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Ghrelin binding to serum albumin and its biological impact

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A B S T R A C T

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.

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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

spectrometry (MALDI TOF MS), and its concentration was estimated using a calculated molar absorption coefficient at 280 nm of 36,570 M^{-1} cm^{-1} (Pace et al., 1995).

To further test a potential role of the FA binding sites of albumin in its interaction with ghrelin, a batch of pure and delipidated albumin was saturated with sodium palmitate, which is one of the main dietary fatty acids. For this purpose, a 100 mM sodium palmitate stock solution was prepared adding 0.4 M sodium hydroxide to palmitic acid at 90 °C. The suspension was stirred until complete solubilization, cooled to 40 °C and then one volume of warm ethanol was added. Aliquots of the palmitate stock solution were slowly poured onto delipidated albumin under gentle shaking at 37 °C, to a final molar ratio of sodium palmitate:albumin of 8:1. Finally, free sodium palmitate was removed by dialysis against phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 2.4 mM KH_2PO_4, pH 7.4). The presence of bound palmitate in the final albumin preparation was also confirmed by TLC of Folch extracts.

The pure delipidated serum albumin and the pure albumin saturated with sodium palmitate will be hereafter referred to as albumin and albumin–palmitate, respectively.

2.4. Binding of fluorescent probes to pure albumin

In order to specifically test if F-ghrelin and albumin interact, F-ghrelin (1 μM) or F-desacyl-ghrelin (1 μM) were incubated with albumin (100 μM) in PBS for 10 min at room temperature. In an additional experiment, F-ghrelin (1 μM) was incubated with albumin–palmitate (100 μM) under similar conditions. After incubation, 50 μl of each mixture were subjected to size exclusion chromatography on a Superdex-75 10/300 GL column and analyzed as described in Subsection 2.2. The assays were performed in triplicate. The 280 nm-absorbance peaks were collected as pools, which included albumin (17.5–19.5 min), free F-ghrelin (39.5–42.5 min) or free F-desacyl-ghrelin (36.5–39.5 min), and its fluorescence emission spectrum was recorded in the range of 450 nm–600 nm upon excitation at 490 nm. Fluorescein (1 μM) was incubated with albumin (100 μM) and analyzed as control.

2.5. Ghrelin and albumin interaction detected by mass spectrometry (MS)

Since the use of ghrelin-related fluorescent probes may have some disadvantages, we tested the interaction of non-labeled native full-length peptides and albumin using MS. In particular, ghrelin (12 μM) or desacyl-ghrelin (12 μM) were incubated with albumin (700 μM) in PBS on an ice bath for 10 min. In an additional experiment, ghrelin (12 μM) was incubated with albumin–palmitate (700 μM) under similar conditions. After incubation, each mixture was diluted 1:10 in water, desalted and concentrated using C18 Zip-Tip Pippette Tips (Merck Millipore, USA), mixed with crystallization matrix solution (1 μl of each) and deposited onto a ground steel plate (Bruker Daltonics, USA) using a standard dried droplet method. MS analysis was carried out in a mass spectrometer with MALDI ionization system and TOF-TOF analyzer (New ultrafleXtreme spectrometer, Bruker Daltonics, USA) using 10 mg/ml 2,5-dihydroxyacetophenone (DHAP) in water:acetonitrile 2:1 containing 20 mM diammonium hydrogen citrate as crystallization matrix. Mass spectra in a range between 20,000 and 100,000 Da were acquired in linear mode geometry, using the following settings: linear positive mode, ion source 1: 25 kV, ion source 2: 23 kV, lens: 6 kV, pulsed ion extraction of 450 ns, high gating ion suppression up to 25,000 m/z. Ionization was achieved by irradiation with SmartBeam II laser operating at 2 kHz (30–60% attenuator). Mass calibration was performed externally using the Protein Calibration Standard II (Bruker Daltonics: Trypsinogen, Protein A, Serum Albumin-Bovine). Data analysis was performed using FlexAnalysis 3.4 and BioTools 3.2 software programs (Bruker Daltonics, USA). Samples containing exclusively ghrelin (12 μM), desacyl-ghrelin (12 μM), albumin (700 μM) or albumin-palmitate (700 μM) were processed in parallel and used as controls of the assay.

2.6. Ghrelin and albumin interaction monitored by isothermal titration microcalorimetry (ITC)

To further confirm the interaction between ghrelin and albumin and obtain biophysical parameters of the binding, we performed ITC experiments using a MicroCal VP–ITC calorimeter (Malvern Instruments, UK). For the assay, albumin (37.3 μM) was loaded in the calorimeter cell and a solution of ghrelin (377 μM) was injected from the syringe. Titrations were done at pH 7.4 using PBS buffer in both the syringe and the cell. Instrument settings were: temperature, 25 °C; reference potency, 20 μcal/s; stirring, 307 rpm; injection volume, 7 μl; injection number, 40; injection period, 240 s.

For data analysis, an equation for identical non interacting sites was fit to the first apparent transition, and binding enthalpy (ΔH, kcal mol^{-1}), dissociation constant (K_D, M), and the number of binding sites per monomer (n) were adjusted using the instrument's software. It was assumed that dilution of ghrelin in buffer only produces thermal changes due to dissociation of self-associated ghrelin molecules. It was also assumed that the aggregation state of ghrelin was independent of the concentration in the tested range. The changes in free energy (ΔG, kcal mol^{-1}) and entropy (ΔS, kcal mol^{-1} K^{-1}) associated to the binding were then calculated using the equation $\Delta G = \Delta H - T\Delta S = -RT \ln K_D$. Estimated concentration of ghrelin:albumin complex ([GA]) under equilibrium conditions was calculated as a function of n, total albumin and ghrelin concentrations ($[A_t]$ and $[G_t]$) and K_D by the following equation $a\,[GA]^2 + b\,[GA] + c = 0$, with $a = 1$, $b = -(K_D + [G_t] + n\,[A_t])$, $c = n\,[A_t][G_t]$ (Cantor and Schimmel, 1980). Negative control of the assay was performed with successive injections of PBS into the same buffer loaded in the cell, whereas dilution heat effects were assessed by titrating PBS with the solution of ghrelin.

2.7. Ghrelin stability measured by MS

To assess the effect of albumin on the stability of ghrelin, ghrelin (12 μM) was diluted in PBS alone or containing albumin (700 μM) and incubated on an ice bath. At time zero, each mixture was diluted 1:10 in PBS and incubated at 37 °C. After 3 h, aliquots were collected and transferred to a tube containing 10% v/v trifluoroacetic acid (TFA) to a final concentration of 0.25% v/v TFA. Samples were analyzed by MALDI TOF MS using 10 mg/ml of α-cyano-4-hydroxycinnamic acid (HCCA) in water:acetonitrile 2:1 with 0.1% v/v TFA as crystallization matrix. The spectra were acquired on an ultrafleXtreme spectrometer with FlexControl 3.4 (Bruker Daltonics, USA) data acquisition software. Mass spectra were acquired in the mass range between 2500 and 4000 Da in reflector mode geometry, using the following settings: reflector positive mode, ion source 1: 25 kV, ion source 2: 22.3 kV, lens: 8 kV, reflector 1: 26.5 kV, reflector 2: 13.4 kV, pulsed ion extraction of 130 ns, high gating ion suppression up to 900 *m/z*. Ionization was achieved by irradiation with SmartBeam II laser operating at 2 kHz (30–60% attenuator). Mass calibration was performed externally using the Peptide Calibration Standard (Bruker Daltonics: Angiotensin II, Angiotensin I, Substance P, Bombesin, ACTH clip 1–17, ACTH clip 18–39, Somatostatin 28). Data analysis was performed using FlexAnalysis 3.4 and BioTools 3.2 software programs (Bruker Daltonics, USA). Signal data was plotted in arbitrary units to evaluate

desacyl-ghrelin to ghrelin relative intensity.

