Docosahexaenoic Acid and Eicosapentaenoic Acid Induce Changes in the Physical Properties of a Lipid Bilayer Model Membrane

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We investigated the effect of fatty acids such as stearic acid (SA, 18:0), oleic acid (OA, 18:1), eicosapentaenoic acid (EPA, 20:5), and docosahexaenoic acid (DHA, 22:6) on a dipalmitoylphosphatidylcholine (DPPC) bilayer by determining the phase transition temperature, fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH), and detergent insolubility. Treatment with unsaturated fatty acid broadened and shifted the phase transitions of the DPPC bilayer to a lower temperature. The phase transition temperature and the value of fluorescence anisotropy of DPH at 37 °C decreased progressively with increasing treatment amounts of unsaturated fatty acid. A large amount of the DPPC bilayer treated with unsaturated fatty acid was dissolved in Triton X-100, obtaining a low level of detergent insolubility. These modifications of the bilayer physical properties were most pronounced with DHA and EPA treatment. These data show that unsaturated fatty acids, particularly DHA and EPA, induce a marked change in the lipid bilayer structure. The composition of fatty acids in the DPPC bilayer was similar after treatment with various unsaturated fatty acids, suggesting that the different actions of unsaturated fatty acids are attributed to change in the molecular structure (*e.g.*, kinked conformation by double bonds). We further explored the change in physical properties induced by fatty acids dispersed in a water-in-oil-in-water multiple emulsion and found that unsaturated fatty acids acted efficiently on the DPPC bilayer, even when incorporated in emulsion form.

Key words docosahexaenoic acid; eicosapentaenoic acid; unsaturated fatty acid; emulsion; physical property; dipalmitoylphosphatidylcholine (DPPC) bilayer

Dietary intake of n-3 polyunsaturated fatty acids, such as docosahexaenoic acid (DHA, 22:6) and eicosapentaenoic acid (EPA, 20:5), is linked to the prevention of diseases such as cancer[1,2] and heart disease,[3—5] and is needed for neurological and brain development.[6] We have recently attempted to use these functional fatty acids as an absorption enhancer and have reported that intestinal absorption of insulin[7—9] and vancomycin[10] increase when administered with these fatty acids in a water-in-oil-in-water (W/O/W) multiple emulsion. We have also reported that the enhancement effect of DHA and EPA is much stronger than that of other unsaturated fatty acids, such as oleic acid, and does not induce any tissue damage.[7—9] Although the mechanism responsible remains unclear, the effect of DHA and EPA may be attributed to their actions on the transcellular pathway rather than the paracellular pathway because the tissue membrane resistance values do not change with treatment.[10]

Administering an emulsion into the intestinal lumen should cause the fatty acids contained in the emulsion to be taken up readily into the lipid bilayer of the intestinal mucosa, causing a change in the lipid packing. In general, the structural change in the lipid bilayer is a critical factor for transcellular pathway. There are many reports that perturbation of the lipid-packing order increases drug permeability.[11—13] In addition, recent studies have shown that altering membrane fluidity influences carrier-mediated transport, such as P-glycoprotein-mediated efflux.[14—16] Because DHA and EPA have multiple double bonds, they should change the lipid packing drastically. Such changes in the lipid bilayer structure should cause modifications of the biological membrane function, and it is important to identify the action of fatty acids on the lipid bilayer structure.

We investigated changes in the physical properties of the lipid bilayer by treating model bilayers with various fatty acids such as stearic acid (SA, 18:0), oleic acid (OA, 18:1), EPA, and DHA. Because the plasma membrane structure is too complex, we used dipalmitoylphosphatidylcholine (DPPC) liposomes as a model of the lipid bilayer. We assessed the structural changes in the lipid bilayer induced by fatty acids by measuring changes in the phase transition temperature, fluorescence anisotropy, and detergent insolubility. To evaluate whether fatty acids incorporated in the formulation act successfully on the lipid bilayer, we also performed the same experiments using a W/O/W multiple emulsion containing fatty acids.

Experimental

Materials DPPC, SA, gelatin, triolein, sorbitan monooleate (Span 80), DL-α-tocopherol, Triton X-100, phospholipid C-Test kit, and nonesterified fatty acid (NEFA) C-Test kit were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). OA and polyoxyethylene sorbitan monooleate (Tween 80) were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Egg yolk phospholipids (phosphatidylcholine and phosphatidylethanolamine) were purchased from Nippon Oil & Fats Co., Ltd. (Tokyo, Japan). DHA (purity 99.0%) and EPA (purity 99.0%) were provided by Nippon Suisan Kaisya, Ltd. (Tokyo, Japan). All other chemicals were of analytical grade and commercially available.

