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Stability, liposome interaction, and *in vivo* pharmacology of ghrelin in liposomal suspensions

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ABSTRACT

Ghrelin is an appetite-stimulating peptide hormone. It is a pharmacologically interesting peptide because of its involvement in e.g. appetite and metabolism, but it has a very short half-life in the body. Ghrelin carries a Ser-3-octanoyl group, and it has previously been suggested that acylated peptides can bind to or be incorporated into liposomes. Therefore, neutral dipalmitoylphosphatidylcholine (DPPC) liposomes and phosphatidylcholine:cholesterol (PC:Chol) (70:30) liposomes as well as negatively charged dipalmitoylphosphatidylcholine:dipalmitoylphosphatidylserine (DPPC:DPPS) liposomes (70:30) were prepared, and ghrelin was added. The chemical and physical stability of ghrelin was examined. Affinity capillary electrophoresis (ACE) revealed an interaction between ghrelin and the negatively charged (DPPC:DPPS) liposomes, whereas only very small affinities were discerned in the other liposomal formulations of ghrelin. Differential scanning calorimetry showed no changes in phase transitions (T_m). *In vivo* pharmacokinetics following subcutaneous administration of ghrelin in buffer and in the liposomal formulations was examined in rats. The PC:Chol formulation had a longer-lasting effect as compared to the ghrelin buffer solution and the other two liposomal formulations. The prolonged effect of the PC:Chol formulation is suggested not to be caused by association between ghrelin and the liposome.

1. Introduction

Ghrelin is the endogenous peptide hormone ligand for the ghrelin receptor. The identity and structure of ghrelin was discovered in 1999 (Kojima et al., 1999). It is a 28 amino acid-long peptide, and in its pharmacologically active form it is acylated at the Ser-3 position, typically with an octanoyl moiety. The sequence is dominated by the presence of many basic amino acids, and ghrelin is positively charged at physiological pH (Fig. 1).

The peptide is primarily secreted from cells in the stomach but also from e.g. the small intestine and colon. The biological role of ghrelin is related to growth and energy metabolism. It stimulates appetite and growth hormone release (e.g. Dimaraki and Jaffe, 2006; Kojima and Kangawa, 2008), and high serum ghrelin levels are related to obesity and diabetes (Arora and Anubhuti, 2006; Higgins et al., 2007). Ghrelin is secreted in a pulsatile pattern, where it increases in the hours before each meal and decreases shortly after initiation of the meal (Cummings et al., 2001). The physiological effects of ghrelin might be linked to a peak or burst of plasma ghrelin.

Ghrelin is presently being investigated as a potential therapeutic peptide; a possible drug for the clinical management of anorexia and cachexia. Long-term infusions of ghrelin in rats result in increases in food intake and fat mass (Strassburg et al., 2008), and likewise in a rat model of cancer cachexia (DeBoer et al., 2007). Cancer-related anorexia and cachexia are difficult to manage clinically, and since anorectic/cachectic patients do respond to appetite stimulants (Yavuzsen et al., 2005), ghrelin was an obvious drug candidate. Clinical trials of ghrelin administration in anorexia and cachexia have, however, not been very promising. Ghrelin administration to anorectic subjects has shown weak or no effect on food intake or appetite in small studies (Akamizu et al., 2008; Miljic et al., 2006) and similar results for administration to patients suffering from cancer-related anorexia or cachexia (Strasser et al., 2008). The question of the clinical applicability of ghrelin has not been resolved yet, but ghrelin and ghrelin agonists are still under clinical investigation in a number of diseases (Kamiji and Inui, 2008).

Ghrelin has a brief biological half-life. In man, the half-life of total (acylated and deacylated) ghrelin is approx. 25–30 min (Akamizu and Kangawa, 2006; Vestergaard et al., 2007). The short half-life of ghrelin is partly due to the deacylation of the peptide; the reported half-life of acylated ghrelin was reported to be 9–13 min (Akamizu and Kangawa, 2006).

