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Resveratrol modulates ATPase activity of liposome-reconstituted ABCG1

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ABSTRACT

ABCG1 is a half-sized transporter with an unquestionable importance in cholesterol homeostasis. So far, its expression and thus its activity was suggested to be regulated at transcriptional level by LXR and PPAR agonists including polyphenols. However, it is unknown whether there are other mechanisms of up-regulation of ABCG1 activity. In the present work resveratrol was shown to induce a nearly twofold increase in ATPase activity of reconstituted ABCG1. Evidence is presented for the first time suggesting that resveratrol is able to activate ABCG1 activity by an alternative mechanism that involves an indirect interaction.

1. Introduction

ABC-transporters belong to a superfamily of proteins that includes close to fifty members in humans that can be subdivided into seven different groups [1]. Interestingly, just a few ABC-transporters were confirmed to be committed to cholesterol transport [2]. Among them, ABCG1 seems to have a crucial role in cholesterol homeostasis [3,4].

ABCG1 expression, and thus its activity, is known to be regulated by LXR and PPARs [5,6]. Indeed, synthetic LXR agonists are able to increase ABCG1 at both mRNA and protein levels [7]. On the other hand, there are natural compounds such as polyphenols that can up-regulate ABCG1 also via activation of LXR and PPAR receptors [8,9].

Besides these effects at transcriptional level, there are accumulating data showing that resveratrol like other polyphenols may interact with membranes as well [10]. In fact, polyphenols are able to change the physochemical properties of the membranes upon insertion, either fluidifying or increasing membrane viscosity depending on the nature of the polyphenol [11]. Pioneering work carried out in our lab unquestionably demonstrated that membrane protein activity could be modulated by the regulation of membrane fluidity [12].

Interestingly, the activity of some ABC-transporters can be modulated by the addition of polyphenols as a consequence of a direct interaction [13]. In this regard, Bobrowska-Hägerstrand et al showed that several stilbenes were powerful inhibitors of ABCB1 [14]. As a matter of fact, this direct regulation was mainly proven in ABC-transporters involved in drug efflux and multiresistance. Indeed, there is no information on the possible modulation of the activities of other ABC-transporters like those involved in cholesterol metabolism.

To explore the effect of a polyphenol such as resveratrol on ABCG1, we expressed human ABCG1 in yeasts, reconstituted it into proteoliposomes and examined its ATPase activity, previously reported as a reliable way to assess reconstituted ABC-transporters activity [15]. This methodology was carried out in order to have a simple system to test our hypothesis i.e. that resveratrol may modulate ABCG1 activity not only at transcriptional level but also by direct interaction with either the transporter or the membrane where the transporter is inserted. We conclusively show that resveratrol does regulate ABCG1 activity in the micromolar range of concentrations.

2. Materials and methods

2.1. Heterologous expression and purification of ABCG1

2.1 kb fragment of the long isoform of *ABCG1* was amplified by RT-PCR from macrophage cDNA, inserted into the pEGFP-N3 vector

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(Clontech) and then sub-cloned into the yeast shuttle vector pCHm3H10C [16]. The final plasmid was called pCH-ABCG1-H10C (Supplementary Fig. S1) and was used to transform competent *Saccharomyces cerevisiae* BJ2168, which is a protease deficient strain [17]. For this purpose, a modification of the classical lithium acetate protocol was followed [18], where sodium acetate was used instead. Colonies were picked after three to five days of incubation at 30 °C in S.D. medium [19]. The presence of pCH-ABCG1-H10C was confirmed by yeast colony PCR [20]. Finally, the expression was analyzed by SDS–PAGE [21]. Gels were stained following a fast Colloidal Coomassie Blue protocol [22]. ABCG1-expressing yeasts were grown till late exponential phase and collected by centrifugation at 6000×*g* for 10 min. The membrane-rich fraction was collected at 50,000×*g* after disruption with French Press and resuspended in 20 mM phosphate buffer pH 7.4, 500 mM NaCl, 10% (v/v) glycerol, 10 mM imidazole and 1 mM β-mercaptoethanol containing 1% dodecyl-β-D-maltoside (DDM). Detergent-solubilized proteins were loaded in a plastic column with IMAC Sepharose™ High Performance R10 charged with Ni^{2+} (GE Healthcare) previously equilibrated with the same buffer containing 0.1% DDM. ABCG1 was eluted in 4 fractions of 1 ml each with the same buffer containing 200 mM imidazole. The samples were kept at 4 °C up to 2 weeks.