2.8. Ghrelin and albumin-ghrelin effects on voltage gated calcium currents

In order to test the bioactivity of albumin-bound ghrelin, we used an *in vitro* setting in which we quantified the ghrelin-induced activation of GHSR by recording its inhibitory action of the calcium currents. Here, 80% confluent HEK 293 T cells were co-transfected with GHSR, the calcium channel $Ca_V2.2$ subunit (#AF055477) and the calcium channel auxiliary subunits $Ca_V\alpha_2\delta_1$ (#AF286488) and $Ca_V\beta_3$ (#M88751) in a 0.1:1:1:1 M ratio, respectively, plus a small amount of eGFP to identify transfected cells, using Lipofectamine 3000 (Invitrogen, USA). Ca_V clones used for this study are a gift from Dr. Diane Lipscombe (Brown University, USA). Whole-cell patch clamp recordings were performed on transfected HEK 293 T cells at room temperature. The internal pipette solution contained (mM): 115 Cs-methanesulfonate, 10 CsCl, 5 NaCl, 10 HEPES, 20 tetraethylammonium chloride, 4 Mg-ATP, 0.3 NaGTP, 0.6 EGTA, pH 7.2 with CsOH. The external solution contained (mM): 2 $CaCl_2$, 140 choline chloride, 1 $MgCl_2$, 10 HEPES, pH 7.4 with CsOH. Calcium currents were triggered by 20 ms square voltage pulses to 10 mV from a resting potential of −100 mV; currents were recorded every 10 s in order to allow the recovery of the channels from inactivation. Calcium channel currents were recorded before treatment until signal stabilization and then recorded in the presence of ghrelin (0.25 μM; $n = 12$ or 0.044 μM; $n = 4$), ghrelin plus albumin (0.25 μM and 12.5 μM, respectively; $n = 7$) or ghrelin plus albumin−palmitate (0.25 μM and 12.5 μM, respectively; $n = 6$) for at least 5 times. Ionic current was recorded with an Axopatch 200 amplifier (Molecular Devices, USA). Data was sampled at 20 kHz and filtered at 10 kHz (−3 dB) using pClamp8.2 software. The recording borosilicate glass electrodes used in these measurements display resistances of 2−4 MΩ when filled with internal solution. Series resistances lower than 6 MΩ were admitted and compensated 80% with a 10-μs lag time. Currents leak were subtracted online using a P/-4 protocol. An extra set of cells exposed to either albumin or albumin−palmitate without ghrelin were included as negative controls of the inhibition of the calcium currents.

2.9. Albumin and albumin-ghrelin effects on food intake in mice

To test the bioactivity of the albumin-ghrelin complex, we quantified its orexigenic effect in mice. This study was performed with adult (10−12 weeks old) C57BL/6 wild-type mice, which were generated at the IMBICE animal facility. Animals were kept with regular chow and water available *ad libitum* and housed in a 12 h light/dark cycle. The study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Research Council, and all efforts were made to minimize animal suffering. All experimentation received approval from the Institutional Animal Care and Use Committee of the IMBICE (approval ID 10-0112). Animals were individually housed two days before the experiment. In the morning of the experimental day, mice were divided in 6 experimental groups, which were subcutaneously (SC) injected with 150 μl of PBS alone ($n = 13$) or containing either: albumin (90 μM; $n = 6$), ghrelin (9 μM; $n = 16$ or 0.27 μM; $n = 10$) or ghrelin and albumin (9 μM and 90 μM, respectively; $n = 15$). In order to test if the bioactivity of ghrelin is affected by the presence of albumin–palmitate, two other sets of mice were injected with albumin-palmitate (90 μM; $n = 6$) or ghrelin and albumin-palmitate (9 μM and 90 μM, respectively; $n = 11$). The orexigenic effect of this dose of ghrelin was fully characterized in our previous studies (Cabral et al., 2014). Mice were exposed to a pre-weighed amount of rodent's food that was then weighed again 30 min later in order to determine food intake.

2.10. Statistical analyses

Data are expressed as the *mean* ± SEM. Equality of variance was analyzed using Bartlett's test. *T*-test was performed in order to compare data from size exclusion chromatography. One-way ANOVA followed by the Newman-Keuls test was used to compare ghrelin's effects on food intake. Patch clamp data were analyzed using Kruskal-Wallis test and Dunn's multiple comparisons test. Differences were considered significant if $P < 0.05$.

3. Results

3.1. F-ghrelin co-elutes with a plasma protein fraction containing albumin

In order to identify plasma protein fractions with the capacity to interact with F-ghrelin (Fig. 1), we fractionated plasma alone, F-ghrelin alone, or plasma preincubated with F-ghrelin samples by size exclusion chromatography while continuously monitoring the absorbance at 280 nm to detect the presence of proteins in each fraction. In the collected fractions, we also measured the fluorescence emission at 520 nm (emission wavelength of free fluorescein). In plasma chromatograms, we detected three main peaks at elution times 16, 18 and 20 min by 280 nm absorbance and no fluorescence peaks (Fig. 1A). In F-ghrelin samples, we detected a coincident 280 nm absorbance and fluorescence peak at fraction 40 (Fig. 1B). In plasma plus F-ghrelin samples, we detected three main peaks at elution times 16, 18 and 20 min in the 280 nm absorbance profile, as seen for the plasma alone samples, as well as three peaks at fractions 15, 18 and 40 in the fluorescence profile (Fig. 1C). In the 280 nm absorbance profile of the chromatograms obtained with the controls of lipoproteins and immunoglobulins, HDL fraction was detected as one peak at elution time 16 min while immunoglobulins were detected as two overlapped peaks at elution times 15 and 16 min. SDS-PAGE separation and further Coomassie staining of each fraction of the plasma plus F-ghrelin eluate showed that fractions 15−17 correspond mainly to high-molecular-weight proteins, consistent with immunoglobulins (~150−170 kDa) and lipoproteins (*i. e.*, ~300 kDa for HDL), while fraction 18 mainly showed a protein band with the expected molecular weight of albumin (Fig. 1D). Thus, F-ghrelin co-elutes with a plasma protein fraction containing albumin.

