J Membrane Biol (2011) 239:131–135
DOI 10.1007/s00232-010-9332-8

The Negative Effect of Soy Extract on Erythrocyte Membrane Fluidity: An Electron Paramagnetic Resonance Study

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Received: 19 July 2010 / Accepted: 15 November 2010 / Published online: 4 December 2010

Abstract A decrease of erythrocyte membrane fluidity can contribute to the pathophysiology of hypertension. Soy products, which are used as alternative therapeutics in some cardiovascular conditions, contain various isoflavones (genistein, daidzein, and their glucosides, genistin and daidzin), which can incorporate cellular membrane and change its fluidity. The aim of this study was to examine the effects of soy extract (which generally corresponds to the soy products of isoflavone composition) on erythrocyte membrane fluidity at graded depths. We used electron paramagnetic resonance spectroscopy and fatty acid spin probes (5-DS and 12-DS), the spectra of which are dependent on membrane fluidity. After being treated with soy extract, erythrocytes showed a significant ($P = 0.016$) decrease of membrane fluidity near the hydrophilic surface, while there were no significant changes of fluidity in deeper hydrophobic membrane regions. These results suggest that soy products containing high levels of genistein and isoflavone glucosides may not be suitable for use in hypertension because they decrease erythrocyte membrane fluidity.

Keywords Soy extract · Isoflavone glucoside · Genistein · Erythrocytes · Electron paramagnetic resonance · Membrane fluidity · Spin probes

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Introduction

Microcirculation is a complex and integrated system in which erythrocytes represent the main definers of blood viscosity. The mechanical properties of the erythrocyte membrane, such as fluidity, seem to be of crucial importance for the ability of erythrocytes to pass through the smallest blood vessels. Some previous data have pointed out higher microviscosity of the erythrocyte membrane in spontaneously hypertensive rats in comparison to controls (Orlov et al. 1982). Hence, a link has been proposed between the alterations of mechanical properties of the erythrocyte membrane and the pathophysiology of hypertension (Postnov and Orlov 1984). Further investigations have confirmed this, showing that erythrocyte membrane fluidity is significantly lower in both spontaneously hypertensive rats and patients with essential hypertension than in normotensive controls (Tsuda et al. 1987), which led to the conclusion that decreased fluidity of the erythrocyte membrane contributes to the pathophysiology of hypertension and other cardiovascular diseases (Zicha et al. 1999).

Soy products contain non-nutrient constituents known as isoflavones, which gained significant attention from the scientific community as a result of their potentially beneficial effects on human health. It has been documented that isoflavones can lower the concentration of cholesterol in the serum (Setchell 1985) and slow down the development of atherosclerotic plaques (Raines and Ross 1995), so soy products are increasingly consumed as alternative

therapeutic agents for cardiovascular conditions. However, some isoflavones found in soy may show negative effects in hypertensive and patients with some other cardiovascular conditions. We recently investigated the effects of two main soy isoflavones, genistein and daidzein, on erythrocyte membrane fluidity. It was observed that genistein provokes a decrease of fluidity of erythrocyte membrane, whereas daidzein shows opposite effects to genistein (Ajdžanović et al. 2010), a finding that recommends daidzein for application in hypertensive patients. However, soy products contain both of these isoflavones as well as their β-glucosides, genistin and daidzin, so it is important to determine the effects of such mixture of isoflavones on the mechanical properties of erythrocyte membrane. It should be stressed that the mechanisms by which isoflavone glucosides reach the systemic circulation from dietary sources are still the subject of controversy. Although the ruling opinion indicates their initial hydrolysis in the intestines (Scalbert and Williamson 2000; Day and Williamson 2001), some authors suggest the glucosides are absorbed intact (Andlauer et al. 2004).

The aim of our study was to determine the ability of commercial soy extract (a mix of aglucones and glucosides, which generally corresponds to soy products of isoflavone composition) to modulate erythrocyte membrane fluidity. Such information could be important for the anticipation of effects of soy products consumption in cardiovascular conditions, including hypertension and decreased luminal diameter of blood vessels, and for elucidating the mechanisms of isoflavone transport across cellular membranes. We used electron paramagnetic resonance (EPR) spectroscopy and lipophilic spin probes that intercalate the membrane and whose EPR spectra are dependent on membrane fluidity.

