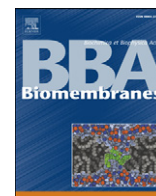




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Acylated and unacylated ghrelin binding to membranes and to ghrelin receptor: Towards a better understanding of the underlying mechanisms

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ABSTRACT

The *O*-octanoylation of human ghrelin is a natural post-translational modification that enhances its binding to model membranes and could potentially play a central role in ghrelin biological activities. Here, we aimed to clarify the mechanisms that drive ghrelin to the membrane and hence to its receptor that mediates most of its endocrinological effects. As the acylation enhances ghrelin lipophilicity and that ghrelin contains many basic residues, we examined the electrostatic attraction and/or hydrophobic interactions with membranes. Using various liposomes and buffer conditions in binding, zeta potential and isothermal titration calorimetry studies, we found that whereas acylated and unacylated ghrelin were both electrostatically attracted towards the membrane, only acylated ghrelin penetrated into the headgroup and the lipid backbone regions of negatively charged membranes. The *O*-acylation induced a 120-fold increase in ghrelin local concentration in the membrane. However, acylated ghrelin did not deeply penetrate the membrane nor did it perturb its organisation. Conformational studies by circular dichroism and attenuated total reflection Fourier transformed infrared as well as *in silico* modelling revealed that both forms of ghrelin mainly adopted the same structure in aqueous, micellar and bilayer environments even though acylated ghrelin structure is slightly more α -helical in a lipid bilayer environment. Altogether our results suggest that membrane acts as a “catalyst” in acylated ghrelin binding to the ghrelin receptor and hence could explain why acylated and unacylated ghrelin are both full agonists of this receptor but in the nanomolar and micromolar range, respectively.

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Abbreviations: ATR-FTIR, attenuated total reflection Fourier transformed infrared; CD, circular dichroism; Chol, cholesterol; DMPC, dimyristoyl-phosphatidylcholine; DMPG, dimyristoyl-phosphatidylglycerol; DOPC, dioleoyl-phosphatidylcholine; DPC, dodecyl-phosphocholine; DPH, 1,6-diphenylhexatriene; EIA, enzyme immunoassay; GOAT, ghrelin *O*-acyltransferase; hAG, human acylated ghrelin; hUAG, human unacylated ghrelin; ITC, isothermal titration calorimetry; LUVET, large unilamellar vesicles produced by extrusion technique; MLV, multilamellar vesicles; PAMPA, parallel artificial membrane permeability assay; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; TFE, trifluoroethanol; TMA-DPH, 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene

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1. Introduction

O-acylation of medium chain length fatty acids is a novel type of natural peptide modification which was discovered a decade ago by Kojima and co-workers [1]. In 1999, ghrelin was isolated from stomach extracts as the endogenous ligand of the orphan growth hormone secretagogue receptor 1a (GHS-R1a), now called the ghrelin receptor. Ghrelin is a 28-aa peptide hormone which possesses a unique post-translational modification: an *O*-acylation at its Ser³ residue. The main acylated forms of ghrelin are modified with an *n*-octanoic acid and to a lesser extent with decanoyl and possibly deconoyle fatty acids [1,2]. The enzyme that catalyzes the acylation of ghrelin is a membrane-bound *O*-acyl transferase called ghrelin *O*-acyltransferase (GOAT) [3,4]. Ghrelin has been discovered thanks to its growth hormone releasing activity [1]. Since then, numerous biological activities have been attributed to ghrelin. The orexigenic effects are the ones that generated the highest interest among endocrinologists. Yet, ghrelin also affects energy and glucose homeostasis,

cell proliferation and differentiation, gastrointestinal, cardiovascular, pulmonary and immune functions as well as bone physiology. Another naturally occurring variant of ghrelin is unacylated ghrelin also called des-acyl ghrelin. This form is the most abundant in the plasma. Even though unacylated ghrelin has long been thought to be devoid of physiological activities, it now appears that acylated and unacylated ghrelin present similar and opposite actions (see [5–7] for review). In the nanomolar range, the acylation of ghrelin is required for efficient binding and activation of the ghrelin receptor that mediates most of its endocrinological activities [1]. However, some other effects of ghrelin have been attributed to the activation of a yet unidentified receptor that could bind acylated and/or unacylated ghrelin [7]. Unacylated ghrelin is also a full agonist of the ghrelin receptor but in the micromolar range [8]. As ghrelin effects seem to be receptor mediated, the interaction of ghrelin with cell membranes is potentially important for all its biological activities.

In 1986, Sargent and Schwyzer were the first to propose a “membrane catalysis” model in which the membrane lipid phase acts as a mediator for peptide–receptor interactions by speeding up the reactions [9]. It relies on evidence, that for any ligand approaching a cell, the probability of contact with the lipid phase is much higher than with one of its receptors. This model is still relevant today even though it has been refined over the years [10,11]. The known and foreseen advantages for peptide–receptor binding associated with the peptide–membranes interactions include favourable kinetics, increased local concentration of the peptide in the vicinity of the receptor, peptide conformation and orientation change, as well as positioning of the peptide at the proper depth for receptor interaction and in some cases diffusional advantage due to the reduction of dimensionality. These peptide–membrane–receptor interactions can be divided into four molecular steps: (i) electrostatic attraction/membrane adsorption of the peptide; (ii) peptide penetration into the headgroup region or in the hydrophobic backbone; (iii) conformational change of the peptide; (iv) peptide–receptor docking [10,12,13]. When basic peptides such as ghrelin interact with acidic lipids, their interactions can either be purely electrostatic like for polylysines or a mix of hydrophobic and electrostatic interactions which occurs for most peptides [12,13].

As we already mentioned, there has been a lot of interest for ghrelin regarding to its therapeutical potential. However, besides the octanoyl role in binding and activation of the ghrelin receptor, little is known about the other roles of this unusual moiety [14]. The acylation of ghrelin increases its lipophilicity [15]. Moreover, as we have previously shown, it also increases its binding extent to model membranes [16]. We can therefore hypothesize that this modification could enhance the binding of ghrelin to biological membranes and hence to its receptor as well as its transmembrane and transepithelial transport [17,18].

The aims of this paper are thus focused on the roles of the octanoyl moiety and the basic residues of ghrelin in the extent of ghrelin binding to membranes and on the underlying mechanisms of ghrelin–lipid interactions in order to give new insights about the differential binding of acylated and unacylated ghrelin to the ghrelin receptor, as well as its role on ghrelin transmembrane and transepithelial transport.

2. Material and methods

2.1. Material

Synthetic human acylated ghrelin (hAG) and human unacylated ghrelin (hUAG) were purchased from NeoMPS (Strasbourg, France). Peptide HPLC purity was >97%, according to the manufacturer. Bovine brain sphingomyelin (SM), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) (DMPG), cholesterol (Chol), phosphatidylserine (PS), trifluoroethanol (TFE) and furosemide were obtained from Sigma–Aldrich, St. Louis, MO,

USA. Egg yolk phosphatidylcholine (PC) (Grade 1), wheat germ phosphatidylinositol (PI) (Grade 1) and egg yolk phosphatidylethanolamine (PE) (Grade 1) were obtained from Lipid Products, Redhill Surrey, UK. Acetonitrile, HPLC grade for far UV, was purchased from Acros organics. Trifluoroacetic acid (TFA), HPLC grade was purchased from Fisher Scientific. All other reagents were of ACS or analytical grade. Peptide quantities are always expressed as the net peptide content.

2.2. Buffers

As the ester bond of acylated ghrelin is chemically unstable and converts into unacylated ghrelin at basic pH, even at pH 7.4 [16], most experiments were conducted at pH 6. Unless otherwise stated, experiments were performed in 12.5 mM Na-citrate 14 mM NaCl pH 6 buffer ($I_c = 0.06$ M), referred as citrate buffer hereafter. In absence of buffering the peptide solution pH is about 4.5. Two other buffers, 12.5 mM Na-citrate 46.2 mM NaCl pH 4 buffer ($I_c = 0.06$ M) and 12.5 mM Na-citrate 103.2 mM NaCl pH 6 buffer ($I_c = 0.15$ M), were used for the pH and ionic strength influence studies, respectively.

2.3. Preparation of liposomes

We prepared large unilamellar vesicles by extrusion (LUVET) using a Thermobarrel extruder (Lipex Biomembranes Inc., Vancouver, Canada) as previously described [19,20]. The *z*-average diameters of the liposomes extruded on either 100 nm (LUVET₁₀₀) or 200 nm (LUVET₂₀₀) pore size filters, as determined using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK), were 100 ± 2 nm and 135 ± 12 nm, respectively. The phospholipid concentrations were determined by phosphorus assay [21]. When liposomes contained cholesterol, the total lipid concentration was calculated assuming similar recovery of phospholipids and cholesterol. Liposomes were stored under nitrogen at 4 °C and used within 5 days.