3.2. F-ghrelin binds to serum albumin

To test the capacity of purified serum albumin to bind F-ghrelin (Fig. 2), samples of albumin alone or preincubated with F-ghrelin were subjected to size exclusion chromatography. Here, we took advantage of the fluorescein labeling of the probe in order to monitor the fractions containing either albumin-bound or free F-ghrelin by fluorescence. For this purpose, pools collected at the elution times containing albumin or the free probe were analyzed as described above. In albumin samples, we found a major peak at 18 min in the 280 nm absorbance profile, with no detectable 520 nm fluorescence (Fig. 2A). In albumin plus F-ghrelin samples, we found a 280 nm absorbance profile similar to the one seen for the albumin alone samples, except for a small peak at F-ghrelin elution time (40 min); in addition, we found fluorescence in the fractions containing the peaks eluting at 18 and 40 min, which correspond to albumin-bound and free F-ghrelin, respectively (Fig. 2B). The proportion of albumin-bound F-ghrelin, estimated by the ratio of fluorescence registered for the peaks at 18 and 40 min,

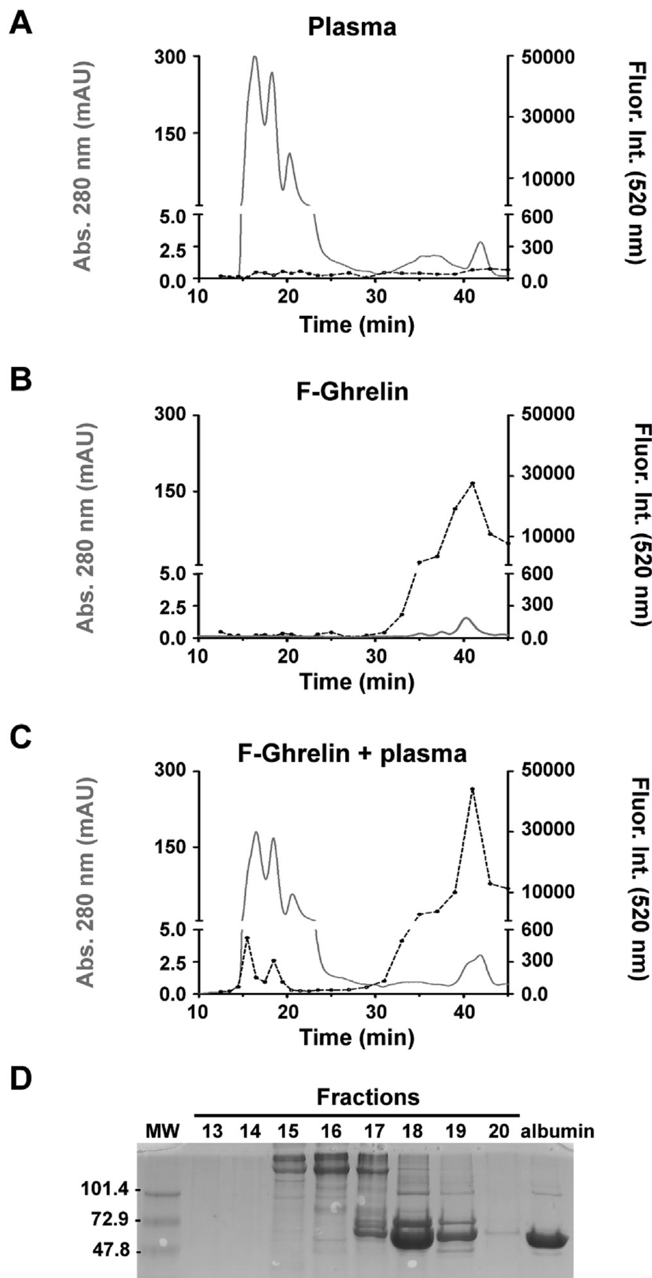

Fig. 1. Size exclusion chromatography analysis of the interaction between F-ghrelin and plasma proteins. Representative chromatograms obtained with (A) plasma ($n = 3$), (B) F-ghrelin (2 μg, $n = 3$) and (C) F-ghrelin plus plasma (2 μg, $n = 3$) are shown. Samples were incubated at room temperature for 10 min before analysis by size exclusion chromatography on a Superdex-75 column. Absorbance was continuously monitored at 280 nm for protein detection (gray continuous line), and the emission of fluorescence at 520 nm was measured in each collected fraction (black dashed line). (D) SDS-PAGE analysis of F-ghrelin plus plasma eluate. Fractions are indicated by the collection time; pure human serum albumin (*albumin*) was also included. The gel was stained with Coomassie Brilliant Blue.

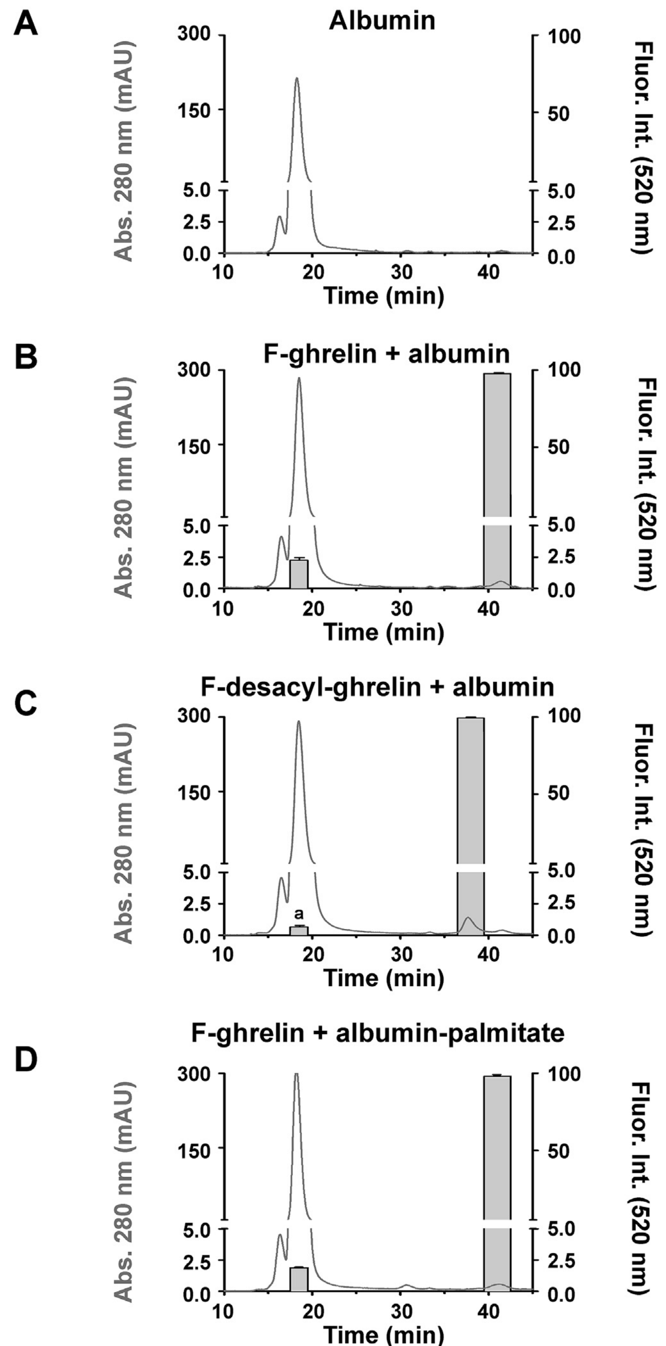

Fig. 2. Size exclusion chromatography analysis of the interaction between F-ghrelin and albumin. Representative chromatograms obtained with (A) albumin (332 μg, $n = 3$), (B) F-ghrelin (0.16 μg) plus albumin (332 μg, $n = 3$), (C) F-desacyl-ghrelin (0.16 μg) plus albumin ($n = 3$), and (D) F-ghrelin (0.16 μg) plus albumin-palmitate (equivalent to 332 μg of delipidated albumin, $n = 2$) are shown. Samples were incubated at room temperature for 10 min before separation on a Superdex-75 column. The 280 nm-absorbance peaks were collected as pools, which included albumin (17.5–19.5 min), free F-ghrelin (39.5–42.5 min) or free F-desacyl-ghrelin (36.5–39.5 min). Gray continuous lines indicate the absorbance at 280 nm, and gray-filled bars represent the emission of fluorescence at 520 nm by the pools of fractions corresponding to the albumin peak (17.5–19.5 min) and the free fluorescent probe peaks (39.5–42.5 min for F-ghrelin and 36.5–39.5 min for F-desacyl-ghrelin).

was 2.2 ± 0.2%.

