Research Communication

Differential inhibition of human erythrocyte acetylcholinesterase by polyphenols epigallocatechin-3-gallate and resveratrol. Relevance of the membrane-bound form

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Abstract

The activity of acetylcholinesterase (AChE) from human erythrocytes was tested in the presence of the phenolic compounds resveratrol and epigallocatechin-3-gallate (EGCG). Even though the stilbene barely changed this enzymatic activity, EGCG did inhibit AChE. Importantly, it preferentially acted on the membrane-bound enzyme rather than on its soluble form. Actually, it was shown that this flavonoid may bind to the red blood cell membrane surface, which may improve the interaction between EGCG and AChE. Therefore, caution should be taken when screening AChE inhibitors. In fact, testing compounds with the soluble form of the enzyme may underestimate the activity of some of these potential inhibitors, hence it would be advisable not to use them as a sole model system for screening. Moreover, erythrocyte AChE is proposed as a good model for these enzymatic assays. © 2016 BioFactors, 00(00):000000, 2016

Keywords: *acetylcholinesterase; epigallocatechin-3-gallate; resveratrol; inhibition; membranes*

1. Introduction

Acetylcholinesterase (EC 3.1.1.7) is a key enzyme in the cholinergic system that acts as a terminator for the transmission of nerve impulses. Three main variants of AChE are formed by alternative "splicing," that is, AchE-S, the main isoform, present in the nervous system, and in skeletal muscle. AChE-R, which is a soluble monomeric AChE isoform that is expressed in embryonic and tumor cells and induced mainly in the brain as a response to psychological stress and AChE inhibition [1]. The third major variant is present only in erythrocytes in humans (AChE-E). The latter protein forms dimers and is associated with red blood cell membranes by glycosylphosphatidylinositol [2,3]. All three isoforms have a globular core of 543 amino acids, where the active site is [4]. In addition, AChE contains a second binding site called "peripheral anionic site," involved in the allosteric modulation of the catalytic activity, mediating the interaction of AChE with inhibitors [5].

Besides its role in the nerve impulse, alternative functions unrelated to its enzymatic activity have been proposed for AChE [6]. Because of the multiple functions of this enzyme, inhibitors have been proposed for a variety of applications in medicine. The most notorious one is in Alzheimer's disease, which is characterized by a gradual decrease in cholinergic neurons and a consequent loss of acetylcholine in the brain. AChE inhibitors have been proposed as possible palliative treatment for this disease. At present, there are three AChE inhibitors already approved: galantamine, rivastigmine, and donepezil, usually being more effective at the initial stages of the disease [7]. Tacrine, the first inhibitor of its kind was discontinued a few years ago.

Additional Supporting Information may be found in the online version of this article.

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AChE inhibitors have also been proposed for other pathologies. For instance, neostigmine and pyridostigmine derivatives were used in myasthenia gravis with good results [8]. Among other diseases that may be treated, it can be cited the autoimmune autonomic ganglionopathy and the postural tachycardia syndrome [9]. Parkinson's disease patients may also benefit from AChE inhibitors [10]. In addition, acetylcholinesterase inhibitors were proposed as a tool to combat leishmaniasis, a disease that globally affects millions of people [11]. *Leishmania braziliensis*, the major etiologic agent of this disease cannot synthesize choline, which is essential for synthesis of phosphatidylcholine. Therefore, growth and development of the parasite may be affected by inhibiting AChE activity [12]. For these reasons, it is certainly important to find new inhibitors of AChE and characterize them. Among natural products that have promising perspective in medicine, polyphenols constitute an interesting group of compounds that deserves attention. These compounds are secondary metabolites with phenolic groups in their structures that are mainly produced by plants. Many beneficial properties have been described for polyphenols so far. For instance, they can act as antioxidants not only because they scavenge reactive oxygen species but also because they can activate signaling pathways and key protective enzymes [13,14]. The antimicrobial activity is another important feature that is being actively studied nowadays [15,16] as well as the antiallergic and anti-hypertensive activities [17,18]. Besides, anti-cancer properties were also reported, which are based on their abilities to inhibit xenobiotic pumps [19,20]. According to the number of phenolic rings and other structural elements, polyphenols can be classified into several groups. The main families are flavonoids, phenolic acids, phenolic alcohols, stilbenes, and lignans [21,22]. Our lab is focused on the study of the modulation of membrane protein activities by polyphenols [23] as well as the analysis of the interaction of phenolic compounds with membrane model systems [24]. In this regard, epigallocatechin-3-gallate (EGCG), a flavonoid derivative which is a major constituent of the green tea, proved to be a very efficient membrane stabilizer [24]. On the other hand, resveratrol, a stilbene that is the most studied polyphenol present in grapes and wines, showed a promising property as enhancer of ATPase activity of ABCG1 [23]. Currently, both of them are subject of great interest due to their potential beneficial impact in human health, although conclusive evidence of the beneficial properties in humans is still elusive [25,26].

