

Article

Extra Virgin Olive Oil Phenols Vasodilate Rat Mesenteric Resistance Artery via Phospholipase C (PLC)-Calcium Microdomains-Potassium Channels (BK_{Ca}) Signals

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Abstract: Recent evidence suggests that the reason Extra Virgin Olive Oil (EVOO) lowers blood pressure and reduces the risk of developing hypertension is partly due to minor components of EVOO, such as phenols. However, little is still known about the mechanism(s) through which EVOO phenols mediate anti-hypertensive effects. The aim of the present study was to investigate the mechanisms of action of EVOO phenols on mesenteric resistance arteries. A pressure myograph was used to test the effect of EVOO phenols on isolated mesenteric arteries in the presence of specific inhibitors of: (1) BK_{Ca} channels (Paxillin, 10⁻⁵ M); (2) L-type calcium channels (Verapamil, 10⁻⁵ M); (3) Ryanodine receptor, RyR (Ryanodine, 10⁻⁵ M); (4) inositol 1,4,5-triphosphate receptor, IP3R, (2-Aminoethyl diphenylborinate, 2-APB, 3 × 10⁻³ M); (5) phospholipase C, PLC, (U73122, 10⁻⁵ M), and (6) GPCR-G_{αi} signaling, (Pertussis Toxin, 10⁻⁵ M). EVOO phenols induced vasodilation of mesenteric arteries in a dose-dependent manner, and this effect was reduced by pre-incubation with Paxillin, Verapamil, Ryanodine, 2-APB, U73122, and Pertussis Toxin. Our data suggest that EVOO phenol-mediated vasodilation requires activation of BK_{Ca} channels potentially through a local increase of subcellular calcium microdomains, a pivotal mechanism on the base of artery vasodilation. These findings provide novel mechanistic insights for understanding the vasodilatory properties of EVOO phenols on resistance arteries.

Keywords: mesenteric artery; BK_{Ca} channels; Ca²⁺ microdomains; PLC; GPCR-G_{αi}



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1. Introduction

Systemic arterial hypertension is the most common preventable risk factor for cardiovascular disease (CVD) [1] and is a leading cause of morbidity, disability, and mortality in the world, with an estimated 17.92 million deaths in 2015 [2]. It is well-established that healthy lifestyle choices, such as diet, exercise, and abstinence from smoking, are associated with a lower risk of CVD [3,4]. In this regard, daily consumption of Extra Virgin Olive Oil (EVOO), a key component of the Mediterranean diet [5], is associated with beneficial effects on human health [6] and, in particular, with reduced cardiovascular risk [7,8]. Epidemiological and clinical studies indicate that daily intake of at least two tablespoons of EVOO can lower blood pressure, thereby decreasing the risk of developing

hypertension [9–11]. The chemical composition of EVOO includes two different fractions: the saponifiable fraction, which represents 98–99% of EVOO and is characterized by a high content of monounsaturated fatty acid (MUFA), and the unsaponifiable fraction which represents 1–2% of EVOO and includes phenols [12].

The phenolic compounds derive from the secondary metabolism of plants and are chemically characterized by having one or more aromatic rings with one or more hydroxyl groups. The amounts of the phenolic components (150–700 mg/kg) in EVOO depend on many different factors including the cultivars, the degree of maturation, the climate, the production method and the storage [13]. Different groups of phenolic compounds can be found in EVOO, such as secoridoids, phenolic acids, phenolic alcohols, hydroxyisochromans, flavonoids and lignans [14].

Some studies have associated the anti-hypertensive effect of EVOO to its acidic component, especially oleic acid [15,16]; however, a growing body of evidence suggests that the phenolic component is the major contributor [17–19] to the biological effects.

Previously, we showed that EVOO phenols exert a vasodilatory effect on rat resistance mesenteric artery (MA) [20], which may underpin the anti-hypertensive properties of EVOO phenols. We demonstrated that EVOO-phenol-induced vasodilation is endothelium-independent and occurs through activation of the large-conductance calcium-activated potassium (BK_{ca}) channels in the vascular smooth muscle cells (VSMCs), but does not involve signaling pathways mediated by nitric oxide (NO)[−], prostanoids, or the cyclic nucleotides cAMP and cGMP [20]. The present study will further clarify the mechanism of action of EVOO phenols in MA by investigating the pathways involved in the activation of the BK_{ca} channels.

2. Materials and Methods

2.1. Extra Virgin Olive Oil Phenols

The phenolic extract used in this work is derived from crude EVOO. The liquid/liquid partitioning method was used with methanol/water (80:20) [21]. The use of methanol/water 80:20 (*v/v*) was reported as an efficient extraction solvent [21] and it is used in the official method of phenolic compounds determination. The extraction was performed through the dispersing device UltraTurrax (IKA®-Werke GmbH & Co. KG, Staufen, Germany) for 2 min at 6000 rpm. The obtained emulsion was centrifuged at 4000 rpm for 15 min at room temperature. The hydroalcoholic fraction then was evaporated and later recovered using acetonitrile for residue solubilization and hexane to remove some minor lipid components. The qualitative and quantitative determination of phenolic compounds was accomplished by high performance liquid chromatography (HPLC-UV) [22]. Oleacein (3,4-DHPEA-EDA) is the most abundant phenolic compound in EVOO as well as oleuropein derivatives.