2.2. Reconstitution and ATPase activity

Unilamellar phosphatidylcholine (PC) liposomes with different cholesterol composition were obtained from multilamellar vesicles by extrusion using a Mini-Extruder with a 200 nm pore diameter membrane filter (Avanti Polar Lipids). Typically, 100 μg of protein (approximately 350 μl of pure detergent-solubilized ABCG1) was mixed with 250 μl of unilamellar liposomes suspension (1 mM final lipid concentration) and dialyzed for 24 h at 4 °C with 50 mM Tris–HCl buffer pH 7.4 containing 100 mM NaCl and 5 mM β-mercaptoethanol. Cholesterol was added either at 40% molar or at 0.1% molar.

Lipid concentration of resulting proteoliposomes was typically around 2 μmol/ml as estimated by Ames-Chem method [23]; whereas protein concentration was around 0.20–0.25 μg/μl as estimated by Lowry method [24]. Reconstituted ABCG1 was used within the day of preparation. For ATPase activity assays, reactions were performed in 50 mM Tris–HCl buffer pH 7.4, 6 mM Na_2ATP and 8 mM $MgCl_2$ at 37 °C in a final volume of 30 μl. Resveratrol (Sigma) was added at final concentrations of 0.05, 0.1, 0.5 and 1 mM. Reactions were initiated upon addition of 10 μl of reconstituted ABCG1 and stopped by the addition of 30 μl of 10% SDS and vigorous shaking. Samples were then boiled for 10 min and ATPase activity of ABCG1-enriched fraction was estimated spectrophotometrically by measuring inorganic phosphate [25].

2.3. Fluorescence spectroscopy. Analysis of membrane-bound ABCG1-resveratrol interaction

ABCG1 was reconstituted into liposomes as described above, containing 1,6-diphenyl-1,3,5-hexatriene (DPH) (Invitrogen) in lipid:DPH ratio of 1000:1. Membrane fluidity was determined by steady-state fluorescence anisotropy (r) in the presence or absence of resveratrol. The steady-state DPH fluorescence anisotropy was determined adjusting the excitation and emission wavelengths at 360 and 450 nm, respectively. Anisotropy was calculated as $r_{DPH} = (I_{||} - I_{\perp})/(I_{||} + 2I_{\perp})$, being $I_{||}$ the fluorescence intensity recorded with the analyzing polarizer oriented parallel while $I_{\perp}$ is the fluorescence measured when polarizer was set perpendicular to the excitation beam [26]. On parallel experiments, solvent penetration in the lipid bilayer was assessed by measuring the 6-dodecanoyl-2-dimethylaminonaphthalene (laurdan) emission red shift as a consequence of membrane water content. These shifts were quantified as generalized polarization (GP) as follows: $GP = (I_{\lambda 440} - I_{\lambda 490})/(I_{\lambda 440} + I_{\lambda 490})$ [27]. For these measurements, liposomes were prepared as described above containing laurdan in lipid:laurdan ratio of 800:1. For tryptophan emission spectra analysis, excitation wavelength was set at 280 nm while the emission spectra were recorded from 320 to 380 nm. Increasing concentrations of resveratrol from 0.005 to 0.1 mM were added to proteoliposomes from a stock solution. All fluorescence measurements were carried out using an ISS PC1 spectrofluorometer L-format equipped with a thermostatic cuvette holder set at 37 °C.

3. Results

3.1. Modulation of the ATPase activity by resveratrol

ABCG1 was successfully expressed in *S. cerevisiae* BJ2168 and purified by affinity chromatography as described in Section 2 (see Supplementary Fig. S2). After reconstitution into proteoliposomes, ATPase activity measurement was chosen as a tool to assess changes in protein activity [28]. Based on the protocol followed, at least half of the NBD domains would be oriented outwards since the large N-terminal domain may help to insert the protein in that position in preformed liposomes [29,30]. Fig. 1 shows that ATPase activity of ABCG1 reconstituted in PC liposomes was greatly improved by the addition of the polyphenol. In fact, there was nearly a threefold increase in ATPase activity upon addition of 50 μM resveratrol. Since Dr. Ueda's group recently showed that 40% cholesterol was optimal for ABCG1 activity [28], liposomes with this composition were also prepared. As reported by Hirayama et al., ATPase activity was higher in that condition as compared to activity of ABCG1 reconstituted in PC liposomes [28] and similar to the activity in PC liposomes plus 50 μM resveratrol (see Supplementary Fig. S3). Importantly, the presence of different concentrations of resveratrol did not further increase ATPase activity in liposomes prepared with 40% cholesterol (Fig. S3).