Interestingly, Ebrahimi and Schluesener described phenolic compounds as reversible inhibitors of AChE [27]. Moreover, Vila-Nova et al. reported that some polyphenols were AChE inhibitors and at the same time leishmanicidal agents [28]. Since erythrocyte-associated AChE was studied in detail in our lab for many years, it was decided to analyze the possible inhibition of this isoform by phenolic compounds. Moreover, it is interesting to note that AChE-E was also proposed as a viable biomarker to evaluate the neurotoxicity as well as the hematological perturbations induced by different substances, from plant products to pesticides and metal contaminants, such as lead [29–32].

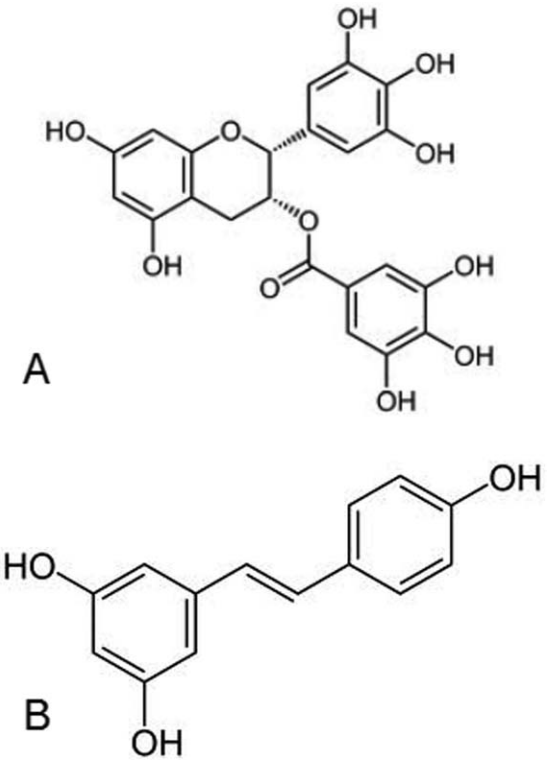


FIG 1 *Chemical structure of the polyphenols (−)-epigallocatechin-3-gallate, EGCG (A) and resveratrol (B).*

In this work, the possible inhibition of AChE-E by resveratrol and EGCG were studied. Even though the stilbene did not show any significant inhibition of AChE, EGCG turned out to be an interesting inhibitor of the erythrocyte-associated enzyme. Surprisingly, EGCG inhibited the membrane-bound enzyme more efficiently than the soluble form of AChE-E, that is the enzyme released from the membranes by Triton X-100. Furthermore, we showed that EGCG was able to closely interact with the surface of red blood cell membranes, hence we postulated this interaction to be a crucial step in the inhibition of AChE-E by EGCG.

