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Enterodiol is Actively Transported by Rat Liver Cell Membranes

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Abstract
The interaction of enterodiol and the well-described polyphenol epigallocatechin gallate (EGCG) with hepatic membranes has been matter of interest in the last few years. On one hand, EGCG is only able to bind to the phospholipid polar head groups, as it has been already described in synthetic lipid bilayers and erythrocyte membranes but cannot get inserted into the hydrophobic core or be transported into the lumen of membrane vesicles. On the other, enterodiol has no interaction with non-energized membranes either, but it is able to interact and even be transported upon addition of ATP. In fact, the ATPase activity undergoes a twofold increase in the presence of enterodiol but not in the presence of EGCG. This is the first report on the transport of enterodiol by liver membranes, and it may help explain the rather high blood concentrations of this estrogenic enterolignan compared to EGCG, which is extensively metabolized by the intestine and the liver. The present results suggest that a fraction of enterodiol may escape the liver inactivation by being pumped out from the hepatocytes to the bloodstream.

Keywords Polyphenols · Membrane binding · ATPase activity · Lignans

Introduction

Polyphenols are metabolites that are mainly produced by plants but they can also be formed in the intestine by microbial biotransformation (Heinonen et al. 2001; Monagas 2010). Based on their chemical structures, there are several groups of phenolic compounds including phenolic acids, stilbenes, polyphenolic amides, lignans, and flavonoids, the largest subclass which can be subdivided in a number of subgroups (Tsao 2010). Nowadays, phenolic compounds are attracting great attention due to the multiple beneficial properties for human health that have been ascribed to them, such as prevention of cardiovascular diseases, cancers, neurodegenerative diseases, diabetes, or osteoporosis (Scalbert et al. 2005; Rodrigo et al. 2014). Polyphenols can interact with various target proteins but also with lipid bilayers. As a matter of fact, the interaction of several polyphenols has been characterized using membrane model systems (Tsuchiya et al. 2002; Oteiza et al. 2005; de Athayde Moncorvo Collado et al. 2016). It is conceivable that some of the properties observed could be explained, at least partially, by the ability of phenolic compounds to get associated to membranes (Pérez-Fons et al. 2010). In this regard, we showed that resveratrol, a stilbene present in grapes, is able to enhance the ATPase activity of the ABC transporter ABCG1 by inducing changes of the lipid bilayer where this protein is inserted (de Athayde Moncorvo Collado et al. 2013). Furthermore, it was recently proved that epigallocatechin gallate (EGCG) also modifies the activity of a membrane-bound protein upon binding to the phospholipid head groups of the red blood cell membranes (Salazar et al. 2017). However, an inhibition of the protein activity, i.e., erythrocyte acetylcholinesterase, is observed in this case. Indeed, the final outcome of the polyphenol-membrane interaction may differ depending on the nature of the phenolic compound, the interplay with membrane proteins, and, undoubtedly, the penetration into the membrane or binding to the lipid surface. For instance, it had been already demonstrated that EGCG mainly interacted with the surface of the membranes in model systems (de Athayde Moncorvo Collado et al. 2016; Ulrih et al. 2010). Thus, it was not completely unexpected to find a similar result in ghosts of red blood cells, although a deeper insertion of EGCG in red blood cells has also been reported (Cyboran et al. 2015). As mentioned above, the consequence of this association is the

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inhibition of the acetylcholinesterase activity, which turns out to be a good model for the neuronal isoform (Salazar et al. 2017). On the contrary, resveratrol can certainly get inserted into the membranes, mostly cholesterol-poor phospholipid bilayers (de Athayde Moncorvo Collado et al. 2013; Brittes et al. 2010) and it can also interact with proteins (Ghorbani et al. 2015; Maliszewska et al. 2016). In the case of ABCG1 proteoliposomes, resveratrol interacts with both lipid and protein, but only the interaction with phospholipids is responsible for the enhancement of ATPase activity (de Athayde Moncorvo Collado et al. 2013).

