ELSEVIER

Biochimica et Biophysica Acta 1285 (1996) 91–100

BBA

Binding of daidzein to liposomes

Jukka Y.A. Lehtonen [a], Herman Adlercreutz [b], Paavo K.J. Kinnunen [a,*]

[a] *Department of Medical Chemistry, Institute of Biomedicine, University of Helsinki, P.O. Box 8 (Siltavuorenpenger 10), FIN-00014 Helsinki, Finland*

[b] *Department of Clinical Chemistry, Meilahti Hospital, University of Helsinki, Helsinki, Finland*

Received 5 March 1996; revised 8 July 1996; accepted 11 July 1996

Abstract

Turbidity and differential scanning calorimetry measurements revealed the plant derived antineoplastic isoflavone, daidzein, to bind to large unilamellar liposomes. Comparing different unsaturated phospholipids most pronounced aggregation due to daidzein was observed for phosphatidylinositol (PI) while the inclusion of cholesterol strongly attenuated the aggregation. Interestingly, aggregation was not observed for the structurally very closely related isoflavone, genistein. The extent of aggregation was nonlinearly dependent on the content of PI in egg phosphatidylcholine (eggPC) vesicles. The saturated dimyristoyl phospholipids, phosphatidylserine, phosphatidylcholine, phosphatidic acid, as well as phophatidylglycerol were also extensively aggregated by daidzein at 10°C, i.e., below their main phase transition temperature whereas their aggregation at 35°C in the fluid phase was strongly reduced. Vesicle aggregation could be accompanied by membrane fusion, however, neither contents mixing nor lipid mixing of the LUVs (large unilamellar vesicles) was observed in the presence of daidzein. Strong perturbation of the thermal phase behaviour of both dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylserine (DMPS) multilamellar vesicles by daidzein was revealed by differential scanning calorimetry. More specifically, for DMPC increasing quantities of daidzein progressively decreased both the main transition temperature T_m and its enthalpy whereas for DMPS a decrease in ΔH was not observed, thus indicating the modes of interaction of daidzein with these phospholipids to differ. Our results indicate daidzein to reside in the polar headgroup/interfacial region of PI and PS membranes. The interactions of daidzein with phospholipids could represent an additional contributor to the growing list of effects of this isoflavone on cellular functions.

Keywords: Drug–lipid interaction; Isoflavonoid; Liposome; Aggregation; Phosphatidylinositol

Abbreviations: DMPS, 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine; DMPA, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidic acid; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; eggPG, egg phosphatidylglycerol; eggPC, egg phosphatidylcholine; brainPS, bovine brain phosphatidylserine; PI, yeast phosphatidyl inositol; DSC, differential scanning calorimetry; T_m, main phase transition temperature; T_p, pretransition temperature; ΔH_m, main phase transition enthalpy; ΔH_p, pretransition enthalpy; LUVs, large unilamellar vesicles; MLVs, multilamellar vesicles; A_{400}, absorbance at 400 nm; PLC, phospholipase C; PKC, protein kinase C; ANTS, 8-aminonaphthalene-1,3,6-trisulfonate; DPX, *p*-xylylenebis(pyridium bromide); Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-diacylphosphatidylethanolamine; NBD-PE, *N*-(7-nitro-2-1,3-bentzoxadiazol-4-yl-phosphatidylethanolamine

* Correspondig author. Fax: +358 0 1918276; e-mail: Paavo.Kinnunen@Helsinki.Fi.

PII S0005-2736(96)00154-X

1. Introduction

Plants provide a rich source of pharmaceutically active agents, ranging from toxins and carcinogens to chemopreventive compounds. Epidemiological studies have revealed the traditional soy-based Asian diet to be associated with a diminished risk for cancer [1–4]. It has long been known that soybeans contain isoflavones, mainly genistein (5,7,4′-trihydroxyisoflavone) and daidzein (7,4′-dihydroxyisoflavone) [5] (see Fig. 1. for the chemical structures). These compounds were identified in human biological fluids [6–8] and particularly high concentrations were found in the plasma and urine of subjects on traditional Japanese diet [9,10]. Importantly, the levels are high enough to suggest that these plant-derived components may exert cancer protective effects in vivo, as follows. These compounds have numerous biological activities in vitro [31,32], among the most interesting being inhibition of proliferation of various cancer cell lines and induction of differentiation of leukemic cells [11–26,64,65]. Genistein and to a much lesser degree also daidzein are inhibitors of protein tyrosine kinases [27–29]. Genistein as well as to some extent also daidzein were shown to inhibit malignant angiogenesis at physiological concentrations in vitro [30]. To this end, several anticancer compounds such as adriamycin, daunomycin, taxol and ET-18-OCH_3 (an antineoplastic etherphospholipid) interact with membranes and this property has been suggested to play a role in their mechanisms of action [33–36].