2. Experimental procedures

2.1. Chemicals

Acetylthiocholine iodide (ATC), resveratrol, epigallocatechin-3-gallate (EGCG), gallic acid, catechin, and quercetin were purchased from Sigma-Aldrich, Argentina (Fig. 1 shows the chemical structures of resveratrol and EGCG). Polyphenols were prepared as methanolic solutions, kept under nitrogen at −20°C and the concentration was routinely checked by the Folin method, with gallic acid as a standard [33]. Alternatively, quercetin was also used as a standard when the concentration of EGCG stock solution was checked. 5, 5′-dithiobis-2-nitrobenzoic acid (DTNB) was purchased from ICN, octadecyl

rhodamine B Chloride (R18), *N*,*N*,*N*-trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl) phenylammonium *p*-toluenesulfonate (TMA-DPH), and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Life Technologies Argentina. Fluorescent probes were prepared as methanolic solutions and stored at −20°C. All other reagents were of the highest purity grade commercially available.

2.2. Membrane preparation and solubilization

After giving informed consent and signing consent documents according to our Institutional Ethics Committee, human blood was obtained by venous puncture with Na_2EDTA as anticoagulant (1 mg/mL blood) from healthy volunteers. All the subjects were residents of San Miguel de Tucumán (Northwestern Argentina). Plasma and cells were separated by centrifugation (2,000*g*, 10 min). Plasma and white cells were discarded and erythrocytes were washed with 10 volumes of 0.9% NaCl three times. Afterward, erythrocytes were hemolyzed with 30 volumes of hemolysis buffer (5 mM phosphate buffer, pH 7.4, containing 1 mM Na_2EDTA). Hemoglobin-free membranes or "ghosts" were obtained by repeated centrifugations at 10,000*g*, 20 min each cycle. All steps were performed at 0–4°C. Even though ghosts can preserve full acetylcholinesterase activity nearly 2 months when stored at 4°C, they were used within a week [34].

The solubilization of AChE from erythrocyte membranes was carried out by diluting membrane suspension 20 times with the same buffer containing the non-ionic detergent Triton X-100 (Sigma-Aldrich) at a final concentration of 0.04%. After gently mixing for 15 min at room temperature, sample was centrifuged for 1 h at 10,000*g* at 4°C and the pellet was discarded [32]. The total activity measured in 500 μL of ghost membrane suspension was 160 IU, with a protein content of 0.95 mg. On the other hand, total activity of the Triton X-100 containing sample was 237 IU (500 μL, 2.6 mg protein).

2.3. Acetylcholinesterase inhibition by phenolic compounds

The inhibition of AChE-E by phenolic compounds was determined by the Ellman's method, using ATC as a substrate [35]. Briefly, ghost membranes or solubilized samples were diluted in 5 mM phosphate buffer, pH 7.4 containing 1 mM EDTA and 0.33 mM DTNB. Then, different concentrations of polyphenols were added (from 3 μM to 200 μM) and preincubated for 2 min at 37°C. For control reactions, the addition of polyphenols was replaced with the same volume of methanol. ATC was then added to the mixture and absorbance was measured at 412 nm in a Beckman DU-7500 spectrophotometer upon reaction of the released thiocholine with DTNB. Parallel reactions performed without enzyme and/or without substrate were used to correct absorbance readings. Kinetics were followed at 37°C during 3 min.

2.4. Membrane order changes induced by polyphenols

Possible changes in membrane order induced by phenolic compounds were estimated by steady-state fluorescence anisotropy using two fluorescent probes, that is, TMA-DPH and DPH. DPH molecule is oriented with its symmetry axis normal to the plane of the membrane. Since absorption and emission transition moments are basically collinear, even small displacements of the symmetry axis result in depolarization of fluorescence emission. Therefore, DPH is an excellent probe for studying the order degree of the hydrophobic core of lipid bilayers. TMA-DPH works in a similar way but it is "anchored" to the membrane interface, hence it senses the order at the glycerol backbone region and the fatty acyl chain regions closer to the lipid–water interface. If EGCG get inserted into the membrane bilayer, changes in the order might be detected as variations in the anisotropy values of either DPH or TMA-DPH, depending on the penetration depth [24,36,37].