After characterizing the interaction of different phenolic compounds as the mentioned resveratrol and EGCG, alongside the citric flavanone naringenin and the mammal enterolignan enterodiol in liposomes and Langmuir monolayers, we decided to tackle the interaction of polyphenols with a more complex membrane model. In particular, we chose EGCG because it was already studied by our group and others and enterodiol because it has not been studied in detail so far. Moreover, these two compounds represent polyphenols that are metabolized at different degrees. Catechins are well known to be extensively inactivated by enterocytes and hepatocytes (Donovan et al. 2001). Furthermore, EGCG also undergoes liver as well as small intestine metabolism (Mereles and Hunstein 2011). EGCG and enterodiol belong to two different families of polyphenols; flavan-3-ol and lignans, respectively (see structure in Fig. 1). Liver membranes were chosen as a model, as they can be obtained by a straightforward protocol with very good yields. Besides, several ATPases and ABC transporters are expressed in these membranes, which is an interesting feature because it allows us to study more closely the effect of the membrane-polyphenol interaction on the ATPase and other activities (Wlcek and Stieger 2014).

On this regard, it has recently been reported that mammal enterolignans plasma levels can be regulated, at least partially, by a mammary gland ABCG2-mediated mechanism (García-Mateos et al. 2017). Among other polyphenols that were already tested with ABC transporters is naringenin. Indeed, this flavanone acts as a competitive inhibitor for ABCC1 but at the same time, it stimulates the ATPase activity and enhances the transport of reduced glutathione, presumably because it would be co-transported with GSH (Leslie et al. 2001). In the same trend, Tammela et al. demonstrated that naringenin can also be actively transported in Caco2 cells, whereas other polyphenols cannot (Tammela et al. 2004). Lastly, it has been reported that several stilbenes can inhibit the transport activity of the ABC transporter ABCC1, although resveratrol itself proved to be ineffective in doing so (Bobrowska-Hägerstrand et al. 2006). However, experimental evidence suggests that unconjugated resveratrol derivatives can be efficiently effluxed by ABCG2 as well, whereas conjugated resveratrol can be pumped out the cells via ABCC2 (Maier-Salamon et al. 2013).

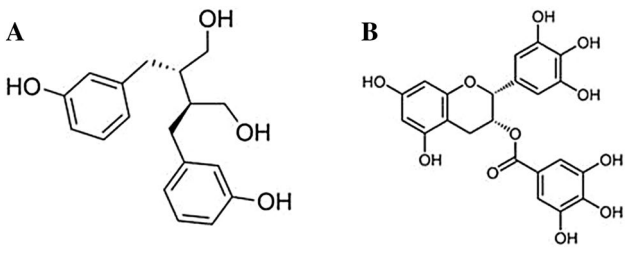

Fig. 1 Chemical structure of enterodiol (Ed, **a**) and epigallocatechin-3-gallate (EGCG, **b**)

In the present work, we show that enterodiol can be transported by hepatic membranes but EGCG cannot. In addition, the transport of enterodiol would significantly increase the ATPase activity. Interestingly, this lignan does not significantly interact with liver membranes in the absence of ATP. The interplay between this enterolignan and membranes of the mentioned tissues might have important consequences on human health. Active transport of these compounds into the bloodstream might extend their bioavailabilities, which could be trascendent for their multiple bioactive features. It seems to be clear that compounds potentially capable of interacting with nonspecific transporters pose as promising candidates for studies such as the reversal of multidrug resistance (MDR), a condition that severely impairs the treatment of several diseases of worldwide impact.

Materials and Methods

Chemicals

Enterodiol, EGCG, naringenin, and resveratrol were purchased from Sigma-Aldrich. Stock solutions were prepared in methanol and dilutions for experimental procedures were performed in the same solvent. Concentration was routinely checked by the Folin method using appropriate standards of known concentrations. Octadecyl rhodamine B chloride (R18), 1,6 diphenyl-1,3,5-hexatriene (DPH), and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH) were purchased from Molecular Probes/Thermo Fisher Scientific. Stock solutions of fluorescent probes were prepared in methanol and kept at − 20 °C. Na_2ATP (Sigma-Aldrich) and all other reagents were of the highest purity available.

