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Biomimesis of Linolenic Acid Transport through Model Lipidic Membranes by Differential Scanning Calorimetry

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Multienoic fatty acids, such as linolenic acid, show their ability to interact with and to penetrate into model biomembranes by biomimetic experiments performed to support the absorption route followed by *n*−3 fatty acid in cells. The thermotropic behavior of model biomembranes, that is, dimyristoylphosphatidylcholine multilamellar or unilamellar vesicles, interacting with linolenic acid was investigated by differential scanning calorimetry. When dispersed in liposomes during their preparation, the examined biomolecule was found to interact with the phospholipid bilayers by modifying the gel to liquid-crystal phase transition of lipid vesicles; this modification is a function of the fatty acid concentration. Calorimetric analysis was also performed on samples obtained by leaving the pure *n*−3 acid in contact with lipid aqueous dispersions (multilamellar or unilamellar vesicles) and then examining the thermotropic behavior of these systems for increasing incubation times at temperatures higher than the transitional lipid temperature. Linolenic acid (LNA) was able to migrate through the aqueous medium and successively to interact with the vesicle surface and to penetrate into the model membranes, following a flip-flop mechanism, with a faster and higher effect for unilamellar vesicles, caused by the larger lipid surface exposed, compared to the multilamellar ones, although due to the lipophilic nature of LNA, such a transfer is hindered by the aqueous medium. The relevance of the medium in LNA absorption has been well clarified by other biomimetic transfer experiments, which showed the LNA transfer from loaded multilamellar vesicles to empty vesicles. Taken together, the present findings support the hypothesis of a passive *n*−3 acid transport as the main route of absorption into cell membranes.

KEYWORDS: Linolenic acid; differential scanning calorimetry; membrane transport

INTRODUCTION

Fatty acids, a nonhomogeneous category of biomolecules, are necessary for normal physiological function and good health. Although all fatty acids carry out many important roles, only some of them are *essential* because they cannot be completely synthesized by the human body and must be obtained from food.

α-Linolenic acid (LNA), synthesized in plants, fungi, and bacteria, belongs to the *n*−3 series of multienoic fatty acids (MEFA) and is considered to be an *essential* fatty acid, because mammals, including humans, lack the enzymes required to insert unsaturated fatty acid chains at positions beyond C-9 along the chain. Therefore, LNA must be obtained through food chain sources, including vegetable oils, such as olive (*1*) and seed oils, and marine lipids, that is, from fatty fish, which obtain it from phytoplankton. LNA develops a bitter taste, when in the emulsified form, with a low threshold (*2*), which can contribute to the sensory attributes of the food product (*3*).

LNA is associated with several human health benefits and positive biological activities. Epidemiological investigations suggest an important role for LNA in the prevention of coronary heart disease and cancer. In fact, *n*−3 fatty acid intake is associated with a 50% reduction in the risk of primary cardiac arrest (*4*) and with a strong protective effect of the LNA-enriched Mediterranean diet on cardiovascular diseases and their resulting mortality (*5*). Furthermore, the level of LNA, inversely related to the risk of cancer, influences peroxidation, an intermediate step toward cell death (*6*).

The biological activity of such a class of unsaturated fatty acids can be better understood by investigating their interactions with biomembranes in order to obtain information about their solubility in lipid membranes; this is a prerequisite for their permeation through the cell wall, which regulates absorption during food gastrointestinal transit as well as biomembrane uptake from the bloodstream.

The effect exerted by LNA on the thermotropic behavior of L-α-dimyristoylphosphatidylcholine (DMPC) vesicles [multilamellar (MLV) and unilamellar (LUVs)] provides biomimetic information on membrane interaction and on its mechanism of migration. In our study this interaction was investigated by

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differential scanning calorimetry (DSC) experiments, a nonperturbative technique employed to investigate thermal effects exhibited by a vesicle-interacting biomolecule during heating or cooling processes. The biomolecule under investigation exerts its effects when dispersed in the lipid bilayer during liposome preparation or in contact with empty MLVs or LUVs, allowing the evaluation of some parameters, such as lipophilicity, solubility, and the role of substituents in the molecule, modulating its interaction and molecular penetration into cell membranes and thus leading to speculations about mechanisms involved in its in vivo absorption.