Fluorescent probes were added under constant vortexing to membrane suspensions in 5 mM phosphate buffer (pH 7.4) containing 1 mM EDTA, in a 1:800 probe:phospholipid molar ratio. Samples were incubated at room temperature for 30 min in the dark in the presence of phenolic compounds at a final concentration of 50 μM or with the same volume of methanol for control conditions. Fluorescence measurements were carried out using an ISS PC1 spectrofluorometer, L-format equipped with a thermostatic cuvette holder set at 37°C. The steady-state DPH fluorescence anisotropy was determined adjusting the excitation and emission wavelengths at 360 and 450 nm, respectively, while the steady-state TMA-DPH fluorescence anisotropy was measured adjusting the excitation and emission wavelengths at 360 and 435 nm, respectively.

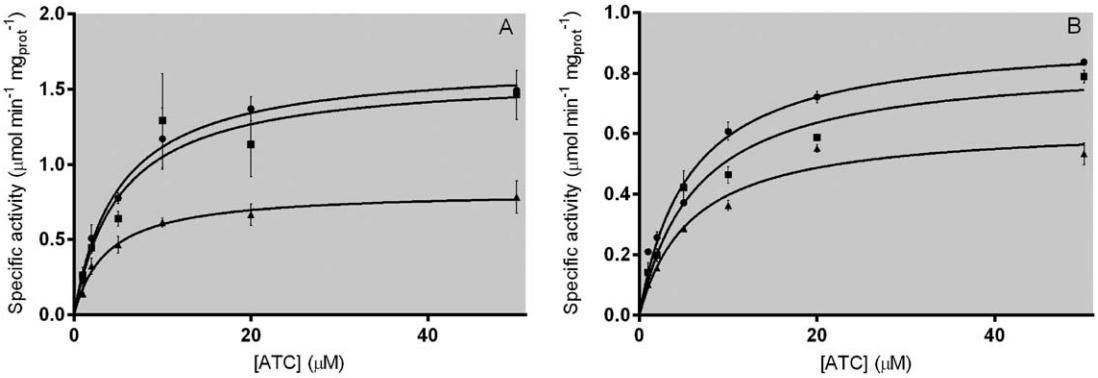
Steady-state anisotropy (r) was calculated by Vinci software (ISS) software as follows:

$$r = \frac{I_{vv} - I_{vh}}{I_{vv} + 2 \cdot I_{vh}},$$

where I_{vv} is the fluorescence intensity recorded with both the analyzing and the excitation beam polarizers vertically oriented, whereas I_{vh} is the fluorescence intensity recorded in crossed polarizers condition, in which excitation beam polarizer is vertically oriented and the emission channel is horizontally oriented. In addition to anisotropy values, total fluorescence intensities were also recorded, looking for possible quenching of the fluorescence as another way to detect interactions of EGCG with red blood cell membranes [38,39]. Each measurement was done by triplicate.

2.5. EGCG binding to ghost membrane surface

As mentioned above, DPH and TMA-DPH fluorescence measurements were carried out as a way of estimating the possible interaction of EGCG with either the deep hydrophobic core of membranes or the glycerol backbone region and the fatty acyl chain zone closer to the lipid–water interface, respectively. Besides, for assessing the possible binding of EGCG to the phospholipid head groups at the erythrocyte plasma membrane, quenching of the R18 fluorescence was measured upon addition of EGCG.



	Control	EGCG	Resv
Vmax	1.681	0.8249	1.597
Km	5.064	3.707	5.227

	Control	EGCG	Resv
Vmax	0.9237	0.6302	0.8397
Km	5.616	5.874	6.382

FIG 2

Determination of the kinetic constants in saturation plots. (A) membrane-bound AChE-E, (B) Triton X-100 solubilized AChE-E. V0 was measured at different concentrations of ATC in control conditions (•) as well as in the presence of 25 μM EGCG (▲) or 25 μM resveratrol (■). Values shown are mean ± standard deviation of three independent assays. Kinetic parameters and curve fit were performed using Prism GraphPad, values significantly different from control are noted with "" ($\alpha = 0.01$).*

As a matter of fact, Kitano et al. investigated the location of EGCG incorporated into multilamellar vesicles of phosphatidylcholine by analyzing the quenching of the fluorescence of two different anthroyloxystearic acids. In fact, not only they were able to estimate how deep EGCG can get into de membrane, but also they demonstrated that this flavonoid works fine as quencher of fluorescent probes [40].

