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Biophysical characterization of genistein–membrane interaction and its correlation with biological effect on cells — The case of EYPC liposomes and human erythrocyte membranes

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ABSTRACT

With application of EPR and ^{1}H NMR techniques genistein interaction with liposomes formed with egg yolk lecithin and with erythrocyte membranes was assessed. The present study addressed the problem of genistein localization and its effects on lipid membrane fluidity and protein conformation. The range of microscopic techniques was employed to study genistein effects on HeLa cells and human erythrocytes. Moreover, DPPH bioassay, superoxide anion radical test and enzymatic measurements were performed in HeLa cells subjected to genistein. The gathered results from both EPR and NMR techniques indicated strong ordering effect of genistein on the motional freedom of lipids in the head group region and the adjacent hydrophobic zone in liposomal as well as in red blood cell membranes. EPR study of human ghost showed also the changes in the erythrocyte membrane protein conformation. The membrane effects of genistein were correlated with the changes in internal membrane arrangement of HeLa cells as it was noticed using transmission electron microscopic and fluorescent techniques. Scanning electron and light microscopy methods showed that one of the aftermaths of genistein incorporation into membranes was creation of echinocytic form of the red blood cells with reduced diameter. Genistein improved redox status of HeLa cells treated with H_2O_2 by lowering radicals' level.
In conclusion, the capacity of genistein to incorporate, to affect membrane organization and to change its biophysical properties is correlated with the changes inside the cells.

1. Introduction

Genistein constitutes an important component in the majority of people's daily diet. The common source of it is soybean [1]. This naturally occurring flavonoid has a lot of beneficial properties on human health and because of that it is one of the most studied isoflavones [2]. There is Q3 a great interest in flavonoids' potential health benefits that result mainly from their anticancer activity as well as from their ability to reduce heart diseases [3]. Many studies have shown that flavonoids, including genistein, can inhibit the growth of various cancer cell lines including: leukemia, lymphoma, prostate, breast, lung and head and neck cancer cells, both in vitro and in vivo [4,5]. The fact that flavonoids can block or reverse carcinogenesis, makes them promising cancer chemopreventive agents. Genistein is known from its antioxidant, radical scavenging and antimicrobial activities. It has also estrogenic properties and is able to prevent osteoporosis [6–8].

Abbreviations: DPPH• radical, 1,1-diphenyl-2-picrylhydrazyl radical; EPR spectroscopy, electron magnetic resonance spectroscopy; EYPC, 1,2-diacyl-sn-glycero-3-phosphocholine from egg yolk; NMR spectroscopy, nuclear magnetic resonance spectroscopy; 5-SASL, 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy free radical; 16-SASL, 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy free radical; SAR, superoxide anion radical; Tempo, 2,2,6,6-tetramethyl-1-piperidinyloxy free radical, 4-maleimido Tempo

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Ability to interact, permeate and alter biophysical parameters of the membrane is of crucial importance for biological effects of flavonoids. The knowledge concerning drug–membrane interactions is very important since the membrane is the first barrier for the drug on the way into a cell. Moreover, antioxidant activity, transport quality through the cell membrane, and other membrane related-processes such as signal transduction, electron transfer and protein and lipid translocation are coupled with ability of flavonoids to incorporate into membranes [9–14].

In recent years the interactions of flavonoids, including genistein, with the membrane are a frequently appearing subject of articles [15–18] but very few of them applied EPR technique. In our previous work on the saturated DPPC liposomes we have provided evidence of genistein interaction with membrane and revealed molecular mechanism of genistein interaction with lipid membrane [13].

Taken into account the antioxidant activities of genistein, its widespread occurrence in food and its beneficial effects on human health we decided to examine the action of genistein on purified lipids made of EYPC and on biological membranes of human erythrocytes. We decided to examine EYPC liposomes because these model membranes are unsaturated and resemble natural membranes due to high amount of phosphatidylcholine in them. In turn, ghost of human red blood cells are natural membranes that have trilamellar composition that consists of lipid bilayer and underlying protein network. In this regard we wanted to characterize the effects of genistein on the biophysical properties of membranes to extend the knowledge concerning genistein–membrane interactions and to get holistic estimation of such relationship. We also wanted to further correlate these effects with some of its biological properties. Our investigations were performed at physiological pH to maintain the same conditions that are found in vivo.

