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Antiaggregant effects of *Arbutus unedo* extracts in human platelets

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Abstract

Platelet hyperaggregability plays a pivotal role in the pathogenesis of cardiovascular diseases. Thrombin evokes aggregation through Ca^{2+} mobilization, tyrosine phosphorylation and generation of reactive oxygen species (ROS). We have investigated the antiaggregant properties of *Arbutus unedo* extracts in human platelets. Changes in cytosolic Ca^{2+} concentration and intracellular oxidants production were registered by espectrofluorimetry using fura-2 and dichlorodihydrofluorescein, respectively, platelet aggregation was assessed by aggregometry and protein tyrosine phosphorylation was detected by Western blotting. Platelet treatment with increasing concentrations (0.015–1.5 mg/mL) of crude aqueous, ethyl acetate or diethyl ether extracts reduced platelet aggregation evoked by thrombin (0.5 U/mL) and show a potent ROS scavenger activity, preventing thrombin-evoked endogenous generation of ROS. Treatment with *Arbutus unedo* extracts did not alter thrombin-evoked Ca^{2+} release from the intracellular stores but reduced store-operated Ca^{2+} entry induced by thrombin or by selective depletion of the two Ca^{2+} stores in platelets, the dense tubular system and the acidic stores. In addition, platelet treatment with extracts reduced both basal and thrombin-stimulated protein tyrosine phosphorylation. We conclude that *Arbutus unedo* extracts show antiaggregant actions due to attenuation of Ca^{2+} mobilization, ROS production and protein tyrosine phosphorylation and might be used for the treatment and/or prevention of cardiovascular diseases.

Keywords: Thrombin; Platelets; Aggregation; Calcium mobilization; Tyrosine phosphorylation; Antioxidants; *Arbutus unedo*

1. Introduction

Platelet hyperaggregability, a phenomenon responsible for the cardiovascular complications associated to several pathologies, such as coronary artery disease and type 2 diabetes mellitus, is a clinical parameter that is gaining relevance in keeping with changes in nutritional behavior of many Mediterranean populations. Paradoxically, in this region traditional medicinal plant extracts constitute a rich source of bioactive chemicals with non-adverse antiaggregant effects (Jouad et al., 2001; Mekhfi et al., 2004).

Arbutus unedo (Ericaceae) is a plant that is widely distributed in the Mediterranean region and North Africa. The ethanol and methanol extracts of *Arbutus unedo* leaves display a potent antioxidant activity (Pabuccuoglu et al., 2003). It has been shown in an inquiry realized by our laboratory that *Arbutus unedo* is frequently used in the traditional medicine system of Oriental Morocco as a natural remedy for hypertension and diabetes (Ziyyat et al., 1997). Experimental investigations have shown that the aqueous extract of the plant exhibited antihypertensive (Ziyyat and Boussairi, 1998) and vasorelaxant (Ziyyat et al., 2002) activities. Recently, in a screening study, we have shown that the aqueous extract of *Arbutus unedo* inhibited *in vitro* rat platelet aggregation induced by thrombin and ADP (Mekhfi et al., 2006). Several compounds have been isolated from *Arbutus unedo*, including aromatic acids, iridoids, monoterpenoids, phenylpropanoids, sterol, triterpenoids and flavonoids (see Carcache-Blanco et al., 2006). Spectrophotometric determination indicates that the leaves are richer in flavonoids than fruits. Among the flavonoids identified by thin-layer chromatography are quercitrin, isoquercitrin, hyperoside and rutin (Males et al., 2006).

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It is known that physiological agonists, such as thrombin, induce platelet aggregation by increasing the cytosolic-free calcium concentration ($[Ca^{2+}]_c$), which consists of two components: release of Ca^{2+} from intracellular stores and Ca^{2+} entry through plasma membrane channels (Rosado and Sage, 2000). Thrombin is able to release Ca^{2+} from two stores in human platelets, the dense tubular system (DTS) and the acidic stores (Lopez et al., 2006), which activate two different mechanisms for store-operated Ca^{2+} entry (SOCE) (Rosado et al., 2004a). On the base of the SERCA isoforms expressed in these stores, the DTS show a high sensitivity to thapsigargin (TG), while 2,5-di-(*tert*-butyl)-1,4-hydroquinone (TBHQ) specifically depletes the acidic stores (Wuytack et al., 1994; Cavallini et al., 1995; Lopez et al., 2005). Removal of Ca^{2+} from the cytosol is mediated by Ca^{2+} extrusion mainly by the plasma membrane Ca^{2+}-ATPase (PMCA) and sequestration into the intracellular stores (Lopez et al., 2006). Furthermore, human platelets generate and release reactive oxygen species (ROS) under physiological stimulation (Pignatelli et al., 1998; Seno et al., 2001). These ROS are required for SOCE (Rosado et al., 2004b; Ben-Amor et al., 2006). Finally, a number of studies have reported that tyrosine kinase activity is required for SOCE and aggregation in human platelets (Sargeant et al., 1993, 1994; Miyakawa et al., 1996; Rosado et al., 2000).