Rat Liver Membrane Preparation

Animals were used according to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, US). Livers obtained from adult Wistar rats (INSIBIO-CCT

Tucuman Animal Facility) were perfused with 25 mM tris–HCl buffer (pH 7.4) containing 100 mM NaCl. Afterward, livers were cut into small pieces with a scalpel, connective tissue was separated, and samples were homogenized in 3 ml of extraction buffer (25 mM tris–HCl pH 7.4, 100 mM NaCl, 15% glycerol, and 1 mM β-mercaptoethanol) using an Omni-Mixer (Sorvall/Thermo Fisher Scientific). Homogenate was centrifuged at 600×*g* for 10 min to remove tissue debris and non-homogenized solids. DNase I (Roche) and PMSF (Sigma-Aldrich) were added and cells were disrupted with a French Press (Thermo Fisher Scientific) using a 4 ml cell at 18,000 psi. Total liver cell extract was centrifuged at 10,000×*g* for 20 min to discard nuclei, mitochondria, and other cellular debris. The remaining suspension was centrifuged for 1 h at 50,000×*g* in a Beckman Coulter Optima Series L preparative ultracentrifuge. After careful resuspension in extraction buffer, protein, phospholipid, and cholesterol concentrations were determined by Lowry, Ames-Chen, and the cholesterol oxidase method (Sigma-Aldrich), respectively. All the procedures were carried out at 0–4 °C and the liver membranes were stored at − 70 °C.

Fluorescence Analyses of Polyphenol-Membrane Interactions

Several assays based on fluorescence measurements were carried out in order to study the association of phenolic compounds with phospholipid head groups, membrane proteins, and hydrophobic/interfacial regions of the lipid bilayer.

Phospholipid Head Groups

Possible interaction of enterodiol and EGCG with the phospholipid head groups of rat liver membranes was estimated by recording the quenching of R18 fluorescence emission (Salazar et al. 2017; Kitano et al. 1997). The probe was added to membrane extracts diluted in reaction buffer (25 mM tris–HCl buffer pH 7.4 containing 100 mM NaCl and 10 mM $MgCl_2$) under constant vortexing (lipid:probe molar ratio of 1000:1). Labeled membranes were then further diluted in microplates to a lipid concentration around 50 µM, condition in which R18 exhibited high fluorescence intensity. Polyphenols were added to final concentrations of 0.1, 0.5, 1, and 5 µM (lipid:polyphenol ratios of 500:1, 100:1, 50:1, and 10:1, respectively). After 5 min, R18 fluorescence intensity was recorded at 580 nm using a Perkin Elmer LS-55 Fluorescence Spectrometer with a 20 nm emission slit. Intensities were normalized against control readings and percent relative fluorescence was calculated. Excitation wavelength was set at 530 nm with a 5 nm excitation slit. R18 quenching determinations were performed at least in triplicate.

Protein–Phenolic Compound Interaction

Tryptophan fluorescence emission was chosen as a tool to assess whether polyphenols could locate in close proximity to membrane-bound proteins (Strugała et al. 2016). Briefly, liver membranes were diluted to a protein concentration of 7.5 µg/ml with reaction buffer, resulting in a lipid concentration around 15 µM. Then, polyphenols were added to final concentrations of 0.1, 0.5, and 1 µM (lipid:polyphenol ratios of 150:1, 30:1, and 15:1). After 5 min, emission spectra were recorded from 316 to 374 nm through a 315 nm cut-off Longpass Filter (Edmund Optics) using quartz cuvettes. The excitation wavelength was set at 295 nm. Control readings were conducted adding the same volume of methanol. Spectra of buffer alone and polyphenols without membrane extract were also recorded to correct fluorescence readings at each condition. Fluorescence intensities at 332 nm were normalized against control readings and relative fluorescence was calculated for each polyphenol concentration. KI was used as tryptophan fluorescence quencher in independent assays as a positive control. Resveratrol was used as a positive control because it has been shown that it can get associated to membrane proteins (de Athayde Moncorvo Collado et al. 2013; Gledhill et al. 2007).

Experiments were performed in triplicate in a photon counting spectrofluorometer at 37 °C.