MATERIALS AND METHODS

Materials. Synthetic DMPC and LNA were obtained from Fluka Chemical Co. (Buchs, Switzerland). Solutions of the lipid were chromatographically pure as assessed by two-dimensional thin-layer chromatography. Lipid concentrations were determined by phosphorus analysis (*7*).

The buffer, consisting of a 50 mM Tris solution, was adjusted to pH 7.4 with hydrochloric acid.

Liposome Preparation. MLVs were prepared in the presence and absence of acid by the following procedure: chloroform/methanol (1:1, v/v) stock solutions of DMPC and LNA were mixed to obtain increasing molar fractions of acid. The solvents were removed under a nitrogen flow, and the resulting films were freeze-dried under vacuum to remove the residual solvents. Liposomes were prepared by adding 50 mM Tris buffer solutions to the film, then heating to 37 °C (this temperature is above the gel−liquid crystalline phase transition), and vortexing three times for 1 min. The samples were shaken for 1 h in a water bath at 37 °C to homogenize the liposomes and to reach a distribution equilibrium between lipid and aqueous phases. Aliquots of 120 μL (5 mg of lipid) were then transferred to a (160 μL) DSC aluminum pan, hermetically sealed under nitrogen flow, and submitted to DSC analysis.

LUVs were obtained by extruding MLVs through polycarbonate membranes of 100 nm pore size in an extruder system (Liposofast Avenstin) (*8*).

DSC Experiments. DSC was performed by using a Mettler TA Star^e system equipped with a DSC 822^e cell. The scan rate was 2 °C/min in the temperature range of 2−37 °C. The reference pan was filled with Tris buffer solution. Indium and palmitic acid were employed to calibrate the transitional enthalpies (ΔH). Data were evaluated from the peak areas using Mettler STAR^e V 6.10 SW software.

The samples were cooled and heated at least three times to achieve reproducibility of the results (the average error was <1% for the peak temperature and <5% for the enthalpy changes). All samples, after calorimetric scans, were removed from the pan, and aliquots were used to determine the amount of phospholipids by the phosphorus assay (*7*).

Biomimetic Permeation Experiments. The possibility that LNA can permeate cell membranes after reaching their surface can be confirmed by carrying out a simple permeation experiment through biomembrane models. Such a kinetic permeation experiment was carried out by leaving a fixed amount of DMPC aqueous suspension (MLVs or LUVs) in contact with a defined amount of pure LNA in the bottom of a DSC crucible, to obtain a 0.09 molar fraction of LNA with respect to the lipids dispersed in the aqueous medium. The samples, hermetically sealed under nitrogen atmosphere in the calorimetric pan, were gently shaken for 10 s and submitted to calorimetric scans in heating, isothermal, and cooling modes by using the following procedure.

(1) A first scan was performed from 2 to 37 °C to detect the eventual interaction of LNA with the model membranes due to the dissolution of the acid in the aqueous medium and its transfer from the solid phase through the aqueous phase to the model membrane surface, remaining in an ordered array.

(2) An isothermal period of 1 h at 37 °C was then used to permit the substance to continue the dissolution and migration processes but also to permeate, if it was able, the lipid layer(s) remaining in a disordered state at a temperature above the lipid transitional temperature.

(3) A cooling scan between 37 and 2 °C was used before the heating program was restarted (first step).

This procedure was run continuously, at least eight times, to detect the variations caused by the interaction of increasing amounts of LNA with model membranes; then, the procedure was carried out for longer incubation periods. No thermal degradation of LNA occurred during the calorimetric scans, as observed when an equal amount of LNA was incubated under nitrogen in the same experimental conditions and the accumulation of hydroperoxides was detected by reverse phase HPLC with UV detection (data not shown).

