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Localization and interaction of genistein with model membranes formed with dipalmitoylphosphatidylcholine (DPPC)

Bożena Pawlikowska-Pawlęga [a,*], Lucjan E. Misiak [b], Barbara Zarzyka [c], Roman Paduch [d], Antoni Gawron [a], Wiesław I. Gruszecki [e]

[a] Department of Comparative Anatomy and Anthropology, Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland
[b] Department of Surface Physics and Nanostructures, Institute of Physics, Maria Curie-Skłodowska University, Pl. M. Curie-Skłodowskiej 1, 20-031 Lublin, Poland
[c] Cell Biology Department, Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland
[d] Department of Virology and Immunology, Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland
[e] Department of Biophysics, Institute of Physics, Maria Curie-Skłodowska University, Pl. M. Curie-Skłodowskiej 1, 20-031 Lublin, Poland

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ABSTRACT

The effect of genistein on the liposomes formed with dipalmitoylphosphatidylcholine was studied with the application of Fourier-transform infrared spectroscopy, nuclear magnetic resonance (^{1}H NMR) and electron paramagnetic resonance techniques. Membranous structures organization of human skin fibroblasts and colon myofibroblasts was also examined using fluorescence and electron microscopy. The strongest rigidifying effect of genistein with respect to polar head groups was concluded on the basis of the effect of the flavonoid on the shape of NMR lines attributed to $-N^+(CH_3)_3$ groups. The rigidifying effect of genistein with respect to the hydrophobic core of lipid membranes was also concluded from the genistein-dependent broadening of the NMR lines assigned to $-CH_2$ groups and terminal $-CH_3$ groups of alkyl chains. EPR data supported ordering effect of genistein of the hydrophobic core in the liquid–crystalline phase (L_α). The analysis of the FTIR spectra of the two-component liposomes showed that genistein incorporates into DPPC membranes via hydrogen bonding between the lipid polar head groups in the C–O–P–O–C segment and its hydroxyl groups. Both fluorescence microscopy and ultrastructural observation revealed changes in membranous structures organization as aftermath of genistein treatment. In conclusion, genistein localized within membranes changes the properties of membrane that can be followed by the changes inside cells being crucial for pharmacological activity of genistein used in cancer or other disease treatment.

1. Introduction

Flavonoids are pigments naturally occurring in plants and mostly derived from benzo-γ-pyrone. They are often incorporated into the people's daily diets [1]. Genistein (Fig. 1) is one of the most popular flavonoids due to the wide spectrum of biological activities and the beneficial effects on human health. It is known from its estrogenic activity, antioxidant and anticancer properties, prevention of osteoporosis and cardiovascular diseases [2,3]. Genistein binds to estrogen receptors α (ERα) and β (ERβ) and due to this property can be used as an alternative to the hormonal therapy for post-menopausal women [4]. It also shows antioxidant properties against oxidative damage of lipids [5].

One of the targets for flavonoids are membranes as well as internal compartments that are limited by these membranes. For this reason the interaction with membranes plays a crucial role in their biological activity and understanding of the effects of flavonoids on biomembranes may help to elucidate the mechanisms of action of flavonoids as anticancer agents and antioxidants. Alteration of lipid bilayer properties may result not only in antioxidant action of flavonoids but also in affecting the activity of integral proteins and hence influence transport and other membrane-related processes [6–11]. Indeed, it was found that genistein inhibited transport by multidrug resistance-related protein (MRP1) and accumulation of daunomycin was increased when cells were treated with 100 μmol/L of genistein in human pancreatic adenocarcinoma cell line [12]. In erythrocytes, genistein decreased the fluorescent dye efflux from erythrocytes and

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; EPR spectroscopy, electron paramagnetic resonance spectroscopy; FTIR spectroscopy, Fourier-transform infrared spectroscopy; L_α, lamellar liquid -crystalline phase; NMR spectroscopy, nuclear magnetic resonance spectroscopy; 16-SASL, 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy free radical