Liposomes for delivery of various drugs have been studied extensively, and drug formulations including liposomes have been

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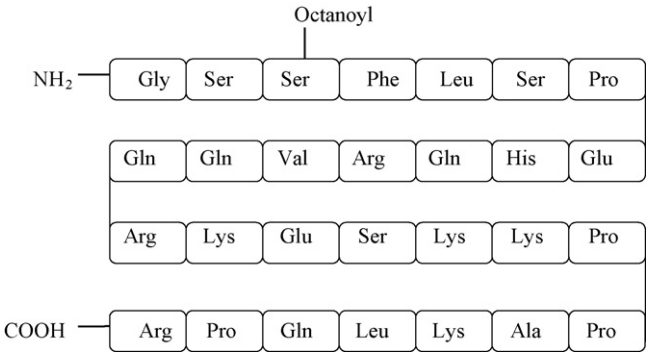

Fig. 1. The primary sequence of human ghrelin.

marketed. Therapeutic agents can be encapsulated in the liposomes, linked covalently to the liposomal surface, or associated externally to the lipid bilayer. Work on drug delivery systems for peptides or proteins, in which the protein is externally associated to liposomes, have been published previously, e.g. using interferon-gamma (Van Slooten et al., 2001) or interferon-alpha (Eppstein and Stewart, 1981) or N-acylated heat shock protein (Quintilio et al., 2000). The binding of acylated peptides to liposomes or membranes has been reported in previous studies on acylated peptides (Pedersen et al., 2001; Yelamos et al., 2006). Ghrelin, having an octanoyl side chain, could associate to the neutral liposomes by the lipophilic octanoyl arm.

In this study, our aim was to study the binding of ghrelin to liposomes and to embed ghrelin in a liposomal drug delivery system that could yield a prolonged biological effect. The many effects of ghrelin have been much studied, but to our knowledge reports of an experimental drug delivery system with a prolonged biological activity of ghrelin have not been published.

By attaching ghrelin to the lipid bilayer of liposomes, the release of ghrelin from the liposomes could theoretically be retarded, and the acyl moiety might be protected from enzymatic hydrolysis due to sterical hindrance. The liposomes chosen for the studies were neutral dipalmitoylphosphatidylcholine (DPPC) liposomes and phosphatidylcholine:cholesterol (PC:Chol) liposomes, and negatively charged DPPC:DPPS liposomes. Ghrelin is positively charged at physiological pH, and in the case of the negatively charged DPPC:DPPS liposomes, electrostatic interactions are thought to cause binding between ghrelin and the liposomes. Ghrelin was not encapsulated, but was expected to associate externally to the liposomes.

We have studied ghrelin in buffer and in the three different liposomal formulations. The chemical and physical stability of ghrelin in the applied buffer were examined using HPLC, optical dispersity and Thioflavin T assay. The interactions between molecules can often be probed using affinity capillary electrophoresis (CE) (Ostergaard and Heegaard, 2006; Ostergaard et al., 2008), and we found that the interaction between ghrelin and a liposomal bilayer could be investigated using the same technique. Further studies on the interaction were carried out using differential scanning calorimetry (DSC). Finally, the formulations were tested *in vivo* by *s.c.* administration in rats.

2. Materials and methods

2.1. Materials

Human ghrelin was obtained from Polypeptide Laboratories (99.9%) (Hillerød, Denmark) and from Global Peptide Services, LLC (>95%) (Fort Collins, CO, USA). Human des-acyl-ghrelin (>95%) was likewise purchased from Global Peptide Services, LLC. Saturated dipalmitoylphosphatidylcholine (DPPC) phospholipid and dipalmitoylphosphatidylserine (DPPS) solution was purchased from Avanti Polar Lipids (Alabaster, AL, USA). ^{3}H-labeled DPPC was from Amersham (Little Chalfont, UK). Egg yolk L-alpha-phosphatidylcholine (99%), HEPES, and potassium chloride were obtained from Sigma–Aldrich (St. Louis, MO, USA). 20% technical grade solution of poly(diallyldimethylammonium chloride) (PDMAC) Mw 400,000–500,000 was obtained from Aldrich-Chemie (Steinheim, Germany). All materials were used as received. Purified water from a Milli-Q deionization unit (Millipore, Bedford, MA, USA) was used. A 10 mM HEPES-buffer containing 50 mM KCl adjusted to pH 5.5 was used throughout the studies involving ghrelin and the liposomes. For all experiments in a series, except for the CE experiments, a single ghrelin stock solution was used.