Preparation of DPPC Liposome DPPC dissolved in chloroform was pipetted into a flask, and the chloroform removed by evaporation at room temperature under a nitrogen stream. This procedure resulted in the formation of a thin lipid film on the inside wall of the flask. The film was stored overnight in a vacuum desiccator to ensure the complete evaporation of chloroform. Ten milliliters of phosphate-buffered saline (PBS, pH 7.4) was added to the flask and the DPPC was hydrated for 30 min. The suspension was sonicated for 10 min at about 60 °C using a bath-type sonicator. The liposome suspension was prepared at a total lipid concentration of 10 mM and stored at room temperature until used in the experiments.

Differential Scanning Calorimeter Measurement Fatty acid suspensions and W/O/W multiple emulsions containing fatty acids were used as the

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treatment solutions. Fatty acid suspensions were prepared by suspending designated amounts of fatty acids such as SA, OA, EPA, and DHA in a mixture of methanol and PBS (1 : 1, v/v). W/O/W multiple emulsions were prepared by a two-step emulsification procedure using a homogenizer (Ace Homogenizer, Nihonseiki Kaisha, Tokyo, Japan) according to the method reported previously.[7] Briefly, purified water containing 5% gelatin and 3% Tween 80 were used for the inner and outer aqueous phases, respectively. The oily phase was composed of 0.06% DL-α-tocopherol, 5% egg yolk phospholipids (phosphatidylcholine : phosphatidylethanolamine, 7 : 3), 10% fatty acid, and 20% Span 80. An appropriate amount of triolein was added to adjust the weight of the oily phase. The weight ratio of each phase was, inner aqueous phase : oily phase : outer aqueous phase, 1 : 4 : 15. Each emulsion was freshly prepared just before the experiments.

Fatty acids were applied to the DPPC bilayer as described previously.[17] Two hundred and fifty microliters of treatment solution was added to 1000 μl of DPPC liposome suspensions and this mixture was incubated for 2 h at 37 °C. Samples were centrifuged at 13000 rpm for 2 min to separate the supernatant and pellet. The obtained pellets of known weight (approximately 5 mg) were placed in aluminum pans for the differential scanning calorimeter (DSC) measurement. DSC measurements were performed with a Thermo Plus DSC 8230 (Rigaku Co. Ltd., Tokyo, Japan). The scan rate was set at 1 °C/min. The phase transition temperature was determined as the peak temperature.

Fluorescence Anisotropy Measurement The DPPC bilayer was labeled with DPH by adding 10 μl of 10 mM freshly prepared DPH stock solution in tetrahydrofuran to 1000 μl of liposome suspension and then incubating at 37 °C for 2 h in the dark to complete the labeling. The treatment with fatty acids in DPH-labeled liposomes was performed as described above. The pellets were collected by centrifugation at 13000 rpm for 2 min. The obtained pellets were resuspended in PBS and the absorbance at 400 nm was set $<$0.50 to permit the measurement of fluorescence. The fluorescence anisotropy of DPH in the DPPC bilayer was measured with a fluorescence spectrophotometer (Hitachi F-450, Hitachi Co. Ltd., Tokyo, Japan) at an excitation wavelength of 351 nm and an emission wavelength of 430 nm. The steady-state fluorescent anisotropy was calculated using the following equation:

$$r = \frac{I_{VV} - I_{VH}}{I_{VV} + 2I_{VH}}$$

where r is anisotropy, and I_{VV} and I_{VH} are the intensity measured in directions parallel and perpendicular to the polarized exciting light, respectively.

Detergent Resistance Studies After application of fatty acids to the DPPC liposome suspension, 750 μl of 10% Triton X-100 solution was added to 1250 μl of a DPPC liposome suspension treated with fatty acid, and the sample was incubated at 25 °C for 2 h. The sample was centrifuged at 13000 rpm for 2 min to separate the supernatant and pellet, and the supernatant was removed and resuspended in an equal volume of fresh PBS. The sample was diluted 100 times with PBS and the optical density (OD) at 400 nm was measured using a spectrophotometer (U-best 30, JASCO, Tokyo, Japan). The OD at 400 nm of a freshly prepared DPPC liposome suspension was also measured and this value was used as the initial level. Detergent insolubility was calculated as the OD after the addition of Triton X-100 divided by the initial level.