Thus in the present paper we applied the spin label electron paramagnetic resonance (EPR) in order to examine genistein effect on the lipid fluidity and the conformational changes of membrane proteins. Nuclear magnetic resonance (^{1}H NMR) measurements were also carried out to gain a deeper insight into the genistein–membrane relationship. Transmission, scanning electron and fluorescence microscopy techniques were additionally performed to address the problem of influence of genistein on human cervix carcinoma and red blood cells. Additionally antioxidant tests were performed with usage of DPPH assay, superoxide anion radical examination together with the investigation of chosen enzymes' activities in HeLa 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 labels: 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy free radical (5-SASL), 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy free radical (16-SASL), 2,2,6,6-tetramethyl-1-piperidinyloxy (Tempo), 4-maleimido-Tempo, 1,2-diacyl-*sn*-glycero-3-phosphocholine from egg yolk (EYPC) and 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. Human ghost isolation

Erythrocyte membranes were obtained by hypotonic lysis according to the procedure of Dodge et al. [19] at 4 °C and then suspended in PBS, pH 7.4. Protein concentration was evaluated by the method of Bradford [20] using BSA as a standard.

2.3. Nuclear magnetic resonance (NMR) measurements

For the ^{1}H NMR spectroscopy measurements, mixtures of phospholipids and isoflavonoid were co-dissolved in chloroform/ethanol mixture (55:1 v:v) at the respective concentration [13]. The lipid concentration in the sample was 3.2×10^{-2} M and of the flavonoid was 3.2×10^{-4} M. After evaporating the solvents under a stream of nitrogen and then by vacuum (4 h), the samples were hydrated with D_2O and vigorously shaked (1 h) on a shaker at room temperature. Then the lipid suspension was sonicated (8 × 3 s) to yield a homogenous lipid dispersion at 4 °C. Shortly before measurements 4 mM praseodymium trichloride ($PrCl_3$) was added. ^{1}H NMR spectra were performed on a Bruker Avance 300 NMR spectrometer using 5-mm probe with pulsed field gradient capabilities. The ^{1}H NMR parameters were as follows: spectral window 3906 Hz, digital resolution 0.238 Hz, pulse width 6.0 ms, acquisition and delay time were 2.09 s and 3 s, respectively, and acquisition temperature 333 K. Relatively high temperature of NMR has been selected in order to compare present data with the previous experiment performed with model membranes formed with DPPC.

2.4. Electron paramagnetic resonance (EPR) measurements of liposomes and human ghosts

In our work we examined the effect of genistein on egg yolk lecithin liposomes representing unsaturated type of membranes. The concentration of the lipid (EYPC) 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 EYPC (100 mg/ml of chloroform) was prepared by mixing solutions of respective compounds and evaporating solvent, first in a stream of nitrogen and subsequently by vacuum (3 h). EYPC samples were hydrated with the phosphate buffer (100 mM, pH 7.4) then manually agitated at room temperature and the lipid suspension was sonicated in buffer to yield homogenous lipid dispersion. 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 at the X band and equipped with variable temperature-stabilizing unit under 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, and scan range 3200–3300 G. The n-SASL spin labels applied in the study are commonly used to monitor the fluidity of model membranes [13,21]. In the spectra, the maximum splitting value $2T'_{||}$, an empirical parameter related to an order parameter of the alkyl chain and the rate of the alkyl motion of the spin label in a lipid core, reflecting the fluidity of a membrane, was analyzed.

In ghosts of human red blood cells we have applied Tempo and maleimido Tempo spin labels. Before measurements the ghosts were incubated with genistein solutions (0 and 50 μg/ml) in isotonic PBS solution. The suspensions of erythrocytes membranes in a PBS (pH 7.4) was vigorously vortexed in a glass tube with a film of Tempo spin label. The concentration of the label was 2.5×10^{-4} mol per 1 mg of total protein in the erythrocyte membranes. Maleimido spin label examines the changes in protein conformation of the membranes [12]. The membranes were incubated with various concentrations of genistein (10, 25, 50 μg/ml) in the dark for 1 h at 37 °C. After incubation, the membranes were washed three times with PBS and then labeled with maleimido Tempo spin label for 12 h at 4 °C, in a ratio of 1 mg per 25 mg of membrane protein. We have examined the changes of partition coefficient B/A using polar spin label Tempo. High field spectra of Tempo spin label show two peaks: one correspondent to a relatively mobile fraction of spin label in the water phase (A) and the fraction of spin label immobilized within the membrane (B). This parameter is also connected with the membrane fluidity [12,22].

In the studies the changes of W/S ratio have been monitored to examine the changes in protein conformation. Maleimido tempo binds

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