In order to get insights into the regions of F-ghrelin interacting with albumin, we used fluorescein and F-desacyl-ghrelin probes. In albumin plus fluorescein samples, we found a fluorescence peak at the free fluorescein elution time (63 min) and no fluorescence associated to the albumin fraction (not shown). In albumin plus F-desacyl-ghrelin samples, we also found a peak of albumin-bound probe, besides the free probe peak that eluted at 38 min (Fig. 2C). The fluorescence of F-desacyl-ghrelin associated to the albumin fraction represented 0.7 ± 0.2% of the total fluorescence. This percentage is significantly lower than that of the F-ghrelin fluorescence associated to albumin ($P < 0.01$; Fig. 2B) suggesting that the

octanoyl moiety of the peptide is involved in the interaction. To test if FA binding sites of albumin participate in F-ghrelin binding, we analyzed samples of F-ghrelin preincubated with albumin complexed with sodium palmitate. We found that the binding of F-ghrelin to albumin–palmitate was reduced by ~20% as compared to the binding to delipidated albumin. Although this difference is not statistically significant ($P > 0.05$; Fig. 2D), it may suggest that one or more of the FA binding sites of albumin are involved in the interaction.

Then, we used MS analysis to test the interaction between albumin and ghrelin (Fig. 3). Here, we used the full-length ghrelin peptide rather than the fluorescent probe used in size exclusion chromatography assays to avoid any possible interference. We detected a peak of 66.46 ± 0.07 kDa in albumin alone samples (Fig. 3A), whereas we also detected a second peak of 69.78 ± 0.07 kDa in albumin plus ghrelin samples (Fig. 3B). The shift in the *m/z* value between peaks was 3.32 kDa, which corresponds to the mass of a molecule of ghrelin (3.31 kDa). We only found a peak of 66.46 ± 0.07 kDa and failed to detect peaks at higher *m/z* values in albumin plus desacyl-ghrelin as well as in albumin-palmitate plus ghrelin samples (Fig. 3C, D). These results confirm that ghrelin binds to albumin and also suggest that both the octanoyl moiety of the peptide and the free FA binding sites of albumin are important for the interaction.

In order to further characterize the interaction ghrelin–albumin, we performed ITC. The titration of albumin with ghrelin elicited an exothermic effect, whereas the control injection of ghrelin into buffer alone was accompanied by an endothermic effect (Fig. 4A). Thus, the overall exothermic titration profile allowed the stepwise monitoring of the ghrelin–albumin binding process (Fig. 4B). The profile of the integrated heat peaks failed to show a clear binding saturation along the titration up to a two-fold molar excess of ghrelin (Fig. 4C). The binding parameters obtained by fitting the apparent transition were $n = 1.6$, $K_D = 4.2 \times 10^{-6}$ M, $\Delta H = -3.8$ kcal mol^{-1}, $\Delta S = 0.012$ kcal mol^{-1} K^{-1}. These results confirm that ghrelin binds to albumin and provide estimated thermodynamic parameters of the interaction.

3.3. Ghrelin binding to albumin stabilizes the octanoyl group of ghrelin

In order to test if the ghrelin–albumin interaction affects the stability of the hormone, we incubated ghrelin in the absence or the presence of albumin and analyzed the peptides present in the samples after 3 h of incubation by MS. At time zero, we detected an intense peak of $m/z = 3313.8$ Da (ghrelin theoretical average $MH^+ = 3313.8$ Da) and a smaller peak of $m/z = 3187.8$ Da (desacyl-ghrelin theoretical average $MH^+ = 3187.7$ Da). Ghrelin peaks represented 96 ± 2 and 93 ± 6% of the total area of the analyzed peaks, in the absence or presence of albumin, respectively (Fig. 5, left panels). After 3 h incubation, ghrelin peak decreased to the 48 ± 5% of the total area of the analyzed peaks ($P < 0.05$, vs. the percentage of ghrelin peak at time zero) in the absence of albumin while it represented 98 ± 1% of the total area of the analyzed peaks in the presence of albumin (Fig. 5, right panels). These results suggest that the ghrelin-albumin interaction prevents the des-octanoylation of the hormone.

3.4. Ghrelin binding to albumin impacts on its biological activity

Finally, we tested whether the ghrelin–albumin interaction impacts on the bioactivity of the hormone. First, we tested *in vitro* the ability of ghrelin, in the presence or the absence of albumin, to inhibit calcium currents in HEK 293 T cells co-expressing GHSR and calcium channels. As previously reported (Soto et al., 2015),