2.4. Binding to liposomes by ultracentrifugation

Human acylated and unacylated ghrelin were used to assess the binding to LUVET₁₀₀ or LUVET₂₀₀ at lipid/peptide molar ratios of 12:1 to 375:1 by ultracentrifugation. Liposomes were either made of Chol/PC/SM/PI/PE (5.7:4.0:1.8:2.7:2.4, M/M) [22], DMPC/PI (10.3:1, M/M), DMPC/PI (5.15:1, M/M), DMPC/DMPG (5.15:1, M/M) or DMPC alone. Peptide concentration was 100 or 200 µg/ml. The liposomes/peptide (either acylated or unacylated ghrelin) mixture (~160 µl) was incubated for 3 h at 4 or 37 °C and then ultracentrifuged at 4 °C (250,000 × *g*) for 1 h. In controls, liposomes were replaced by the equivalent volume of buffer. 100 µl of the supernatants, that contained the free peptides, were then collected. Controls and supernatants were quantified by HPLC/UV as previously described [16]. The binding percentage was calculated as follows: ((control concentration – supernatant concentration) / control concentration) × 100. Acylated and unacylated ghrelin binding rates at 37 °C to Chol/PC/SM/PI/PE LUVET₂₀₀ at a lipid/peptide molar ratio of 375:1, as determined by ultracentrifugation ($98.7 \pm 1.0\%$ and $47.7 \pm 2.1\%$, respectively), were confirmed by equilibrium dialysis of ¹²⁵I-human Tyr⁴-acylated ghrelin and ¹²⁵I-human Tyr⁴-unacylated ghrelin (Eurogentec, Herstal, Belgium), $96.8 \pm 1.9\%$ and $46.9 \pm 3.4\%$, respectively, $p > 0.05$ (unpaired *t*-test).

2.5. Binding to erythrocytes by centrifugation

Blood samples were collected in citrate tubes from 6 healthy volunteers who gave informed consent (3 male and 3 female). Erythrocytes were isolated by centrifugation (10 min, 330 × *g*, 20 °C) and then washed 3 times with PBS. After addition of acylated ghrelin or unacylated ghrelin (200 µg/ml) to the erythrocytes (45% V/V), the erythrocytes were incubated for 3 h at 4 °C and then centrifuged at

4 °C (330×g) for 10 min. Thereafter the supernatants were collected and quantified by UV after an RP-HPLC separation, as previously described [16].

2.6. Fluorescence polarization

The effect on membrane fluidity was studied by measuring the degree of fluorescence polarization of 1,6-diphenylhexatriene (DPH) and 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) (Invitrogen, Carlsbad, CA, USA) in Chol/PC/SM/PI/PE (5.7:4.0:1.8:2.7:2.4, M/M) LUVET₂₀₀ at 4, 37 and 55 °C according to the method of Shinitzky and Barenholz [23]. Samples preparation was adapted from [24]. Lipid/peptide molar ratio was 240:1, whereas lipid to DPH and TMA-DPH molar ratios were 315:1 and 477:1, respectively. Fluorescence polarization measurements and determination of the degree of polarization were performed as previously described [20].

2.7. Zeta potential determination

The vesicles electrophoretic mobility was determined using a Zetasizer Nano ZS. For the electrophoretic mobility measurements at constant peptide concentration (200 µg/ml) the samples were prepared as follows: DMPC or Chol/PC/SM/PI/PE (5.7:4.0:1.8:2.7:2.4, M/M) LUVET₂₀₀ were added to the peptide solution (or buffer for controls) at a lipid/peptide molar ratio of either 240:1 or 60:1, with a final mixture volume of about 160 µl. After 3 h incubation at 37 °C, 1 ml of citrate buffer was added prior to the sample measurement. For electrophoretic mobility measurements at different peptide concentrations, the peptide (1 mg/ml stock concentration) was added to 1 ml of 61.5 µM Chol/PC/SM/PI/PE (5.7:4.0:1.8:2.7:2.4, M/M) LUVET₁₀₀ and incubated for 1 h at 37 °C and then measured at the same temperature. The zeta potentials were calculated using Oshima's analytical expression of Henry's equation described in Section 2.12.

2.8. Infrared spectroscopy measurement

Attenuated total reflection Fourier transformed infrared (ATR-FTIR) spectroscopy was used to evaluate any secondary structure change of acylated and unacylated ghrelin in presence of DMPC/DMPC (5.15:1, M/M) LUVET₂₀₀ regarding to their structure in solution. Before sample preparation, the trifluoroacetate (TFA) counterions, which strongly associate with the peptides, were replaced by chloride counterions by three successive lyophilisations against HCl 10 mM [25]. These were followed by 2 successive lyophilisations in water in order to remove the excess of HCl. This allowed the elimination of a strong C=O stretching absorption band due to TFA near 1673 cm⁻¹ [26]. Spectra were recorded on a Bruker Equinox 55 at room temperature as previously described [27]. About 265 µl of either peptide alone (200 µg/ml) or of a lipid/peptide mixture (at a 50:1 lipid/peptide molar ratio), in NaCl 3 mM was spread out on the plate. Samples were flushed with N₂ saturated with D₂O for 4 h. The secondary structure changes were monitored by analysis of the amide I vibrational bands (1600–1700 cm⁻¹) as described in [28]. The effects of the peptides on the orientation of the phospholipids acyl chains were assessed as previously described [29]. In brief, we monitored the transition dipole moment of the $\gamma_w(\text{CH}_2)$ peak at 1202 cm⁻¹ as it lies along the lipid hydrocarbon chains. The angle between the germanium crystal and the dipole was calculated from the dichroic ratio R_{ATR} . The dichroic ratio (R_{ATR}) was calculated by determining the ratio of the peak absorbance as follow: $R_{\text{ATR}} = A(90^\circ)/A(0^\circ)$.

2.9. Circular dichroism

Far-UV circular dichroism (CD) spectra (185–260 nm) of the peptides were recorded on Jasco-710 spectropolarimeter. A quartz cell with a path length of 0.05 or 0.02 cm was used. All measurements were made at room temperature (22 °C). The parameters used were as follows: bandwidth, 1.0 nm; step resolution, 0.5 nm; scan speed, 50 nm/min; response time, 2 s. Each spectrum was obtained from an average of 4 scans. The contribution of the buffer was subtracted. Peptide concentration was 200 µg/ml.

2.10. PepLook method (Boltzmann–Stochastic method)

In order to explore conformational possibilities of the peptide in a hydrophobic environment we used the Boltzmann–Stochastic *in silico* method, PepLook [30]. This method requires several successive steps of calculation. At each step, a random population of 10,000 conformations of ghrelin (acylated or not) is generated and the energy of all conformations is calculated using the force field described elsewhere [30]. The first step uses a set of 64 pairs of Φ/Ψ of angles with equal probability. In the next steps, the probabilities of Φ/Ψ values per residue vary according to whether they had previously contributed to exclusively poor or, exclusively good structural solutions for the peptide, respectively. The calculation was iterated up to when the probability of all Φ/Ψ angles remains constant. Then, the 99 models of lower energy were further minimized using a Simplex method [31,32] with a precision of 5 degrees and a maximum of 1000 steps. The structure with the best energy (called the “prime”) was considered.

2.11. Isothermal titration calorimetry

The heat flows resulting from the binding of acylated and unacylated ghrelin to Chol/PC/SM/PI/PE (5.7:4.0:1.8:2.7:2.4, M/M) LUVET₁₀₀ were measured by high-sensitivity isothermal titration calorimetry (ITC) using a Microcal VP-ITC (Microcal, Northampton, MA) with a reaction cell volume of 1.4565 ml [33] and Milli-Q water as a reference. Solutions were degassed for 10 min under vacuum prior to use. Peptide-into-lipid and lipid-into-peptide titrations were performed under constant stirring (305 rpm). After an initial delay of 1800 s and a 2 µl initial injection, 5 µl (in 5 s) or 10 µl (in 7.1 s) of either peptide (200 µM) or lipids (12.2 to 48.4 mM) were injected from the 300 µl syringe into the sample cell containing either the lipids (12.2 to 18.0 mM) or the peptide (10 to 100 µM). The interval between each injection was 4 min. The heats of dilution were determined in control experiments by injection of either the peptides or the lipids into the buffer and were subtracted from the heats determined in the corresponding peptide-into-lipid or lipid-into-peptide experiment. All experiments were performed at 37 °C in duplicate.

2.12. Binding model

The binding of ghrelin to lipids can be described by its overall or apparent binding coefficient, K_{app} , that includes both the electrostatic attraction and the chemical partition equilibria. This apparent binding coefficient is linked to the degree of peptide binding, X_b^i , and to the bulk molar concentration of the peptide, C_f^i , as follows:

$$K_{\text{app}} = X_b^i / C_f^i \quad (1)$$

where X_b^i is the number of moles of peptides bound, $n_{\text{dep},b}^i$, per mole of lipids that the peptides can theoretically reach, i.e. 50% of the total lipids in the case of LUVET if the peptide does not cross the bilayer [34]. i represents the i th external lipid/peptide molar ratio L_{ext}/P .