Transmembrane Biomimetic Transport Experiments. The previous experiment allowed us to investigate only if the aqueous medium can hinder the uptake of the low water soluble LNA by the lipid membranes and if the larger exposure surface of the LUV lipid can increase the extent of the absorption. To evaluate the possibility that the cellular absorption of LNA can be helped by the presence of a lipophilic medium where LNA can be molecularly dispersed, a transmembrane transport of the acid was carried out. The previous procedure was applied to samples obtained by mixing equal aliquots (60 μL each containing 2.50 mg of DMPC) of 0.12 molar fraction loaded MLVs with empty ones; in such a way the calorimetric experiment should show two different calorimetric peaks, the first one attributable to the empty liposomes (falling at higher temperatures) and the second one due to the loaded vesicles (0.12 molar fraction) at lower temperatures. By submitting the samples to the procedure reported in the previous section, it was possible to follow the progressive transfer of the LNA from the loaded to the unloaded vesicles, with the subsequent shift of the two calorimetric peaks, respectively, at lower and higher temperatures and the appearance of a single peak only if a complete LNA transfer occurred (final total molar fraction = 0.06). This process is seen by comparing the resulting calorimetric curve with that obtained by direct preparation of the 0.06 LNA molar fraction in MLVs.

RESULTS AND DISCUSSION

DMPC vesicles, as a synthetic simplified membrane model of bilayer biomimicking the lipid structure of cell membranes, show temperature-dependent thermotropic behavior (*9*). In fact, the DMPC acyl chains change with a temperature increase from an ordered to a disordered configuration, through the $L_\beta-L_\alpha$ or gel-to-liquid crystal phase transition, characterized by two important thermodynamic parameters, that is, the enthalpy change (ΔH) and the phase transition temperature (T_m) (*9*−*12*).

The thermotropic behavior is influenced by the presence of molecules dissolved in the lipid bilayer, such as LNA, that act as a solute in a solvent and can lower the T_m or cause changes in the ΔH of the process (*13*, *14*). The temperature decrease is proportional to the amount of biomolecules dissolved in the lipid bilayer and is related to the their lipophilicity. DMPC vesicles were chosen as a simple membrane model to investigate the LNA biomimetic interaction and penetration into biological membranes. Calorimetric heating curves of DMPC MLVs, in the presence of different molar fractions of LNA, are reported in **Figure 1**. The fatty acid interacts with the DMPC liposomes, causing a shift of the endothermic peak, associated with the gel to liquid-crystal phase transition, typical for lipid vesicles, toward a low temperature. The decrease of the T_m of the calorimetric peaks is related to the amount of LNA present in the aqueous dispersion, with a lowering effect controlled by increasing the LNA molar fraction.

The biomimetic interaction between LNA and DMPC liposomes was interpreted in terms of a *fluidifying* effect, due to the introduction of lipophilic biomolecules into the ordered structure of the lipid bilayer (*9*, *13*, *15*−*20*). LNA acts as a spacer in such a structure, causing a destabilization of the lipid mosaic with a decrease in the T_m of the gel-to-liquid crystal