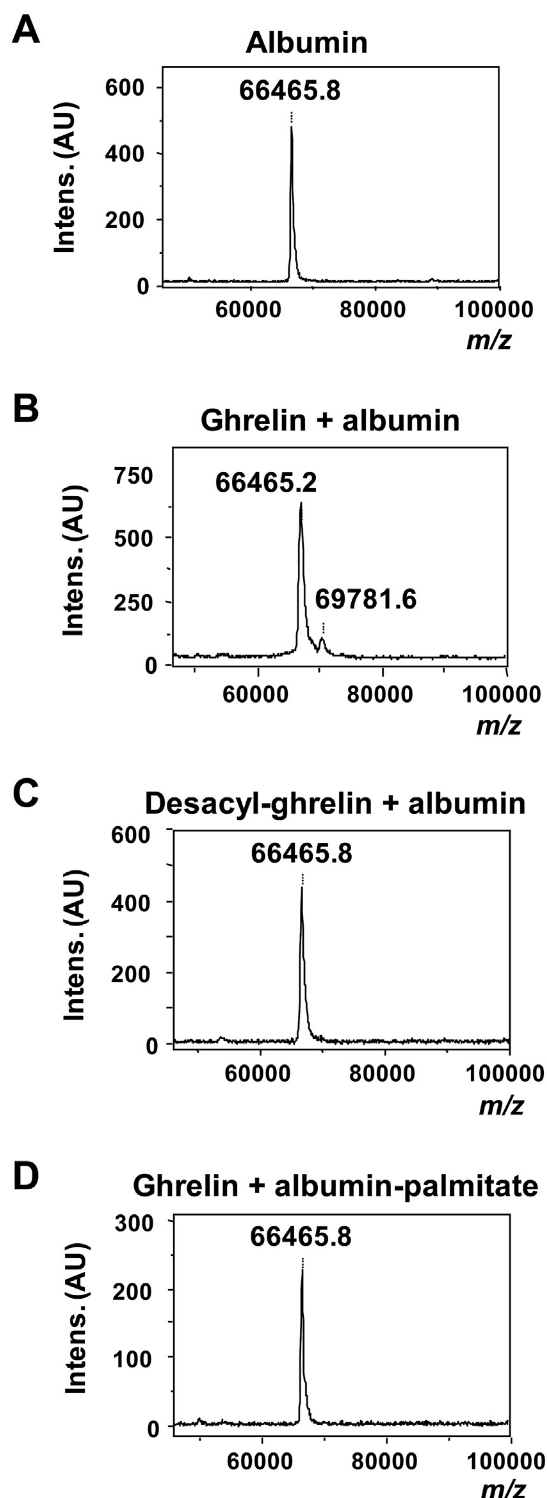

Fig. 3. Determination of ghrelin-albumin complex by MALDI TOF MS. Albumin was ice-incubated with ghrelin or desacyl-ghrelin and crystallized with DHAP matrix for mass spectra acquisition. (A) Albumin molecular ion peak ($m/z = 66.76 \pm 0.07$ kDa) is shown in the albumin sample spectrum ($n = 3$). (B) The formation of a complex between ghrelin and albumin is evidenced by the detection of a second peak with a mass increase of 3.32 kDa with respect to the molecular ion peak of albumin in the ghrelin plus albumin sample spectrum ($n = 3$). The spectra acquired for (C) desacyl-ghrelin plus albumin sample ($n = 3$) and (D) ghrelin plus albumin-palmitate sample ($n = 3$) are also shown. .

0.25 μM ghrelin inhibited calcium currents (Fig. 6A). In contrast, 0.25 μM ghrelin pre-incubated with 12.5 μM albumin failed to affect calcium currents. Based on the estimated K_D and the equation indicated in subsection *2.6*, we calculated that the concentration of free ghrelin present under this experimental condition is 0.044 μM. When tested *in vitro*, this concentration of ghrelin failed to affect calcium currents in the absence of albumin (−4 ± 3% of calcium current inhibition, $P = NS$ vs. no ghrelin). Interestingly, we found that 0.25 μM ghrelin preincubated with 12.5 μM albumin–palmitate significantly inhibited calcium currents, similarly as seen for 0.25 μM ghrelin alone. Importantly, albumin or albumin–palmitate alone failed to affect calcium currents (data not shown).

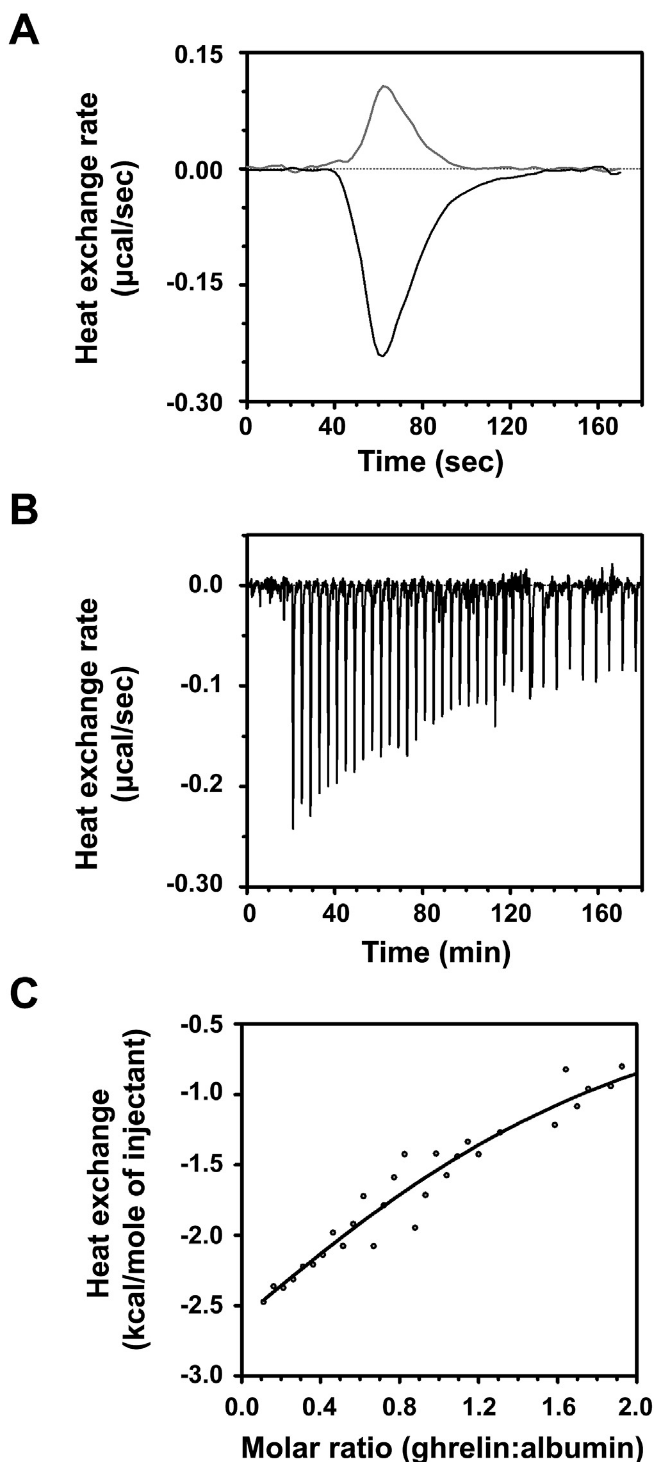

Fig. 4. Binding of ghrelin to serum albumin followed by ITC. PBS or serum albumin in PBS were loaded in the stirred cell and an aliquot (7 μl) of ghrelin solution was automatically injected every 6 s. (A) Time course of heat exchange after a single injection of ghrelin into serum albumin (black line) or ghrelin into PBS (gray line). (B) Heat exchange along the titration of serum albumin with ghrelin. (C) The integrated experimental heat change, corrected for the heat of dilution represented (circles) as a function of ghrelin:albumin molar ratio; full line indicates the best fit for the first apparent transition.

We then tested the impact of ghrelin–albumin interaction on the orexigenic effects of the hormone in mice. As expected, ghrelin alone induced a significant increase of the food intake, as compared to vehicle-treated mice ($P < 0.05$ vs. food intake in PBS-injected mice; Fig. 6B). In contrast, the administration of a combination of ghrelin and albumin failed to affect food intake in mice ($P = NS$ vs. food intake in albumin-treated mice, and $P < 0.05$ vs. food intake in ghrelin alone-treated mice). Of note, the food intake in ghrelin-albumin-treated mice was increased as compared to food intake found in mice treated with vehicle alone ($P < 0.05$, Fig. 6B). Here, we also used the estimated K_D to calculate the concentration of free ghrelin present in the solution of ghrelin and albumin used to treat the mice. The concentration of free ghrelin was estimated as a ~3% of the total ghrelin, and its administration, as a dose of ghrelin alone, failed to affect food intake in an independent set of mice (47 ± 6 mg, $P = NS$ vs. food intake in vehicle-treated mice). Importantly, the dose of ghrelin that failed to increase food in the presence of albumin was able to fully increase food intake in the presence of albumin–palmitate ($P < 0.05$ vs. food intake in albumin–palmitate-injected mice, Fig. 6B).