Materials and Methods

Fresh blood was obtained from four healthy volunteers between the ages of 30 and 35 years, using tubes containing 0.072 ml of 7.5% K_3EDTA as the anticoagulant per 3 ml of blood (Vacuette EDTA; Greiner Bio-One, Kremsmünster, Austria). The erythrocytes (erythrocyte membranes) were spin labeled as described previously (Spasojević et al. 2005). Fresh blood erythrocytes were washed three times with isotonic phosphate-buffered saline (NaCl 8.8 g/L, Na_2HPO_4 1.2 g/L, NaH_2PO_4 0.43 g/L, pH 7.4) by centrifugation at 3,500$\times g$ for 10 min at 4°C. The hematocrit for fresh blood was ~40%, and all samples were adjusted to the same hematocrit before incubation. Strong methanol solutions of commercial soy extract, which contained 40% of isoflavones—genistein (≥10%), daidzein (≥15%), genistin (≥3%), daidzin (≥5%), glicitin (≥1%), and glicitein (≥0.5%), according to the manufacturer's specifications (Nutraceutica, Monterenzio, Italy)—were added to the washed erythrocytes to obtain the final isoflavone concentration of 0.1 mg/ml. This concentration was selected to mimic in vivo levels in the blood of humans exposed to elevated concentrations when nutritional supplements are used for therapeutic purposes (Doerge and Sheehan 2002; Ajdžanović et al. 2010). The structures of genistein, daidzein, genistin, and daidzin are presented in Fig. 1. Aliquots of methanol were added to control samples. The final proportion of methanol was 0.5% in all samples. Samples were incubated at 37°C for 10 min.

To determine the reversibility of potential changes in the fluidity of erythrocyte membranes, a part of each sample was taken and gently washed three times with phosphate-buffered saline. Ethanol solutions of the fatty acid spin-probes 5-DS and 12-DS (2-(3-carboxypentyl)-2-tridecyl-4,4-dimethyloxazolidine-3-oxyl and 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyloxazolidine-3-oxyl; Molecular Probes, Junction City, OR) were applied on the walls of tubes. The amount of DS to be used was calculated to obtain the optimal spin-label/membrane-lipid ratio of approximately 1:100 (Cooper et al. 1992). After the ethanol had evaporated, the sample was added and gently mixed. Samples were placed in Teflon tubes with a wall thickness of 0.025 mm and an internal diameter of 0.6 mm (Zeus Industries, Raritan, NJ) and inserted into quartz capillaries. The incubation and EPR measurements were performed in air. EPR spectra were recorded using a Varian E104-A EPR spectrometer (Palo Alto, CA) operating at X-band (9.1 GHz) and adjusted to the following settings: modulation amplitude, 2 G; modulation frequency, 100 kHz; microwave power, 10 mW; scan range, 100 G; scan time, 4 min; time constant, 0.25 s. The temperature was controlled at 20°C during the measurements.

Spectra were recorded and analyzed using EW software (Scientific Software, Bloomington, IL). The order parameter (S), which is reciprocally proportional to fluidity, was calculated as shown previously (Gaffney 1976). All procedures related to the use of human blood in this study

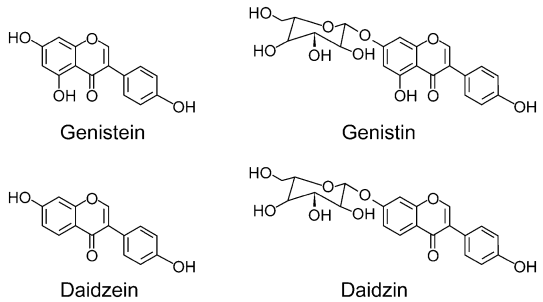

Fig. 1 The chemical structures of genistein, daidzein, and their glucosides, prevalent in soy extract

conformed to the recommendation provided in the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The data are presented as means ± standard deviation (SD) for at least five separate experiments. The significances of differences were calculated by a nonparametric two-tailed Mann–Whitney test. Means were considered significantly different at $P < 0.05$.

Results

We used two spin probes, 5-DS and 12-DS, for this study to explore the effects of soy extract on erythrocyte membrane at graded depths. EPR with 5-DS provides information about the fluidity near the hydrophilic membrane surface (Bahri et al. 2005; Grammenos et al. 2009), while 12-DS has a nitroxide (EPR active) structure far down the lipid acid chain (at carbon 12 from the carboxyl group), thus providing information on the fluidity of deeper membrane regions (Bahri et al. 2005, 2007). Figure 2 shows characteristic EPR spectra of untreated erythrocytes or erythrocytes treated with soy extract, labeled with 5-DS (Fig. 2a) or 12-DS (Fig. 2b).