Hydrophobic Core and Interphase of Lipid Bilayer

Modulation of membrane order degree in the presence of the aforementioned polyphenols was assessed by measuring DPH and TMA-DPH fluorescence anisotropies; thus analyzing the hydrophobic core and the interfacial region, respectively (de Athayde Moncorvo Collado et al. 2016). Briefly, TMA-DPH or DPH was added to a diluted liver cell membrane suspension (final lipid concentration was 15 µM) in reaction buffer under constant vortexing. Final lipid:probe molar ratio was 800:1. Labeled membranes were incubated for 30 min at 4 °C in the dark and then thermostated at 37 °C. Polyphenols were added to a lipid:polyphenol molar ratio of 100:1 (0.15 µM). Control assays were carried out adding the same volume of methanol. Excitation/emission wavelengths were set at 360 and 450 nm for DPH whereas for TMA-DPH they were set at 360 and 435 nm, respectively. Total fluorescent intensities were recorded in a photon counting spectrofluorometer and the steady-state fluorescence anisotropy (r) values were calculated by Vinci software (ISS) as described in

previous works. Determinations were performed in three replicate sets of 8 readings for each point.

ATPase Activity Measurement

Mg^{2+}-dependent total ATPase activity was measured in the absence and in the presence of polyphenols. Assays were carried out in reaction buffer at 37 °C in a final volume of 30 µl. Rat liver membranes were diluted to obtain a final protein and lipid concentration of 0.25 mg/ml and 0.45 mM, respectively (typically 1:200). Reactions were initiated by the addition of 6 mM Na_2ATP to membrane suspensions that were preincubated with each polyphenol (lipid:polyphenol molar ratio of approximately 100:1). ATPase activity was estimated spectrophotometrically by measuring the release of inorganic phosphate as a result of ATP hydrolysis using an UV/Visible Beckman DU 7500 spectrophotometer. In control reactions, ATP, polyphenols, and membranes were replaced by double distilled water, methanol, and buffer, respectively. Resveratrol was used as a positive control, based on our previous finding in proteoliposomes (de Athayde Moncorvo Collado et al. 2013). Reactions were conducted as three independent experiments carried out in triplicate.

Polyphenol Active Transport into Rat Liver Membrane-Derived Inside–Out Vesicles

In order to determine whether active transport of polyphenols could modify the distribution of these compounds between membrane and the aqueous solution, their concentrations were measured after separation of both phases in the absence and in the presence of ATP. It is important to note that membrane samples were obtained by French press, which implies that resulting vesicles would be mostly inside–out. Therefore, ATP-binding domains would be exposed and readily available for the ATP added. Consequently, upon addition of ATP polyphenol molecules would be transported into the lumen of the inside–out vesicles, thus being trapped inside them. In the absence of ATP, however, only association of phenolic compounds to the membranes would be observed.

For transport assays, 250 µl of rat liver membranes suspended in reaction buffer were incubated for 10 min with each polyphenol at a final lipid:polyphenol molar ratio of 100:1. Then, 1.5 mM Na_2ATP was added and samples were further incubated for 30 min. Suspensions were centrifuged and supernatants were carefully collected for polyphenol determination. For EGCG readings, 200 µl aliquot of supernatants was placed in a microplate and absorbance was measured using a Molecular Devices Spectra Max Plus spectrophotometer at 328 nm. A calibration curve was constructed with standard of known concentration. For enterodiol determinations, interfering proteins were eliminated with trichloroacetic acid. Suspensions were centrifuged at 14,500×*g* for 10 min and supernatants were carefully collected. Fluorescence intensities were read at 322 nm with the aid of a 315 nm cut-off Longpass Filter (Edmund Optics) using a 150 µl quartz cuvette. Excitation wavelength was set at 270 nm. A calibration curve was constructed by reading fluorescence intensity of enterodiol standards of known concentrations. Naringenin was used as a positive control, since it was already reported that this flavanone can be transported upon addition of ATP (Leslie et al. 2001; Tammela et al. 2004). In this case, the absorbance at 315 nm was measured in the samples.

Polyphenol association to membrane-derived vesicles was calculated according to:

$$\%\text{Polyphenol}_{\text{membrane}} = [\text{polyphenol}]_{\text{added}} - [\text{polyphenol}]_{\text{supernatant}}/[\text{polyphenol}]_{\text{added}} \times 100.$$

Absorbance or fluorescence intensities of membrane extracts incubated with methanol and water were also measured. Na_2ATP did not exhibit significant absorbance between 315 and 328 nm and no fluorescence emission was detected at the conditions described above. A control of ATP and polyphenols was also included. Experiments were performed at least three times and in triplicate.