However, as the peptide chemical (hydrophobic) partition is of high interest to us, we used another binding model described by Seelig et al. [35,36]. In this model, the chemical partition coefficient K_p is not assumed anymore to be linked to the bulk concentration of the peptide but rather to its concentration near the membrane surface, C_M^i , as follows:

$$K_p = X_b^i / C_M^i \quad (2)$$

The X_b^i have been calculated here from the binding experiments by ultracentrifugation of ghrelin–Chol/PC/SM/PI/PE LUVET mixtures at L_{ext}/P molar ratios at which the membrane is not yet saturated by the peptide i.e. at $L_{ext}/P \geq 10$, as follows:

$$X_b^i = n_{pep,b}^i / \gamma n_L^i = (C_0^i - C_f^i) / \gamma C_L^i \quad (3)$$

where n_L^i is the total number of moles of lipids, C_0^i is the total or control molar peptide concentration, C_f^i is the free molar peptide concentration, γ is the proportion of external lipids (50%) and C_L^i is the total molar lipid concentration.

The peptide membrane concentration, C_M^i , is correlated to the free peptide concentration, C_f^i , as well as to the peptide effective charge, z_{eff} , and to the membrane surface potential, ψ_0^i , according to the Boltzmann relation [37] as follows:

$$C_M^i = C_f^i \exp(-z_{eff} F \psi_0^i / R T) \quad (4)$$

where F is the Faraday constant (C/mol), R is the gas constant (J/K mol) and T is the temperature (K).

Unfortunately, direct measurement of ψ_0^i cannot be performed. However, ψ_0^i is related to the zeta potential, ζ^i , and to the surface charge density, σ^i , the first being related to the electrophoretic mobility, u_e^i , and the latter being connected to X_b^i .

We first calculated ζ^i , from the measured u_e^i , of the ghrelin–Chol/PC/SM/PI/PE LUVET mixtures at different L_{ext}/P molar ratios, using the Oshima's analytical expression of Henry's equation [38]:

$$\zeta^i = \frac{3 \eta}{2 \epsilon_r \epsilon_0 f(\kappa R_s)} u_e^i \quad (5)$$

with

$$\kappa = \sqrt{\frac{2000 e^2 N_A I}{\epsilon_r \epsilon_0 k_B T}} \quad (5a)$$

and

$$f(\kappa R_s) = 1 + \frac{1}{2} \left[1 + \left(\frac{2.5}{\kappa R_s [1 + 2 \exp(-\kappa R_s)]} \right) \right] \quad (5b)$$

where ζ^i and u_e^i are respectively expressed in (V) and ($m^2 V^{-1} s^{-1}$), η is the dispersant viscosity (Pa.s), ϵ_r is the relative permittivity of water (74.4 at 37 °C), ϵ_0 is the vacuum electric permittivity (F/m), κ is the reciprocal of the Debye screening length (m^{-1}), R_s is the radius of the liposomes (m), e is the elementary charge (C), N_A is the Avogadro's number (mol^{-1}), I is the buffer ionic strength (M) and k_B is the Boltzmann constant (J/K).

From the ζ^i we could then calculate the ψ_0^i using the Debye–Hückel approximation for spherical double layers [39,40]:

$$\psi_0^i = \frac{\zeta^i}{\frac{R_s}{R_s + x} \exp(-\kappa x)} \quad (6)$$

where x is the distance of the hydrodynamic plane of shear from the liposomes surface (m).

In our model we consider the sodium binding to the membrane as described by Seelig et al. [35]. However, we neglected the peptide penetration area into the lipid bilayer. The surface charge density σ^i was therefore related to X_b^i as follows:

$$\sigma_i = \sigma_0 + \sigma_k = - \frac{e X_{PI} (1 - X_{Na}^i)}{A_L} + \frac{e z_{eff} X_b^i}{A_L} \quad (7)$$

with

$$X_{Na}^i = \frac{K_{Na} C_{M,Na}^i}{1 + K_{Na} C_{M,Na}^i} \quad (7a)$$

where σ_0 is the surface charge density in the absence of the peptide (C/m^2), σ_k is the increment of charge density due to the addition of peptide to the liposomes (C/m^2), X_{Na}^i is the molar fraction of PI with a Na^+ bound and $C_{M,Na}^i$ is the Na^+ membrane concentration (M). The molar fraction of PI is $X_{PI} = 0.164$. The mean lipid molecular area, $A_L = 5.26 \times 10^{-19} m^2$, was determined using a Langmuir film balance by air/water surface compression isotherms at 37 °C of a Chol/PC/SM/PI/PE (5.7:4.0:1.8:2.7:2.4, M/M) monolayer at a lateral pressure of 32 mN/m which corresponds to the monolayer–bilayer equivalent pressure [41]. K_{Na} is the Na^+ binding coefficient (M^{-1}). This value was calculated, for each plane of shear distance x , from the electrophoretic mobilities of the liposomes in 12.5 mM Na-citrate 14 mM NaCl pH 6 buffer in the absence of the peptide, where $X_b^i = 0$, and hence by the combination of the Eqs. (7) and (7a). For $x = 2 \text{ \AA}$, $K_{Na} = 2.35 M^{-1}$, this value is in good agreement with the previously reported value for PI containing liposomes [42].

The surface charge density σ^i , creates a surface potential ψ_0^i which can be calculated using the Grahame equation based on the Gouy–Chapman theory [37]:

$$\sigma_i = \text{sign}(\psi_0^i) \left\{ 2000 \epsilon_r \epsilon_0 RT \sum_j C_{j,eq} \left[\exp\left(\frac{-z_j F \psi_0^i}{RT}\right) - 1 \right] \right\}^{1/2} \quad (8)$$

where $C_{j,eq}$ is the molar concentration of the j th electrolyte in the bulk aqueous phase and z_j the signed valency of this j th electrolyte.

The experimental values of u_e^i and X_b^i were obtained with Chol/PC/SM/PI/PE LUVET and ghrelin in 12.5 mM Na-citrate 14 mM NaCl pH 6 buffer as described above. The unknowns in the above equations that are needed to determine K_p from u_e^i and X_b^i are the surface potential ψ_0^i , the plane of shear distance x and the peptide effective charge z_{eff} . As no analytical solution exists, we developed a numerical solution. For each plane of shear distance x , from 0 to 7.5 Å with a 0.5 Å step, we computed a solution and the best fit to the experimental data was found for $x = 2 \text{ \AA}$, as found in many other systems [43–45]. First, we determined the K_{Na} as described above and hence X_{Na}^i . As there are 2 independent ways to calculate σ^i from the experimental data, i.e. either from the u_e^i by the combination of Eqs. (5), (6) and (8), or from the X_b^i by the combination of Eqs. (3) and (7), Eqs. (7) and (8) were thus equalized to obtain z_{eff} by a one-parameter linear regression. On the basis of this z_{eff} value we calculated C_M^i using Eq. (4) and hence K_p by linear regression of $K_p C_M^i = X_b^i$.

2.13. Parallel Artificial Membrane Permeability Assay (PAMPA)

The commercial PAMPA “sandwich” from pION Inc. (P/N 110163, Woburn, MA, USA) is composed of a receiver 96-well filter plate deposited on a donor 96-well microtiter plate. On the filter we deposited 4 μ l of either a 20% (w/v) *n*-dodecane solution of a lecithin mixture (pION, P/N 110669) containing excess negative charges, or a 2% w/v DOPC *n*-dodecane solution (pION P/N 110615). The donor and receiver chambers were both filled with 200 μ l of 12.5 mM Na-citrate 103.2 mM NaCl pH 6 buffer. The donor chamber also contained

200 µg/ml of peptide. Incubation lasted for 18 h at 25 °C, after which the receiver chamber was quantified. Acylated and unacylated ghrelin were analyzed using specific enzyme immunoassays (EIA) kits (A05106 and A05119, SPI-bio, Montigny le Bretonneux, France). Carbamazepine and furosemide at 25 µg/ml (2.5% DMSO) were used as controls and quantified by UV following a RP-HPLC separation (carbamazepine P_{app} was 2.76 ± 0.11 and $2.74 \pm 0.05 \times 10^{-6}$ cm/s with DOPC and lecithin, respectively, whereas furosemide P_{app} was $0.45 \pm 0.02 \times 10^{-6}$ cm/s with lecithin and not detectable in the acceptor well with DOPC and thus its P_{app} was $<0.19 \times 10^{-6}$ cm/s).