3.2. Membrane physico-chemical properties

50–100 μM resveratrol induced slight but statistically significant changes in the viscosity of PC acyl chains of ABCG1 containing liposomes as it can be estimated from anisotropy values (Fig. 2). On

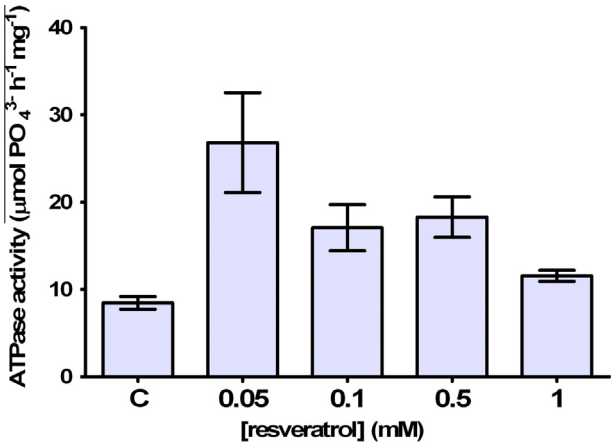

Fig. 1. Resveratrol enhances ATPase activity. ABCG1 was reconstituted into unilamellar liposomes as described in Section 2. Proteoliposomes were incubated with increasing concentrations of resveratrol (0.05–1 mM) in the presence of Na_2ATP. Residual phosphate was detected spectrophotometrically as described in Section 2. Experiments were performed at least three times and in triplicate; values are expressed as specific activityaboratory of Molecular Cell Biology. Insti± standard deviation.

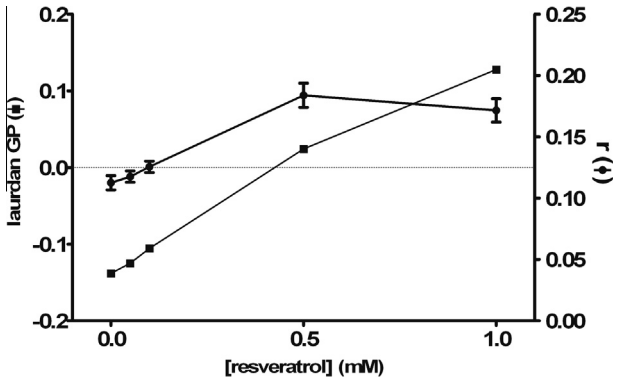

Fig. 2. Fluorescence anisotropy and solvent accessibility changes induced by resveratrol. ABCG1-containing proteoliposomes were prepared with the addition of the fluorescent probes DPH or laurdan. Proteoliposomes were incubated 15 min at 37 °C with increasing concentrations of resveratrol (from 0.05 to 1 mM) and measurements were carried out as described in Section 2. Excitation and emission wavelengths were adjusted at 360 and 450 nm, respectively for DPH anisotropy studies (●) while laurdan emission was recorded at 440 and 490 nm in order to calculate GP (■) (λ_{exc} = 350 nm). Experiments were performed at least three times by triplicate. Values are expressed as mean ± standard deviation.

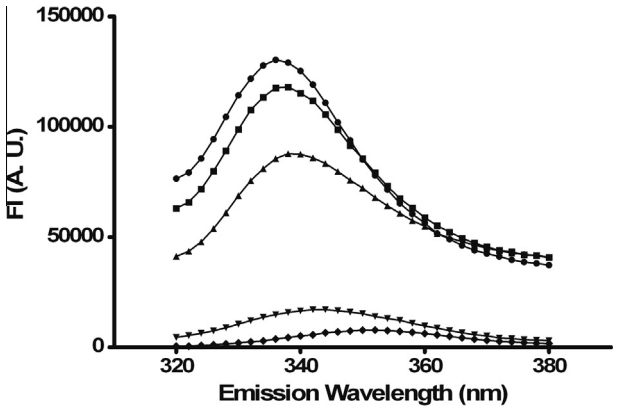

Fig. 3. Resveratrol-ABCG1 interaction assessed by tryptophan emission spectrum analysis. ABCG1-containing unilamellar proteoliposomes were prepared and incubated 15 min at 37 °C with increasing concentrations of resveratrol: 0 (●), 5 μM (■), 10 μM (▲), 50 μM (▼) and 100 μM (♦). Tryptophan fluorescence spectra (λ_{em} = 280 nm) were obtained. Values are representative of three independent experiments.

the other hand, laurdan generalized polarization analysis confirms and complements the information provided by the DPH fluorescence anisotropy determinations. In fact, Fig. 2 also shows that there is a concomitant increase in GP values i.e. a blue shift of laurdan emission fluorescence upon addition of the polyphenol, strongly suggesting that resveratrol induces changes in PC membranes. Taken together, these results allow us to hypothesize that resveratrol decreases the water accessibility to the membrane interphase and increases membrane order [31]. Interestingly, 40% cholesterol would render in membranes with nearly the same anisotropy and GP values as compared to PC liposomes plus 50 μM resveratrol. The addition of resveratrol did further increase these values.