R18 was added to membrane suspensions under constant vortexing, at a 1:1,000 probe:phospholipid final molar ratio, in which high fluorescence intensity was achieved. R18 fluorescence emission spectra were recorded upon addition of EGCG from a 1 mM stock solution. Fluorescence measurements were carried out in microplates with a Perkin Elmer LS-55 Fluorescence Spectrometer. Excitation wavelength was set at 540 nm (2.5 mm slit), while the emission spectra were taken from 560 nm to 650 nm (15 mm slit).

2.6. EGCG measurement

EGCG was diluted in 5 mM phosphate buffer (pH 7.4) containing 1 mM EDTA and ghost membranes were added. Samples were incubated 20 min at 37°C and then centrifuged at 15,000*g* for 30 min. Supernatants were carefully collected and the concentration of the unbound EGCG was spectrophotometrically quantified at 400 nm by the $AlCl_3$ method [41,42] as modified by Pękal and Pyrzynska for catechins. This procedure involves the sequential addition of 5% (w/v) $NaNO_2$, 2% (w/v) $AlCl_3$, and 1 M NaOH [43]. Calibration curve was constructed with known concentration of EGCG (Supplementary material, Fig. S1).

2.7. Miscellaneous

Phospholipid concentration in membranes was estimated by the Ames method [44]. Protein was determined by Bradford assay [45].

3. Results

3.1. Ghost membranes solubilization

Membranes were purposely prepared from different healthy volunteers each time. AChE-E constitutes not only a suitable model for studying the enzymatic activity of membrane-bound AChE isoforms but it gives also a straightforward way for obtaining a model for soluble AChE variant. Actually, the addition of Triton X-100 at a final concentration of 0.04% did not abolished the activity but allowed us to work with a soluble form of the enzyme, as it was previously shown not only in erythrocytes but also in neurons [34,46,47]. Importantly, the AchE-specific activity was highly reproducible.

3.2. Membrane-bound and soluble AChE-E are inhibited by EGCG but not by resveratrol

As shown in Fig. 2A, AChE-E showed the expected kinetic in control conditions. The addition of resveratrol did not induce any significant change in V_{max} nor in K_m even at final concentrations as high as 200 μM. On the other hand, EGCG did inhibit AChE-E activity from 3 μM onwards (33% inhibition at this concentration). V_{max} was greatly reduced in the presence of this flavonoid, while no significant change was observed for K_m ($\alpha = 0.01$).

As expected, the solubilized AChE-E displayed a similar kinetic behavior. No significant changes were measured upon incubation with resveratrol. In turn, EGCG was still able to inhibit soluble AChE-E, but its effect on V_{max} was reduced as compared with the inhibition displayed on the membrane-bound AChE (Figs. 2A and 2B). To better analyze the differences between membrane-bound AChE-E and solubilized AChE-E, inhibitory concentration 50% (IC50) was calculated. As given in Table 1, the IC50 of EGCG for the membrane-associated enzyme was 18.5 μM, whereas the IC50 for the Triton X-100-solubilized form was 38 μM. Interestingly, related

TABLE 1 *IC50 values of polyphenols for inhibitory activities on AchE-E forms*

IC_{50} (μM)	Membrane-bound form	Triton X-100-treated form
EGCG	18.5	38
Resveratrol	>100	>100
Gallic acid	68.5	>100
Catechin	>100	>100

molecules such as catechin and gallic acid displayed significant lower inhibitory activities as compared with EGCG.

3.3. EGCG can bind to ghost membrane surface

Ghosts were incubated with the fluorescent probe R18, which was properly diluted to avoid self-quenching, obtaining a highly fluorescent membrane sample. Labeled membranes were incubated with different concentrations of EGCG. As shown in Fig. 3A, the presence of this compound did quench R18 fluorescence emission, which indicated a close association of EGCG with membrane surfaces. The decrease in the emission intensity was observed even at the lowest concentration tested. On the other hand, EGCG did not significantly quench R18 fluorescence when membranes were solubilized with Triton X-100 (Fig. 3B). In this condition the probe is no longer associated with membranes. This result strongly suggests that EGCG does interact with red blood cell membranes, at least with the polar head of phospholipids.