The calculated order parameters (S) of erythrocyte membrane labeled with 5-DS and 12-DS are summarized in Table 1. Considering that the order parameter is reciprocally proportional to the membrane fluidity, a significant decrease of membrane fluidity near the hydrophilic surface was detected in erythrocytes treated with the soy extract. On the other hand, there were no significant changes of fluidity in deeper, hydrophobic membrane regions as determined using 12-DS. The observed change of fluidity in the membrane layer near the surface was reversible, as the order parameter of membrane of erythrocytes that were treated with the soy extract and subsequently washed showed a value that is not significantly different in comparison to the control.

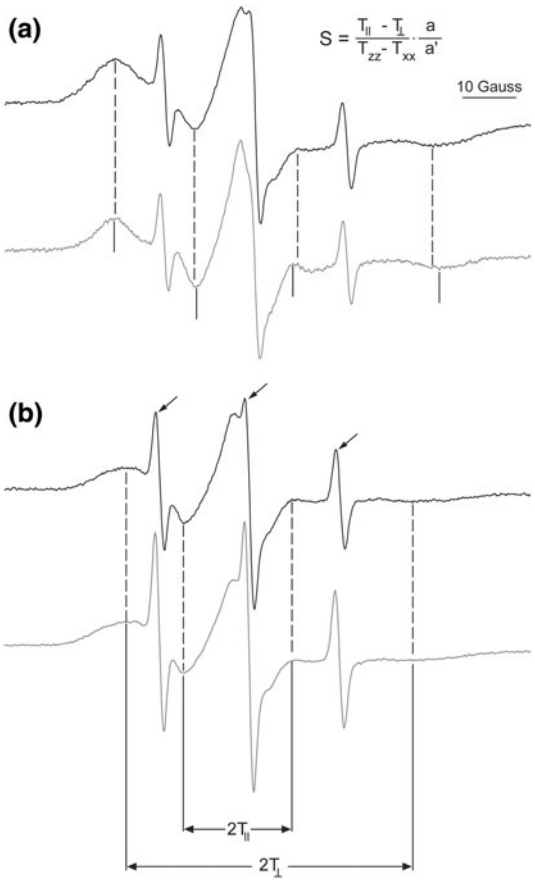

Fig. 2 Characteristic EPR spectra of **a** erythrocytes labeled with 5-DS without (control; *dark trace*) or with soy extract (0.1 mg isoflavones/ml; *pale trace*). **b** Erythrocytes labeled with 12-DS without (control; *dark trace*) or with soy extract (0.1 mg isoflavones/ml; *pale trace*); S, order parameter; $2T_{II}$, outer hyperfine splitting; $2T_{\perp}$, inner hyperfine splitting; a, isotropic hyperfine coupling constant in crystal [$a = 1/3(T_{xx} + T_{yy} + T_{zz})$]; a′, isotropic hyperfine coupling constant in membrane [$a' = 1/3(T_{II} + 2T_{\perp})$]; T_{xx}, T_{yy}, T_{zz}, hyperfine constants (for 5-DS they were taken to be $T_{xx} = T_{yy} = 6.1$ G, $T_{zz} = 32.4$ G; for 12-DS $T_{xx} = 6.26$ G, $T_{yy} = 5.81$ G, $T_{zz} = 33.46$ G) (Gaffney 1976). *Dashed lines* marking parameters in control spectra are shown to emphasize the differences between controls and treated samples. Three *narrow lines* originate from the DSs in the solution (*arrows*) (Gaffney 1976; Spasojević et al. 2005)

Discussion

Soy extract, at physiologically relevant concentrations, shows different effects on erythrocyte membrane fluidity, at graded depths. The extract provoked a decrease of membrane fluidity near the hydrophilic surface without a significant change of fluidity in deeper regions of the membrane. The main isoflavone constituents of soy extracts are genistein, daidzein, and their 7-*O*-*β*-D-glucoside forms, genistin and daidzin. They are long and rigid molecules, with the complex three-dimensional organization (Fig. 1), and as such, they are in principle hydrophobic. The reversibility of the changes of membrane fluidity indicates that the soy extract isoflavones are not covalently bound to membrane molecules, but rather intercalate into the membrane. However, their specific chemical properties define different positioning inside the membrane. Daidzein has two hydroxyl groups that are far away from each other. Hence, it does not have a defined hydrophilic moiety and intercalates deep into the hydrocarbon core of the membrane, interrupting its orderly structure. Pertinent to this, we have recently demonstrated using EPR and 12-DS that daidzein can provoke an increase of fluidity of deeper layers of erythrocyte membrane (Ajdžanović et al. 2010). Such effects were not observed here because the final concentration of daidzein was approximately seven times

Table 1 Order parameters (S) obtained by using 5-DS and 12-DS in human erythrocytes exposed to the soy extract

Samples	Control	Soy extract	Soy extract + wash
5-DS (mean ± SD)	0.698 ± 0.010	0.729 ± 0.009	0.719 ± 0.011
Statistical significance[a]	–	0.016	NS
12-DS (mean ± SD)	0.597 ± 0.013	0.591 ± 0.010	0.595 ± 0.007
Statistical significance	–	NS	NS

[a] Relative to control

lower (~0.015 mg/ml) than the previously used concentration (0.1 mg/ml; Ajdžanović et al. 2010).

