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Endothelium-dependent vascular relaxing effects of different citrus and olive extracts in aorta rings from spontaneously hypertensive rats

Noemi López-Carreras [a], Julian Castillo [b,c], Begoña Muguerza [d,e,*], Amaya Aleixandre [a]

[a] Department of Pharmacology, Facultad de Medicina, Universidad Complutense, Madrid, Spain
[b] Department of Research and Development, Nutrafur S.A, Murcia, Spain
[c] Department of Food Technology and Nutrition, Universidad Católica San Antonio, Murcia, Spain
[d] Nutrigenomic Group, Department Biochemistry and Biotechnology, Universidad Rovira i Virgili, Tarragona, Spain
[e] Technological Center of Nutrition and Health (CTNS), TECNIO, CEICS, Reus, Spain

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Naringenin (PubChem CID: 932)
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ABSTRACT

Three citrus fruit extracts (orange, grapefruit and lemon), an olive leaf extract and a citrus–olive mixed extract (Citrolive), which was obtained from olive leaf and bitter orange fruits, were characterized by HPLC and investigated for their endothelium-dependent vascular relaxing ability. Subsequently, aorta rings from SHR were mounted in tissue baths. They were pre-contracted with methoxamine and exposed to the extracts. Intact, endothelium-denuded, L-NAME-, indomethacin- and sirtinol-treated preparations were used. All extracts exhibited endothelium-dependent relaxations that were totally reversed by L-NAME and partly blocked by sirtinol. Indomethacin also decreased the relaxing effect of high doses of lemon and olive extracts, but the arterial relaxations caused by low doses of the different extracts and those caused by high doses of Citrolive extract (a mixed extract) were potentiated by indomethacin. This study confirms Mediterranean plants as an excellent source of functional compounds, showing the relaxing effect of the assayed plant extracts in aorta rings from SHR. Moreover, the results obtained demonstrate the endothelium-dependent vascular relaxing effect of the studied extracts. Endothelial NO release seems implicated in the effect of all extracts and prostacyclin probably participates in the effect of lemon and olive extracts. Nevertheless, enhanced vasoconstrictor endoperoxides seem to be of special importance in the endothelial tissue of SHR, and the release of these products may impair, at least in part, the endothelium-dependent vascular relaxation caused in these animals by orange, grapefruit, lemon, olive, and in particular, Citrolive extracts.

1. Introduction

According to the World Health Organization (WHO), cardiovascular disease (CVD) is the leading cause of death worldwide and represents 30% of all deaths worldwide. In fact, the number of people who die of CVD is estimated to increase to 23.3 million by 2030 (WHO, 2011). The Mediterranean diet, which is rich in fresh fruits and vegetables, has been associated with a lower incidence of CVD, partly because of its high proportion of bioactive compounds such as vitamins and polyphenols.

Flavonoids in particular are low-molecular-weight polyphenol molecules that display a broad range of biological effects, including anti-inflammatory and antioxidant effects (Fraga, Galleano, Verstraeten, & Oteiza, 2010). Flavonoids are widely present in the plant kingdom, including a wide variety of edible Mediterranean plants such as citrus species and olives (*Olea europaea*). The basic structure of flavonoids is a 2-phenyl benzopyrone, in which the three-carbon bridge between the phenyl groups is usually cyclized oxygen (Corradini et al., 2011; Hughes, Croley, Metcalfe, & March, 2001). The flavonoids include several subfamilies according to their degree of unsaturation and the degree of oxidation of the oxygenated heterocycle: flavanones, flavones, flavonols, isoflavones, flavanols and anthocyanidins (Corradini et al., 2011; Hughes et al., 2001). Specifically, four types of flavonoids (flavanones, flavones, flavonols and anthocyanins) occur in citrus (the last only in

* Corresponding author at: Biochemistry and Biotechnology Department, Rovira i Virgili University, C/ Marcel·lí Domingo s/n, 43007 Tarragona, Spain.
E-mail address: begona.muguerza@urv.cat (B. Muguerza).