The lipophilicity of daidzein readily suggests that this compound should interact with biological membranes. Certain flavonoids, i.e., quercetin, hesperetin, and naringenin were recently demonstrated to partition into liposomal bilayers as revealed by differential scanning calorimetry [66]. In the present study, daidzein was found to associate with phospholipids, as shown by turbidity and by differential scanning calorimetry measurements. Binding of this compound to large unilamellar vesicles PI resulted in their aggregation whereas this was not observed for the structurally closely related isoflavone, genistein. Our results further show that the structural and physical requirements for daidzein induced aggregation are best met by liposomes composed of either fluid PI or gel phase DMPS. Daidzein also alters the physical properties of liposomal membranes, evident as changes in the thermal phase behaviour upon DSC. The possible consequences of the lipid association of daidzein are discussed.

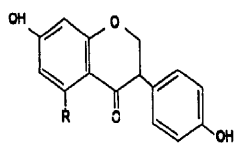


Fig. 1. The chemical structures of daidzein (R = H) and genistein (R = OH).

2. Materials and methods

2.1. Materials

DMPS was from Alexis (Läufelfingen, Switzerland), and brainPS, PI, DMPC, DMPG, eggPG, sphingosine, and cardiolipin were from Sigma. EggPC as well as the labelled phospholipids, NBD-PE and Rh-PE, were from Avanti Polar lipids (Alabaster, AL, USA). No impurities were detected in any of the above lipids upon thin-layer chromatography on silicic acid coated plates (Merck, Darmstadt, Germany) using chloroform/methanol/water (65:25:4, v/v) as the solvent system and examination of the plates after iodine staining. The concentrations of the nonfluorescent phospholipids were determined by phosphorus assay [37]. Daidzein was synthesized as described previously [7] and genistein was from Karl Roth GmbH (Karlsruhe, Germany).

2.2. Liposome preparation

Lipids were dissolved in chloroform and after mixing of the desired compositions the solvent was removed under a stream of nitrogen. The dry lipid residues were then maintained under reduced pressure for one hour and subsequently hydrated in 20 mM Tris, 0.1 mM EDTA, pH 7.4, at least 10°C above the gel → liquid crystalline transition temperature (T_m) of the phospholipid in question. To obtain large unilamellar vesicles (LUVs) the dispersions were extruded through Millipore (Bedford, MA, USA) 0.1 μm pore size polycarbonate filters using a LiposoFast low pressure homogenizer (Avestin, Ot-

tawa, Canada) essentially as described [38,39]. Resulting dispersions were optically clear.

2.3. Turbidity measurements

As a qualitative estimate of liposome aggregation absorbance was measured at 400 nm using a Shimadzu Graphicord spectophotometer and quartz cuvettes with an optical path of 1 cm. We observed daidzein to adsorb to cuvettes and therefore their careful cleaning was important. Unless otherwise specified experiments were performed at 25°C in 20 mM Tris, 0.1 mM EDTA, pH 7.4 and at a lipid concentration of 50 μM. Daidzein as well as genistein were added as ethanol solution (2 mg/ml). Daidzein but not genistein has a tendency to crystallize in ethanol solution. Therefore, to ensure complete solubility solutions used were heated and vigorously mixed prior to use. As a control ethanol was added to vesicles and caused no turbidity changes whatsoever. Likewise, the addition of daidzein to buffer in the absence of lipid had no influence on A_{400}.

2.4. Lipid mixing

For the resonance energy transfer assay, 1 mol% each of NBD-PE and Rh-PE were incorporated into liposomes [40]. Under these conditions, NBD fluorescence was quenched by Rh. These labelled liposomes were subsequently mixed in an overall phospholipid stoichiometry of 1:9 with vesicles containing no fluorescent lipids. NBD fluorescence at 530 nm was recorded continously using excitation at 455 nm. Lipid mixing would be evident as an increase of fluorescence resulting from the dilution of the fluorescent lipids into unlabelled liposomes which decreases the efficiency of resonance energy transfer between NBD and Rh.

2.5. Aqueous contents mixing

The assay for monitoring the mixing of the aqueous contents upon vesicle fusion is based on the collisional quenching of ANTS by DPX upon merging of the vesicles [41]. Vesicles were prepared in either 25 mM ANTS/45 mM NaCl/0.1 mM EDTA, 20 mM Tris, and pH 7.4 or 90 mM DPX/0.1 mM EDTA, 20 mM Tris, pH 7.4. There was no significant osmotic gradient across the membrane during the measurement [41]. Vesicles containing either ANTS or DPX were subsequently mixed in the cuvette. The fluorescence scale was set such that vesicles encapsulating both ANTS and DPX represented 0% fluorescence (complete fusion) whereas vesicles containing only ANTS yielded 100% fluorescence (no fusion). Excitation was at 360 nm and emission at 530 nm.