Determining the Composition of Fatty Acids in DPPC Bilayers Treated with Fatty Acids Application of unsaturated fatty acids to DPPC liposomes was performed using fatty acid suspensions. The treatment amount of each fatty acid was fixed at 30 mol% relative to the amount of DPPC. The pellets were collected by centrifugation at 13000 rpm for 2 min and the pellets were resuspended in an equal volume of fresh PBS. DPPC and fatty acid concentration were determined using a phospholipid C-Test kit and a NEFA C-Test kit, and the composition of fatty acids in DPPC bilayer was calculated.

Results and Discussion

Changes in the Physical Properties of the DPPC Bilayer Induced by Treatment with Fatty Acids Figure 1 shows the DSC curves of the DPPC bilayer treated with various fatty acids. For the DPPC bilayer treated with the mixture of PBS and methanol (control), the sharp endothermic peak due to the phase transition from the gel phase to the liquid-crystalline phase was observed at 40.8 °C. This temperature is lower than the phase transition temperature of pure

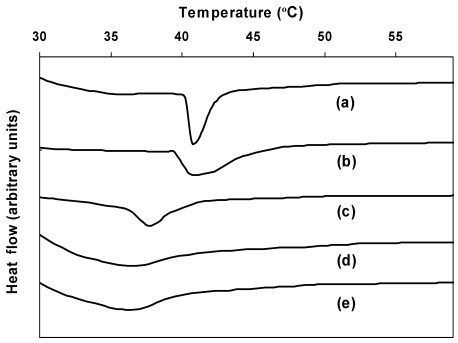

Fig. 1. Differential Scanning Calorimetry Thermograms of the DPPC Bilayer after Treatment with Fatty Acids

Thirty mole percent of fatty acid relative to the amount of DPPC was applied to the DPPC bilayer in the form of a suspension. The temperature was scanned at 1 °C/min. (a) Control, (b) SA, (c) OA, (d) EPA, and (e) DHA.

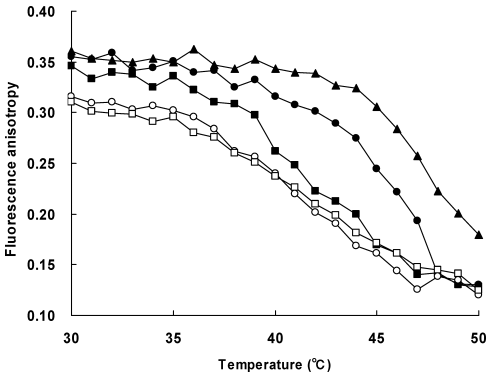

Fig. 2. Fluorescence Anisotropy of DPH in the DPPC Bilayer after Treatment with Fatty Acids

Thirty mole percent of fatty acids relative to the amount of DPPC was applied to the DPPC bilayer in the form of a suspension. The temperature was scanned at 1 °C/min. (●) Control, (▲) SA, (■) OA, (○) EPA, and (□) DHA.

DPPC reported by other researchers,[11,18] and the reduction in phase transition temperature occurred probably because of the effect of methanol in the treatment solution. Tran *et al.* reported a similar effect induced by ethanol.[19] In addition, we had already confirmed in a preliminary study that DPPC liposomes without any treatment (blank) had a phase transition at about 42 °C (data not shown). Treatment with unsaturated fatty acids, such as OA, EPA, and DHA, shifted the phase transition temperature to a lower value and broadened the sharp endothermic peak (Fig. 1). The phase transition temperatures of the DPPC bilayer treated with 30 mol% of fatty acids were 41.3 °C for SA, 37.5 °C for OA, 36.3 °C for EPA, and 36.2 °C for DHA.

Figure 2 shows the effects of fatty acids (30 mol%) on the fluorescence anisotropy of DPH in the DPPC bilayer. Treatment with unsaturated fatty acids shifted the phase transition of the DPPC bilayer to a lower temperature and broadened the phase transitions compared with those of the control. These results were consistent with those obtained from the DSC study (Fig. 1). Treatment with unsaturated fatty acids markedly decreased the values of fluorescence anisotropy of DPH in the gel phase.

We investigated further the effects of the amount of fatty acids used in the treatment on the phase transition temperature and fluorescence anisotropy of DPH. Increasing the molar fraction of unsaturated fatty acids induced a notable

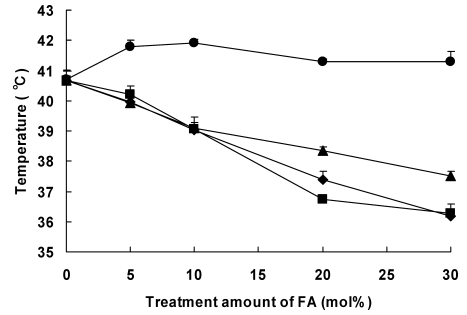

Fig. 3. Effect of the Treatment Amount of Fatty Acids on the Phase Transition Temperature of the DPPC Bilayer

An adequate amount of fatty acids was applied to the DPPC bilayer in the form of a suspension. Each value represents the mean±S.D. of three experiments. (●) SA, (▲) OA, (■) EPA, and (◆) DHA.