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blood oranges) (Horowitz & Gentili, 1977). *Olea europaea* also shows a characteristic polyphenol profile, with three types of flavonoids (flavones, flavonols and flavanols) basically present in this plant as well as other classes of non-flavonoid polyphenolic compounds such as oleuropeosides (oleuropein and verbascoside) and substituted phenols (e.g., hydroxytyrosol, caffeic acid) (Benavente-García, Castillo, Lorente, & Alcaraz, 2002; Benavente-García, Castillo, Lorente, Ortuño, & Del Rio, 2000). The most abundant polyphenol compound of olive leaves extracts is oleuropein, followed by its derivative demethyloleuropein, the flavone-7-glucosides of luteolin and apigenin, and the phenolics verbascoside and hydroxytyrosol (Benavente-García et al., 2000, 2002).

The protective effects of polyphenols on CVD could be attributed, at least in part, to the beneficial effects of these compounds on the endothelial function (Vita, 2005). Endothelial tissue regulates vascular tone and exerts finely tuned control over cardiovascular homeostasis, with nitric oxide (NO) being one of the best-characterized vasodilator endothelial factors. NO is synthesized in the endothelial cells by a Ca^{2+}-dependent constitutive isoform of the NO synthase enzyme (eNOS), which can be up-regulated by increased arterial blood pressure or by insulin and other hormones such as adiponectin, estrogens or thyroid hormone (Vanhoutte, Shimokawa, Tang, & Feletou, 2009). The diet is also an important factor in the up-regulation of eNOS. The consumption of some classes of flavonoids is known to potentiate NO endothelium-dependent relaxation (Duffy et al., 2001; Fisher, Hughes, Gerhard-Herman, & Hollenberg, 2003; Schroeter et al., 2006; Stein, Keevil, Wiebe, Aeschlimann, & Folts, 1999; Yamamoto, Suzuki, & Hase, 2008). Prostaglandin I2 (PGI2), also known as prostacyclin, is an important vasodilator endothelial factor that is synthesized in this tissue by cyclooxygenase (COX).

The relaxing effects of some particular polyphenols on isolated rat arteries have previously been investigated (Chen & Pace-Asciak, 1996; Woodman & Chan, 2004). Those studies showed that these compounds could cause endothelium-dependent effects at physiological concentrations and suggested the involvement of NO in the responses. In addition, aqueous extracts of a variety of vegetables, fruits, teas, nuts, herbs and spices were tested *in vitro* for their endothelium-dependent relaxing ability (Fitzpatric, Hirschfield, Ricci, Jantzen, & Coffey, 1995). The vascular relaxation observed for many of the extracts that were studied was also largely endothelium and NO-dependent, although other mechanisms also appeared to be involved. Aqueous extracts of fruits such as apple, guava and plum were shown to be particularly strong relaxing agents, but aqueous citrus extracts such as grapefruit and orange extracts exhibited moderate activity (Fitzpatric et al., 1995). In addition, arterial and *in vivo* studies carried out by our research group demonstrated the involvement of endothelial relaxing factors, primarily NO, in the vascular relaxing and antihypertensive effects of some polyphenol-rich extracts such as cocoa and grape seed extracts (Quiñones, Muguerza, Miguel, & Aleixandre, 2011; Quiñones, Sánchez, Muguerza, Miguel, & Aleixandre, 2011; Quiñones et al., 2010, 2014).

The aim of the present study was to characterize the endothelium-dependent vascular relaxing effect of some extracts obtained from Citrus species and *Olea europaea*. The extracts studied were three citrus extracts (orange, grapefruit and lemon extracts), an olive leaf extract and a mixed extract (Citrolive), which was obtained from olive leaf and citrus species. All of these extracts had previously been characterized for their flavonoid and polyphenol profile and were subsequently assayed in aorta rings obtained from spontaneously hypertensive rats (SHR).