4. Discussion

To our knowledge, the present work provides the first evidence that ghrelin and serum albumin interact. It had been shown that plasma ghrelin does not circulate as a free peptide, but rather bound to two pools of larger molecules (Patterson et al., 2005). However, the identity of the molecules that bind most of the circulating ghrelin was unknown. In line with previous findings, our chromatographic separations of plasma plus F-ghrelin samples showed that F-ghrelin is detected in two plasma fractions confirming that ghrelin binds to fractions of high (>100 kDa) and middle (~60–90 kDa range) molecular-weight. The affinity of F-ghrelin for these plasma components must be strong, otherwise the dilution and diffusion effects during the chromatography would have impeded their co-elution. We found that F-ghrelin co-eluted with either a fraction containing high-molecular-weight entities (Fraction 15), which includes immunoglobulins and lipoproteins such as HDL, or a fraction that mainly contains serum albumin (Fraction 18). Although we cannot exclude the possibility that the fraction 18 contains another ghrelin-binding protein, the well-known fact that serum albumin is one of the most abundant plasma proteins that serves as a carrier for several endogenous molecules (Ryan et al., 2011) strongly point to its interaction with ghrelin. Unfortunately, the direct detection of albumin–ghrelin complexes in plasma samples by standard MS techniques is extremely challenging due to both the complexity of the sample and the relative low amount of the complexes of interest.

In order to confirm and further characterize ghrelin-albumin interaction, we then tested pure and delipidated serum albumin. Size exclusion chromatography studies indicated that F-ghrelin binds to albumin. Here, a higher proportion of albumin-bound F-ghrelin was found, as compared to the proportion found in plasma samples, which was expected since delipidated albumin should be free of ligands. MS data also indicated that ghrelin and albumin interact. In addition, both experimental systems suggested that the

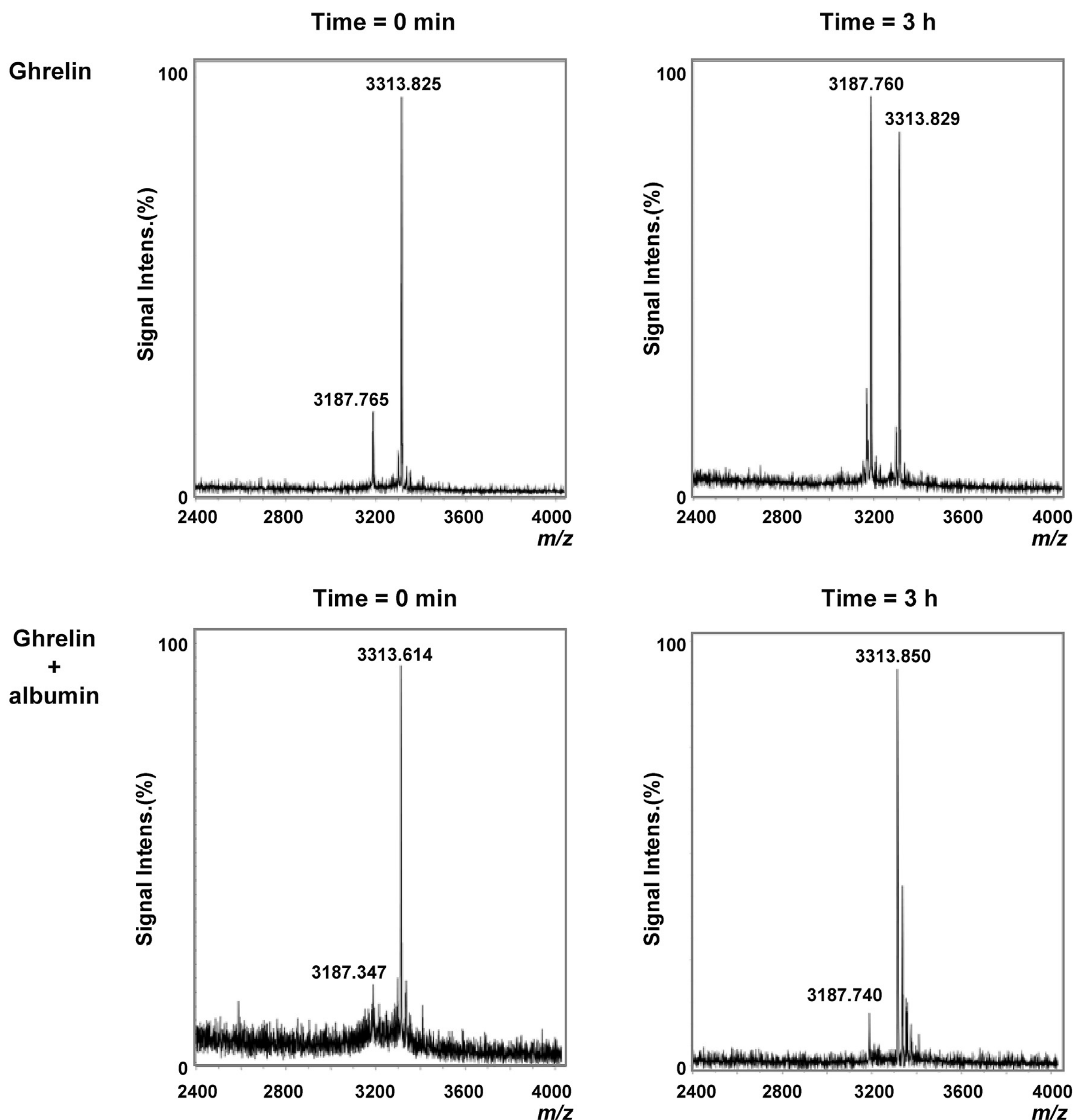

Fig. 5. Effect of the binding to albumin on the stability of ghrelin octanoyl group. Ghrelin was incubated alone or in the presence of albumin at 37 °C. During incubation, samples were collected at 0 min and 3 h and analyzed by MALDI TOF MS using HCCA as crystallization matrix. Peaks at 3187 and 3313 *m/z* values correspond to desacyl-ghrelin and octanoylated ghrelin, respectively (see the text). Upper and lower panels show representative spectra acquired at the indicated time with ghrelin samples and ghrelin plus albumin samples, respectively. The proportion of ghrelin that is deacylated increases with incubation time only in samples incubated without albumin.

octanoyl moiety is important for the interaction. This observation is interesting since this octanoylated region has been also shown to constitute the active core of ghrelin and attach the hormone to membranes (GroBauer et al., 2010; Bednarek et al., 2000). The middle region of ghrelin may also interact with lipophilic regions of albumin since a theoretical study has suggested that the Arg^{15} to Ser^{18} sequence of ghrelin could bind to lipid membranes (Beevers and Kukol, 2006). We also found that the pre-treatment of albumin with sodium palmitate impairs the binding of ghrelin suggesting that sites for non-esterified FA are important for the ghrelin-albumin interaction. Crystallographic analyses of FA-serum albumin complexes have revealed the presence of seven FA binding sites, which would be potential candidates to interact with ghrelin (Petitpas et al., 2001; Simard et al., 2005). Importantly, NMR spectroscopic analyses of the binding of ^{13}C-labeled FA to serum albumin have shown that the affinity of these sites for FA depends on the interaction with the lipid carboxyl group (Simard et al., 2005, 2006). Thus, the binding of ghrelin to albumin might also occur at sites that overlap with sites for FA, or that suffer conformational changes by the bound palmitate that, in turn, reduces hormone binding (Ghuman et al., 2005).

