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Effects of verbascoside, a phenylpropanoid glycoside from lemon verbena, on phospholipid model membranes

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ABSTRACT

Phenylpropanoid glycosides are water-soluble compounds widely distributed, most of them deriving from medicinal herbs. Among them, verbascoside or acteoside has exhibited a wide biological activity, being free radical scavenging the most representative one. Moreover, antitumor, antimicrobial, anti-inflammatory, anti-thrombotic and wound healing properties have been previously described. Herein, the interaction of verbascoside with phospholipid membranes has been studied by means of differential scanning calorimetry, fluorescence anisotropy and dynamic light scattering. Verbascoside showed stronger affinity for negatively charged membranes composed of phosphatidylglycerol (PG) than for phosphatidylcholine (PC) membranes. This compound promoted phase separation of lipid domains in PC membranes and formed a stable lipid complex with and approximate phospholipid/verbascoside ratio of 4:1. Despite its hydrophilic character, verbascoside's caffeoyl moiety was located deep into the hydrophobic core of PC membranes and was almost inaccessible to spin probes located at different depths in PG membranes. This compound affected the ionization behavior of the PG phosphate group and most likely interacted with the vesicles surface. The presence of verbascoside decreased the particle size in PG unilamellar vesicles through the increase of the phospholipid head group area. A localization of verbascoside filling the upper region of PG bilayers close to the phospholipid/water interface is proposed. These effects on membranes may help to understand the mechanism of the biological activity of verbascoside and other similar phenylpropanoid glycosides.

1. Introduction

Verbascoside belongs to the phenylpropanoid glycosides family and it is structurally characterized by the caffeic acid moiety and 4,5-hydroxyphenylethanol (hydroxytyrosol) bound to a β-D-glucopyranoside, through ester and glycosidic links, respectively, with a rhamnose in sequence (1–3) to the glucose molecule (Fig. 1) (Korkina et al., 2007). Several biological properties have been described for both, this compound and its aglycone (Korkina, 2007), such as anti-inflammatory (Deepak and Handa, 2000; Díaz et al., 2004; Hausmann et al., 2007), antimicrobial (Avila et al., 1999), antitumor (Ohno et al., 2002), and antioxidant (Liu et al., 2003; Siciliano et al., 2005; Valentao et al., 2002; Wong et al., 2001).

Many studies have pointed out the relationship between the biological activity of phenolic compounds and their affinity and distribution in lipid membranes (Yang et al., 2001; Zhang et al., 2006). We have previously shown that some phenolic compounds, such as galloylated catechins (Caturla et al., 2003), seco-iridoids from olive leaves (Caturla et al., 2005), diterpenes (Pérez-Fons et al., 2006), and norlignans (Laporta et al., 2007a) promoted significant perturbations on phospholipid model membranes, which suggests a membrane-related mechanism laying beneath their activity. Therefore, the biological activity of verbascoside might be also related to its capability to modulate membrane-dependent cellular processes. For instance, verbascoside activity has been related with membrane-related processes such as cell signaling (Lee et al., 2004) and mitochondrial functionality (Koo et al., 2006; Sheng et al., 2002; Zhang et al., 2008), although the molecular basis of these effects still remains unclear. Moreover, it has been reported that the antimicrobial activity of verbascoside against *Staphylococcus aureus* occurs through leucine uptake inhibition, then its mechanism could also be membrane related (Avila et al., 1999).

Abbreviations: 16-NS, 16-doxyl-stearic acid; 5-NS, 5-doxyl-stearic acid; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]; DPH, 1,6-diphenyl-1,3,5-hexatriene; DSC, differential scanning calorimetry; HPLC-DAD–ESI-MS/MS, high performance liquid chromatography with diode array detection coupled to electrospray and ion-trap mass spectrometry; IP, gel–fluid intermediate phase; K_P, phospholipid/water partition coefficient; LUVs, large unilamellar vesicles; MLVs, multilamellar vesicles; PC, phosphatidylcholine; PG, phosphatidylglycerol; T_c, onset temperature of the gel to liquid-crystalline phase transition; T_m, gel to liquid-crystalline phase transition temperature; T_m^{off}, offset temperature of the IP region; T_m^{on}, onset temperature of the IP region.

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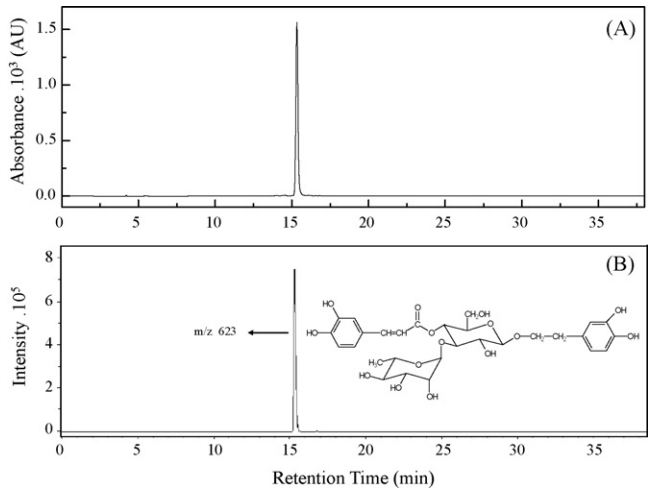

Fig. 1. HPLC profiles of purified verbascoside with diode array at 330 nm (A) and base peak chromatogram of 50–800 *m/z* obtained by HPLC–ESI-MS/MS (B). Insert shows the chemical structure of the phenylpropanoid verbascoside.