In addition, we measured the concentration of EGCG in the supernatants after interaction with ghost membranes. For this purpose, we quantified EGCG by a modified $AlCl_3$ method and found that the remaining concentration of EGCG in the supernatant was reduced by 50% upon addition of ghosts as compared with control samples where no membranes were present (Fig. 4). This result is in agreement with the R18 measurements and strongly suggest that EGCG may interact with red blood cell membranes under our working conditions.

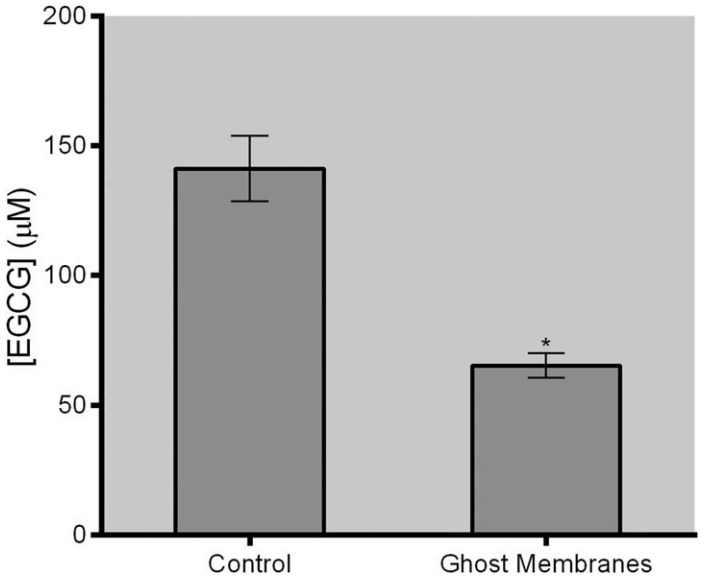


FIG 4 *EGCG measurement. The flavonoid was incubated in the presence or the absence (control) of ghost membranes at 37°C. Afterward, samples were centrifuged and EGCG was measured by the AlCl3 method. Values shown are mean ± standard deviation of at least four independent assays. Values significantly different from control are noted with "*" (α = 0.01).*

3.4. EGCG does not modulate membrane physicochemical properties

EGCG was incubated with membranes and fluorescence anisotropy of DPH and TMA-DPH was measured as described in Experimental procedures. As shown in Fig. 5A, fluorescence anisotropy values of either probe did not show any significant change in the order degree. Thus, no significant penetration of this compound into neither the interface nor the hydrophobic core occurred. Furthermore, no change in the total

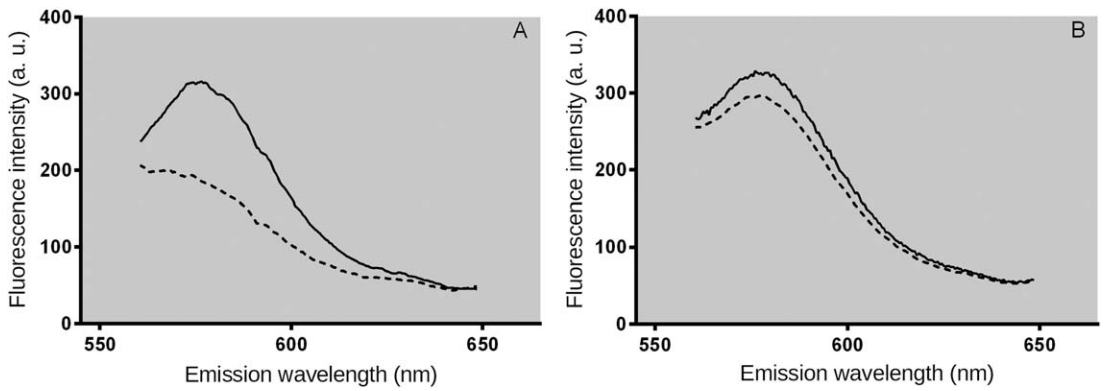


FIG 3 *Fluorescence spectra of R18-labeled ghost membranes (A) in the absence (full line) or presence of 5 μM EGCG (dashed line). Same measurements were performed after treating membranes with Triton X-100. (B) Excitation wavelength was set at 540 nm. Spectra are representative of three independent assays.*