The outcomes of chromatography and MS experiments displayed some discrepancies. MS failed to detect interactions between either desacyl-ghrelin and albumin or ghrelin and albumin–palmitate, whereas size exclusion chromatography data revealed small amounts of albumin-bound F-desacyl-ghrelin and a decreased, although significant, capacity of albumin–palmitate to bind F-ghrelin. These discrepancies may arise from a number of reasons. Size exclusion chromatography experiments were performed with the fluorescent, ghrelin-related 18-residue probes that resemble the N-terminal part of the hormones and their biological actions (Fernandez et al., 2016; Cabral et al., 2013). F-ghrelin is very

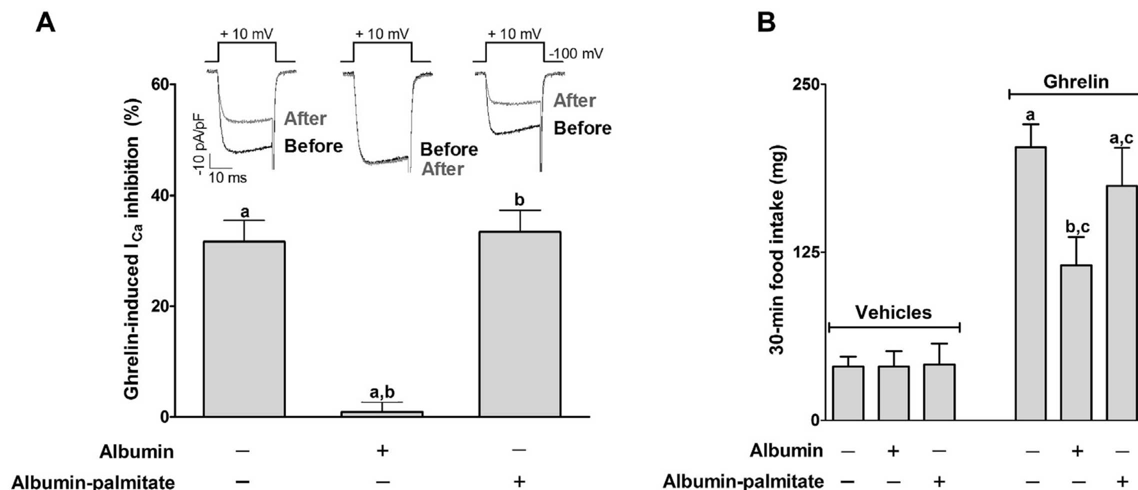

Fig. 6. Impact of the binding to albumin on ghrelin-induced effects: food-intake and inhibition on CaV2.2 presynaptic calcium channel current (I_{Ca}). (A) Representative traces of ghrelin effect on I_{Ca} from HEK 293 T cells expressing $Ca_V2.2$, $Ca_V\alpha_2\delta_1$, $Ca_V\beta_3$ and GHSR1a in a 1:1:1:0.1 M ratio, before and after adding ghrelin ($n = 12$), ghrelin plus albumin ($n = 7$) or ghrelin plus albumin-palmitate ($n = 6$). Averaged percent of I_{Ca} inhibition for each condition is shown below the corresponding trace, data are expressed as *mean* ± SEM, (a and b) indicate significant difference among groups ($P < 0.01$) with Kruskal-Wallis test and Dunn's multiple comparisons test. Both albumin and albumin-palmitate controls had no effect on I_{Ca} (albumin = -2 ± 2, $n = 6$; albumin-palmitate = 5 ± 2, $n = 4$; Wilcoxon Signed Rank Test against 0, $P = 0.3125$ and $P = 0,1250$). (B) 30 min food-intake in mice subcutaneously treated with vehicle alone (n = 13) or containing ghrelin (n = 16), albumin (n = 6) or albumin plus ghrelin (n = 15), albumin-palmitate alone (n = 6) or containing ghrelin (n = 11). Data are expressed as mean ± SEM; (a) indicates $P < 0.05$ vs. food intake in sample vehicle-injected group, (b) indicates $P < 0.05$ vs. food intake in ghrelin alone-treated mice and (c) indicates $P < 0.05$ vs. food intake in vehicle-injected group, with Newman-Keuls Multiple Comparison Test.

stable for binding studies because the octanoyl moiety is covalently coupled through an amide bond, which is resistant to esterases. However, fluorescein itself can bind to albumin with low affinity and such interaction partially quenches its fluorescence (Nagataki and Matsunaga, 1985). Thus, quantitative data obtained with the fluorescent probes should be interpreted with caution. Moreover, size exclusion separation of molecules during chromatography in the liquid phase and under steady flow conditions may affect the binding equilibrium. The lack of the C-terminal residues of ghrelin in the probes may also impact in the interaction with albumin. Conversely, MALDI TOF MS analysis, which measures the *m/z* ratio of ionized molecules after desorbing them from the crystallized sample, does not allow us to discard that a weak interaction between desacyl-ghrelin and albumin or ghrelin and albumin–palmitate exist since the absence of peaks corresponding to complexes present in low proportion may be due to a suppression of their ionization and/or detection caused by the predominant species in the mixture (issue known as signal suppression phenomenon, Yanes et al., 2005). Alternatively, desacyl-ghrelin and albumin or ghrelin and albumin–palmitate complexes may be bound with low affinity and have been dissociated during the procedure. A weak interaction between palmitate and some of the FA binding sites of albumin may also explain why *m/z* peaks corresponding to albumin–palmitate complexes are not evident in the albumin–palmitate samples (Simard et al., 2006; Ashbrook et al., 1975; Berde et al., 1979). In this regard, some peaks corresponding to albumin partially saturated by palmitate may be present in the spectrum but masked by the albumin peak particularly because even the maximum expected shift for albumin–palmitate, which is $m/z = 1787.8$ (=255.4 Da × 7 palmitate molecules), may be hard to resolve by this experimental strategy. Finally, it is important to acknowledge that although the current study was performed using human serum albumin and fluorescent probes based on human ghrelin sequence, ghrelin full-length forms were based on the murine sequence—which differs at positions 11 and 12 from the human sequence—since *in vivo* experiments were carried out in rodent models. Thus, we cannot rule out that this interspecies difference contributes to the discrepancies of the above described outcomes.