Miscellaneous

Graphics and statistical analysis were performed using Prism GraphPad software. Fluorescence readings were carried out in an ISS PC1 Photon Counting Spectrofluorimeter L format equipped with polarizers for both the excitation and emission beams for anisotropy determinations and a cuvette holder thermostatized at 37 °C by means of an external circulating bath (Cole Parmer), unless stated otherwise.

Results

Rat Liver Cell Membrane Isolation

Membrane extraction and isolation was carried out as described in Materials and methods. On the crude extract, protein concentration was typically in the order of 50 mg/ml, while lipid was in the order of 95 mM (around 65 mM for phospholipids and 30 mM cholesterol). Every membrane preparation consistently displayed significant and highly reproducible ATPase activity, even after storage at − 70 °C for up to 3 months. Once thawed and reconstituted, membranes were kept at 4 °C and ATPase activity remained almost unchanged for up to 5 days.

EGCG Closely Interacts with Phospholipid Head Groups

Interaction between polyphenols and phospholipid head groups of rat liver membrane extracts was assessed by determining the fluorescence quenching of R18. The probe was added in low concentrations in order to achieve high fluorescence readings. Presence of phenolic compounds in close proximity to the probe would render in a decrease of fluorescence emission. As it can be observed in Fig. 2a, EGCG is able to quench R18 emission even at the lowest concentration tested (lipid:polyphenol molar ratio of 500:1), whereas enterodiol is unable to significantly quench R18 emission even at the highest concentration tested.

In order to examine whether polyphenols could locate in close proximity to membrane-bound proteins, tryptophan fluorescence spectra was recorded. As it can be seen in Fig. 2b, tryptophan emission is barely quenched in the presence of enterodiol and EGCG even at the highest concentrations. On the contrary, resveratrol efficiently quenchs Trp fluorescence as expected. It can be concluded that neither enterodiol nor EGCG would locate nearby membrane bound proteins, at least close to tryptophan residues. No significant spectral shift is observed for any of the phenolic compounds employed in the experiments, which strongly suggests that no conformational changes in membrane-bound proteins take place upon addition of these polyphenols.

In addition, a possible modulation of membrane order degree caused by the presence of polyphenols was assessed by determining fluorescence anisotropy of DPH and TMA-DPH. No significant change is detected in the hydrophobic core or in the interfacial region (data not shown).

Enterodiol Enhances ATPase Activity of Rat Liver Cell Membranes

Assays for determining total Mg^{2+}-dependent ATPase activity were performed as described in Materials and methods. As shown in Fig. 3, specific ATPase activity is in the order of 100 µmol/ml/h of inorganic phosphate released from ATP hydrolysis. ATPase activity increases twofolds in the presence of enterodiol and approximately 40% in the presence of resveratrol (positive control). As expected, EGCG fails to modify ATPase activity at the tested conditions.

Enterodiol is Actively Transported into Inside–Out Vesicles Derived from Rat Liver Cell Membranes

In order to establish whether active transport of these compounds could result in a different distribution between membranes and the aqueous medium, the amount of polyphenol associated to inside–out vesicles was assessed

