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Ghrelin binding to serum albumin and its biological impact

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ABSTRACT

Ghrelin is an octanoylated peptide hormone that plays a key role in the regulation of the body weight and glucose homeostasis. In plasma, ghrelin circulates bound to larger proteins whose identities are partially established. Here, we used size exclusion chromatography, mass spectrometry and isothermal titration microcalorimetry to show that ghrelin interacts with serum albumin. Furthermore, we found that such interaction displays an estimated dissociation constant (K_D) in the micromolar range and involves albumin fatty-acid binding sites as well as the octanoyl moiety of ghrelin. Notably, albumin-ghrelin interaction reduces the spontaneous deacylation of the hormone. Both *in vitro* experiments—assessing ghrelin ability to inhibit calcium channels—and *in vivo* studies—evaluating ghrelin orexigenic effects—indicate that the binding to albumin affects the bioactivity of the hormone. In conclusion, our results suggest that ghrelin binds to serum albumin and that this interaction impacts on the biological activity of the hormone.

1. Introduction

Ghrelin is a 28-residue peptide hormone mainly secreted into the bloodstream by endocrine cells located in the stomach (Kojima et al., 1999). Ghrelin is the only known protein modified with an O-linked octanoyl group. The octanoylation is catalyzed by ghrelin *O*-acyl transferase within the Golgi apparatus and takes place at the Ser^3 residue of the hormone (Yang et al., 2008). This post-translational modification is crucial for the binding of ghrelin to its specific receptor, the growth hormone secretagogue receptor (GHSR), and, as a consequence, for its biological effects (Kojima and Kangawa, 2005). Ghrelin was initially described as an endogenous growth hormone secretagogue and, soon after, also recognized as the only known orexigenic peptide hormone (Kojima et al., 1999; Nakazato et al., 2001). Subsequent studies have reported a huge variety of effects for ghrelin, highlighting its physiological relevance (Kojima and Kangawa, 2005). The fact that ghrelin is conserved in all vertebrates indicates that this hormone is necessary for life, particularly under low energy balance conditions when plasma ghrelin levels dramatically increase (Kojima and Kangawa, 2005; Goldstein et al., 2011). Indeed, the ghrelin-induced elevation of growth hormone levels during severe calorie restriction in mice is essential for preserving blood glucose and preventing death (Zhao et al., 2010).

Plasma ghrelin concentration needs to be tightly regulated as small changes in hormone levels could have crucial physiological impact. For instance, the blunted postprandial decrease of plasma ghrelin detected in obese people has been associated to the pathophysiology of obesity because it would increase the time people feel hungry (English et al., 2002; Le Roux et al., 2005; Yang et al., 2009). Importantly, ghrelin circulates bound to larger molecules in plasma (Patterson et al., 2005), and such interactions add another level of complexity to the regulation of ghrelin biological activity. Hormone-binding protein complexes play a key role in the endocrine systems because they serve as circulating reservoirs, ensure ubiquitous distribution of hormones and protect them from rapid inactivation or excretion (Melmed et al., 2011). Previous size

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exclusion chromatography studies clearly indicate that plasma ghrelin is distributed in at least two pools, which are associated with entities of very different molecular mass (Patterson et al., 2005). The smaller pool of plasma ghrelin is detected in association with high molecular weight entities, which likely include lipoproteins and immunoglobulins. In this regard, ghrelin has been found associated with some plasma lipoprotein subfractions, including triglyceride-rich lipoproteins, low-density lipoproteins (LDL), very high-density lipoproteins (VHDL) and high-density lipoproteins (HDL; Beaumont et al., 2003; De Vriese et al., 2007). Notably, these lipoproteins are also associated with some esterases that can mediate ghrelin's deacylation, a mechanism that generates the des-octanoylated form of ghrelin named desacyl-ghrelin (De Vriese et al., 2004; Satou et al., 2010; Soares and Leite-Moreira, 2008). Although desacyl-ghrelin seems to have intrinsic biological activity (Delhanty et al., 2014), the fact that no specific receptor has been reported for it favored the notion that des-octanoylation is a mechanism for the inactivation of ghrelin (Delporte, 2013). In addition, plasma ghrelin is found bound to anti-ghrelin autoantibodies, which may play a role in its transport (Fetissov et al., 2008; Takagi et al., 2013). Interestingly, the majority of plasma ghrelin is found associated with the fraction of smaller molecular size, which is in the molecular weight range of albumin (Patterson et al., 2005); however, an interaction between serum albumin and ghrelin has not been previously reported.

Serum albumin is the most important plasma carrier for small lipophilic compounds, including a large variety of drugs, hormones and nutrients such as fatty acids (FA; Bhattacharya et al., 2000). Albumin displays multiple binding sites, including seven FA binding sites (Ghuman et al., 2005), which could potentially interact with ghrelin. Therefore, we hypothesized that serum albumin would be a good candidate for binding ghrelin through its lipid-like octanoyl group. Interestingly, serum albumin also possesses a putative esterase-like activity (De Vriese et al., 2007; Kragh-Hansen et al., 2002), which has been thought to contribute to ghrelin's deacylation in plasma (De Vriese et al., 2007). This possibility, which would have profound relevance on the half-life of circulating ghrelin, has not been examined. Thus, the present study was designed to test if ghrelin interacts with serum albumin, as well as to get insight into the biological impact of such interaction.