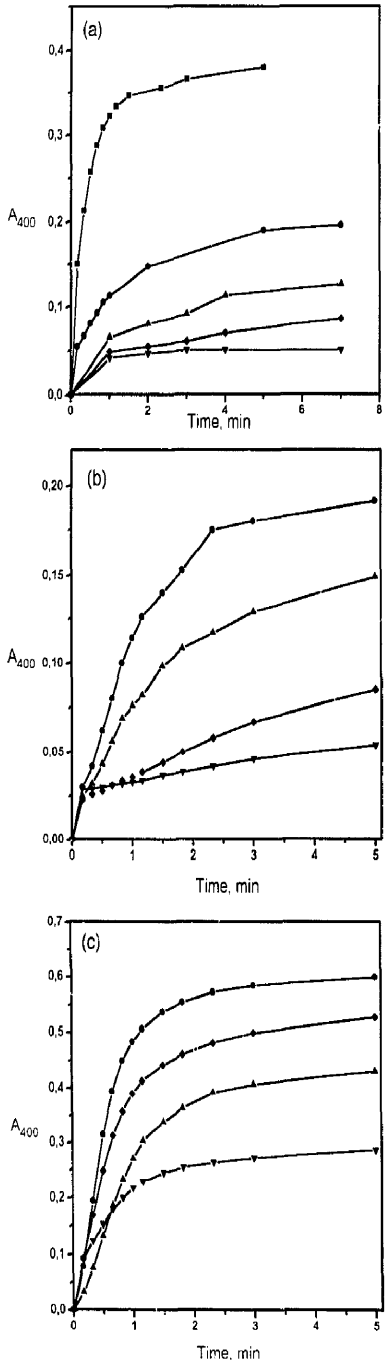
2.6. Differential scanning calorimetry (DSC)

Liposomes were prepared by evaporating the drug/lipid mixtures in chloroform to dryness under a stream of nitrogen, the dry lipid residues were then maintained under reduced pressure for overnight and thereafter hydrating the samples with the buffer above the main transition temperature of the lipid in question to obtain a lipid concentration of 0.7 mM. After hydration samples were cooled to 0°C and allowed to equilibrate at least overnight. DSC measurements were performed using a high sensitivity calorimeter DASM-4 (Biopribor, Pushchino, Russia) at a heating rate of 0.5C°/min. The transition enthalpies were determined by planimetry of the peaks using the internal electrical power calibration signal as a reference.

3. Results

3.1. Aggregation of LUVs

Addition of daidzein to PI LUVs caused an increase in absorbance at 400 nm, indicating vesicle aggregation, Fig. 2a. This effect of daidzein was not specific for PI and aggregation was observed also for other unsaturated phospholipids, the extent of drug-induced turbidity increase diminishing in the sequence PI > PS > PG > PA > PC, Fig. 2a. Binding of daidzein to PI was rapid and maximal increase in turbidity was reached within approximately six minutes after the addition of the drug. In contrast to the above phospholipids, no turbidity changes were observed for vesicles composed of cardiolipin or sphingosine (data not shown). On the timescale (30 min) of our measurements and at drug/lipid stoichiometry of 2.4:1 the structurally closely related isoflavone,



genistein, had essentially no effect on the absorbance of the vesicles composed of any of the lipids usde in the present study (data not shown).

We then studied the possibility that the phase state of the phospholids could be important for daidzein induced vesicle aggregation. For this purpose daidzein was added at a drug/lipid ratio of 2.4:1 to LUVs of different dimyristoyl phospholipids, Fig. 2b. At 35°C, i.e., above the main transition temperature, most intensive aggregation was evident with DMPS. The timescale of aggregation (maximal increase in turbidity within 10 min) was comparable to brainPS. When aggregation was measured at 10°C (below the T_m values of the phospholipids used) the extent of turbidity increase for these dimyristoyl lipids was considerably higher (approx. 3-fold) than in the liquid crystalline state, thus indicating more vigorous aggregation, Fig. 2c. This would suggest that it is the properties of the membrane surface that determine the aggregation of vesicles. Again, of the phospholipids studied DMPS exhibited the most intense aggregation, the absorbance increasing from 0.2 to 0.6 within 5 minutes. Similar effect was also observed for DPPC ($T_m \approx 41$°C). For this lipid in the gel phase daidzein induced a rapid aggregation of vesicles whereas at 50°C the extent of absorbance increase due to daidzein was strongly attenuated.

We then proceeded to study the dependency of aggregation of brainPS and PI LUVs on [daidzein]. Lipid concentration was held constant at 50 μM while increasing amounts of daidzein were added, Fig. 3. Aggregation of these vesicles by daidzein appeared to be critically dependent on the drug/lipid

Fig. 2. (a) Time courses of daidzein induced turbidity changes for large unilamellar vesicles composed of yeastPI (■), brainPS (●), eggPG (▲), eggPA (◆), or eggPC (▼). The concentration of lipids was 50 μM in 20 mM Tris, 0.1 mM EDTA, pH 7.4, and measurements were conducted at 25°C. Daidzein was dissolved in ethanol (2.0 mg/ml) and was added to yield a 2.4:1 drug/lipid molar ratio (i.e., 120 nmol of daidzein in 15.5 μl ethanol). The sample was subsequently vigorously mixed by vortexing prior to the measurement of A_{400}. (b) Aggregation of LUVs composed of DMPS (●), DMPG (▲), DMPA (◆), or DMPC (▼) by daidzein at 35°C. The daidzein/lipid ratio as well as other conditions were otherwise identical to those described for panel a. (c) Lipids and experimental conditions were as for panel b except that temperature was 10°C.