2. Material and methods

2.1. Extracts and reagents

All the extracts used in this study were kindly provided by Nutrafur S.A. (Murcia, Spain). The orange, grapefruit and lemon extracts were obtained from immature fruits of *Citrus sinensis* (sweet orange), *Citrus paradise* (grapefruit) and *Citrus limon*, (lemon), respectively. The olive extract is an olive leaf extract obtained from *Olea europaea*. Finally, Citrolive is a mixed extract obtained in specific proportions from olive leaf and *Citrus aurantium* (bitter orange).

All commercial standards used for quantitative analysis by HPLC-DAD were purchased from Extrasynthese (Genay, France), except vanillin, vanillic acid and caffeic acid which were obtained from Sigma Chemical Co. (Madrid, Spain). Organic solvents (high performance liquid chromatography [HPLC]-grade) were obtained from Scharlab (Barcelona, Spain) and Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q advantage A10 system (Madrid, Spain).

NW-nitro-L-arginine methyl ester (L-NAME), sirtinol and indomethacin were purchased from Sigma (Barcelona, Spain).

2.2. Chromatographic analysis and quantification of polyphenol compounds

For the elucidation and quantification of the main polyphenols present in each extract, we modified a previously published method (Benavente-García et al., 2000). All of the samples were dissolved in dimethyl sulfoxide (DMSO) at a ratio of 5 mg/mL, and the resulting solutions were filtered through a 0.45 mm nylon membrane. The high performance liquid chromatography (HPLC) equipment was a Hewlett–Packard Series HP 1100 equipped with a diode array detector. The stationary phase was a C18 LiChrospher 100 analytical column (250 mm × 64 mm i.d.) with a particle size of 5 mm (Merck, Darmstadt, Germany) warmed at 30 °C. The flow rate was 1 mL/min, and the absorbance changes were monitored simultaneously at 280 and 340 nm. The mobile phases for chromatographic analysis were (A) acetic acid: water (2.5:97.5) and (B) acetonitrile. A linear gradient was run from 95% (A) and 5% (B) to 75% (A) and 25% (B) for 20 min; changed to 50% (A) and (B) for 20 min (40 min, total time); changed to 20% (A) and 80% (B) for 10 min (50 min, total time), and finally re-equilibrated for 10 min (60 min, total time) to the initial composition.

2.3. General protocol in rats

Untreated male SHR were used for this study. The animals were purchased from Charles River Laboratories (Barcelona, Spain) and maintained at a temperature of 23 °C, with 12-h light/dark cycles and free access to water. They were fed solid standard diet for rats (A04 Panlab, Barcelona, Spain), *ad libitum*, until they were sacrificed.

The rats were sacrificed by decapitation after 27–30 weeks of life, and their aorta was extracted. Excess fat and connective tissue were removed from the aorta, and the tissue was cut into rings (approximately 4 mm in length). The aortic rings were mounted between two steel hooks in isolated tissue chambers containing Krebs–Henseleit solution with the following composition (mmol/L): NaCl, 118.2; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; and glucose, 10.0. The medium was maintained at 37 °C and was continuously bubbled with a 95% O_2-5% CO_2 mixture, which gave a pH of 7.4. An optimal resting tension of 2 g was applied to all aortic rings. This tension was adjusted every 15 min during a 60- to 90-min equilibration period before adding the drugs. Isometric tension was recorded by using an isometric force displacement transducer connected to an acquisition system (Protos 5, Panlab, Spain). After the equilibration period, the rings were first contracted by 80 mM KCl to verify their functionality, and when the contraction had reached the steady state (approximately 15 min after the administration), the preparations were washed until the basal tension was recovered. The rings were then exposed to 10^{-5} M methoxamine, and dose–response curves (10^{-6}–10^{-1} mg/ml) for the different extracts (orange, grapefruit, lemon, olive and Citrolive) were prepared in the methoxamine-pre-contracted rings. Relaxant responses to the extracts were expressed as a percentage of the pre-contraction induced by methoxamine. The previously described procedure was applied to intact and endothelium-denuded tissue. The procedure was