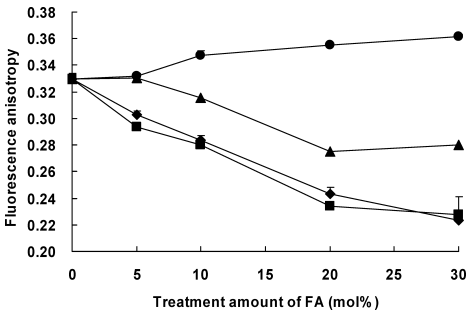

Fig. 4. Effect of the Treatment Amount of Fatty Acids on Fluorescence Anisotropy of DPH in the DPPC Liposome at 37 °C

An adequate amount of fatty acids was applied to the DPPC bilayer in the form of a suspension. Each value represents the mean±S.D. of three experiments. (●) SA, (▲) OA, (■) EPA, and (◆) DHA.

progressive decrease in the phase transition temperatures and the values of fluorescence anisotropy of DPH (Figs. 3, 4). The modifications induced by DHA and EPA were most pronounced. These changes are caused by the interaction between fatty acids and the DPPC bilayer. DPPC, which has two 16-carbon saturated chains, forms a substantially more tightly packed lipid bilayer. Once applied to the DPPC bilayer, unsaturated fatty acids are taken up into the lipid bilayer in a dose-dependent manner, reducing the van der Waals interactions between the phospholipid hydrocarbon chains because of their kinked structure.[13] The decreases in the phase transition temperature and the value of fluorescence anisotropy reflect this mechanism. Conversely, SA treatment caused a slight increase in the phase transition temperature and the values of fluorescence anisotropy of DPH (Figs. 3, 4). Because SA is a saturated fatty acid composed of a longer hydrocarbon chain than DPPC, the packing of the DPPC bilayer might be tighter after treatment with SA.

We also assessed the detergent insolubility of the DPPC bilayer treated with fatty acids. This method is based on the observation that ordered lipid domains tend to resist solubilization by nonionic detergents, such as Triton X-100, whereas disordered fluid domains dissolve in these detergents. The insoluble membrane fraction can be regarded as tightly packed domains that were present in the sample before the addition of detergent. London *et al.* used detergent insolubility as an index of the formation of ordered lipid domains (lipid raft) in a model lipid bilayer.[20,21] We found that fatty acid treatment influenced the distribution of the ordered

Table 1. Detergent Insolubility of the DPPC Bilayer after Treatment with Fatty Acids

	Detergent insolubility $(OD_{400\,nm}+TX\text{-}100)/(OD_{400\,nm}-TX\text{-}100)$
Control[a)]	0.501±0.027
SA	0.612±0.016
OA	0.092±0.006
EPA	0.030±0.002
DHA	0.030±0.004

a) Mixture of PBS and methanol (1 : 1 v/v%). Thirty mole percent of fatty acids relative to the amount of DPPC was applied to the DPPC bilayer in the form of a suspension. Each value represents the mean±S.D. of three determinations. TX-100, Triton X-100.

Table 2. Composition of Fatty Acids in the DPPC Lipid Bilayer after Treatment with Unsaturated Fatty Acids

	Fatty acid (mol%)	DPPC (mol%)
OA	27.0±0.3	73.0±0.3
EPA	26.4±0.4	73.6±0.4
DHA	25.9±0.2	74.1±0.2

Thirty mole percent of fatty acids relative to the amount of DPPC was applied to the DPPC bilayer in the form of a suspension. Each value represents the mean±S.D. of three determinations.

lipid domain in the membrane. Table 1 shows the detergent insolubility of the DPPC bilayer treated with 30 mol% of fatty acids. Considerable amounts of insoluble membrane fractions were obtained from the control and SA treatment conditions (Table 1), indicating that these membranes were composed of a large amount of ordered and rigid regions. In contrast, the DPPC bilayer treated with unsaturated fatty acids was nearly completely dissolved in Triton X-100. The lowest level of detergent insolubility was observed with DHA and EPA treatment, suggesting that the tightly packed membrane of the DPPC bilayer was altered in the disordered fluid membrane by treatment with unsaturated fatty acids, particularly DHA and EPA.