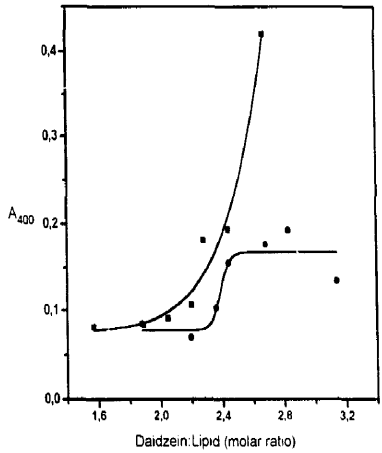


Fig. 3. Aggregation of yeastPI (■), and brainPS (●) vesicles at varying concentrations of daidzein. Lipid concentration was 50 μM. Absorbance at 400 nm was measured after a 15 min of incubation at 25°C. Conditions were otherwise as for Fig. 2.

ratio and a steep increase in turbidity occurs for PI upon reaching 2.2:1 stoichiometry. Below this stoichiometry only minor turbidity changes upon increasing [daidzein] were evident and as judged by absorbance the extent of aggregation of brainPS and PI vesicles was significantly reduced and comparable to that of eggPC LUVs.

3.2. Lipid mixing and contents mixing

To determine whether bilayer fusion occured during the daidzein-induced aggregation of vesicles both lipid as well as contents mixing assays were employed as described under Section 2. However, no evidence for these processes was obtained thus suggesting lack of fusion of bilayers during the aggregation of the vesicles (data not shown).

3.3. Dependency of aggregation on the acidic phospholipid content in eggPC

As shown above, unsaturated PI and brainPS vesicles were aggregated by daidzein whereas this was not the case for eggPC liposomes. Accordingly, we then characterized the dependency of daidzein-induced aggregation of LUVs on the content of PI, brainPS, or eggPG in eggPC. For this purpose, vesicles with different acidic phospholipid/eggPC molar ratios were prepared. Both yeastPI and brainsPS LUVs showed marked sensitivity on their content in eggPC, Fig. 4. Varying the amount of PI in eggPC between 40 and 90 mol% caused clearly nonlinear changes in the daidzein induced aggregation. Interestingly, for brainPS/eggPC vesicles more vigorous aggregation was seen at lower contents of brainPS, reaching maximum at 65 mol% of the acidic lipid. For eggPG only rather small variation in the drug-induced aggregation of the different eggPG/eggPC mixtures was observed, Fig. 4.

3.4. Effect of cholesterol and ionic strength

Interestingly, already 10 mol% of cholesterol in a PI matrix strongly reduced the extent of daidzein induced vesicle aggregation, evident as a ≈ 50% attenuation in absorbance increase at 400 nm, Fig. 5. However, increase in the cholesterol content up to 30 mol% does not lead to significant further reduction in aggregation. Increasing concentrations of NaCl de-

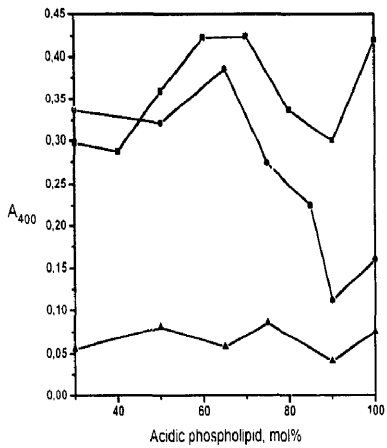


Fig. 4. Aggregation by daidzein of mixed eggPC LUVs containing yeastPI (■), brainPS (●) or eggPG (▲), as a function of the mol% of the acidic phospholipid of the total lipid. Absorbance was recorded after a 15 min of incubation at 25°C. Experimental conditions were otherwise as described for Fig. 2.

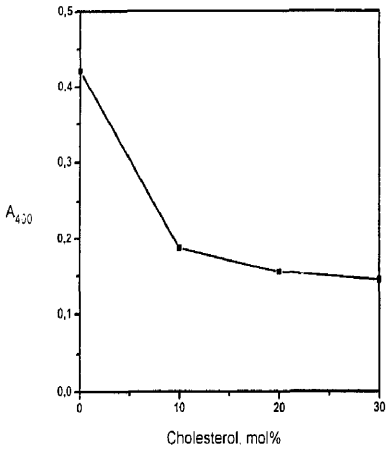


Fig. 5. Influence of the content of cholesterol on the aggregation of yeastPI LUVs by daidzein. A_{400} was measured after a 15 min incubation at 25°C. Conditions were otherwise as described in the caption for Fig. 2.

creased aggregation of yeastPI LUVs by daidzein, Fig. 6. More specifically, 50 mM NaCl reduced the extent of aggregation by 50% whereas at higher salt concentrations no further decrease coud be observed.