* Corresponding author at: Maria Curie-Skłodowska University, Faculty of Biology and Biotechnology, Institute of Biology and Biochemistry, Department of Comparative Anatomy and Anthropology, Akademicka 19, 20-033 Lublin, Poland. Tel.: +48 81 537 59 28; fax: +48 81 537 59 01.
E-mail addresses: bozka1996@o2.pl (B. Pawlikowska-Pawlęga), lucjan.misiak@poczta.umcs.lublin.pl (L.E. Misiak), basiaza@vp.pl (B. Zarzyka), rpaduch@poczta.umcs.lublin.pl (R. Paduch), agawron@poczta.umcs.lublin.pl (A. Gawron), wieslaw.gruszecki@umcs.pl (W.I. Gruszecki).

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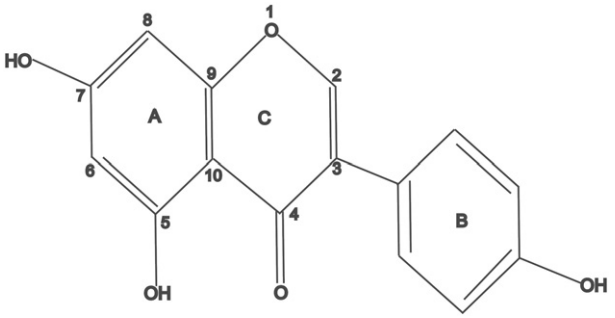

Fig. 1. The chemical formula of genistein.

thus inhibited MRP1 transport activity [13,14]. It was found that direct interaction with substrate-binding site of MRP1 is responsible for its inhibiting properties [15].

Genistein–lipid interactions were studied by several research groups. The interaction of genistein with the liposomes composed of different lipids was examined by authors who used calorimetry and turbidity measurements [16]. Fluorescence polarization anisotropy measurements were used in the study of the influence of genistein on the fluidity of liposome membranes [17]. Maniewska et al., using fluorescence spectroscopic and calorimetric investigation, have demonstrated that genistein affects lipid bilayers [18]. The alteration of bilayer fluidity by genistein was found by the research group of Łania-Pietrzak et al. [19].

The studies concerning effects of genistein on lipids did not however reveal detailed mechanisms responsible for genistein-membrane interactions. Investigation of such molecular interaction seems to be important from biological and medical standpoints. Thus in the present paper we applied the spin label electron paramagnetic resonance (EPR) measurements and nuclear magnetic resonance (^{1}H NMR) to study the effect of genistein on dynamic and structural properties of liposomes made of DPPC. Fourier-transform infrared spectroscopic (FTIR) investigation was also performed to address the problem of incorporation of genistein into lipid membranes. Using the electron and fluorescence microscopic techniques we have also investigated how genistein affects normal colon myofibroblast cells and human skin fibroblasts cells, considering especially membranous structures organization of these cells.

2. Materials and methods

2.1. Chemicals

Genistein (Sigma Chemical Co., USA) dissolved in ethanol (Merck, Germany) was used in the studies. The solution was kept in the dark. Spin label 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy free radical (16-SASL), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and tris (hydroxymethyl)aminomethane (TRIS) were purchased from Sigma Chemical Co. Deuterium oxide (D_2O) was purchased from ARMAR Chemicals Co. (Switzerland). Spin labels were dissolved in absolute ethanol and stored at 4 °C. All other chemicals were of the best quality available.