2.14. Transport experiments

Mono-cultures of Caco-2 cells and co-cultures of Caco-2 and Raji cells were grown on/under inverted 12-wells inserts as previously described by des Rieux et al [46,47]. 400 µl of acylated or unacylated ghrelin in HBSS pH 6 (100 µg/ml) were added to cell monolayer apical compartments of mono- and co-cultures. Basolateral compartments were filled with 1200 µl of HBSS pH 7.4. Cell monolayers were incubated for 105 min at 37 °C. Then, 30 µl of 1% TFA was added to acidify the basolateral compartments of acylated ghrelin samples (final pH ~5.7). Basolateral solutions were sampled and stored at -80 °C before quantification. Amounts of acylated and unacylated ghrelin transported were measured in duplicates using the specific EIA kits described above. For acylated ghrelin samples, both acylated and unacylated ghrelin were dosed. The *in vitro* model functionality was assessed by the transport of control nanoparticles [47].

2.15. Statistical analysis

Unless otherwise stated, all average results are represented as mean \pm S.D. Differences between 2 groups were analyzed by unpaired t-test with Welch's correction, whereas differences between three or more groups were analyzed by one-way ANOVA combined with the Tukey's test. Differences from 0 were analyzed with one sample t-test. A probability level <0.05 was considered significant.

3. Results

3.1. Binding of ghrelin to model and biological membranes

To determine if acylated and unacylated ghrelin bind to membranes, we assessed the binding extent of these peptides to model plasma membranes (liposomes) or to biological membranes (ery-

throcytes) by (ultra)centrifugation. As displayed in Fig. 1A both acylated (hAG) and unacylated (hUAG) ghrelin bound to liposomes and to erythrocytes. The extent of binding of ghrelin to both liposomes and erythrocytes was enhanced by its acylation from ~30 to ~90% (Fig. 1A). Interestingly, acylated and unacylated ghrelin bound to erythrocytes to the same extent than to the Chol/PC/SM/PI/PE liposomes, which mimic the plasma membrane [22,48–50], at a lipid/peptide molar ratio of 240:1. Ghrelin has been shown to circulate in the bloodstream associated with lipoproteins [51,52] in the plasma compartment. However, to our knowledge, we were the first to investigate the fate of ghrelin in the erythrocytes fraction. The acylation of ghrelin enhanced its binding extent to erythrocytes but also to human serum albumin ($49.2 \pm 2.8\%$ for hAG and $18.8 \pm 1.1\%$ for hUAG). Knowing that (i) the erythrocytes represent 45% of the blood volume, (ii) albumin has a pseudo-esterase activity [53] and (iii) other studies highlighted the implication of esterases in the desoactanoylation of ghrelin [54], it could partly explain why desacylghrelin is the major form of ghrelin in the plasma [55]. This would, however, require further investigation.

The binding extent of acylated and unacylated ghrelin to Chol/PC/SM/PI/PE liposomes also depended on the lipid/peptide molar ratio. The binding extent increases faster for acylated ghrelin and, in our conditions, reached a 100% plateau that was not observed for unacylated ghrelin (Fig. 1B). At all lipid/peptide molar ratios, the binding extent of acylated ghrelin to the Chol/PC/SM/PI/PE liposomes was significantly higher ($p < 0.001$, except at the lipid/peptide molar ratio of 12:1 where $p < 0.01$) than that of unacylated ghrelin (e.g. $77.0 \pm 0.7\%$ vs. $12.0 \pm 0.8\%$, for hAG and hUAG, respectively, at a lipid/peptide molar ratio of 60:1). The size of the liposomes extruded either through 100 or 200 nm pores, did not influence the binding of the peptides (data not shown) [16]. The apparent binding coefficients determined from these results will be calculated and analyzed together with the chemical partition coefficients (see Section 3.4.).

3.2. Electrostatic adsorption studies

As acylated and unacylated ghrelin are highly charged peptides at pH 6 and 7.4 (theoric charges of ~-5.5 and ~-5.0, respectively), we first investigated the possible electrostatic interactions between ghrelin and liposomes. For that purpose, we used liposomes of different compositions. By comparison with the binding to negatively charged liposomes (Fig. 2A), acylated ghrelin only slightly bound to zwitterionic liposomes made of DMPC ($7.7 \pm 1.3\%$) whereas unacylated ghrelin did not (Fig. 2A). As the presence of an acidic phospholipid

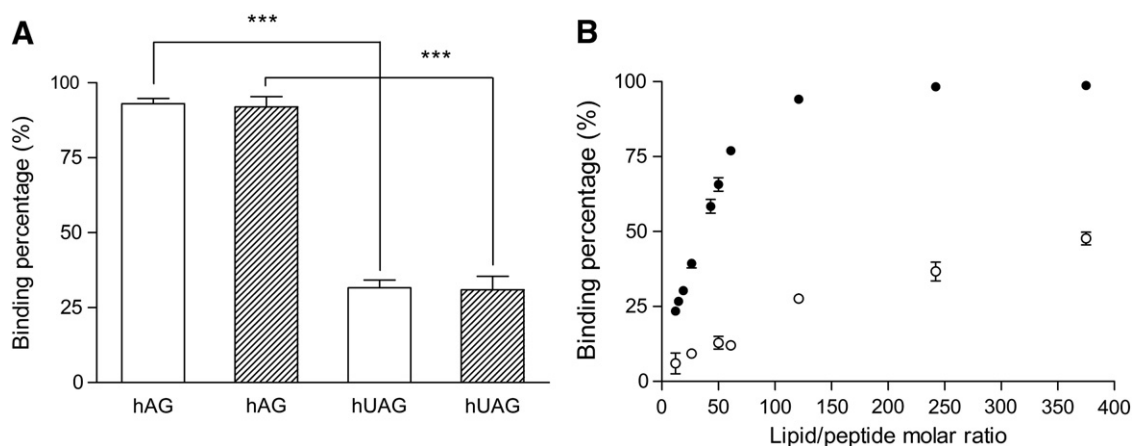


Fig. 1. Acylation influence on ghrelin binding to model and biological membranes. (A) Binding of acylated ghrelin (hAG) and unacylated ghrelin (hUAG) at 200 µg/ml to Chol/PC/SM/PI/PE (5.7:4.0:1.8:2.7:2.4, M/M) LUVET₂₀₀ (empty bars) in Na-citrate 12.5 mM NaCl 14 mM pH 6 buffer at a lipid/peptide molar ratio of 240:1 or to erythrocytes (45%, V/V) (hatched bars) in PBS pH 7.4. T = 4 °C (n = 3–6). Bars represent the mean values \pm S.D. *** $p < 0.0001$. (B) Binding of hAG (●) and hUAG (○) to Chol/PC/SM/PI/PE (5.7:4.0:1.8:2.7:2.4, M/M) LUVET₂₀₀ in Na-citrate 12.5 mM NaCl 14 mM pH 6 buffer at increasing lipid/peptide molar ratios, T = 37 °C (n = 3–6). Circles represent the mean values \pm S.D.