2.2. Animals

Male Sprague-Dawley rats (*n* = 16) were used at 18–23 weeks of age. Rats were housed at the University of Calabria under controlled conditions on a 12 h light/dark cycle; commercial chow and tap water were provided *ad libitum*. All procedures were conducted in accordance with the European Guidelines for the care and use of laboratory animals (Directive 2010/63/EU) and approved by the Italian Institutional Animal Care (n.295/2016-PR). Animals were euthanized with isoflurane and killed by cervical transection; the abdominal cavity was opened immediately, and a section of the mesentery was excised 5 cm distal to the pylorus. The mesenteric tissue was placed in a Sylgard-lined Petri dish containing cold (4 °C) HEPES physiological saline solution (PSS) at pH = 7.4.

2.3. Rat Isolated Mesenteric Artery Preparation

Third-order MA were dissected free from the surrounding adipose and connective tissues and cut into segments of about 3 mm in length. Vessels were cannulated in the chamber of a small arteriograph (Instrumentation and Model Facility, University of Vermont, Burlington, VT, USA), superfused with HEPES-PSS at 37 °C and pressurized using

a pressure-servo system (Living Systems Instrumentation, St. Albans City, VT, USA). Intraluminal diameter was measured using a video dimension analyzer (Living Systems Instrumentation) and recorded on LabView software.

2.4. Experimental Protocol

All arteries were pressurized at an intraluminal pressure of 50 mmHg (as it approximates in vivo conditions) and exposed to K^+ -rich (60×10^{-3} M) HEPES-PSS, and Acetylcholine (10 μ M), to test artery viability and endothelium integrity, respectively. Vessels that did not elicit reproducible responses to either potassium or acetylcholine were excluded from the study. MA were then equilibrated for 30 min in HEPES-PSS at 37 °C and pre-constricted with phenylephrine to produce a 40–60% reduction in lumen diameter [23]. Increasing concentrations (0.1 \div 10 μ M) of EVOO phenols were tested on pre-constricted MA, and the resulting changes in diameter were recorded once dilation stabilized at each concentration. At the end of each experiment, vessels were treated with the relaxing solution, HEPES-PSS without Ca^{2+} and containing the phosphodiesterase inhibitor, papaverine (10^{-4} M), to induce maximal vasodilation. To investigate molecular mechanism(s) underlying the EVOO phenol-induced vasodilation, a dose-response curve for phenols (0.1 \div 10 μ M) was tested in arterial segments pre-incubated for 30 min with one of the following inhibitors, prior to exposure to phenylephrine: (1) Paxillin (10 μ M) [20], a selective blocker of BK_{Ca} ; (2) Verapamil (10 μ M) [24], a L-type calcium channel blocker; (3) Ryanodine, (10 μ M) [25], an inhibitor of Ryanodine receptor (RyR); (4) 2-APB (3×10^3 μ M) [26], an inhibitor of inositol triphosphate receptor (IP3R); (5) U73122 (10 μ M) [27], an inhibitor of phospholipase C (PLC); and (6) Pertussis Toxin (10 μ M) [28], a blocker of the subunit $G_{\alpha i}$ of G-protein-coupled receptors (GPCR).

2.5. Drug and Solutions

The physiological salt solution HEPES-PSS contained the following (in mmol/L): sodium chloride 141.8, potassium chloride 4.7, magnesium sulfate 1.7, calcium chloride 2.8, potassium phosphate 1.2, HEPES 10.0, EDTA 0.5, and dextrose 5.0. The solution was prepared in deionized water and titrated with sodium hydroxide (10 M) to a physiologic pH of 7.4.

K^+ -rich (60×10^{-3} M) HEPES-PSS has the same composition as the standard solution, HEPES-PSS, except that NaCl was replaced by an equimolar concentration of KCl.

Chemicals were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), Santa-Cruz (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), Cayman Chemical Co (Cayman Chemical, Ann Arbor, MI, USA), unless otherwise specified. All drugs tested were administered from stock solutions prepared daily, except for EVOO phenols stock solutions that were frozen in small aliquots.

2.6. Data Analysis and Statistics

Data are expressed as means \pm Standard Error Media (SEM), where n is both the number of arteries studied and animals used. The vasodilatory effects of EVOO phenols are expressed as percentage of maximal diameter, which was determined in the presence of the relaxing solution. The vasorelaxing efficacy was evaluated as the maximal vasodilatory response (E_{max}); while the potency was expressed as EC_{50} , calculated as the dilator concentration that caused 50% of the maximum response. Furthermore, we compared the area under the dose-response curve (AUC) at the different experimental conditions. Statistical analysis was performed with paired Student's t -test. $p < 0.05$ was considered significant.