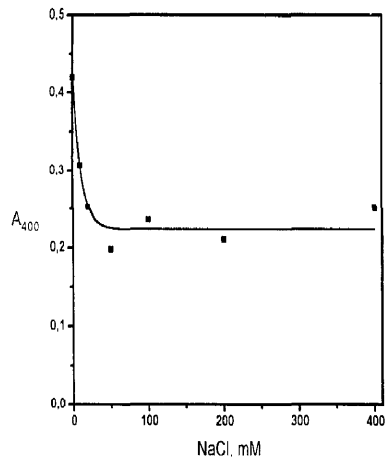


Fig. 6. Effect of [NaCl] on the aggregation of yeastPI vesicles by daidzein, measured as increased absorbance after a 15 min incubation at 25°C. Experimental conditions were as described for Fig. 2.

3.5. Differential scanning calorimetry

Upon a temperature scan from 10°C to 30°C the presence of daidzein at a 1:1 drug/lipid ratio decreased the T_m of DMPC from 25.0 to 23.5°C. Likewise, increasing contents of daidzein progressively

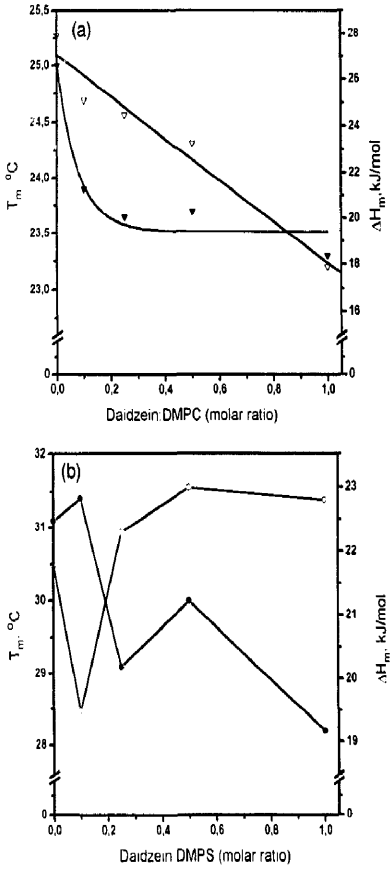


Fig. 7. (a) Variation of the main transition temperature T_m and ΔH as a function of the content of daidzein in DMPC. The filled symbols (▼●) indicate the transition temperatures (scale on the left) and the open symbols (▽○) the enthalpy (scale on the right). The experimental conditions were otherwise as described for Fig. 2. (b) Summary of effects of daidzein on the phase behaviour of DMPS. The symbols and conditions were as for panel a.

reduced the main transition enthalpy ΔH_m from 28 to 18 kJ/mol, Fig. 7a. The main transition peak was also broadened, revealing reduction in transition cooperativity. Pretransition of DMPC which occurs at 15°C was abolished already at 1:10 drug/lipid ratio. The above results suggest that daidzein could at least partly reside in the hydrocarbon region of DMPC MLVs.

Although of smaller magnitude, effects quantitatively similar to those described above for DMPC were observed also for DPPC. In brief, the transition temperatures and enthalpies decreased for both the pretransition and main transition. Accordingly, at the drug/lipid ratio of 0.65:1 T_m of DPPC decreased from 41.6°C to 41.3°C while ΔH_m was reduced from 39.4 kJ/mol to 32.5 kJ/mol. For comparison, at a 0.65:1 drug/lipid ratio, genistein, a close structural analog of daidzein, reduced the T_m of DPPC to 40.7°C and ΔH_m to 33.0 kJ/mol.

Both daidzein and genistein reduced the enthalpy as well as temperature of the pretransition of DPPC in a concentration dependent manner. Thus, daidzein at a drug/lipid stoichiometry of 1:1 suppressed T_p of DPPC from 35.5°C to 33.5°C and its enthalpy from 7.4 kJ/mol to 3.5 kJ/mol. With genistein, these values were 32°C and to 4.3 kJ/mol, respectively.

Under the conditions of our calorimetry scans DMPS revealed an endotherm at 31.2°C. This transition temperature decreased along with increasing daidzein content to 28°C, Fig. 7b. In contrast to DMPC, the enthalpy of DMPS MLVs is slighly increased upon the inclusion of increasing quantities of daidzein (up to 1:1 drug/lipid ratio).

4. Discussion

The present results clearly demonstrate daidzein to incorporate into phospholipid bilayers, affecting their thermal phase properties and causing the aggregation of liposomes composed of acidic phospholipids. Aggregation studies comparing different phospholipids revealed that DMPS and PI exhibit most intensive aggregation by daidzein whereas above the T_m for DMPC no change in the absorbance occurs. Aggregation was not observed for the structurally closely related isoflavone, genistein. Interestingly, increasing [daidzein] decreases the T_m as well as the ΔH_m of DMPC while for DMPS T_m decreases and the enthalpy is increased. These data demonstrate that daidzein interacts with both DMPC and DMPS but the modes of interaction are different. At pH 7.4 daidzein is partly deprotonated and bears a net negative charge. Therefore, the association of daidzein with PS and PI is probably not electrostatic in nature. Likewise, membrane association of daidzein could enhance its deprotonation.