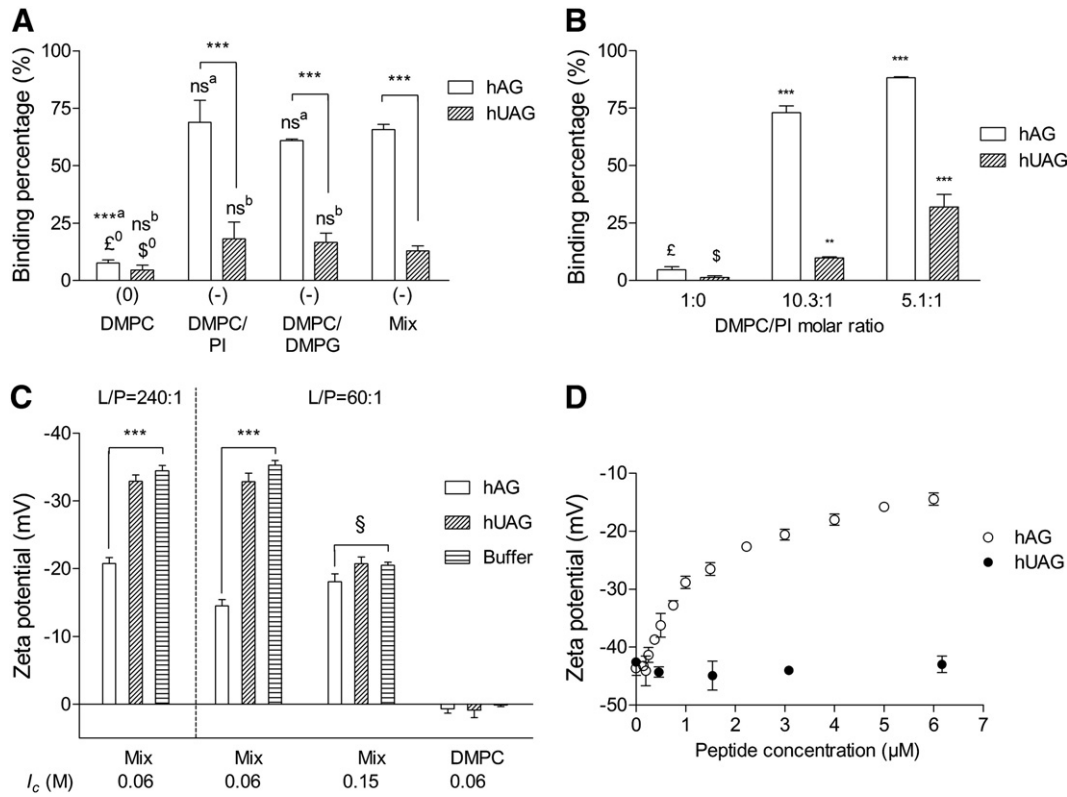


Fig. 2. Electrostatic adsorption of ghrelin to liposomes. Studies conducted at 37 °C with acylated ghrelin (hAG) and unacylated ghrelin (hUAG) in Na-citrate 12.5 mM NaCl 14 mM pH 6 ($I_c = 0.06$ M) buffer ($n = 3-6$), unless otherwise stated. Mix = Chol/PC/SM/PI/PE (5.7:4.0:1.8:2.7:2.4, M/M). (A) Binding of hAG and hUAG to LUVET₂₀₀ with different charges at a lipid/peptide molar ratio of 50:1. DMPC/PI and DMPC/DMPG molar ratios of 5.15 to 1. $\$^0 p > 0.05$, $\epsilon^0 p < 0.05$ compared to 0; ns^a $p > 0.05$, $***^a p < 0.0001$ compared to Mix hAG; ns^b $p > 0.05$ compared to Mix hUAG. (B) Binding of hAG and hUAG to DMPC/PI LUVET₂₀₀ with increasing charges at a lipid/peptide molar ratio of 200:1. For DMPC, $\$ p > 0.05$, $\epsilon p < 0.05$ compared to 0; $***^a p < 0.0001$ compared to all other groups; $**^b p < 0.001$ compared to hUAG 1:0. (C) Zeta potential of Mix LUVET₂₀₀ after addition of peptide (200 μ g/ml) or buffer at a lipid/peptide molar ratio of either 240:1 or 60:1. Mix ($I_c = 0.15$ M) buffer = Na-citrate 12.5 mM NaCl 103.2 mM pH 6. $\$ p < 0.05$; $***^a p < 0.0001$. Only significant differences with the buffer within each condition tested are indicated. (D) Zeta potential of Mix LUVET₁₀₀ as a function of hAG or hUAG concentrations. Lipid total concentration was 60.5 μ M. Peptide concentrations are the total peptide concentrations. $V = 1$ ml.

seemed to be important for the binding of ghrelin and as some peptides specifically bind to a specific head [56,57], we investigated whether acylated and unacylated ghrelin could bind to different acidic lipids such as PI, PG and PS. The binding of acylated and unacylated ghrelin to the liposomes was essentially charge dependent as both bound to negatively charged liposomes whatever the acidic phosphatidyl head carrying the negative charge: PI, DMPG (Fig. 2A) or PS (data not shown). Moreover, when doubling the number of acidic phospholipids in the liposomes composition (Fig. 2B), there was an increase in the bound peptide concentration. This concentration increase was larger for unacylated ghrelin (3.8-fold) than for acylated ghrelin (1.3-fold) (Fig. 2B). The acylation of ghrelin induced a highly significant increase (e.g. from $16.8 \pm 6.2\%$ to $66.1 \pm 7.4\%$ at the neutral lipids to PI molar ratio of 5.15:1) in the binding extent of ghrelin to all negatively charged liposomes (Fig. 2A and B).

Zeta potential (ζ) measurements revealed that, in any case, unacylated ghrelin did not affect the ζ of the liposomes (Fig. 2C and D). However, even though acylated ghrelin did not affect the ζ of neutral liposomes (DMPC), the ζ of negatively charged liposomes made of Chol/SM/PC/PI/PE became less negative in presence of acylated ghrelin, e.g. from -34 ± 1 mV to -21 ± 1 mV at a lipid/peptide molar ratio of 240:1 in 12.5 mM Na-citrate 14 mM NaCl buffer (Fig. 2C and D). The ζ of DMPC/PI (5.15:1; M/M) liposomes at lipid/peptide molar ratios of 200:1 and 50:1 were similar to those of Chol/SM/PC/PI/PE liposomes shown in Fig. 2C in presence of acylated and unacylated ghrelin (data not shown).

The electrostatic forces which influence the binding of the peptides were also investigated by examining the pH (4 and 6) and ionic strength effects (0.06 and 0.15). At pH 4, the theoretical charge

(~ 7.3) of ghrelin is increased compared with pH 6 (~ 5.5) whereas the liposomes charge is not much affected as the intrinsic phosphate pK_a of phospholipids is ranging from 1.2 to 2.5 [58]. Acylated ghrelin binding to Chol/PC/SM/PI/PE liposomes was unaffected by lowering the pH whereas it decreased with the increase in ionic strength (Fig. 3). Unacylated ghrelin binding to the liposomes increased while

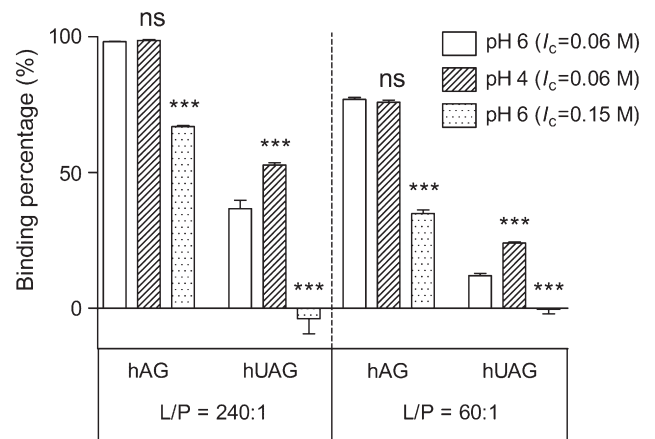


Fig. 3. Ionic strength and pH influence on ghrelin binding to liposomes. Acylated ghrelin (hAG) and unacylated ghrelin (hUAG) binding to Chol/PC/SM/PI/PE (5.7:4.0:1.8:2.7:2.4, M/M) LUVET₂₀₀ at $T = 37$ °C ($n = 3-6$). pH 4 ($I_c = 0.06$ M) = 12.5 mM Na-citrate 46.2 mM NaCl pH 4 buffer; pH 6 ($I_c = 0.06$ M) = 12.5 mM Na-citrate 14 mM NaCl pH 6 buffer; pH 6 ($I_c = 0.15$ M) = 12.5 mM Na-citrate 103.2 mM NaCl pH 6 buffer. ns $p > 0.05$, $***^a p < 0.0001$ vs. pH 6 ($I_c = 0.06$ M) for each peptide and each lipid/peptide ratio; $***^b p < 0.0001$ for hAG vs. hUAG within each condition tested.

lowering the pH and was suppressed at higher ionic strength (Fig. 3). Why was the binding of acylated ghrelin at pH 4 unaffected? There are several possible explanations, among which: (i) all the acylated ghrelin electrostatic binding sites of the liposomes are already saturated at pH 6; (ii) the hydrophobic interaction forces are stronger than the electrostatic interactions forces. In the case of increased ionic strength, more screening ions are present and the distance that separates the plane of shear and the vesicle surface diminishes. This might explain why there was less peptide bound and a lower increase of the zeta potential for acylated ghrelin (Fig. 2C) due to a reduced amount of peptide inside that plane. Indeed, as there are more screening ions in the case of increased ionic strength, the membrane negative surface potential is reduced which facilitates the adsorption of anions and hinders the binding of cations [58]. Based on these hypotheses, hydrophobic interactions have been investigated (see Section 3.4.).

3.3. Lipid–peptide interactions: effect of and on membrane fluidity

To determine if the binding of ghrelin to lipids was affected by the lipid state or affected the membrane fluidity, we investigated (i) the binding of ghrelin to liposomes exhibiting a phase transition; and (ii) the change of membrane fluidity.

The lipid state influence experiments were conducted at 4 and 37 °C with DMPC, DMPC/PI and DMPC/DMPG liposomes. The transition temperatures of DMPC, DMPC/PI (5.15:1, M/M) and DMPC/DMPG (5.15:1, M/M) liposomes (MLV) in 12.5 mM Na-citrate 14 mM NaCl pH 6 buffer were 20.1 °C, 17.6 °C and 22.9 °C, respectively, as determined by differential scanning calorimetry (from 0 to 55 °C at 1 °C/min). The binding experiments carried out by ultracentrifugation revealed no significant difference in acylated and unacylated ghrelin binding to these liposomes, at a lipid/peptide molar ratio of 50 to 1, below (4 °C) and above (37 °C) T_c ($p > 0.05$).