2.2. HPLC

For HPLC studies, a Beckman Gold system (Beckman Coulter Inc., USA) was used with a Waters 717plus autosampler. The column was a LiChrospher® 100 RP-18e (5 μm). The elution system consisted of solvent A (0.1% TFA in Milli-Q water) and solvent B (0.1% TFA in acetonitrile) in a linear gradient of 10–60% of B over 30 min at a flow rate of 0.5 ml/min with UV detection at 210 nm. Sample volume was 100 μl.

2.3. Dynamic light scattering and zeta potential

Size measurements of the unilamellar liposomes were performed using a Zetasizer Nano ZS (Malvern, UK). The dynamic light scattering data was collected at an angle of 90° using a helium laser as the light source. The apparatus was calibrated using polystyrene latex spheres of known size (220 nm). The average value of three measurements is reported.

2.4. Turbidity (OD_{340}) and Thioflavin T measurements

OD_{340} and Thioflavin T fluorescence were measured using a Fluostar Optima plate reader (BMG Labtech GMBH, Offenburg, Germany) and 96-well plates. Sample volume in the wells was 200 μl. For the turbidity measurements, the absorbance at 340 nm was measured (in triplicate). For the Thioflavin T assay, 25 μl 1 mM Thioflavin T solution was added to the wells, and the excitation/emission wavelengths were set to 480 nm/450 nm (triplicate).

2.5. Preparation of liposomes

Liposome suspensions of DPPC, PC:Chol (70:30, w/w), and DPPC:DPPC (70:30, w/w) were prepared by the hydrated film method. The target lipid concentration was 2 mM, and a surplus of 30 mol% of the lipids was weighed. Lipids were dissolved in chloroform, stripped thrice with absolute ethanol, and evaporated *in vacuo* overnight. The resulting lipid films were hydrated with the buffer at 51 °C (64 °C for the DPPC:DPPS liposomes) and the suspensions were extruded 10 times through two stacked 100 nm pore size polycarbonate filters to yield unilamellar liposomes with a narrow size distribution. The lipid concentrations were determined by monitoring the radioactivity of incorporated ^{3}H-DPPC in the liposomes or by using a Phospholipids C enzymatic assay for phospholipid determination (Wako Chemicals GMBH, Neuss, Germany). The suspensions were sterilized by passing through a MiniSart 0.20 μm filter (Sartorius, Germany) and were characterized with respect to polydispersity, size, and zeta potential. The liposomal preparations were stored at 5 °C for up to a week.

2.6. Differential scanning calorimetry

Differential scanning calorimetry was carried out using a VP-DSC differential scanning calorimeter (MicroCal, LLC, Northampton, MA). Temperature scans were performed from 25 to 90 °C at a scan rate of 1 °C/min with buffer in the reference cell, and the samples were degassed before use. Baselines were created in Origin 7.0 (OriginLab, Northampton, MA) using a progress baseline.

2.7. Capillary electrophoresis

A Beckman PACE 5510 CE instrument with diode array detection (Beckman, Fullerton, CA) was used for the capillary electrophoresis experiments. Capillaries with dimensions of 37 cm (30 cm effective length) × 50 μm ID were used. Fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) were conditioned with 1 M sodium hydroxide for 30 min, water for 15 min, 1% PDMAC solution in water for 30 min, and water for 30 min prior to use in order to obtain a capillary wall surface which was positively charged. The coated capillary thus generated was flushed with water for 5 min and 0.1% PDMAC solution for 5 min before the start of a series of experiments. Between measurements the capillary was rinsed for 2 min with 0.1% PDMAC and for 1 min with the HEPES-buffer. Polyvinyl alcohol (PVA) coated capillaries were obtained from Agilent Technologies (Germany). Between samples, the PVA capillaries were flushed with 10 mM phosphoric acid for 2 min and the buffer for 1 min. The separation voltage was set to 11 kV, the capillary cassette temperature was 25 °C, and detection was at 200 nm. Samples were introduced into the capillaries by hydrodynamic (0.5 psi) or electrokinetic (10 kV) injection for 5–50 s. The electropherograms were recorded and analyzed with Beckman P/ACE Station software Version 1.21. The quantitation of binding was done by comparing peak heights or areas of ghrelin-liposome samples with those of ghrelin standards having a known concentration.

