-0677, GHRP-6, hexarelin, ghrelin AA1-10, ghrelin AA1-14) diluted in water. To observe urine matrix effect, some competition experiments were done by diluting GHRP-6 in urine. Due to the possibility that ghrelin is secreted during exercise a competition experiment of comparison between pre- and post-exercise urines was included. The dissociation constant ($K_d$) and the receptor concentration ($B_{max}$) were calculated from a saturation curve using non-linear regression with GraphPad Prism 4 program (GraphPad, San Diego, California, USA). Competition curves were also analyzed with GraphPad.
software and inhibition constant \((K_i)\) values for various secretagogues and various matrices (water and urine) were calculated.

**Radiolabelling** – Ghrelin, desoctanoyl ghrelin and truncated ghrelin variants 1-10 and 1-14 were labelled with \(^{125}\text{I}\) following the traditional protocol. In brief, 1 to 10 µg of purified material were re-suspended in 10 µl sodium phosphate (10 mM, pH 8.5). To this solution were added 10 µl chloramine T (1M) followed by 0.5 mCi Na\(^{125}\text{I}\) and the mixture incubated for 90´´ at room temperature. The reaction was quenched by adding 10 µl sodium metabisulfite. The radiolabeled peptide was purified by reversed phase HPLC on a Vydac C\(_4\) column (15 cm * 4.6 mm i.d.) using a gradient from 5% MeCN (in 0.1% TFA) to 95 % MeCN over 20 minutes. A flow-rate of 1 ml/min was employed and the effluent was monitored for both UV absorbance (228 nm) and \(\gamma\)-radiation. The specific activity of the radiolabeled material was estimated at ca. 400 Ci/mmol.

**Results and Discussion**

In order to ensure that the transfected and selected HEK293 cells truly expressed a functional receptor experiments were designed to measure the natural function, i.e. liberation of Ca\(^{2+}\) from the cellular reservoirs. In a first attempt fluorescence microscopy was employed. With this approach a selected set of cells could be monitored continuously in time. The addition of ghrelin was followed by an increase in fluorescence emission that was detected in time (figure 2). Individual time frames were processed and the fluorescence signal integrated to generate a two-dimensional graph (figure 2).

![Figure 2](image)

Figure 2. Fluorescence microscopy analysis of the interaction between ghrelin and its receptor expressed in HEK293 cells. On the left a morphological image of the plated cells is included. In the center panel the fluorescence image of the cells is given: in green the resting cells, in red and yellow those cells that upon interaction with ghrelin release intracellular calcium provoking increase in fluorescence. On the right the fluorescence integral of a particular number of cells (inside the box in the center panel) over time is given (ascending curve) in comparison to non-transfected cells (descending curve).
From a comparison between transfected and wild-type cells the stable expression of the GHSR1a could be corroborated. Moreover, this observation led to the suggestion that a whole-cell assay could possibly be employed for the screening protocol. For this, we employed flow cytometry, rather than fluorescence microscopy. Also in this case a clear signal could be observed for the transfected cells when compared with the wild type (figure 3). In order to allow a standardised interpretation a software package was developed to interpret the information obtained from the fluorescence reader. Amongst other things the software determined the exact time-point were the ghrelin was added and aligned distinct fluorograms accordingly. Furthermore, sets of data-points were averaged out in sets of 10 consecutive points to compute a mean graph of the different cells. With this interpretation (figure 3) the maximum fluorescence at different concentrations was determined to construct a concentration-vs-fluorescence plot (figure 3).

Figure 3. Flow cytometry plots (upper part) of HEK293 cells (wild type on the left and transfected cells on the right) in time. To the left of the blank interval the baseline fluorescence was established. At the gap, ghrelin was added to the cells. To the right of the blank interval the fluorescence, corresponding to the intracellular calcium release was determined. Lower left: overlayed integrated graphs of the flow cytometry plots. Lower right: dose-response (fluorescence) curve for ghrelin demonstrating that a linear relationship exists between activation of the receptor through ghrelin and intracellular Ca\textsuperscript{2+} release.