The change of membrane fluidity was assessed by the anisotropy change of fluorescent probes, DPH and TMA-DPH, which are located in the bilayer lipid compartment. The polarization of DPH and TMA-DPH in Chol/PC/SM/PI/PE liposomes (LUVET₂₀₀), at a 240 to 1 lipid/peptide molar ratio, were not statistically affected ($p > 0.05$) by the presence of acylated or unacylated ghrelin at any of the investigated temperatures (data not shown).

3.4. Hydrophobic interactions

The overall partition coefficient of ghrelin into membranes, K_{app} , which takes into account the hydrophobic (K_p) and electrostatic (K_{el}) contributions, was quite easily determined from binding experiments. However, quantitative analysis of ghrelin hydrophobic interactions with negatively charged membranes was not straightforward due to the electrostatic interactions. Therefore we used a binding model, adapted from Seelig et al. [35,36,44] and described in Section 2.12, in order to calculate the chemical (hydrophobic) partition coefficient K_p from the binding and zeta potential measurements at 37 °C.

The acylated ghrelin apparent binding coefficient (Eq. (1)) to Chol/PC/SM/PI/PE liposomes in pH 6 citrate buffer ($I_c = 0.06$ M) varied from 775 to 8000 M^{-1} whereas the unacylated ghrelin apparent partition coefficient varied from 75 to 175 M^{-1} at external lipid/peptide molar ratios of 6:1 to 188:1. Although ghrelin theoretical charge is $z = +5.5$, we found that its effective charge perceived by the membrane was smaller ($z_{eff} = +2.0$), as it has already been shown for other basic peptides in presence of acidic lipids [44,59,60]. The effective charge is the number of charges of the peptide that are available for the binding. The acylated ghrelin hydrophobic partition coefficient, found after correction for electrostatic effects by means of the Gouy–Chapman theory, was 74 M^{-1} . However, with our method we were unable to determine the hydrophobic partition coefficient of unacylated ghrelin as on the one hand, the heats in lipid into peptide titration experiments

were too small compared to the blanks, and on the other hand addition of unacylated ghrelin, even at lipid/peptide ratios as low as 1.2, to negatively charged liposomes did not induce any change in the zeta potential of these liposomes.

Thermodynamically, membrane partitioning is not only characterized by its partition coefficient, K , but also by the Gibbs energy of reaction, ΔG , the reaction enthalpy, ΔH , and the reaction entropy, ΔS . The Gibbs energy of reaction, ΔG , depends upon the partition coefficient as follows: $\Delta G = -RT \ln 55.5 K$, where 55.5 accounts for the cratic contribution [61]. As the Gibbs energies of electrostatic and hydrophobic interactions are additive, the electrostatic contribution, K_{el} , can be calculated from the K_{app} and the K_p . Therefore for acylated ghrelin as we had $K_{app,max} = K_p K_{el,max} = 8000 M^{-1}$ and 300 M^{-1} in pH 6 citrate buffer (at an external lipid/peptide molar ratio of 120:1) with ionic strengths of 0.06 and 0.15 M, respectively, and a hydrophobic partition coefficient, $K_p = 74 M^{-1}$, thus $K_{el,max}$ were 108 M^{-1} and 4 M^{-1} at the ionic strengths of 0.06 and 0.15 M, respectively. This is a hint that the electrostatic contribution at physiological ion concentration ($I_c = 0.15$ M) is rather small compared to the hydrophobic contribution but rather similar at a lower ionic strength.

For acylated ghrelin, hydrophobic binding alone led to $\Delta G_p = -5.1$ kcal/mol, whereas electrostatic and hydrophobic bindings together yielded a $\Delta G_{app,max} = -8.0$ kcal/mol. The binding of acylated and unacylated ghrelin to Chol/PC/SM/PI/PE liposomes, as measured by peptide-into-lipid isothermal titration calorimetry at high lipid/peptide ratios, were exothermic and gave rise to overall reaction enthalpies of -4.22 ± 0.20 kcal/mol and -0.19 ± 0.04 kcal/mol, respectively. Since $\Delta G = \Delta H - T\Delta S$, the reaction entropy for acylated ghrelin binding to Chol/PC/SM/PI/PE liposomes was positive.

3.5. Peptide conformation and lipid orientation studies

To determine if the acylation of ghrelin modify the conformation of ghrelin in aqueous solutions, lipids and membranes, the conformation of ghrelin was investigated by (i) *in silico* modelling in a hydrophobic medium, (ii) ATR-FTIR and, (iii) CD spectroscopy.

Our *in Silico* 3D model of acylated and unacylated ghrelin in a hydrophobic medium revealed an α -helix (Pro7–Ser18) surrounded by more flexible arms. As it can be seen on Fig. 4, the acylation did not much affect the overall peptide structure.

As displayed in Table 1 acylated and unacylated ghrelin structure, as determined by CD spectroscopy, were similar whatever the environment was: water, buffer, micelles (below, at, and above the micellar concentration) or a hydrogen bound stabilizing environment like trifluoroethanol (TFE). This α -helix content increased with the TFE content from $4 \pm 1\%$ in absence of TFE to $54 \pm 5\%$ in 100% TFE (Table 1 and Fig. 5). Acylated and unacylated ghrelin also adopted an α -helix structure in negatively charged micelles made of sodium dodecyl sulfate (SDS), e.g. $30 \pm 8\%$ in SDS 40 mM, whereas their conformation did not change in zwitterionic micelles made of dodecyl-phosphocholine (DPC), e.g. $6 \pm 1\%$ in DPC 5 mM, in comparison with water ($4 \pm 1\%$) and pH 6 buffer ($8 \pm 2\%$) (Table 1 and Fig. 5).

Acylated and unacylated ghrelin conformations, as determined by ATR-FTIR, slightly changed towards the α -region in presence of negatively charged DMPC/DMPG liposomes (Fig. 6). However they did not induce any change in lipid orientation (data not shown). This α -shift was more important for acylated ghrelin than for unacylated ghrelin (Fig. 6).

3.6. Transmembrane and cellular transport studies

As ghrelin strongly binds to membranes, we tested whether ghrelin could cross lipid bilayers and/or model epithelia and how the acylation influenced these phenomena. The ability of acylated and unacylated ghrelin to diffuse passively through lipid bilayers was assessed by PAMPA with lipid bilayers composed of either DOPC

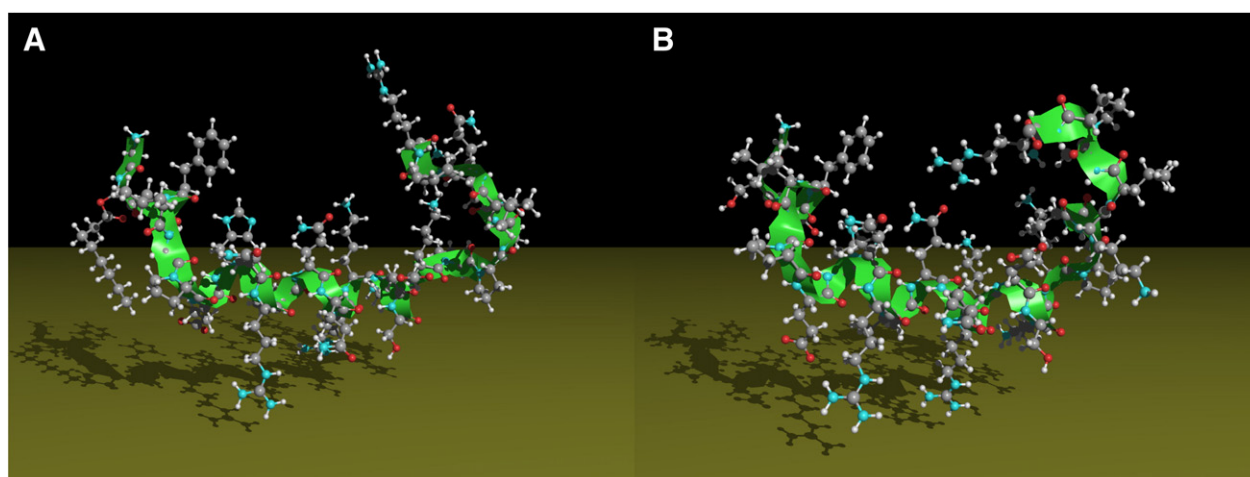


Fig. 4. *In silico* 3D model of ghrelin in a hydrophobic medium. (A) Acylated ghrelin and (B) unacylated ghrelin.

or a lecithin mixture with an excess of negative charges. Neither acylated nor unacylated ghrelin was detected in the receptor compartments ($LOQ=10\text{ pg/ml}$; $n=6$). The calculated P_{app} was thus below $2.5 \times 10^{-12}\text{ cm/s}$ for both peptides. Experiments to demonstrate the ability of ghrelin to cross intestinal epithelia were conducted on monocultures of Caco-2 and co-cultures of Caco-2/M-like cells. Both acylated and unacylated ghrelin were able to cross cell monolayers (Fig. 7). As acylated ghrelin is easily converted into unacylated ghrelin both peptides were dosed in the receptor compartments. M-like cells presence in the *in vitro* cell co-culture model resulted in a 4.3-fold increase in the transport of acylated ghrelin and a 2.9-fold increase for unacylated ghrelin, in comparison with cell monolayers consisting exclusively of Caco-2 cells. This increase was even more pronounced for intact acylated ghrelin (5.7-fold). The transport of unacylated ghrelin was higher than acylated ghrelin transport in mono-cultures but not in co-cultures.