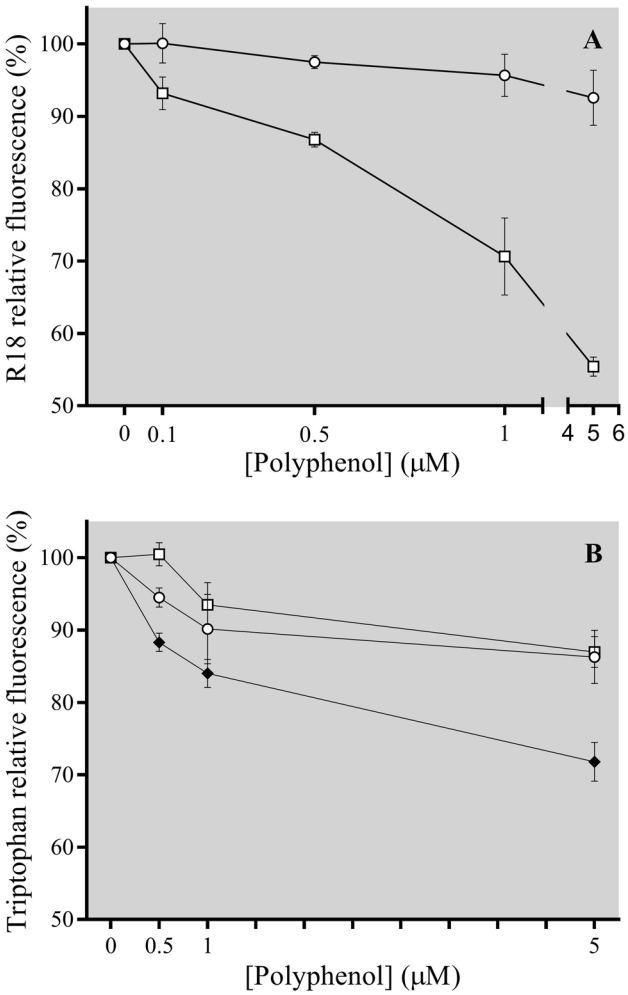

Fig. 2 Polyphenol-rat liver derived membrane interaction assessed by fluorescence techniques. **a** Quenching of R18-dyed membranes in the presence of different concentrations of enterodiol (open circle) and EGCG (open square) after 5 min of incubation, shown as relative fluorescence intensity (readings without polyphenol represent 100%). Each point was determined by triplicate, values shown are mean ± standard deviation. **b** Quenching of tryptophan fluorescence of membrane bound proteins in the absence or in the presence of increasing concentrations of enterodiol (open circle), EGCG (open square), and resveratrol (closed diamond, positive control), shown as relative fluorescence intensity (readings without polyphenol represent 100%). Each point was determined by triplicate, values shown are mean ± standard deviation

in the absence and in the presence of ATP. As it can be seen in Fig. 4, no significant change is detected in the distribution of EGCG upon addition of ATP. In fact, the association of this compound with membrane vesicles is approximately 60% regardless the presence or absence of ATP. This result rules out an active transport of EGCG in these conditions. Surprisingly, enterodiol association to the membrane fraction greatly increases, from 15 to nearly 85% after ATP addition, evidencing that an active transport of this polyphenol takes place. In turn, the amount of

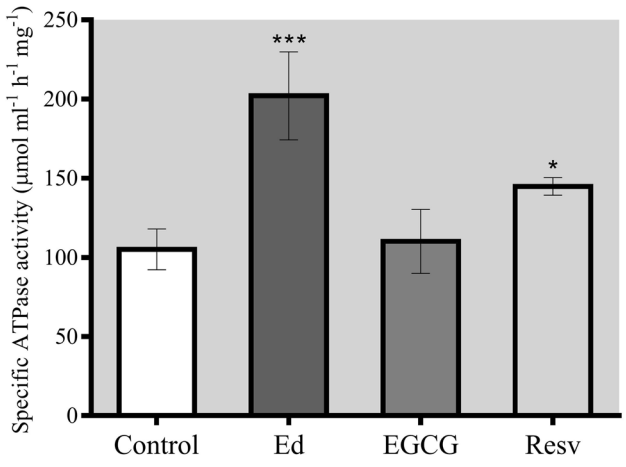

Fig. 3 Effect on total ATPase activity of rat liver derived membranes by polyphenols. Mg^{2+} dependent specific ATPase activity was measured in the absence (white) and in the presence of enterodiol, EGCG, and resveratrol (Resv, positive control) in gray scale, from darker to lighter, respectively, in a lipid:polyphenol molar ratio of 100:1 at 37 °C. Values shown are mean ± standard deviation. Each assay was performed by triplicate, results shown are representative of three independent experiments. One-way ANOVA and Dunett's multiple comparisons test (each group compared to Control) were performed and a score from * to *** was assigned to significantly different groups (α = 0.01)