2.8. In vivo pharmacology

Thirty male Sprague–Dawley rats (aged 6–7 weeks, approximately 300 g; Charles River, Germany) were used. Rats were housed three per cage for 1 week and subsequently transferred to individual cages with access to food and water *ad libitum*. From the arrival date, rats were kept under 12/12 L/D cycle lights in temperature and humidity controlled rooms. After 1 week of singly housing, rats received a mock *s.c.* injection daily with saline (0.9%) to accustom them to the experimental procedure and to reduce injection related stress 3 days prior to experiment start. Rats were assigned randomly into five weight-matched groups, to which were administered the buffer, ghrelin in the buffer and ghrelin with the three liposome formulations, respectively. Ghrelin was administered *s.c.* at 200 μg/kg. 200 μl blood samples were drawn from the tail vein at time points −15, 0, 15, 30, 60, 90, 120 and 240 min relative to the injection of test solution. Blood samples were kept on ice, centrifuged (4000 rpm for 10 min), and plasma was transferred to vials containing 5 μl 1N HCl and 5 μl 2 mg/ml solution of phenylmethylsulfonyl fluoride in isopropanol. Acylated active ghrelin in the plasma samples was measured by a RIA assay (Linco Research, Inc., MO, USA) according to the manufacturer's protocol.

3. Results and discussion

Many peptides and proteins, naturally occurring as well as man-made synthetic entities, are acylated with fatty acids. Examples of endogenous fatty acid-modified proteins include T-cell receptors CD4 and CD8 (Bijlmakers, 2008), retinal transducin α (Neubert et al., 1992), and neuronal protein PDS-95 (Craven et al., 1999). The fatty acids confer interesting properties to the peptide or protein. For example, a fatty acid chain can cause a protein to self-associate to composite entities (Havelund et al., 2004); it can enhance binding to membranes (Bijlmakers, 2008); it can increase the physical stability of a protein (Olsen and Kaarsholm, 2000); or it can induce binding to other proteins such as albumin (Home and Kurtzhals, 2006).

Depending on the structure of the covalent bond, the fatty acid modifications can have a transitory existence as the Ser-3 ester group in ghrelin, or they can be very resilient amide groups as in detemir (Whittingham et al., 1997) and liraglutide (Agerso et al., 2002). Hydrolysis of the ester group in ghrelin yields des-acyl ghrelin with consequent loss of ghrelin receptor activity (Hosoda et al., 2000). Under the conditions employed during these studies, the octanoyl group was shown to be intact by HPLC using a previously published method (Ishimaru et al., 2003). Des-acyl-ghrelin was under the detection limit after 3 days at ambient temperature.

Peptides and proteins may denature and aggregate or fibrillize during heating or freezing, or on exposure to salts (Kendrick et al., 2002; Wang, 1999). Ghrelin was in this study found to be physically and chemically stable to rotation at room temperature for 3 days in the HEPES-buffer used. No formation of fibrils or aggregates was detected by Thioflavin T fluorescence and optical dispersion studies (data not shown).

The liposomal suspensions were duly made and characterized. The size, polydispersity, and zeta potentials of the liposomes, both before and after addition of ghrelin, are reported in Table 1. The anticipated interactions between the peptide and the various liposomes were evident from the changes in size observed on addition of the ghrelin. As expected, addition of the net positively charged ghrelin to the negatively charged DPPC:DPPS liposomes led to larger clusters of liposomes, along with a rise in zeta potential. Titration of DPPC:DPPS suspension with 0.24 mg/ml ghrelin gave an almost linear increase in the average diameter from approximately 80 nm without ghrelin to approximately 180 nm at a 0.25 mg/ml ghrelin concentration (Fig. 2). Similar clustering of negatively charged liposomes have been reported with e.g. divalent cations (Mora et al., 2000).

For the neutral DPPC liposomes another effect was observed. It was a characteristic trait of the DPPC liposomes, as opposed to PC:Chol liposomes, that they self-associated. Even in freshly

Table 1
Size, polydispersity, and zeta potential before/after addition of 0.24 mg/ml human ghrelin (in duplicate, averaged).