In the present study, we have investigated the effect of crude aqueous, diethyl ether and ethyl acetate extracts from *Arbutus unedo* leaves on oxidants production, Ca^{2+} mobilization, tyrosine phosphorylation and aggregation evoked by thrombin or the pharmacological tools TG and TBHQ in platelets from healthy human donors.

2. Materials and methods

2.1. Materials

Fura-2 acetoxymethyl ester (fura-2/AM), calcein/AM and 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) were from Molecular Probes (Leiden, The Netherlands). Apyrase (grade VII), EGTA, aspirin, bovine serum albumin, thrombin and TG were from Sigma (Madrid, Spain). TBHQ was from Alexis (Nottingham, UK). Anti-phosphotyrosine monoclonal antibody (4G10) was from Upstate Biotechnology (Madrid, Spain). Horseradish peroxidase-conjugated ovine anti-mouse IgG antibody (NA931) was from Amersham (Buckinghamshire, UK). Enhanced chemiluminescence detection reagents were from Pierce (Cheshire, UK). Hyperfilm ECL was from Amersham (Arlington Heights, IL, USA). All other reagents were purchased from Panreac (Barcelona, Spain).

2.2. Plant material

Arbutus unedo leaves (Ericaceae) were collected on may 2004 from Tazzeka Mountain, Taza (Morocco). The collected plant was identified by Prof. B. Haloui, from the Biology Department, Faculty of Sciences (Oujda, Morocco), where a voucher specimen (no. 14 ZL) is deposited.

Dried and powdered leaves from *Arbutus unedo* (50 g) were infused into 500 mL of boiled distilled water for 30 min. After decantation and filtration, the filtrate was evaporated at 50 °C to give a crude residue (yield: 19.74%). For the preparation of extracts enriched in flavonoids, dried and powdered leaves (200 g) were first degreased with petroleum ether (450 mL) using the Soxlhet refluxing apparatus for 10 h. After filtration, the degreased vegetal material was then extracted with a mixture of acetone (250 mL) and water (360 mL) under refluxing conditions during 10 h. After filtration, the filtrate was concentrated *in vacuo* to remove all acetone, and the resulting aqueous solution was washed twice with petroleum ether (100 mL) to remove lipids and chlorophylls. The remaining aqueous solution was then extracted with diethyl ether (3 × 100 mL) and then, with ethyl acetate (3 × 100 mL) (El Haouari et al., 2006). The extraction yield was 0.84% and 5.21% for genins and heterosidic flavonoids, respectively.

2.3. Platelet preparation

Fura-2-loaded platelets were prepared as described previously (Rosado et al., 2004a). For measurements of $[Ca^{2+}]_c$ platelet-rich plasma was incubated at 37 °C with 2 μM fura-2/AM for 45 min. Cells were collected by centrifugation and resuspended in HEPES-buffered saline (HBS) containing (in mM): 145 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 1 $MgSO_4$, pH 7.45, supplemented with 0.1% (w/v) bovine serum albumin and 40 μg/mL apyrase.

2.4. Cell viability

Cell viability was assessed using calcein and trypan blue. For calcein loading, cells were incubated for 30 min with 5 μM calcein-AM at 37 °C, centrifuged and the pellet was resuspended in fresh HBS. Then, resting cells or treated with the tested extract (1.5 mg/mL) were incubated at 37 °C for 10 min. Fluorescence was recorded from 2 mL aliquots using a Cary Eclipse fluorescence spectrophotometer (Varian Ltd., Madrid, Spain). Samples were excited at 494 nm and the resulting fluorescence was measured at 535 nm. The calcein fluorescence remaining in the cells after treatment with the tested extract was the same as in control, suggesting that under our conditions there was no cellular damage. The results obtained with calcein were confirmed using the trypan blue exclusion technique. Ninety-six percent of cells were viable in our platelet preparations, at least during the performance of the experiments.

2.5. Measurement of intracellular-free calcium concentration ($[Ca^{2+}]_c$)

Fluorescence was recorded from 2 mL aliquots of magnetically stirred cell suspensions (2×10^8 cells/mL) at 37 °C using a Fluorescence Spectrophotometer (Varian Ltd., Madrid, Spain) with excitation wavelengths of 340 and 380 nm and emission at 510 nm. Changes in $[Ca^{2+}]_c$ were monitored using the fura-2 340/380 fluorescence ratio and calibrated according to the method of Grynkiewicz et al. (1985). Ca^{2+} entry was estimated