2. Material and methods

2.1. Materials

Octanoylated murine ghrelin (GSS(octanoyl)FLSPEHQKAQQRKESKKPPAKLQPR) and desacyl-ghrelin (GSSFLSPEHQKAQQRKESKKPPAKLQPR) were purchased from Global Peptide (cat# PI-G-03, and cat# PI-G-04, respectively). F-ghrelin (GSDpr (octanoyl) FLSPEHQRVQQRKESK(fluorescein)) and F-desacyl-ghrelin (GSSFLSPEHQRVQQRKESK(fluorescein)) are 18-residue analogs of human ghrelin and desacyl-ghrelin, respectively, conjugated to fluorescein isothiocyanate through a lysine at their C-termini. Both fluorescent probes were synthesized as previously described (McGirr et al., 2011; Fernandez et al., 2016), and provided by Dr. Leonard Luyt from the Department of Chemistry, The University of Western Ontario, Canada. Pharmaceutical grade human serum albumin was provided by the Laboratorio de Hemoderivados de la Universidad Nacional de Córdoba, Argentina. Plasma was obtained from adult (12–16 weeks-old) male Sprague Dawley rats. For this purpose, trunk blood was collected into plastic tubes containing heparin (10 IU/ml of blood) and immediately centrifuged to obtain plasma. HDL fraction isolated from human plasma was provided by Dr. Garda from Instituto de Investigaciones Bioquímicas de La Plata, Universidad Nacional de La Plata, Argentina. HDL was obtained by ultracentrifugal flotation in NaBr (1.21 g/ml density, 44 h, 200 g) followed by size exclusion chromatography on a Sephacryl S-400 column equilibrated and eluted with 10 mM Tris–HCl, 0.15 M NaCl, pH 8.0. Albumin free fluorescein-conjugated goat IgG immunoglobulins were from Cappel-Cooper Biomedical Inc. (USA, cat.#1212-0231). All the reagents were purchased from Sigma-Aldrich (USA) or Biopack (Argentina).

2.2. Binding of F-ghrelin to plasma proteins

Initially, we looked for evidences of an interaction between ghrelin and serum albumin. For this purpose, we used F-ghrelin, which not only displays similar structure-activity relationship to that of the natural ghrelin—*i.e.*, similar receptor binding affinity, half-life, and preserved lipophilic features of the Ser^3 side chain, indicating that the fundamental properties of the N-terminal region of the molecule are not altered—but also it is a stable probe for binding studies because its octanoyl moiety is covalently coupled through an esterase-resistant amide bond (McGirr et al., 2011; Cabral et al., 2013). Therefore, F-ghrelin was considered an appropriate probe for studying our hypothesis that ghrelin interacts with albumin through its N-terminus. Thus, F-ghrelin (12 μM) was incubated with a 1:5 dilution of rat plasma in PBS for 10 min at room temperature. Then, 50 μl of the mixture was loaded onto a Superdex-75 10/300 GL column (GE Healthcare Life Sciences, Sweden) equilibrated in PBS and connected to a LC-net II/ADC FPLC system (Jacso, Japan). The size exclusion chromatography was carried out in PBS at a flow rate of 0.5 ml/min, and absorbance at 280 nm was continuously monitored. Fractions of 0.5 and 1.0 ml were collected in the elution range of 12–24 and 24–39 min, respectively. The fluorescence emission spectrum for each fraction was recorded in the range of 450 nm–600 nm upon excitation at 490 nm with a FS-2 Fluorescence Spectrometer (Scinco Co, Korea). Recovery rate of the method was 93.3%. Fractions eluted from the plasma plus F-ghrelin samples showing significant absorbance at 280 nm were analyzed by SDS-PAGE using 12% polyacrylamide gels (Schägger and von Jagow, 1987) in a Bio Rad Mini-Protean 3 Cell (Bio Rad). Samples were pre-treated at 95 °C for 10 min in sample buffer (0.0625 M Tris-HCl pH 6.8, 20 g/l SDS, 0.02 g/l bromophenol blue, 100 g/l glycerol, 5% β-mercaptoethanol). After electrophoresis, gels were stained with 0.2% Coomassie Brilliant Blue R-250 (Sigma) in 10% acetic acid, 50% methanol for 10 min and then, destained with 5% acetic acid, 25% methanol. The assays were performed in triplicate. In parallel to the F-ghrelin plus plasma samples, samples containing the same dilutions of plasma or F-ghrelin alone were used as controls. In addition, 50 μl of a HDL fraction (~13 mM) or IgG immunoglobulins (2 μg) were used as controls of the immunoglobulin or lipoprotein fractions, respectively.

2.3. Preparation of pure delipidated serum albumin

To test the hypothesis that ghrelin and albumin interact, we prepared pure albumin free of lipids. Briefly, a 20% aqueous solution of human serum albumin of pharmaceutical grade (Cohn Fraction V) was subjected to size exclusion chromatography on a Sephacryl S200 HR column (GE Healthcare Life Sciences, Sweden) followed by ionic exchange chromatography on a Q-Sepharose FF column (GE Healthcare Life Sciences, Sweden), and then to a final batch elimination of hydrophobic ligands employing an Amberlite Monobed Resin (Sigma-Aldrich, USA). The absence of covalent dimers, larger aggregates and hydrophobic compounds was tested by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and thin-layer chromatography (TLC) of Folch extracts (Folch et al., 1987). The purity of the albumin preparation was also confirmed by matrix-assisted laser desorption/ionization time-of-flight mass