Although there are many studies concerning the biological activity of verbascoside, its molecular mechanism and targets are uncertain. A recent study has described the incorporation of verbascoside into mixed multilamellar vesicles through the encapsulation method for cosmetic applications (Sinico et al., 2008). However, to our knowledge, no study on the molecular interaction of verbascoside with biological membranes has been performed to date. Some antioxidants with an apparent hydrophilic nature, such as hypoxoside have shown to be effective antioxidants in lipophilic environments and exerted strong effects in biological membranes (Laporta et al., 2007a, 2007b). Moreover, we have recently shown that verbascoside and lemon verbena extract exhibited strong antioxidant capacity to inhibit lipid peroxidation in a model membrane system such as the thiobarbituric acid reactive substances (TBARS) assay, as well as the Trolox equivalent antioxidant capacity (TEAC) assay performed in lypophilic environment (Funes et al., 2009). Therefore, the study of the interaction of verbascoside with phospholipid membranes may help to understand whether the effect of this compound on the membrane physical properties have some contribution to its lipid antioxidative properties.

In this work, verbascoside has been isolated from a commercial extract of lemon verbena (*Lippia citriodora*) by semipreparative chromatography. The spectroscopic characteristics of verbascoside were studied, and its phospholipid/water partition coefficient was determined in different model membranes. Fluorescence spectroscopy studies were used to study the potential location of verbascoside in the membrane. Besides, verbascoside's effect on the thermotropic properties of model membranes composed of zwiterionic or anionic phospholipids, was studied by differential scanning calorimetry (DSC). Finally a combination of DSC, fluorescence anisotropy-pH titration and particle size studies was used to determine the effects of verbascoside on the phospholipid/water interface of membranes composed of the anionic phospholipid phosphatidylglycerol (PG).

2. Materials and methods

2.1. Materials

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) (DMPG) were obtained from Avanti Polar Lipids (Birmingham, AL, USA). Stock solutions of lipids were prepared in chloroform/methanol (1:1) and stored at −20 °C. The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH), spin labels 5-doxyl-stearic acid (5-NS) and 16-doxyl-stearic acid (16-NS) were obtained from Molecular Probes (Eugene, OR). Lemon verbena (*L. citriodora*) extract (25% verbascoside) was kindly provided by Monteloeder, S.L. (Elche, Spain). Verbascoside standard was obtained from Chromadex Inc. (St. Ana, California; USA). All other compounds were of analytical, spectroscopic or chromatographic reagent grade and were obtained from Merck KGaA (Darmstadt, Germany). Double-distilled and deionized water was used throughout this work.

2.2. Semipreparative HPLC

For the purification of verbascoside an own-developed method was used. Promptly, 2 g of lemon verbena commercial extract were dissolved in 20 ml of distilled water, centrifuged at 2000 × *g* at room temperature and filtered through 0.45 μm nylon filter. The filtered supernatant (11 ml) of the extract was injected into a preparative reverse phase column LiChrospher® 100 RP-18 (15 μm, 250 mm × 25 mm i.d.) from Merck KGaA (Darmstadt, Germany) and subjected to a semipreparative elution. The separation of the compounds from lemon verbena extract was carried out at room temperature with a gradient elution program at a flow rate of 31.25 ml/min. The mobile phase consisted in water: phosphoric acid 0.45N (90:10, v/v) (A) and water:phosphoric acid 0.45N (10:90, v/v) (B). The following multi-step linear gradient was applied: 0 min, 0% B; 12.5 min, 12% B; 22.5 min, 20% B; 25 min, 60% B; 26 min, 0% B and 30 min, 0% B. The UV–vis detection was performed at 330 nm. Semipreparative purification was carried out using a WellChrom (Merck-Knauer, Berlin, Germany) preparative HPLC equipped with two K-1800 pumps, dynamic mixing chamber, manual injector, UV–vis detector and fraction collector. EuroChrom® software version 3.01 (Merck-Knauer) was used for data acquisition and analysis. Major peak appearing in the semipreparative chromatograms and identified as verbascoside was collected and analyzed by analytical HPLC in order to check its purity. Each eluted fraction containing only pure compound was then solvent exchanged to pure methanol through the use of preparative C_{18} cartridges in order to eliminate phosphoric acid and evaporated to dryness by rotatory evaporation. Last traces of solvent were removed by lyophilization for 48 h and after that, the purified compound was weighted and subjected to HPLC analysis to check for possible degradation during the purification process. Verbascoside stocks solutions were prepared in methanol and stored at −20 °C.

2.3. Analytical HPLC-DAD–ESI-MS/MS

Ten milligrams of lemon verbena extract were dissolved in 2 ml of distilled water and centrifuged for 30 min at 11,000 × *g*. Ten microliter of the supernatant were injected in an analytical reverse phase column LiChrospher® 100 RP-18 (5 μm, 250 mm × 4 mm, i.d.) from Merck and subjected to HPLC-DAD–ESI-MS/MS analysis. The chromatographic separation of compounds from lemon verbena extract was performed as previously reported (Funes et al., 2009). Aliquots of the semipreparative purification of verbascoside were analyzed in identical way.

2.4. Spectroscopic measurements and determination of verbascoside partition coefficient in model membranes (K_P)

Fluorescence spectra and quenching measurements were recorded with a SLM-8000C spectrofluorimeter fitted with Glan-Thompson polarizers. All emission spectra were automatically corrected for instrument response at each wavelength. The partition coefficient (K_P) of verbascoside was determined from its intrinsic fluorescence intensity increase upon the incorporation into large unilamellar vesicles (LUVs) composed of DMPC or DMPG