Before/after ghrelin	Size (nm)	Polydispersity	Zeta potential (mV)
2 mM DPPC	120.0/95.1	0.33/0.101	−0.34/8.34
6 mM DPPC	111.3/98.8	0.43/0.11	−1.03/4.95
2 mM PC:Chol	93.4/94.3	0.108/0.124	−1.86/−1.78
6 mM PC:Chol	91.2/90.9	0.095/0.093	−1.46/2.85
2 mM DPPC:DPPS	82.9/3044	0.040/1	−34.3/−20.6
6 mM DPPC:DPPS	81.4/3035	0.059/1	−40.1/−29.4

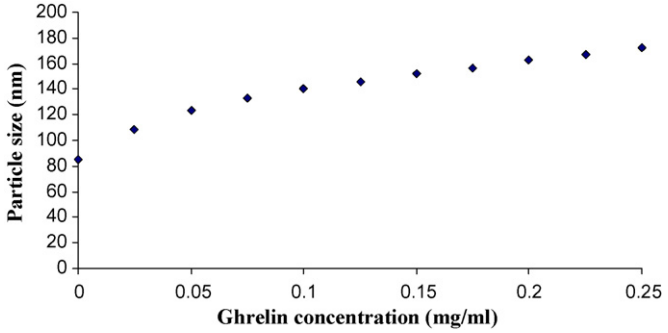

Fig. 2. Size of DPPC:DPPS (70:30) liposomes on addition of 0.24 mg/ml human ghrelin.

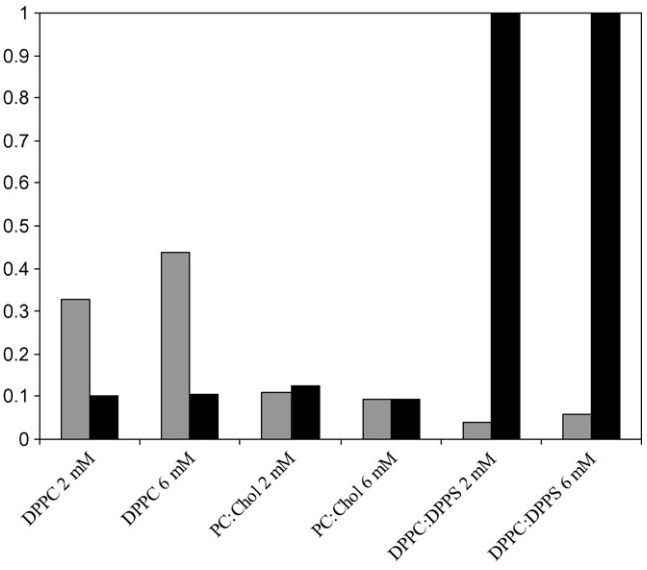

Fig. 3. Polydispersity before (grey columns) and after (black columns) addition of 0.24 mg/ml ghrelin.

prepared DPPC liposomes, the size distribution was broader (the polydispersity was larger), and a cloudy sedimentation was typically observed. On addition of ghrelin, however, the suspension cleared up instantly, the sedimentation disappeared, and the polydispersity decreased in a characteristic manner (Fig. 3 and Table 1). The positively charged peptide would induce an electrostatic repulsion between the vesicles on binding to the liposome, and the decrease in the polydispersity thus indicated that some degree of binding did take place.

The possible binding of ghrelin to the liposomes was investigated using capillary electrophoresis. CE has proved to be well suited for investigating noncovalent interactions between a wide range of molecules (Neubert and Rüttinger, 2003; Ostergaard and Heegaard, 2006; Owen et al., 2005; Rundlett and Armstrong, 2001; Tanaka and Terabe, 2002). Liposomes have also been studied using capillary electrophoresis as reviewed by Owen et al. (2005) and Bilek et al. (2006). In most cases, liposomes have been utilized in studies of drug lipophilicity in relation to membrane permeation properties (Bilek et al., 2006; Ostergaard et al., 2008; Owen et al., 2005).

Affinity capillary electrophoresis was conducted in the pre-equilibrium mode using hydrodynamic injection for 40 s (frontal analysis mode (Ostergaard et al., 2008)) or electrokinetic injection for 5 s. Similar results were obtained using the two approaches. Capillary electrophoresis revealed that ghrelin interacts only to a very limited extent with the PC:Chol (~9% bound) and DPPC (<4% bound) liposomes but interacts more strongly with the DPPC:DPPS (75% bound) liposomes due to the electrostatic interactions with the negatively charged DPPS in the bilayer. The values given in brackets above relate to the fraction of ghrelin bound in liposomal samples containing 120 μg/ml ghrelin and 2 mM lipid in the HEPES-buffer at room temperature. As anticipated, the presence of the octanoyl side chain in ghrelin increases the binding to DPPC:DPPS liposomes as compared to des-acyl-ghrelin. For all liposomal formulations investigated by capillary electrophoresis, the shapes of the ghrelin peaks in the electropherograms indicated that the interaction between ghrelin and the liposomes is characterized by fast binding kinetics, and the binding is readily reversible. Details related to the development of the capillary electrophoresis methods applied will be reported elsewhere.