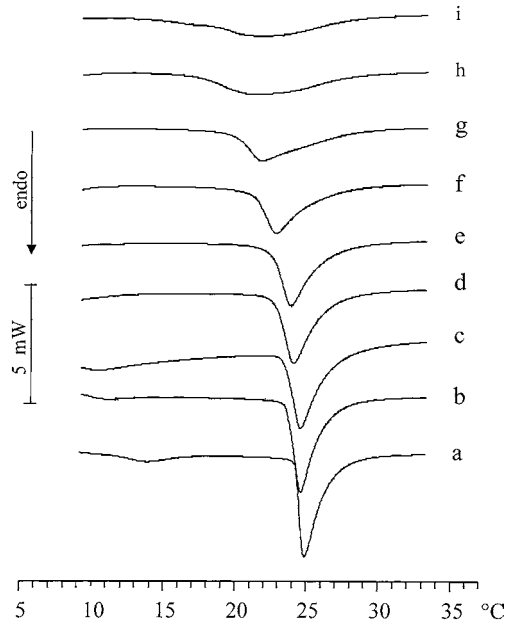

Figure 1. DSC heating curves of hydrated DMPC MLVs in the presence of LNA at the following molar fractions: a, 0.0; b, 0.015; c, 0.03; d, 0.045; e, 0.06; f, 0.09; g, 0.12; h, 0.15; i, 0.18.

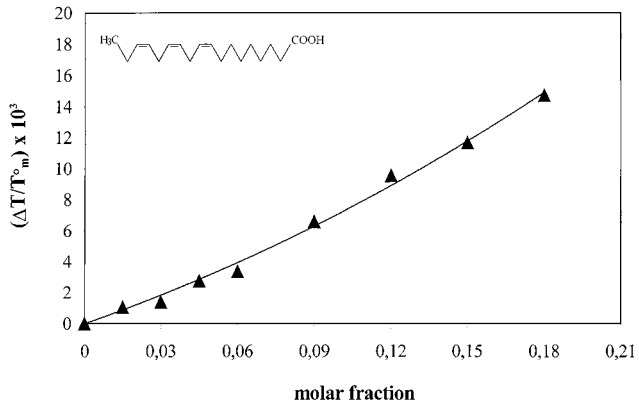

Figure 2. Transition temperature variation (as $\Delta T/T^{\circ}_{m}$) values of multilamellar DMPC vesicles as a function of LNA molar fractions.

phase transition and calorimetric peak broadening. In **Figure 2**, the temperature shifts, obtained from the calorimetric curves, caused by the presence of LNA are expressed as $(\Delta T/T^{\circ}_{m}) \times 10^3$ ($\Delta T = T^{\circ}_{m} - T_m$, where T°_{m} and T_m are, respectively, the T_m of pure DMPC and that of DMPC in the presence of increasing amounts of LNA) and are plotted against LNA molar fractions in the lipid aqueous dispersion. Differences in ΔH, as shown in **Figure 3**, can be due to the localization of LNA inside the lipid bilayer, because a negligible ΔH can be explained only as an interaction between the biomolecules and lipid surface, without greatly involving the acyl chains (*21*). Thus, a perturbing effect on ΔH can be controlled by a deep interaction due to the high lipophilicity of the LNA examined.

LNA, introduced with the food, to elicit its functions, should be partitioned between the aqueous medium and the biological membranes and not only interact with model membranes but also be *absorb* by them and transported inside them. Therefore, empty MLVs or LUVs were left in contact with fixed amounts of pure liquid LNA ($X = 0.09$) at increasing incubation times and at a temperature higher than the lipid T_m. The calorimetric peaks, associated with the gel to liquid-crystal transition of the lipid vesicles, were due to the absorption of such biomolecules

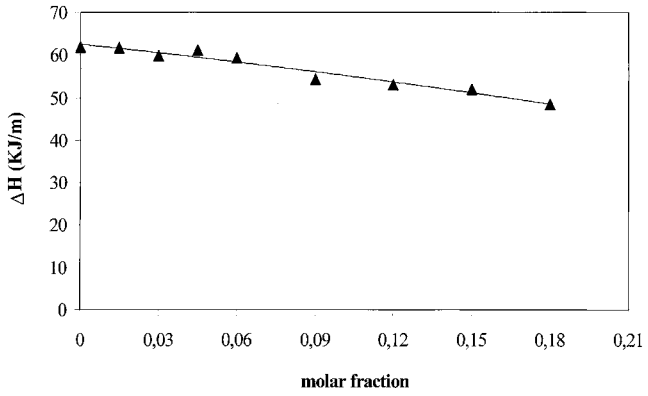

Figure 3. Transitional enthalpy variation (ΔH) values (average of at least three runs), in heating mode, as a function of LNA molar fractions.