On the other hand, the negative effects on the fluidity of the erythrocyte membrane near the surface were observed for both pure genistein (Ajdžanović et al. 2010) and soy extract, although in the current study, the final concentration of genistein was 10 times lower (0.01 mg/ml) than previously (0.1 mg/ml; Ajdžanović et al. 2010). This implies that genistein is not solely responsible for the negative effects of soy extract on the fluidity observed here, and that genistin and daidzin may also play an important role. Genistein contains a defined hydrophilic moiety composed of two hydroxyl groups placed close to each other on the same ring. Thus, when genistein intercalates the membrane, it is positioned near the surface and oriented such that the hydrophilic moiety is at the aqueous phase of the lipid bilayer and the hydrophobic structure extends downward in the hydrocarbon part of the membrane (Ajdžanović et al. 2010). The structures of genistin and daidzin also show a hydrophilic moiety in the form of glucose, so they should be positioned in a fashion similar to genistein.

It has been previously documented that glucose groups of soybean-derived steryl glucosides are projected outward from the surface of liposomes (Shimizu et al. 1996). Because they are positioned near the membrane surface, genistein, genistin, and daidzein decrease the mobility of polar heads of phospholipids and interrupt lateral diffusion, thus provoking a decrease of membrane fluidity. It is important to note that the mechanisms of transport of isoflavone glucosides from intestines, across cellular membranes, and into the circulation are not clear (Scalbert and Williamson 2000; Day and Williamson 2001; Andlauer et al. 2004). We believe that our results may shed some light on this issue. It is known that glycolipids containing two or more sugar moieties in the head group are not able to spontaneously transverse the membrane, so they are usually located only in one specific membrane leaflet (van Meer and Holthuis 2000). However, structures containing only one sugar, such as glucosylceramid, can be found in both membrane leaflets (van Meer and Holthuis 2000). Isoflavone glucosides intercalate and increase the order and rigidity of the outer leaflet of cellular membrane, which is an energetically unfavorable setup that shows decreased entropy in comparison to intact inner leaflet. Therefore, it seems that isoflavone glucosides could be transported across the membrane via entropy-driven flip-flop, analogously to the short-chain phospholipids (Anglin et al. 2010), but further investigations are needed to verify this hypothesis.

In our study, soy extract was used to mimic potential effects of consumption of dietary soy products. The consumption of soy products and soy isoflavones as alternative therapeutics for different cardiovascular conditions has increased significantly over the past few years. Soy isoflavons are considered to induce a decrease of serum cholesterol concentrations (Setchell 1985). In addition, because of their antioxidative activity (Mitchell et al. 1998; Kruk et al. 2005), soy isoflavones could have positive effects in hypertension and atherosclerosis, which are principally associated with oxidative stress (Crimi et al. 2007). However, the pathogenesis of hypertension and some other cardiovascular problems also involves low erythrocyte membrane fluidity and related negative hemodynamic changes (Postnov and Orlov 1984; Tsuda et al. 1987; Kritz et al. 1996; Zicha et al. 1999). Under such settings, a therapy resulting in decreased elasticity of erythrocyte shape is not preferred. Our results imply that precautions should be taken when soy is to be introduced into the diet of hypertensive patients because it may promote hypertension. However, it is known that the isoflavone content and profiles in soy may vary considerably in relation to soy variety and geographic and environmental conditions, as well as to the mode of industrial processing (Cassidy et al. 2000; De Lima Toccafondo Vieira et al. 2008). Hence, soy products showing high levels of daidzein and low levels of genistein, genistin, and daidzin could be developed for application in the diet of patients with cardiovascular diseases.

In conclusion, the number and polarity of functional groups in isoflavone glucosides and genistein determinate the positioning of these compounds at the surface of lipid bilayer, which represents the crucial factor in negative modulation of the erythrocyte membrane fluidity. From the perspective of erythrocyte rheologic behavior, the soy products containing isoflavone glucosides and genistein are

not suitable for the use in patients with cardiovascular conditions, including essential hypertension and atherosclerosis, because they decrease erythrocyte membrane fluidity.

Acknowledgment Supported by the Ministry of Science of the Republic of Serbia (grants 143007B and 143016B).

Conflicts of interest None.

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