4. Discussion

It is known that ghrelin binds to the ghrelin receptor and that the Ser³-acylation is necessary to activate it [1,62]. However, little is known about the role of the octanoyl in ghrelin membrane binding

and transport [14]. Therefore, the aim of this paper was thus to investigate the roles of this ghrelin octanoyl moiety in membrane binding and associated phenomena in order to give new insights about its role in ghrelin receptor affinity.

After showing that the octanoyl significantly enhanced the binding of ghrelin to model and biological membranes, we further studied the underlying mechanisms and possible biological implications. The electrostatic attraction/ membrane adsorption of ghrelin were investigated, as well as the peptides penetration in the headgroup region or in the hydrophobic backbone of the membrane and the conformational change of the peptides. Based on these observed phenomena we will try to explain the differences in acylated and unacylated ghrelin receptor binding.

4.1. Electrostatic–hydrophobic interactions

Knowing that acylated and unacylated ghrelin are highly basic peptides we first investigated the possible interactions between ghrelin and neutral or acidic lipids. Acylated and unacylated ghrelin both interacted electrostatically with the membrane as the presence and the amount of acidic lipids like PI, PS and PG greatly enhanced their binding to liposomes. Our results suggest that electrostatics play a more important role in unacylated ghrelin binding to liposomes than for the acylated form as: (i) unacylated ghrelin did not bind to neutral liposomes whereas acylated ghrelin did (Fig. 2A); (ii) doubling the number of negative charges of the liposomes induced a larger increase in binding for unacylated ghrelin than for acylated

Table 1

Ghrelin structure by circular dichroism (CD). Secondary structural analysis of acylated ghrelin (hAG) and unacylated ghrelin (hUAG) in various environments as measured by CD spectroscopy and calculated using SELCON 3, CONTIN/LL and CDSSTR algorithms from the CDPRO software package. Values represent means of the 3 algorithms \pm SD. Pipes pH 6 = Na-Na₂ Pipes 10 mM pH 6 buffer; DPC, dodecyl-phosphocholine; SDS, sodium dodecyl sulphate; TFE, trifluoroethanol.

Environment	Secondary structure (%)							
	hAG				hUAG			
	Helix	Strand	Turn	Random	Helix	Strand	Turn	Random
Water	4 \pm 1	14 \pm 4	10 \pm 4	71 \pm 8	4 \pm 1	16 \pm 6	11 \pm 5	68 \pm 8
Pipes pH 6	8 \pm 2	16 \pm 4	12 \pm 2	63 \pm 7	5 \pm 1	15 \pm 1	10 \pm 2	69 \pm 2
DPC 0.2 mM	4 \pm 1	12 \pm 1	10 \pm 1	73 \pm 3	4 \pm 0	12 \pm 4	10 \pm 3	74 \pm 7
DPC 1.1 mM	5 \pm 1	12 \pm 2	9 \pm 1	75 \pm 3	4 \pm 0	9 \pm 3	9 \pm 2	78 \pm 6
DPC 5 mM	6 \pm 1	13 \pm 4	10 \pm 2	72 \pm 5	4 \pm 0	10 \pm 4	9 \pm 3	77 \pm 8
SDS 8.1 mM	31 \pm 7	17 \pm 2	19 \pm 4	32 \pm 2	35 \pm 7	12 \pm 6	18 \pm 5	34 \pm 9
SDS 40 mM	30 \pm 8	20 \pm 7	21 \pm 2	28 \pm 1	32 \pm 7	19 \pm 0	18 \pm 2	31 \pm 3
TFE 20%	8 \pm 2	19 \pm 2	12 \pm 1	61 \pm 3	7 \pm 2	17 \pm 1	12 \pm 1	64 \pm 1
TFE 40%	19 \pm 1	17 \pm 6	15 \pm 3	49 \pm 9	16 \pm 1	20 \pm 3	15 \pm 3	48 \pm 5
TFE 50%	19 \pm 1	21 \pm 3	16 \pm 2	44 \pm 4	21 \pm 2	18 \pm 4	14 \pm 2	48 \pm 3
TFE 60%	25 \pm 3	21 \pm 3	18 \pm 3	36 \pm 8	29 \pm 3	12 \pm 3	16 \pm 1	42 \pm 6
TFE 80%	34 \pm 3	16 \pm 2	19 \pm 2	29 \pm 4	38 \pm 2	12 \pm 1	20 \pm 5	30 \pm 3
TFE 100%	54 \pm 5	8 \pm 3	14 \pm 3	23 \pm 5	47 \pm 3	8 \pm 5	15 \pm 3	30 \pm 6

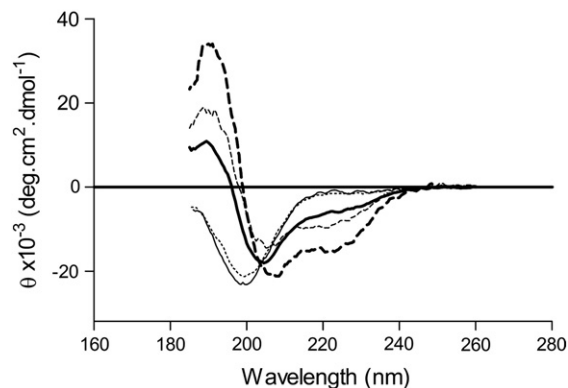


Fig. 5. Representative CD spectra of ghrelin. Acylated ghrelin in water (—), DPC 5 mM (⋯⋯⋯), SDS 40 mM (---), TFE 80% (- - - -) or TFE 100% (— — —) at 22 °C.

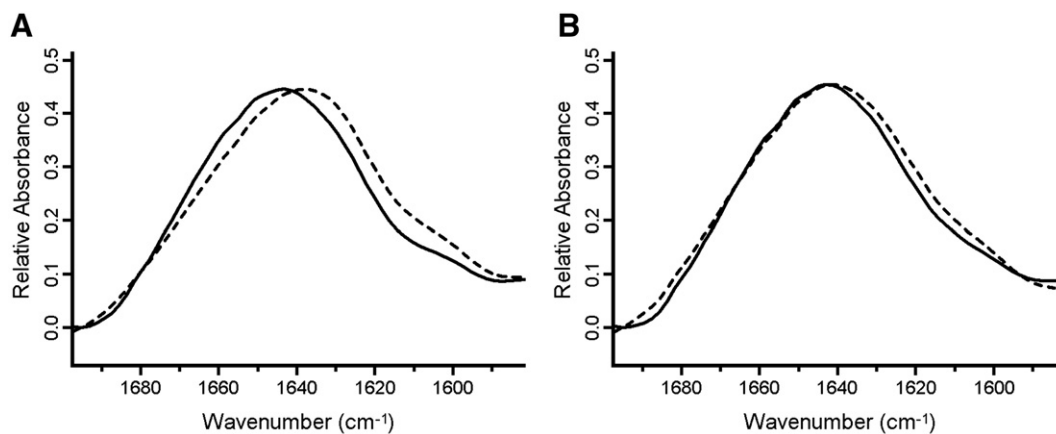


Fig. 6. Acylation influence on ghrelin structure in aqueous and membrane environments by infrared spectroscopy. ATR-FTIR spectra ($1700\text{--}1580\text{ cm}^{-1}$) at room temperature in NaCl 3 mM (—) or in presence of DMPC/DMPG (5.15:1, M/M) LUVET₂₀₀ in NaCl 3 mM (---). (A) Acylated ghrelin; (B) unacylated ghrelin.

ghrelin (Fig. 2B); (iii) increasing the peptide charge by about 2 units only increased the binding of unacylated ghrelin to the acidic liposomes (Fig. 3); (iv) increasing the screening of the liposomes surface charge by addition of NaCl decreased the binding of acylated ghrelin to the liposomes but suppressed it for unacylated ghrelin (Fig. 3).

