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Resveratrol modulates ATPase activity of liposome-reconstituted ABCG1

Alejandro de Athayde Moncorvo Collado [a], Natalia Corbalán [a], László Homolya [b], Roberto Morero [a], Carlos Minahk [a,*]

[a] Instituto Superior de Investigaciones Biológicas (CONICET-UNT), Instituto de Química Biológica "Dr. Bernabe Bloj", San Miguel de Tucumán, Argentina
[b] Laboratory of Molecular Cell Biology, Institute of Molecular Pharmacology, Research Centre for Natural Sciences, Hungarian Academy of Sciences, Pusztaszeri ut 59-67, H-1025 Budapest, Hungary

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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

* Corresponding author. Address: Chacabuco 461, San Miguel de Tucumán T4000ILI, Argentina. Fax: +54 0381 4248921.
E-mail address: cminahk@fbqf.unt.edu.ar (C. Minahk).

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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

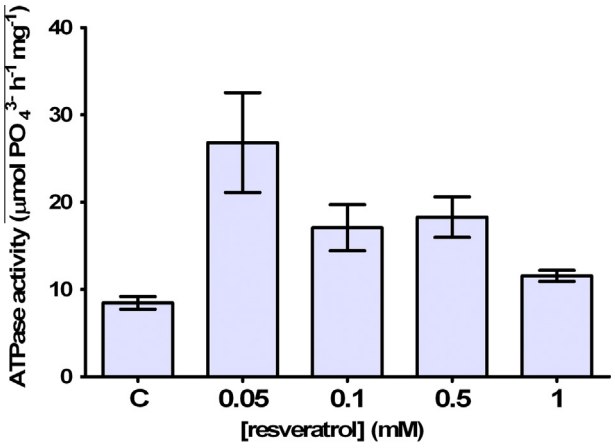

Fig. 1. Resveratrol enhances ATPase activity. ABCG1 was reconstituted into unilamellar liposomes as described in Section 2. Proteoliposomes were incubated with increasing concentrations of resveratrol (0.05–1 mM) in the presence of Na_2ATP. Residual phosphate was detected spectrophotometrically as described 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.