The chemical structure of daidzein readily suggests it to locate in the interfacial region of the membrane. The structure of this region is a complex mixture of water, glycerol, carbonyl, and methylene groups and for a lipid such as dioleoyl phosphatidylcholine it accounts for half of the total thermal thickness of the bilayer [42]. More specifically, the interfacial region resides between the more superficial hydrated polar headgroup region of the amphiphilic lipids adjacent to bulk water and the hydrocarbon interior. Incorporation of amphiphilic tripeptides in the bilayer is driven by hydrophobic interactions while also hydrogen bonding is important for the membrane incorporation of these compounds [43]. Although the limitations of our data make it too preliminary to dwell in more detail on the location of daidzein in bilayers it is rather clear that similar mechanisms should be relevant also for daidzein. Various attracting and repulsing forces govern the aggregation of vesicles and the minimal energy state of the system is determined by their balance. While vesicles are mutually attracted by van der Waals interactions electrostatic repulsion prevails between vesicles composed of phospholipids with a net negative charge. At separation distances of < 10 Å vesicle interaction is dominated by the strongly repulsive hydration force which at small separations is attributed to the energy required to remove water as the vesicle–vesicle separation distance decreases [44]. Hydration, in turn, arises from the interaction of polar headgroup region with the adjacent water layer by hydrogen bonding. Phosphatidylserine and phosphatidylinositol are both negatively charged phospholipids and are both strongly hydrated in the absence of Ca^{2+} [45,46]. The importance of the hydration force in preventing the fusion of liposomes is readily evident by comparing poorly hydrated phospholipids such as phosphatidylethanolamine, or phosphatidylserine in the presence of Ca^{2+} which aggregate and fuse much

more readily when compared to the strongly hydrated lipids, such as phosphatidylcholine [47]. Aggregation of the liposomes by daidzein clearly reveal that the properties of the bilayer surface must be rather drastically influenced by this compound. Changes in lipid hydration could be involved. In keeping with this notion the inclusion of increasing amounts of cholesterol into yeastPI vesicles decreased the extent of their daidzein induced aggregation. Cholesterol has been shown to increase hydration of phospholipids [67]. Also increasing [NaCl] attenuated the extent of vesicle aggregation by daidzein. This effect could be related to changes in the polar headgroup conformation [48].

Aggregation or close approach of vesicles is a prerequisite for the fusion of liposomes. The mechanisms of liposome fusion have been intensively studied [49] and it is clear that the ability of the fusion promoting lipids to form the inverted hexagonal phase H_{II} is closely related to this phenomenon [50,51]. Although PS may, under proper conditions, form the so-called cochleate structures neither brainPS nor yeastPI have been reported to form the H_{II} phase [52]. Interestingly, the absence of contents mixing as well as lipid mixing of brainPS and yeastPI vesicles indicates that they do not fuse due to daidzein. The absence of lipid mixing also indicates that there is no hemifusion of these vesicles which occurs for instance when PS or PA vesicles are aggregated by Mg^{2+} [53,54].

Our results show that the structural and physical requirements for daidzein induced aggregation are best met by liposomes composed of fluid PI and gel phase DMPS. The extent of aggregation also depends in a clearly nonlinear manner on the surface concentration of yeastPI in eggPC vesicles. Consequences of drug-induced changes in the physical properties of membranes are in general poorly understood. Yet, interactions of daidzein with phospholipids could represent an important contributor to the growing list of effects of daidzein on cellular functions. The concentrations of daidzein used in these experiments are similar to those causing inhibition of cell growth [55]. In general binding of peptides and drugs to membranes has been suggested to concentrate drugs to surfaces facilitating the binding of drugs and peptide hormones to their receptors [56–58]. Daidzein might alter the phosphoinositide–protein interactions in membranes [59]. Both PI and PS are though to be located in the inner leaflet of plasma membranes and have been suggested to exist in domains [60]. The inositol phospholipids further serve as important substrates for specific phospholipases C. Protein kinase C requires in addition to Ca^{2+} and diacylglycerol also phosphatidylserine for activity [61–63]. Our results allow us to hypothesize that daidzein could bind in the polar headgroup/interfacial region of membrane domains enriched in PI and PS and subsequently interfere with the association of PI- PLC and PI-kinases and PKC with these acidic phospholipids.

Acknowledgements

We wish to thank B. Rantala for skillful technical assistance. This study was supported by grants from Finnish State Medical Research Council, Biocentrum Helsinki, Sigrid Juselius Foundation (P.K.J.K.), Finnish Medical Foundation (J.Y.A.L.), and by NIH grant/ ROI CA 56289-01 (H.A.).