It was observed that a clear relationship exist between the amount of ghrelin added to the cells and the fluorescence observed, with an evident saturation depending on the amount of cells employed. This behaviour was reproduced for all secretagogues included in the study (not shown) for which an assay based on intact cells could be
feasible. Nevertheless, the main objective was to employ receptor contained in membranes but harvested from cells. This approach should be preferred as it allows a better standardisation of the working conditions, i.e. the amount of protein material (read receptor) employed in each experiment. Furthermore, this approach required the use of labelled material in order to monitor the binding to the receptor. Labelling of ghrelin, desoctanoyl ghrelin, and the truncated variants comprising amino acids 1-10 and 1-14 was set-up with Na\textsuperscript{127}I and derivatised products purified were characterised by means of MALDI-TOF MS, LC-UV and LC-MS, both before and after distinct proteolytic digestion protocols (data not shown). From all experiments it could be deduced that the \textsuperscript{127}I-incorporation occurs exclusively at histidine-9. As this residue is fairly close to the relevant residues for receptor binding, the iodine-containing ghrelin (\textsuperscript{127}I labelled material) employed in a flow-cytometry experiment in order to determine the effect of the incorporation of a bulky residue at this position. The integrated flow-cytometry plots (not shown) from labelled and non-labelled material were perfectly superimposable demonstrating that the labelling does not interfere whatsoever. With the labelling protocol optimised experiments were repeated employing Na\textsuperscript{125}I. With estimations of the absolute amount of peptide labelled, derived from the UV peak integrals, and the radioactivity included in the peptide the specific activity could be estimated. With this material, binding experiments were performed using isolated membranes. Maximum and minimum binding levels were determined for a particular amount of cell extract (absolute amount of protein) to evaluate non-specific binding to either the cell membrane or the filter. Subsequently, the inhibition curves were constructed for each GHS with respect to labelled ghrelin (figure 4).

![Figure 4](image)

Figure 4. Inhibition curves for the four different growth hormone secretagogues. In the experiments a fixed amount of protein (receptor) material was employed and a fixed amount of radioactive (expressed in CPS) ghrelin.

From these curves it becomes evident that ghrelin is the tightest binder with an observed Ki value of 14.4 pM whereas MK-0677 displayed a value of 164.5 pM, and GHRP6 and
hexarelin values of 2.28 and 2.53 nM, respectively. The value observed for ghrelin roughly correspond to the concentration described in biological fluids by others\textsuperscript{2,7-9}. Subsequently, the effect of a urine matrix on the inhibition was studied.

![Figure 5. Inhibition curves for GHRP-6 in a buffered solution (in yellow and with triangles) or in urine (orange with diamonds).](image)

Figure 5 shows an example of an inhibition curve for GHRP-6 in buffer and in urine. Both curves do not overlap exactly and it remains to be investigated whether the displacement is significant and general. Furthermore, an initial experiment was performed where a single individual was submitted to stringent exercise. Urine specimens before and after the sport event were analysed within the inhibition experiment (data not shown) and appear to show that exercise does have an effect on the inhibition experiment. This, finding has prompted the search of a standardised protocol for preparation of the samples prior to the experiments. Furthermore, efforts are focussed on the neutralisation of the endogenous ghrelin present to prevent it from participating in the displacement of the radiolabelled ligand.

**Conclusion**

A screening method for the presence of growth hormone secretagogues was designed based on the ability of these compounds to displace a known, labelled ligand from the receptor. For this purpose the stable expression of the recombinant receptor in HEK293 was established as well a radiolabelling protocols for the ligand-to-displace. During the course of the development the use of fluorescence microscopy and flow cytometry have demonstrated to merit consideration as alternative screening protocols with the basis of the approach in the intracellular calcium release upon activation of the receptor. Future studies are heading in this direction. The proof-of-principle of the screening procedure based displacement/inhibition model has been demonstrated and current efforts aim at
elaborating standard parameters (in terms of sample handling/preparation) for the execution of the protocol.

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**References**