Complementary results such as very weak binding heat in ITC studies and no zeta potential modification further indicate that unacylated ghrelin does not specifically adsorb to negatively charged liposomes and that the nature of their interactions are Coulombic which are long-range electrostatic interaction forces [38]. Indeed, on the one hand the very low enthalpy measured by ITC suggested pure electrostatic interactions [63]. On the other hand (i) zeta potential of negatively charged liposomes was unaffected by unacylated ghrelin even at high concentrations (Fig. 2D) and (ii) the zeta potential is a technique that measures the potential difference between the hydrodynamic plane of shear of the liposomes and the bulk solution; (iii) the Stern layer is located closer within the plane of shear; (iv) specifically adsorbed ions charges are located within the Stern layer [38]. Acylated ghrelin is also electrostatically attracted to acidic

liposomes and results indicate that at least a part of it must be located very close to the surface as the zeta potential is measured near the vesicles surface i.e. 2 to 8.2 Å [43,64,65]. We have also shown that acylated ghrelin also interacts with the lipids with a hydrophobic partition coefficient (74 M^{-1}) comparable to that of penetratin [66], somatostatin analogues [67] and magainin 2 amide [61]. Additionally the binding of acylated ghrelin to negatively charged liposomes is entropy and enthalpy driven as it does for other acylated peptides [68,69]. The calculated $\Delta G = -5.1\text{ kcal/mol}$ is in good agreement with the octanoyl penetration into hydrocarbon region of the membrane [69,70]. However, acylated ghrelin is unlikely to be deeply inserted in the membrane as (i) it did neither interact with DPH nor with TMA-DPH and that both these probes have a deep DPH location within the bilayer [71]; (ii) ghrelin binding to the liposomes was not affected by their the membrane fluidity state; (iii) its hydrophobic partition coefficient is not large [61]. As ghrelin is an endogenous hormone, it is not so surprising that it does not perturb the membrane organization.

Therefore, we hypothesize that the octanoyl penetrates into the hydrophobic backbone while the ghrelin α -helix lies in the headgroup region of the membrane.

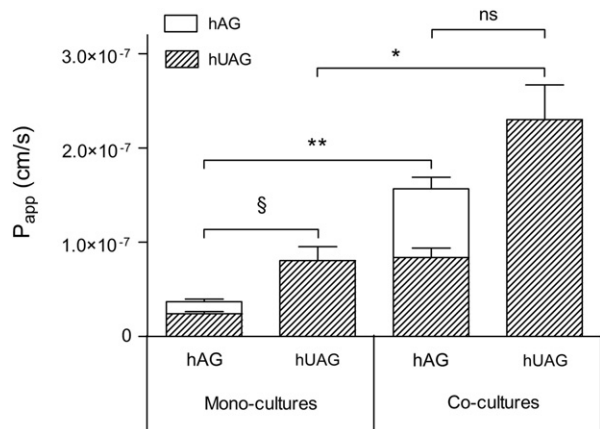


Fig. 7. Influence of acylation and presence of M-like cells on ghrelin transport by human intestinal cells. Transport of acylated ghrelin (hAG) and unacylated ghrelin (hUAG) in mono-culture of Caco-2 cells and co-cultures of Caco-2 and Raji cells at 37 °C ($n=6$). Peptides on the abscissa are the peptides deposited in the donor compartments. Bars represent the means values \pm S.E. of the collected peptides in the receptor compartments that were dosed by EIA. Statistical analysis by unpaired *t*-test. ns, §: comparison of hAG total vs. hUAG on the same cell model, ns: no statistical difference ($p>0.05$), § $p<0.05$; *, **: comparison between the mono- and co-cultures for each peptide, * $p<0.005$, ** $p<0.001$.

4.2. Peptide conformation

Ghrelin does neither disturb the membrane, nor does it cross lipid bilayers as shown in our PAMPA experiments. Nevertheless does ghrelin become structured in contact with membranes, even though it has been reported to adopt a random coil conformation in water [72]? It is known that peptides and proteins with a random coil conformation in water can adopt an α -helical or β -sheet structure in membranes [73–75]. CD and ATR-FTIR spectroscopy studies as well as computational modelling revealed that both acylated and unacylated ghrelin adopt mainly the same structure in any of the environments tested although acylated ghrelin in presence of model membranes adopted a slightly more structured conformation than unacylated ghrelin towards the α -helical region. The absence of structuring effect of DPC in opposition to SDS confirmed the necessity of a negative charge. Our *in silico* 3D model of acylated ghrelin in a hydrophobic medium obtained using the Stochastic procedure Pep-Look algorithm is in accordance with the molecular dynamics simulation studies of Beevers and Kukol in 2006 [72]. Indeed, our simulations also revealed a central α -helix surrounded by more flexible arms that confers a “staple” shape aspect to the peptide (Fig. 4). Our computational modelling is, as far as we know, the first that compared both forms of ghrelin and is in accordance with our experimental results.

4.3. Cellular transport

We have shown that ghrelin is unable to cross lipid bilayers by itself. The reason for this is probably that the octanoyl arm is too short for the peptide to translocate. Indeed, peptides able to cross the bilayer usually need an acyl modification of at least 14 to 16 carbons which roughly corresponds to half the bilayer thickness [76,77]. In Caco-2 cells, the unacylated form of ghrelin was better transported than the acylated form. This is in contradiction with previous publications about the transport of other acylated peptides like tetragastrin and thyrotropin releasing hormone by Caco-2 cells [18,78]. This is not, however, highly surprising in the case of ghrelin. Indeed, Banks et al. already reported in 2002 that, in mice, unacylated ghrelin was transported through the blood brain barrier by an unsaturable mechanism in the blood-to-brain direction whereas acylated ghrelin was transported by a saturable mechanism in the brain-to-blood direction [15]. M-like cells presence not only enhanced the transport of both forms of ghrelin but also the proportion of intact ghrelin. These results are in agreement with previously published results from our laboratory on helodermin transport [79].

4.4. Ghrelin acylation vs. receptor association

As the acylation of ghrelin seems to be essential for efficient membrane binding, we were interested in the possible implications for receptor binding. Indeed, the membrane could act as a catalyst for ghrelin receptor binding as it does for other peptides [9,10,80]. The ghrelin third amino acid residue has to carry a bulky hydrophobic group for its maximum activity mediated by the ghrelin receptor [62,81]. Concerning the amino acid sequence, it has been shown *in vitro* that only the first five amino acids are necessary for full activation of the ghrelin receptor [62]. If we look at the hydrophilicity/hydrophobicity profile of ghrelin, we see that even though ghrelin is predominantly hydrophilic, these five amino acids along with the octanoyl belong to the most hydrophobic portion of the peptide. Moreover, in mammals, the first ten amino acids are identical. Altogether these data indicate that the N-terminal region of ghrelin is of central importance to the activity of ghrelin [82]. Furthermore, mutational analysis of the ghrelin receptor revealed that the ghrelin binding pocket is likely to be located in transmembrane domains III, VI and VII which implies that ghrelin needs access to it [83–87]. Besides, even though electrostatics played a determinant role in ghrelin attraction towards the membrane, acylation induced a 120-fold increase, as compared to unacylated ghrelin, in ghrelin local concentration in the membrane and hence in the vicinity of the receptor [10,67,70]. Moreover, at a physiologically relevant ionic strength the observed decrease of ghrelin binding to the liposomes indicates that unacylated ghrelin is much less likely to encounter the membrane, and hence its receptor, than acylated ghrelin. All these could partly explain the difference in the concentrations necessary for the full activation of the ghrelin receptor by unacylated (μM) and acylated (nM) ghrelin observed by Gauna and co-workers [8]. Even if ghrelin is directed to the membranes, it is unlikely that the octanoyl plays a role in ghrelin secretion as in GOAT-null mice unacylated ghrelin is detected in the bloodstream [3]. We have thus demonstrated that close association of ghrelin with membranes requires Ser³ O-octanoylation as well as the presence of basic residues. These results are in accordance with those obtained for other peptides like the myristoylated alanine-rich C kinase substrate effector domain (MARKS ED) [57,88].

In conclusion, the unacylated ghrelin seems to be only driven towards the membrane by electrostatic attraction between its basic residues and the negatively charged lipids of the membrane, leading to a lower affinity for the membrane than acylated ghrelin. Acylated ghrelin is also electrostatically attracted but in addition, it penetrated into the headgroup and lipid backbone regions. The membrane

probably acts as a “catalyst” for ghrelin binding to its receptor. In this process the O-octanoylation of ghrelin is assumed to (i) partition ghrelin into the lipids; (ii) increase local concentration in the vicinity of the receptor; (iii) bring ghrelin to the membrane where its binding pocket is located; (iv) optimize the conformation of ghrelin for improving its docking to the ghrelin receptor. This latest hypothesis needs to be confirmed. Moreover, ghrelin conformational flexibility may be associated with its various roles in different tissues. However, it seems that O-octanoylation of ghrelin does not help its transport across membranes and epithelia.

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