References

[1] Severson, R.K., Nomura, A.M.Y., Grove, J.S. and Stemmerman, G.N. (1989) Cancer Res. 49, 1857–1860.
[2] Lee, H.P., Gourley, L., Duffy, S.W., Estéve, J., Lee, J. and Day, N.E. (1991) Lancet 337, 1197–1200.
[3] Adlercreutz, H., Fotsis, T., Bannwart, C., Wähälä, K., Brunow, G. and Hase, T. (1991) Clin. Chim. Acta 199, 263–278.
[4] Watanabe, S. and Koessel, S. (1993) J. Epidem. 3, 47–61.
[5] Walz, E. (1931) Justus Liebigs Annln. Chem. 498, 118–155 (in German).
[6] Bannwart, C., Fotsis, T., Heikkinen, R. and Adlercreutz, H. (1984) Clin. Chim. Acta 136, 165–172.
[7] Adlercreutz, H., Musey, P.I., Fotsis, T., Bannwart, C., Wähälä, K., Mäkelä, T., Brunow, G. and Hase, T. (1986) Clin. Chim. Acta 158, 147–154.
[8] Adlercreutz, H., Honjo, H., Higashi, A., Fotsis, T., Hämäläinen, E., Hasegawa, T. and Okada, H. (1991) Am. J. Clin. Nutr. 54, 1093–1100.
[9] Adlercreutz, H., Markkanen, H. and Watanabe, S. (1993) Lancet 342, 1209–1210.
[10] Adlercreutz, C.H.T., Goldin, B.R., Gorbach, S.L., Höckerstedt, K.A.V., Watanabe, S., Hämäläinen, E.K., Markkanen, M.H., Mäkelä, T.H., Wähälä, K.T., Hase, T.A. and Fotsis, T. (1995) J. Nutr. 125, 757S–770S.
[11] Hirano, T., Oka, K. and Akiba, M. (1989) Res. Commun. Chem. Pathol. Pharmacol. 64, 69–78.
[12] Okura, A., Arakawa, H., Oka, H., Yoshinari, T. and Mon-