Studies using differential scanning calorimetry (DSC) on DPPC and DPPC:DPPS liposomes mixed with cationic peptides have shown that a downward shift in T_m can sometimes be observed (Pedersen et al., 2001). DSC on the DPPC:DPPS (70:30) and DPPC liposomal suspensions yielded phase transitions for DPPC:DPPS of 43.9 °C/43.6 °C (with/without ghrelin) and 41.7 °C/41.4 °C, respectively (Fig. 4). The T_m's for the liposomes were comparable to those reported in the literature, e.g. T_m's of 44.4 °C for DPPC:DPPS (70:30) and 41.4 °C for DPPC (Pedersen et al., 2001). The observed small changes in T_m are not significant, and together with the fast on–off kinetics this suggests that ghrelin is associated to the surface of the liposomes and is not inserted deeply into the lipid bilayer of the vesicles. PC:Chol does not have a phase transition in the temperature range scanned and was therefore not studied. Effective binding of ghrelin to lipid bilayers perhaps demands a longer fatty acid chain than the octanoyl entity; reports for other peptide-liposomes systems indicate that longer fatty acid chains (n = 10, 12, 14) give stronger binding (Pedersen et al., 2001; Peitzsch and McLaughlin, 1993).

The results of the *in vivo* studies in rats are shown in Fig. 5. The half-lives were calculated to be as follows: ghrelin in buffer 25.65 ± 5.2 min; DPPC 49.67 ± 8.3 min; DPPC:DPPS 16.21 ± 2.3 min; and PC:Chol 255.9 ± 74 min. The curves for DPPC and DPPC:DPPS show a weak tendency towards a prolongation, whereas PC:Chol demonstrates a very clearly prolonged effect. The active ghrelin displays a characteristic increase after approx. 90 min in all experiments. This increase is assumed to be due to a rise in endogenous ghrelin, since the rats are fasting during the experiments.

In comparison with the CE studies, in which only low affinities were seen for both DPPC and PC:Chol liposomes, the prolonged activity of PC:Chol in rats *in vivo* is suggested not to be caused by a strong affinity between the peptide and the PC:Chol liposomes.

The formulations were given *s.c.*, and the diffusion of liposomes and peptide is a factor for a prolonged effect. Injection of a liposomal suspension into the subcutis places the liposomes in predominantly adipose tissue. From the place of injection, the liposomes must diffuse to blood vessels and lymph vessels. Percutaneous delivery of liposomes depends on the physical state of the liposomes. Fluid

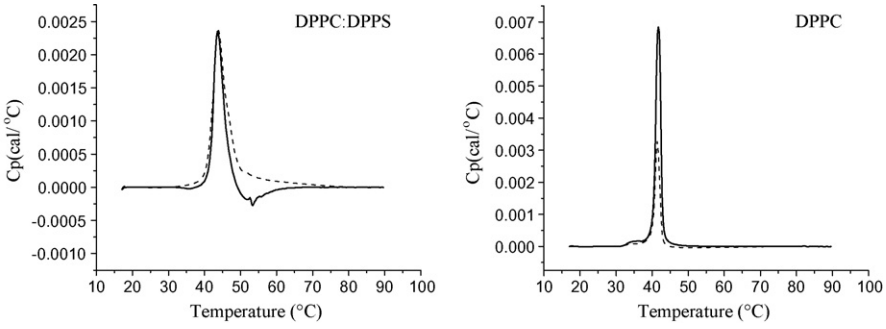

Fig. 4. DSC thermograms of DPPC:DPPS (70:30) and DPPC with (line) and without (dash) ghrelin.