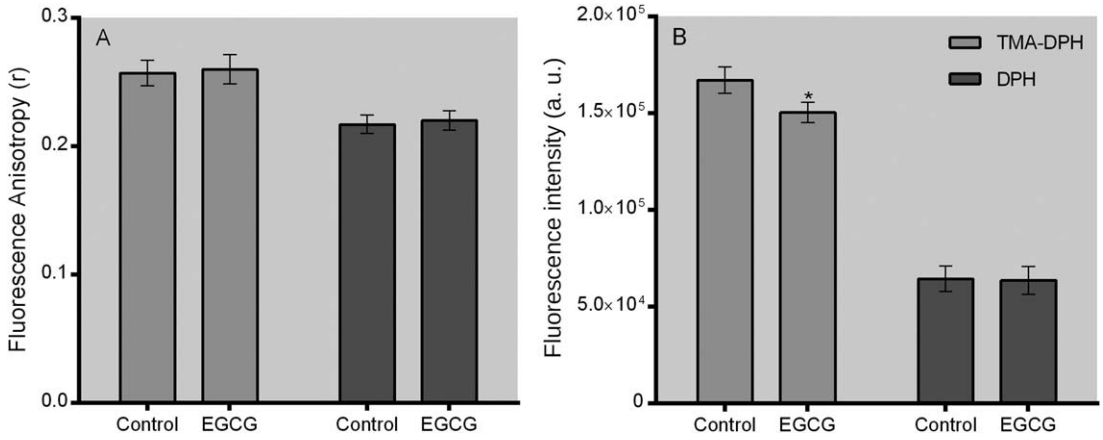


FIG 5

Fluorescence anisotropy (panel A) and total fluorescence intensity (panel B) of DPH and TMA-DPH, recorded at 37°C. Assays were carried out in the absence (Control) or presence of 50 μM EGCG. Values shown are mean ± standard deviation of 10 measures and are representative of three independent assays. Values significantly different from control are noted with "" (α = 0.05).*

fluorescence intensity was observed for DPH upon addition of EGCG, confirming that this flavonoid did not interact with the hydrophobic core of the membranes, at least under our experimental conditions. However, we did find a modest quenching of TMA-DPH fluorescence with EGCG, which was able to reduce 12% the total intensity measured at 435 nm (Fig. 5B). Based on the location of TMA-DPH, this result would support the hypothesis that EGCG would interact with erythrocyte membrane surface but it would not penetrate into the hydrophobic regions of the bilayers.

4. Discussion

The human AChE-E isoform was chosen as a model in this study for two main reasons: on the one hand, a simple and straightforward protocol for obtaining ghosts is available. On the other hand, the membrane-bound enzyme as well as the solubilized AChE-E has high specific activities [34].

The stilbene resveratrol and the flavonoid EGCG share similar abilities to interact with model membranes, as it was recently demonstrated by our group [24]. Moreover, resveratrol was shown to upregulate ATPase activity of ABCG1 by modulating membrane order [23]. Since AChE-E isoform is a membrane-bound protein, activity modulation by membrane-active species such as polyphenols might also occur.