3.3. Resveratrol-ABCG1 interaction

From the data presented above, resveratrol did increase ABCG1 activity and at the same time it would change the physico-chemical properties of the membrane. These results would favor an indirect activation of ABCG1. However, a direct interaction polyphenol-protein could not be ruled out. In order to analyze this possibility, ABCG1 tryptophan spectra were recorded in the presence and in the absence of different concentrations of resveratrol. A marked red shift upon addition of this polyphenol was observed, strongly suggesting that some conformational changes might occur in the presence of resveratrol i.e. tryptophans would become more exposed to the polar environment. The red shift was observed from resveratrol concentrations as low as 5 μM. Interestingly, 50 and 100 μM induced the greatest shifts (Fig. 3). On the other hand, it was seen a marked decrease in the tryptophan quantum yield upon addition of resveratrol, strongly suggesting that this polyphenol would localize in close proximity to ABCG1 tryptophans, quenching their fluorescence. However, when ABCG1 was reconstituted in membranes with 40% cholesterol, same fluorescence changes were seen. Taking into account that no further improvements in activity were observed, resveratrol might not modulate ATPase activity of ABCG1 by direct interaction with this protein.

4. Discussion

It is well known since a long time ago, that there is an inverse correlation between high density lipoprotein cholesterol levels (HDL-C) and the incidence of atherosclerosis [32], because HDL-C are implicated in the removal of cholesterol from peripheral tissues to the liver in the so-called reverse cholesterol transport [33]. The modulation of the activity of the cholesterol transporters involved in this process would represent a promising therapeutic strategy for cardiovascular disease [34]. Actually, LXR activators are being considered as therapeutic drugs because they might increase a variety of molecules involved in cholesterol efflux and transport via LXR up-regulation [35]. We propose an alternative approach i.e. that there are compounds able to enhance cholesterol transporter activities by direct interaction with the transporter itself or with the membrane where the transporter is immersed. We chose ABCG1 as a model protein ABC-transporters because it undoubtedly plays a role in cholesterol homeostasis and its size is suitable for in vitro manipulations [3]. *S. cerevisiae* was selected as the host for heterologous expression because large quantities of recombinant protein can be achieved besides the simple and inexpensive culture conditions and reliable results [36]. Furthermore, the close-related transporter ABCG2 was successfully expressed very recently in *S. cerevisiae* [37]. The authors were able to show that ABCG2 retained its drug-stimulated ATPase activity once purified and reconstituted from yeast membranes. Moreover, they used the same yeast shuttle plasmid backbone, although the poly-His tag was placed at the N-terminal of ABCG2 [37].

The possible regulation of ABCG1 activity by resveratrol in a non-transcriptional way was analyzed. We chose resveratrol as a potential modulator because it was already reported that displays a positive effect on ABCG1 expression [9]. Based on the results presented here, it can be concluded that resveratrol does regulate ABCG1 ATPase activity. Interestingly, the concentration needed to observe the activation of ABCG1 was in the same order of concentrations tested elsewhere.

Regarding the mechanism of action, an indirect interaction is proposed based on our observations. On one hand, fluorescence anisotropy as well as GP changes were observed when resveratrol was added, hence indicating that a perturbation of the membrane was produced. In fact, the results obtained here strongly suggest that an increase in membrane acyl chains order is induced upon addition of the polyphenol. Comparing ATPase activity in the presence of resveratrol with lipid order changes would suggest that there is an optimal membrane viscosity for ABCG1 that is obtained at 50–100 μM resveratrol. Beyond this level no improvements were observed in spite of having even higher viscosity levels.

Hirayama et al. reported that ABCG1 activity was enhanced in the presence of 40% cholesterol, which increases membrane viscosity, again higher values of cholesterol did not have further effects [28]. We confirmed this observation and saw anisotropy values similar to those associated to 50–100 μM resveratrol, where activity was maximal. On the contrary, a direct interaction would not be involved. The addition of resveratrol to ABCG1 proteoliposomes did quench tryptophan fluorescence in great extent and a red shift was observed in tryptophan fluorescence, strongly suggesting that a close interaction of resveratrol with ABCG1 may take place, as reported for other proteins [38,39]. However, these very same changes were also seen in liposomes prepared with 40% cholesterol, where no further enhancement of the activity could be found, ruling out any involvement of the direct interaction ABCG1-polyphenol with ATPase activity. Even though changes in tryptophan fluorescence emission suggest that an interaction polyphenol-protein takes place, these fluorescence changes could also be ascribed to changes in the membrane properties triggered by resveratrol, which in turn would induce protein structural changes.

In summary, we showed for the very first time that resveratrol may have a different way to stimulate ABCG1 ATPase activity. In the present work, evidence is presented supporting the idea that resveratrol might interact with membranes thus enhancing its ATPase activity, particularly, membrane domains with low content of cholesterol, where ABCG1 ATPase activity is relatively low. This is an alternative mechanism, different from transcriptional regulation described so far.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.06.001.

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