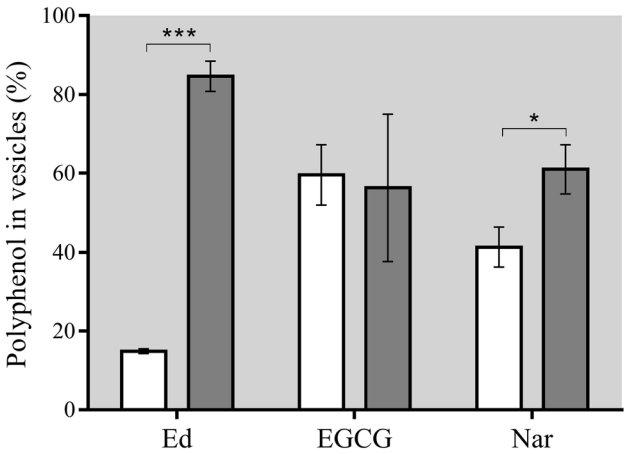

Fig. 4 Polyphenol association to rat liver membranes derived inside–out vesicles. Association of enterodiol, EGCG, and naringenin (Nar, positive control) to membrane fraction separated after 30 min of incubation was calculated as described in "Materials and Methods" section in the absence (white) and in the presence of ATP (gray). Values shown are mean ± standard deviation. Assays were performed by triplicate, graph is representative of three independent experiments. Significant differences between Control and ATP treated groups as determined by *t* test (Holm–Sidak method) are marked with "*" (α = 0.01) and "***" (α = 0.001)

naringenin associated to vesicles significantly increases from 40 to ~ 60% upon addition of ATP, as it was already reported (Leslie et al. 2001).

Discussion

In the present work, we analyzed the interaction of polyphenols with the surface and the interphase of liver membranes. It has been observed that some polyphenols, including resveratrol, naringenin, and, very recently, enterodiol are capable of interacting with ABC transporters (de Athayde Moncorvo Collado et al. 2013; García-Mateos et al. 2017; Leslie et al. 2001; Bobrowska-Hägerstrand et al. 2006). Interestingly, when concentrations ranged from 1 to 10 µM, activity enhancing properties can be detected (Hirano et al. 1989; Duchnowicz et al. 2012). Thus, we maintained the lipid:polyphenol ratio of 100:1 for activity determination assays which resulted in polyphenol final concentration of 5 µM. This value is in the range of physiological concentrations of enterodiol in mouse plasma under a normal diet (García-Mateos et al. 2017; Kuijsten et al. 2006).

Enterodiol significantly increases the ATPase activity in rat liver membranes, even at a higher degree than resveratrol, although this effect is not appreciated with EGCG. It was previously demonstrated that resveratrol enhances ATPase activity of ABCG1 in cholesterol-poor egg PC proteoliposomes by an indirect mechanism that involves a strong increase in membrane order (de Athayde Moncorvo Collado et al. 2013). In the case of enterodiol, the increase in ATPase activity would not involve an interaction with membranes or the associated proteins. On the contrary, some compounds can bind the transporters inhibiting ATPase activity and blocking the transport of different substrates (Aurade et al. 2010). Hence, enterodiol may have a different mechanism. We hypothesized that this polyphenol could be transported by non-specific transporters. To test this hypothesis, the association of polyphenols to rat liver inside–out vesicles was determined, both in the absence and the presence of ATP. Upon addition of ATP, polyphenol molecules would be transported into the lumen of the inside–out vesicles, thus being trapped inside them. In the absence of ATP, EGCG exhibits the highest affinity for membranes, as it is reported in the bibliography (Arora et al. 2000; Caturla et al. 2003). Consistently with the results mentioned above, enterodiol shows poor affinity for membranes. Nevertheless, after the addition of ATP, the distribution of enterodiol significantly changed suggesting that an active transport could take place. So far, active transport of enterodiol has only been reported in mouse mammary gland model by ABCG2, a non-specific transporter that is also expressed in the liver (García-Mateos et al. 2017). Besides, the closely related lignan enterolactone has been described as an ABCG2 competitive inhibitor of cancer drugs efflux in mammal models (Miguel et al. 2014).

The interaction of these and other polyphenols with liver membranes deserves to be further explored as it could have important implications on human health. Evidence that

enterodiol can be actively transported by rat liver membranes is a prime example of this. Furthermore, active transport of enterodiol out of hepatocytes could be responsible, at least partially, of its longer bioavailability compared to other compounds. In this way, free enterodiol would be available for interacting with its target tissues, exerting a wide assortment of health benefits. On the contrary, the low concentration of EGCG in the plasma may be due to the fact that this flavan-3-ol is not actively transported out of hepatocytes and, therefore, it is available for extensive chemical modification.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Statement This article does not contain any studies with human participants or animals performed by any of the authors.

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