Even though resveratrol derivatives were recently described as AChE and specially butirylcholinesterase inhibitors [48], resveratrol itself induced no significant change in the activity of AChE-E in this work. On the other hand, EGCG inhibited AChE-E in a non-competitive way. Interestingly, it was more active on the membrane-bound enzyme rather than on the soluble form (IC50 18.5 μM vs. 38 μM, respectively). Furthermore, it can be proposed that EGCG may interact with membrane surfaces, which would improve its interaction with AChE, enhancing its inhibitory effect. This mechanism may not involve EGCG membrane insertion since no change in physicochemical properties of the membranes was observed. In this regard, we recently demonstrated that EGCG interacted with model membranes in a way dependent on the cholesterol content that is a deeper localization in the membranes in cholesterol-free bilayers, whereas interacting with the polar head groups of the phospholipids and interfacial membrane domain in cholesterol rich membranes [24]. Erythrocyte membranes are known to have high cholesterol content, which may explain why there is no deeper interaction of EGCG with these membranes. Interestingly, EGCG was shown to increase the viscosity of pig ghost membranes when incubated in an isotonic buffer [49]. The discrepancy found with our results may be explained by the differences in the buffer used, since it was already demonstrated that flavonoid membrane penetration is stimulated by the presence of salts and inhibited by the negative charges of the phospholipids [50]. Cyboran et al. incubated ghost membranes with EGCG in isotonic buffer, whereas we used 5 mM phosphate buffer, a much diluted medium. Other authors also reported EGCG-red blood cell membranes interactions. In this regard, Kumar and Maurya described a positive effect on Ca^{2+}-ATPase and Na^{+}-K^{+} ATPase by protecting erythrocyte membranes from oxidative damage [51]. However, prooxidant effect of EGCG on red blood cell membranes was also suggested [52].

It can be envisioned that once EGCG get contact with membranes, it may approach more efficiently to AChE. Furthermore, other compounds might interact with AChE in a similar manner that is a first interaction with some membrane-related structure and then with the membrane-associated AChE, thus inducing a more efficient inhibition in this way.

There are several reports in the literature regarding EGCG and AChE. For instance, it was recently found that EGCG reversed the increase in acetylcholinesterase activity induced by streptozotocin in rats [53]. On the other hand, green tea leaf extract, which contains epigallocatechin-3-gallate and

epicatechin as major constituents, was shown to correct the levels of AChE in aluminum chloride-treated rats. Furthermore, rats treated with green tea leaf extract alone had even higher AChE activity than control rats, possible due to the catechins antioxidant effect [54]. Interestingly, EGCG seemed to increase the inhibitory effect of huperzine A on AChE activity in rats, although EGCG alone was unable to modify brain AChE activity [55,56]. In other report, AChE activity was increased in aged rats fed with this phenolic compound with no change induced in the activity of brain AChE of young rats [57].

Although in this work EGCG induced only a moderate inhibition of AChE-E, membrane binding arises as an important parameter to take into account. Indeed, a number of papers describing new inhibitors use the soluble commercial form of the enzyme [58–61]. This approach may underestimate the activity of some potential inhibitors since physiological relevant variants of AChE are associated with cell membranes. The inhibitory compounds should be tested using other sources of the enzyme such as brain or erythrocyte AChE. As a matter of fact, there are some reports that searched for inhibitors of AChE using the rat brain isoform [62,63], which would be a better approach. We propose AChE-E as a reliable model for testing inhibitors that have to work on the main isoform, AChE-S. As a matter of fact, Srividhya et al. had reported that EGCG was able to increase the activity of solubilized brain AChE [57]. On the contrary, we found that EGCG displayed an important inhibitory effect on AChE activity measured in a membrane-associated fraction of brain homogenate (Supplementary material Fig. S2). This result backs up our findings and supports the use of membrane-bound AChE-E as a good model for *in vitro* studies of brain AChE.

5. Conclusion

Although EGCG had been proposed as a possible AChE inhibitor based on an *in silico* approach [64], the present report warns about that a proper enzyme model should be used to obtain more precise information. Accordingly, we conclude that further studies of AChE inhibitors should consider both forms (i.e., membrane-bound and soluble) to achieve a more accurate notion of the potential as inhibitors of each molecule tested. In this regard, AChE-E seems to represent a closer model to the brain isoform.

This work strongly suggests that the inhibition in AChE-E activity by EGCG is improved by the association of this flavonoid with erythrocyte membrane. Interestingly, this might be the case for other tested species as well, what suggest that a revision of previous studies may reveal unknown inhibitory features of the aforementioned substances.

Conflict of Interests

The authors declare that they do not have conflicts of interest.

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