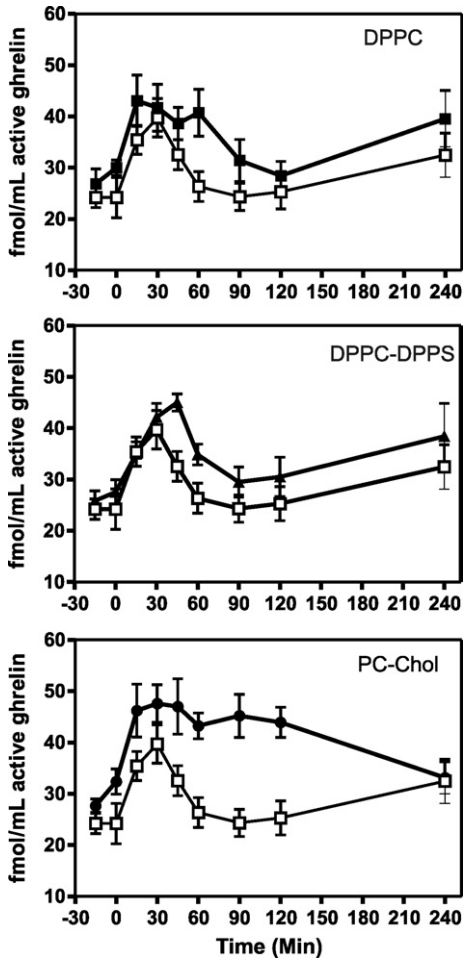

Fig. 5. *In vivo* profile of active ghrelin in plasma measured by RIA after *s.c.* administration of ghrelin in buffer (open squares), with DPPC (filled squares), with DPPC:DPPS (filled triangles), and with PC:Chol (filled circles).

(liquid state) liposomes have been shown to be able to give a good percutaneous absorption (Coderch et al., 2000; Fresta and Puglisi, 1996; Vrhovnik et al., 1998). The behaviour of liquid state liposomes such as PC:Chol (70:30) in tissue could be speculated to be the reason for the different *in vivo* profile of the PC:Chol (70:30) formulation.

Absorption of small (<100 nm) neutral liposomes occurs partly through the lymphatic capillaries. A fraction of the smaller, and also the larger, liposomes will remain at the injection site where they degrade (Oussoren and Storm, 2001). It is interesting that the clustered DPPC:DPPS-ghrelin particles do not show a prolonged release, as these large conglomerates could be expected to be retained at the site of injection. The fraction of liposomes absorbed through the lymphatic system is not affected by the fluidity of the bilayer, nor by the presence of cholesterol or negatively charged lipids (Oussoren et al., 1997). Phosphatidylserine constitutes an exception, tending to give increased lymphatic uptake (Oussoren and Storm, 2001). PC:Chol (50:50; size 30–60 nm) liposomes have been reported to be absorbed through the lymph capillaries and reach the blood circulation after 3 h (Tumer et al., 1983). However, if the liposome and ghrelin bind only weakly, the peptide will diffuse away from the liposome on dilution *in vivo* and permeate the tissue freely. A cautious interpretation of the *in vivo* experiments could be that the DPPC and DPPC:DPPS liposomal formulations behave much like the ghrelin solution under these conditions, whereas the PC:Chol system behaves as expected for a liposomal delivery system absorbed through the lymphatic system. Another explanation could be that ghrelin is more or less protected against enzymatic hydrolysis in the different liposomal environments. However, this was not definitely established in these studies, and further investigations are needed.

4. Conclusions

Liposomal formulations of ghrelin were manufactured and their *in vivo* profile was examined following *s.c.* administration in rats. Of the liposomes examined, ghrelin persisted in plasma for a longer time *in vivo* upon administration of a formulation of ghrelin together with unilamellar PC:Chol (70:30) liposomes as compared to the ghrelin buffer solution and the other two liposomal formulations. Capillary electrophoresis methods were successfully developed and the CE studies showed that there were minor affinities between ghrelin and the neutral PC:Chol (70:30) and DPPC liposomes, respectively. A larger affinity was measured between the peptide and the negatively charged DPPC:DPPS (70:30) liposomes, and this affinity was clearly enhanced by the presence of the octanoyl group. DSC studies, on the other hand, demonstrated that the phase transitions of the lipid bilayers were not perturbed as shown by the unchanged T_m's. In summary, the prolonged effect of the PC:Chol formulation is suggested not to be caused by association between ghrelin and the liposome, and further work is needed to elucidate these factors.

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