Our results confirmed that the unsaturated fatty acids changed the lipid bilayer structure markedly. The modifications of the bilayer physical properties were more pronounced with DHA and EPA than with OA treatment. The order of potency of the modifications of the physical properties induced by unsaturated fatty acids agreed with the order of their absorption enhancement effects.[7—9] We speculate that the potency of the modifications depends on the molecular structure and amount of fatty acids incorporated into the lipid bilayer. To examine the possible mechanism responsible for the differences in potency, we determined the composition of fatty acids in the DPPC bilayer after incubation with unsaturated fatty acids. As shown in Table 2, each fatty acid comprised over 25 mol% of the bilayer lipid component, and the relative composition was nearly identical for all fatty acids tested. This finding indicates that the different actions of the various unsaturated fatty acids can be attributed mainly to changes in the bilayer molecular structure, such as a kinked conformation contributed by the double bond, and not to the amount of fatty acids incorporated into the lipid bilayer.

Effects of Fatty Acids Contained in W/O/W Multiple

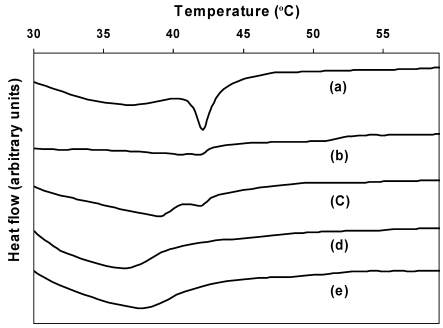

Fig. 5. Differential Scanning Calorimetry Thermograms of the DPPC Bilayer after Treatment with a W/O/W Multiple Emulsion Containing Fatty Acids

The temperature was scanned at 1 °C/min. (a) Control, (b) SA, (c) OA, (d) EPA, and (e) DHA.

Emulsions on Membrane Fluidity Figure 5 shows the DSC curves of the DPPC bilayer treated with emulsions containing various fatty acids. We observed a sharp endothermic peak at about 42 °C and a broad endothermic peak at below 40 °C in the DPPC bilayer treated with the emulsion without fatty acid (control). The sharp endothermic peak reflects the phase transition of pure DPPC domains and the broad endothermic peak reflects the disordered fluid domains that were altered by the treatment with emulsion in the membrane. The emulsion itself induced a marked change in the lipid bilayer structure because the emulsion contains large amounts of unsaturated lipids, such as triolein and egg yolk phospholipids. The endothermic peak at about 42 °C decreased after treatment with the emulsion containing OA and disappeared completely after treatment with the emulsion containing DHA or EPA. Table 3 lists the fluorescence anisotropy values of DPH in the bilayer treated with the emulsions at 37 °C. These values decreased markedly with treatment with emulsion containing unsaturated fatty acids. Table 4 summarizes the detergent insolubility values of the DPPC bilayer treated with emulsion. A large amount of DPPC was dissolved in Triton X-100 in all samples, which showed only very low levels of insolubility. These results demonstrate that unsaturated fatty acids act efficiently on the DPPC bilayer, even when included in emulsion form, which obviously changed the physical properties of the DPPC bilayer.

In conclusion, we clarified the structural changes in a DPPC bilayer induced by treatment with various fatty acids. Treatment with unsaturated fatty acids markedly altered the physical properties of the DPPC bilayer in a dose-dependent manner. DHA and EPA induced the most pronounced modifications of the bilayer physical properties. Treatment with unsaturated fatty acids in the form of an emulsion caused similar modification of the bilayer. The potency order of the fatty acids' effects on the lipid bilayer was consistent with the absorption enhancement effects.[7—9] These results suggest that changes in the lipid bilayer structure contribute to the absorption enhancement effect. We believe that our findings provide significant information about the functions of DHA and EPA.

Table 3. Fluorescence Anisotropy of DPH in the DPPC Bilayer after Treatment with a W/O/W Multiple Emulsion Containing Fatty Acids at 37 °C

	Fluorescence anisotropy
Control[a)]	0.305±0.007
SA	0.346±0.002
OA	0.278±0.005
EPA	0.208±0.006
DHA	0.210±0.005

a) W/O/W multiple emulsion without fatty acids. Each value represents the mean± S.D. of three determinations.

Table 4. Detergent Insolubility of the DPPC Bilayer after Treatment with W/O/W Multiple Emulsions Containing Fatty Acids

	Detergent insolubility $(OD_{400\,nm}+TX\text{-}100)/(OD_{400\,nm}-TX\text{-}100)$
Control[a)]	0.045±0.002
SA	0.101±0.030
OA	0.012±0.005
EPA	0.007±0.003
DHA	0.008±0.008

a) W/O/W multiple emulsion without fatty acids. Each value represents the mean± S.D. of three determinations. TX-100, Triton X-100.

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