den, Y. (1988) Biochem. Biophys. Res. Commun. 157, 183–189.
[13] Kiguchi, K., Constantinou, A.I. and Huberman, E. (1990) Cancer Commun. 2, 271–278.
[14] Honma, Y., Okabe-Kado, J., Kasukabe, T., Hozumi, M. and Umezawa, K. (1990) Jap. J. Cancer Res. 81, 1132–1136.
[15] Constantinou, A., Kiguchi, K. and Huberman, E. (1990) Cancer Res. 50, 2618–2624.
[16] Makishima, M., Honma, Y., Hozumi, M., Nagata, N. and Motoyoshi, K. (1993) Biochim. Biophys. Acta 1176, 245–249.
[17] Kuriu, A., Ikeda, H., Kanakura, Y., Griffin, J. D., Druker, B., Yagura, H., Kitayama, H., Ishikawa, J., Nishiura, T., Kanayama, Y., Yonezawa, T. and Tarui, S. (1991) Blood 78, 2834–2840.
[18] Peterson, G. and Barnes, S. (1991) Biochem. Biophys. Res. Commun. 179, 661–667.
[19] Kondo, K., Tsuneizumi, K. and Oishi, M. (1991) Cancer Res. 51, 5398–5404.
[20] Traganos, F., Ardelt, B., Halko, N., Bruno, S. and Darzynkiewicz, Z. (1992) Cancer Res. 52, 6200–6208.
[21] Schweigerer, L., Christeleit, K., Fleischmann, G., Adlercreutz, H., Wahala, K., Hase, T., Schwab, M., Ludwig, R. and Fotsis, T. (1992) Eur. J. Clin. Invest. 22, 260–264.
[22] Jing, Y., Nakaya, K. and Han, R. (1993) Anticancer Res. 13, 1049–1054.
[23] Matsukawa, Y., Marui, N., Sakai, T., Satomi, Y., Yoshida, M., Matsumoto, K., Nishino, H. and Aoike, A. (1993) Cancer Res. 53, 1328–1331.
[24] Makishima, M., Honma, Y., Hozumi, M., Sampi, K., Hattori, M., Umezawa, K. and Motoyoshi, K. (1991) Leuk. Res. 15, 701–708.
[25] Mousavi, Y. and Adlercreutz, H. (1993) Steroids 58, 301–304.
[26] Higashi, K. and Ogawara, H. (1994) Biochim. Biophys. Acta 1221, 29–35.
[27] Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S-I., Itoh, N., Shibuya, M. and Fukami, Y. (1987) J. Biol. Chem. 262, 5592–5595.
[28] Ogawara, H., Akiyama, T., Watanabe, S.-I., Ito, N., Kobori, M. and Seoda, Y. (1989) J. Antibiotics XLII, 340–343.
[29] Teraoka, H., Ohmura, Y. and Tsukada, K. (1989) Biochem. Intern. 18, 1203–1210.
[30] Fotsis, T., Pepper, M., Adlercreutz, H., Fleischmann, G., Hase, T., Montesano, R. and Schwigerer, L. (1993) Proc. Natl. Acad. Sci. USA 90, 2690–2694.
[31] Keung, W. M. and Vallee, B. L. (1993) Proc. Natl. Acad. Sci. USA 90, 1247–1251.
[32] Keung, W-M. and Vallee, B.L. (1993) Proc. Natl. Acad. Sci. USA 90, 10008–10012.
[33] Tritton, T.R. and Yee, G. (1992) Science 217, 248–250.
[34] Mustonen, P. and Kinnunen, P.K.J. (1991) J. Biol. Chem. 266, 6302–6307.
[35] Maurer, N., Prenner, E., Paltauf, F. and Glatter, O. (1994) Biochim. Biophys. Acta 1192, 167–176.
[36] Balasubramanian, S.V. and Straubinger, R.M. (1994) Biochemistry 33, 8941–8947.
[37] Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468.
[38] Olson, P., Hunt, C.A., Szonka, F.C., Vail, W.J., Papahadjopoulos, D. (1979) Biochim. Biophys. Acta 557, 9–23.
[39] MacDonald R.C., MacDonald, R.I., Menco, B.M., Takeshita, K., Subbarao, N.K. and Hu, L.R. (1991) Biochim. Biophys. Acta 1061, 297–303.
[40] Hoekstra, D. (1982) Biochemistry 21, 2833–2840.
[41] Ellens, H., Bentz, J. and Szonka, F.C. (1985) Biochemistry 24, 3099–3106.
[42] White, S.H. and Wimley, W.C. (1994) Curr. Op. Struct. Biol. 4, 79–86.
[43] Jacobs, R.E. and White, S.H. (1989) Biochemistry 28, 3421–3437.
[44] Lis, L.J., McAlister, M., Fuller, N., Rand, R.P. and Parsegian, V.A. (1982) Biophys. J. 37, 667–672.
[45] Hauser, H. (1984) Biochim. Biophys. Acta 772, 37–50.
[46] Dluhy, R.A., Cameron, D.G. and Mantsch, H.H. (1984) Biochim. Biophys. Acta 792, 182–185.
[47] Wilschut, J., Düzgünes, N., Fraley, R. and Papahadjopoulos, D. (1980) Biochemistry 19, 6011–6021.
[48] Roux, M. and Bloom, M. (1991) Biophys. J. 60, 38–44.
[49] Ellens, H., Siegel, D.P., Alford, D., Yeagle, P.L., Boni, L., Lis, L.J., Quinn, P.J. and Bentz, J. (1989) Biochemistry 28, 3692–3703.
[50] Kinnunen, P.K.J. (1992) Chem. Phys. Lipids 63, 251–258.
[51] Glaser, P.E. and Gross, R.W. (1994) Biochemistry 33, 5805–5812.
[52] Boggs, J.M. (1987) Biochim. Biophys. Acta 906, 353–404.
[53] Leventis, R., Gagne, J., Fuller, N., Rand, R.P. and Silvius, J.R. (1986) Biochemistry 25, 6978–6987.
[54] Wilschut, J., Düzgünes, N. and Papahadjopoulos, D. (1981) Biochemistry 20, 3126–3133.
[55] Barnes, S., Peterson, G., Grubbs, C. and Setchell, K. (1994) In Diet and Cancer – Advances in Experimental Medicine and Biology, Vol. 354 (Jacobs, M.M., eds.), Plenum Press, New York.
[56] Rhodes, D.G., Sarmiento, J.G. and Herbette, L.G. (1985) Mol. Pharmacol. 27, 612–623.
[57] Herbette, L.G., Chester, D.W. and Rhodes, D.G. (1986) Biophys. J. 49, 91–94.
[58] Sargent, D.F. and Schwyzer, R. (1986) Proc. Natl. Acad. Sci. USA 83, 5774–5778.
[59] Verbist, J., Gadella Jr., T.W.J., Raeymaekers, L., Wuytack, F., Wirtz, K.W.A. and Casteels, R. (1991) Biochim. Biophys. Acta 1063, 1–6.
[60] Gascard, P., Sauvage, M., Sulpice, J-C. and Giraud, F. (1993) Biochemistry 32, 5941–5948.
[61] Epand, R.M. and Bottega, R. (1988) Biochim. Biophys. Acta 944, 144–154.
[62] Orr, J.W. and Newton, A.C. (1992) Biochemistry 31, 4661–4667.
[63] Goldberg, E.M., Lester, D.S., Borchardt, D.B. and Zidovetzki, R. (1994) Biophys. J. 66, 382–393.

[64] Higashi, K. and Ogawara, H. (1992) Chem. Pharm. Bull. 40, 157–160.
[65] Setchell, K. D. R. and Adlercreutz, H. (1988) in Role of the Gut Flora in Toxicity and Cancer (Rowland, I., ed.), pp. 315–345, Academic Press, London.
[66] Saija, A., Bonina, F., Trombetta, D., Tomaino, A., Montenegro, L., Smeriglio, P. and Castelli, F. (1995) Int. J. Pharm. 24, 1–8.
[67] Yeagle, P.L., Hutton, W.C., Huang, C.-H. and Martin, B.R. (1977) Biochemistry 16, 4344–4349.