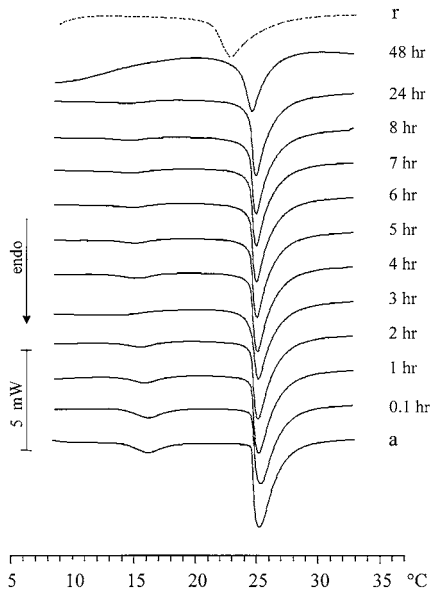

Figure 4. DSC heating curves of empty multilamellar DMPC vesicles alone (curve a) or in the presence of a fixed molar fraction (0.09) of liquid LNA for increasing incubation times. Curve r represents the effect of the 0.09 molar fraction of LNA on the MLV, obtained starting from organic solvent solutions, to be considered as the effect to be reached if the LNA was completely transferred to empty vesicles.

in lipid layers, as determined during the biomimetic DSC experiments (*22*−*27*). The biomimetic transfer kinetics of liquid LNA (molar fraction = 0.09) through the aqueous medium to the MLV and LUV surfaces is shown in **Figures 4** and **5**, respectively, and compared to the curve r obtained from mixing biomolecules and phospholipids in the organic phase to obtain the maximum interaction to be considered as the interaction at infinite time (t_{inf}). LNA biomimetic transfer through the aqueous medium and the successive interaction with lipids appear to be faster and larger in LUVs (**Figure 5**) than in MLVs (**Figure 4**). LUVs, in fact, expose a greater surface area to the uptake process of LNA biomolecules with respect to multilamellar vesicles, as summarized in **Figure 6**, where the temperature variations are related to the transfer incubation times.

Considering the above-reported experiments, it seems that LNA migrates through the aqueous medium and reaches the outer lipid vesicle surface in a very slow process hindered by the hydrophilic medium. The flip-flop mechanism, which could permit penetration of the biological membrane, seems to be slow

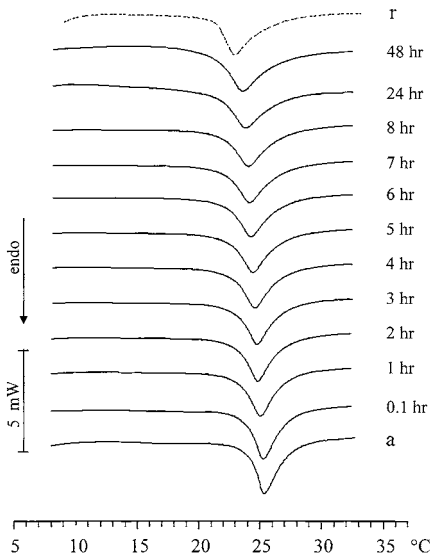

Figure 5. DSC heating curves of empty unilamellar DMPC vesicles alone (curve a) or in the presence of a fixed molar fraction (0.09) of liquid LNA for increasing incubation times. Curve r represents the effect of the 0.09 molar fraction of LNA on the MLV, obtained starting from organic solvent solutions, to be considered as the effect to be reached if the LNA was completely transferred to empty vesicles.