The ITC experiments indicated that binding of ghrelin to albumin is a complex exothermic process, in which additional transitions may occur at high ghrelin:albumin molar ratios. The absence of a clear baseline at the end of the titration impedes the calculation of an accurate K_D value. However, the estimated K_D for the affinity of the first apparent transition remains a valuable experimental indication of the strength of interaction between these molecules in a wide ghrelin:albumin molar ratio range, including the ratios found in plasma under physiological conditions. Thus, we estimated the apparent thermodynamic parameters for the major binding sites adopting a simplified model for the low ghrelin:albumin molar ratios, and found that both enthalpic and entropic components contribute to the binding, suggesting that not only hydrophobic effects drive the interaction. The estimated K_D of the binding sites was 4.2×10^{-6} M, which seems to be fairly reasonable as it is comparable to the K_D reported for albumin towards other hormones or lipophilic compounds (Baker, 2002; Mendel, 1989). For instance, the K_D for albumin-bound testosterone and albumin-bound thyroxin are 1×10^{-4} and 1.4×10^{-6} M, respectively, which are several orders of magnitude greater than the K_D of such hormones for their specific plasma binding globulins (in the 10^{-9}–10^{-10} M range; Melmed et. al., 2011, Zheng et al., 2015). Interestingly, the K_D for autoantibodies-bound ghrelin is in the 10^{-8} M range (Takagi et al., 2013); however, albumin-bound ghrelin likely represents the majority of the circulating ghrelin due to the high concentration of serum albumin in plasma, despite of the higher affinity of the autoantibodies. In particular, plasma concentrations of serum albumin and ghrelin are ~6×10^{-4} and ~2×10^{-10} M, respectively (Ryan et al., 2011; Hassouna et al., 2014); thus, our estimated K_D predicts that albumin displays the ability to bind all the circulating ghrelin under equilibrium conditions (estimated free plasma ghrelin is ~0.4%).

The mathematical analysis of the first transition predicts that each serum albumin molecule can, on average, bind 1.6 ghrelin molecules, assuming that all of them bind to identical and independent sites. However, the occupancy of the serum albumin by ghrelin would be very low under normal conditions given the

enormous disparity in their concentrations in plasma. According to our calculations, albumin would be able to bind most circulating ghrelin even under low energy balance conditions, such as calorie restriction, when plasma ghrelin concentration increases up to 10 times (Goldstein et al., 2011). It is interesting to mention, however, that each serum albumin molecule binds between 0.1 and 2 FA molecules under normal conditions and that the number of albumin-bound FA can rise to 6 or greater during fasting (Brodersen et al., 1990; Fraze et al., 1985). Thus, the interaction between ghrelin and serum albumin may be affected by the metabolic status.

Our observation that the des-octanoylation of ghrelin is reduced in the presence of albumin indicates that ghrelin-albumin interaction impacts on the stability of the hormone. As compared to previous reports (Satou et al., 2015), we detected a significant degree of des-octanoylation of the free hormone in buffer solution. Many reasons may account for this basal quantitative difference since the hydrolysis of the octanoyl moiety of ghrelin strongly depends on many factors including its counter-ion, pH, the solvent, among other (Ishimaru et al., 2004). It is well known that the half-life of exogenously administered ghrelin in circulation is very short, ranging between 8 and 10 min, as a consequence of the rapid deacylation of the hormone by plasma esterases (De Vriese et al., 2007; Satou et al., 2010; Akamizu et al., 2012; Gauna et al., 2004; Mayorov et al., 2008). Since serum albumin was reported to be able to hydrolyze several esterified compounds, including long- and short-chain FA esters (Wolfbeis and Gürakar, 1987), it was hypothesized that an esterase-like activity of serum albumin could also contribute to ghrelin deacylation in plasma (De Vriese et al., 2007). Our observations, however, indicate that the spontaneous deacylation rate of ghrelin is decreased by albumin binding. This result suggests that albumin binding plays a rather protective role on the hormone in plasma prolonging its half-life.

Since the albumin-bound hormones are normally considered active (Melmed et al., 2011), we determined the implications of the ghrelin-albumin interaction on the hormone bioactivity. In both assays, we used ghrelin and albumin combinations in which most of the hormone should be bound to albumin according to our estimated K_D. *In vitro*, we found that the presence of albumin impairs the ability of ghrelin, as compared to ghrelin alone, to inhibit the calcium channels. Interestingly, the concentration of ghrelin estimated to be free under these experimental conditions, which is 17.4% of total ghrelin, also failed to inhibit calcium channels suggesting that no much ghrelin is dissociated during the electrophysiological recordings. This observation may be unpredicted based on the fact that the K_D of albumin-bound ghrelin is much larger than the K_D of GHSR-bound ghrelin, estimated in the 10^{-9}–10^{-10} M range (Holst et al., 2003, 2005; Katugampola et al., 2001). We hypothesize that kinetic aspects, rather than thermodynamic aspects, of the ghrelin-albumin interaction are conditioning the results because the recordings are performed in very short time-frame (less than 60 s). *In vivo*, the orexigenic activity of ghrelin was also reduced in the presence of albumin; although, the blockage of this effect was not as robust as the observed in the *in vitro* system. Here, the dose of ghrelin estimated to be free in the ghrelin-albumin solution, which represents ~3% of total ghrelin, also failed to increase food intake suggesting that some albumin-bound ghrelin is dissociated from the protein during the 30 min period in which the food intake was quantified. Given the complexity of the eating responses, however, the reduced orexigenic activity of exogenously administered albumin-bound ghrelin may be due to a variety of reasons, including alterations of either subcutaneous absorption of the complexes, hepatic clearance or brain accessibility, among others. Even when several aspects of these experiments remain to be clarified, our results indicate that the ghrelin and albumin interaction is relevant enough to impact on the bioactivity of the hormone.

In our opinion, current data may have a variety of implications. At physiological level, our finding indicates that the plasma pool of albumin-bound ghrelin would represent a long-term circulating reservoir of the hormone with relatively extended half-life. To our knowledge, the K_D for the lipoprotein-bound ghrelin has not been estimated; however, a quick esterase-mediated deacylation of ghrelin is predicted for the lipoproteins-bound hormone (De Vriese et al., 2007; Soares and Leite-Moreira, 2008). Thus, current results give rise to the exciting hypothesis that the pools of circulating ghrelin, associated to different carriers, may have different physiological implications. The use of radiolabeled ghrelin probes, which can be detected in very low levels that do not affect the endogenous equilibrium, will likely be helpful to uncover the distribution and the dynamic aspects of these pools of plasma ghrelin *in vivo*. A higher stability for the albumin-bound ghrelin is not unexpected since fatty acid acylation is a current pharmaceutical approach used to manipulate the bioactivity of other peptide hormones. The acylation with saturated FA of the hormones glucagon like-peptide 1 or insulin enables their binding to serum albumin (K_D ~ 10^{-4}–10^{-5} M; Pollaro and Henis, 2010), and such interaction decreases their bioactivity but increases their plasma half-lives, as compared to the unmodified peptides (Pollaro and Heinis, 2010; Kurtzhals et al., 1995). Thus, analogs such as insulin detemir (LysB29(N-tetradecanoyl) des (B30) human insulin) are used as long acting drugs that achieve continuous and steady blood insulin levels. Additionally, our observations may have some practical implications for basic and clinical studies related to ghrelin. For instance, bovine serum albumin is sometimes added as additive to stabilize ghrelin and prevent its adsorption to plastics; however, researchers should be more careful about this practice as it may impact on the outcome of the studies. In addition, it is well-known that the dosage of ghrelin concentrations in blood samples is sometimes problematic as it is markedly affected by the conditions of collection and storage. Thus, the binding of ghrelin to exogenously-added albumin may be considered in order to increase the stability of the hormone in the samples. Future studies will be necessary in order to address all these lines of inquiry.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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