2.2. ^{1}H NMR measurements of DPPC liposomes

For the ^{1}H NMR spectroscopy measurements, mixtures of phospholipids and flavonoid were co-dissolved in chloroform/ethanol mixture (55:1, v:v) at the respective concentration. The concentration of the lipid in the sample was 3.2×10^{-2} M and of the flavonoid 3.2×10^{-4} M. After evaporating the solvents under a stream of nitrogen and then in vacuum (overnight) the samples were hydrated with D_2O (pH 7.4) and vigorously shaken (1 h) on a shaker at the temperature above the main phase transition of lipid (41 °C) until optical homogeneity of the mixture was observed. Then the lipid suspension was being sonicated for 10 min with a 20 kHz sonicator to yield a homogeneous lipid dispersion. NMR data were collected for samples of 0.6 ml vesicles suspension with 4 mM praseodymium trichloride ($PrCl_3$). ^{1}H NMR spectra were acquired on a Bruker Avance 300 NMR spectrometer using 5-mm probe with pulsed field gradient capabilities at a spinning speed of 300 MHz, at pulse length π/2 and acquisition temperature 333 K.

2.3. EPR measurements of DPPC liposomes

Multilamellar liposomes were obtained by shaking [8]. We used saturated DPPC liposomes, which resulted in an ordered phase that was interrupted by an insertion of the flavonoid. The concentration of the lipid (DPPC) in phosphate buffer was 10^{-5} M. The concentration of genistein was 5 mol% and of the spin label 1 mol% with respect to the lipid. Dispersion of multilamellar liposomes of DPPC (73 mg/ml of chloroform) was prepared by mixing solutions of respective compounds, evaporation of solvent, first in a stream of nitrogen and subsequently by vacuum (3 h). DPPC samples were hydrated with phosphate buffer (100 mM, pH 7.4) by vigorous shaking at the temperature above the main phase transition of lipid (41 °C) until optical homogeneity of the mixture was observed. The samples to be measured were placed in a 1.3-mm diameter capillary (Hyland Lab. Inc) and sealed with miniseal wax. EPR spectra were recorded with a SE/X-2547 (Radiopan, Poznań) spectrometer working in the X band and equipped with variable temperature-stabilizing unit in the following conditions: modulation amplitude 5 G in the case of spectra scanning and 10 G for determining an accurate position of the maxima, time constant 0.3 s, scan time 2 min, scan range 3200–3300 G. The n-SASL spin labels applied in the study are commonly used to monitor the fluidity of model membranes [20]. In the spectra the height of the center line (h_o), an empirical parameter related to an order parameter of the alkyl chain and the rate of alkyl motion of the spin label in a lipid core, reflecting the fluidity of a membrane, was analyzed [21]. All data are expressed as means ± SD (n = 3).

2.4. FTIR measurements

Infrared absorption spectra of flavonoid alone and with liposomes (DPPC) were recorded with the Fourier-transform infrared absorption spectrometer equipped with the attenuated total reflection set-up (ATR-FTIR). The concentration of genistein was 1 mol% and of the lipid 10^{-5} M. In the case of DPPC liposomes the samples were hydrated with TRIS buffer in D_2O (100 mM, pH 7.4) by vigorous shaking at a temperature above the main phase transition of lipid (41 °C). The samples were deposited on the ATR crystal element by evaporation from TRIS buffer in D_2O (pH 7.4). The spectra were then recorded with a Vector 33 spectrometer (Brucker, Germany). The internal reflection element was a ZnSe crystal (45° cut) yielding 10 internal reflections. Typically, 10 scans were collected, Fourier transformed and averaged for each measurement. Absorption spectra at a resolution of one data point every 2 cm^{-1} were obtained in the region between 4000 and 400 cm^{-1} using a clean crystal as the background. The instrument was being purged with argon for 40 min before and continuously during measurements. The ATR crystals were cleaned with organic solvent (ethanol). All experiments were done at 21 °C. The spectral analysis was performed with OPUS (Brucker, Germany) and Grams Al software from ThermoGalactic (USA).

2.5. Cell culture

HSFs, [human skin fibroblast cell line derived from freshly excised skin fragments in the Department of Virology and Immunology, UMCS, Lublin, Poland] were cultured in RPMI (1640) medium (Roswell