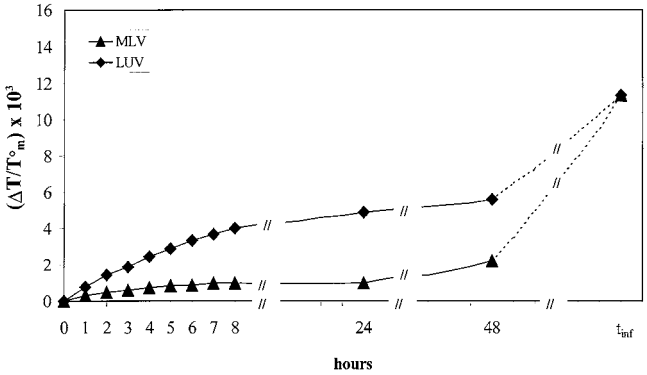

Figure 6. Transitional temperature variations (as $\Delta T/T^{\circ}_{m}$) of empty multilamellar DMPC vesicles and LUVs in the presence of liquid LNA at a fixed molar fraction (0.09) for increasing incubation times. The t_{inf} values represent the effect exerted by the 0.09 molar fraction of LNA on the MLVs and LUVs, obtained starting from organic solvent solutions, to be considered as the maximum interaction between compound and vesicles.

enough that dissolution increases and the uptake processes of LNA are not favored; thus, a complete LNA absorption to reach the effect at T_{inf} is not obtained.

The previously reported considerations could be explained by examining the calorimetric curves, obtained from transmembrane transport experiments, with 0.12 molar fraction loaded MLVs, left in contact with empty multilamellar vesicles (final molar fraction = 0.06, curve r) as reported in **Figure 7**. The curves reveal the higher LNA absorption into the lipidic vesicles due to the uptake process favored by the dispersion in a lipophilic medium. The two calorimetric signals (curve 0.1 h) can be associated with the loaded MLVs at low temperature and with the pure, still empty, MLVs at a high temperature. They show a progressive coalescence effect during the successive scans, with shoulders indicating dishomogeneous zones in lipid vesicles, when LNA was incubated in the calorimetric pan at a temperature higher than the T_m, and thus indicate the transfer from the loaded to the empty vesicles. The value obtained for the curve r demonstrates that the LNA-loaded vesicles were able

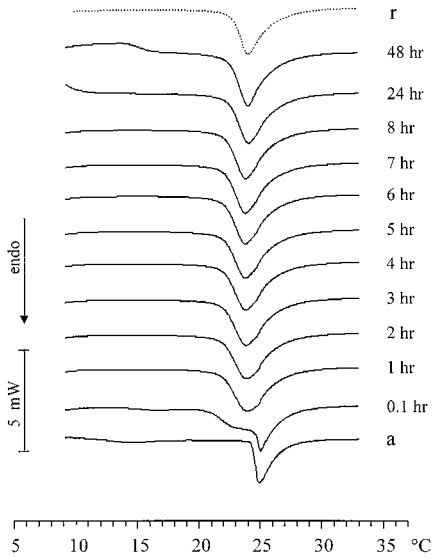

Figure 7. DSC heating curves of empty multilamellar DMPC vesicles alone (curves a) or in the presence of multilamellar DMPC vesicles loaded with LNA at a fixed molar fraction (0.12) for increasing incubation times. Curves r represent the effect of the 0.06 molar fraction of LNA in the MLV, obtained starting from organic solvent solutions, to be considered as the effect to be reached if the LNA was transferred from loaded to empty vesicles so as to obtain an average amount of 0.06 in molar fraction.

to transfer their guest biomolecules, which successively migrate through biomembrane lamellae.

The results of the described in vitro biomimetic experiments permit a deeper understanding of the interaction and penetration through biological membranes by LNA, which may be representative of the omega−3 fatty acid class found in olive drupes and their Mediterranean food derivatives. The relevance of these results lies in the information concerning the kinetic process, the dissolution of LNA in an aqueous medium, its transfer, and mainly its potential absorption through a biological membrane. Also, even if merely speculative, the possible explanation of these findings may provide new insights on the health effects of omega−3 fatty acids contained in Mediterranean foods on human well-being.

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