3. Results

We tested the effect of EVOO phenols (0.1–10 μ M) on MA pre-constricted with phenylephrine. As shown in Figure 1, EVOO phenols induced significant vasodilation ($p < 0.001$) in a dose-dependent manner, with an E_{max} of $97.74 \pm 0.63\%$ and a EC_{50} of 0.6 ± 0.04 μ M. Vehicle condition for EVOO phenols (Ethanol) was assessed and did not show any effect.

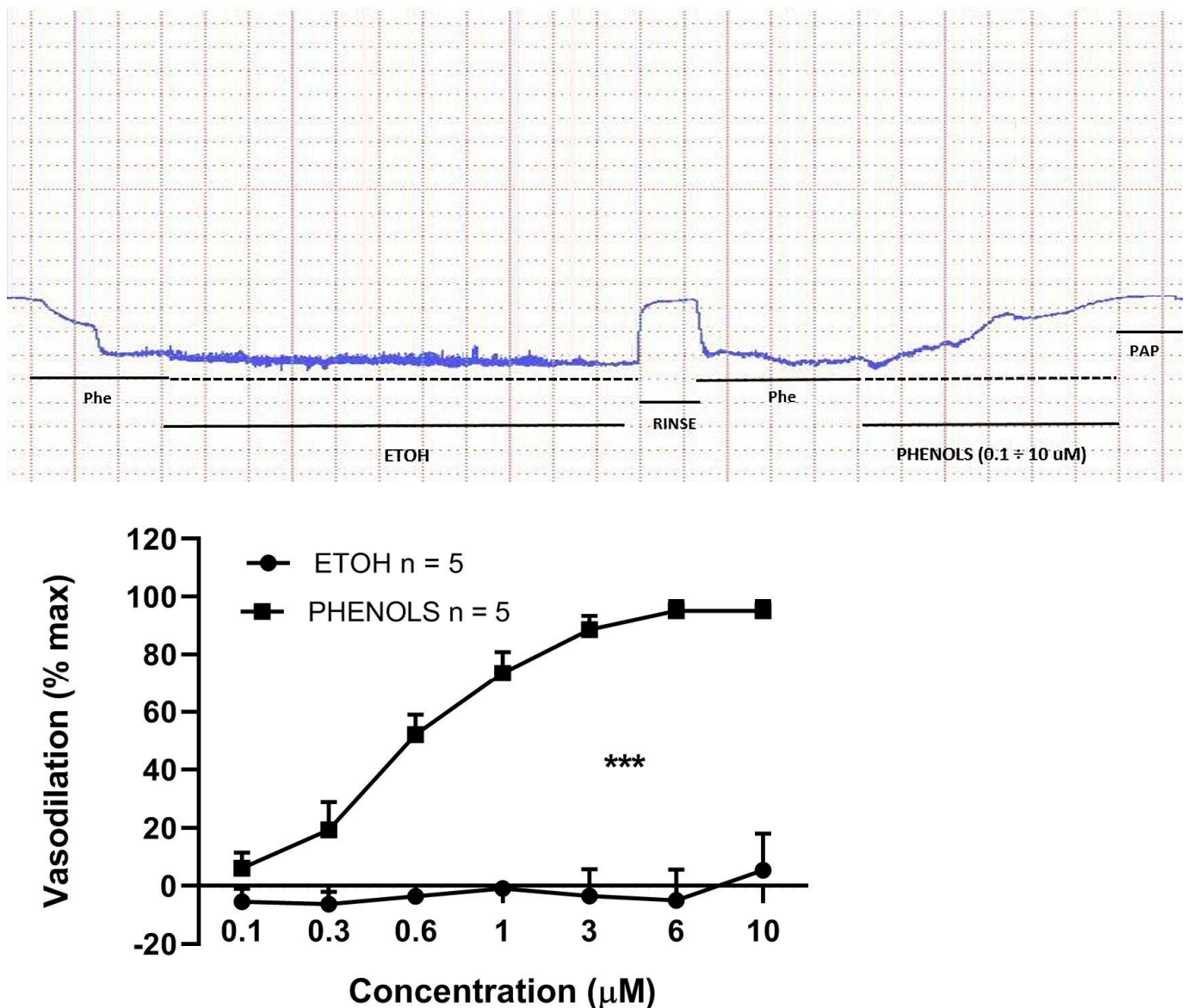


Figure 1. EVOO phenols vasodilation on mesenteric resistance artery. Representative trace showing the effect of Ethanol (EtOH, EVOO phenols vehicle) and EVOO phenols (Phenols, 0.1–10 μM) in phenylephrine pre-constricted mesenteric resistance artery. HEPES-PSS without Ca^{2+} , containing Papaverine (Pap) was added at the end of each experiment to calculate maximum dilation. Several experiments were done and summarized in the figure, which shows a dose-dependent vasodilation for EVOO phenols but not for EtOH. Data are presented as the mean \pm SEM, n (experimental number), *** $p < 0.001$ refers to the area under the curve.

To determine the mechanisms by which EVOO phenols induced vasodilation, a dose-response curve to EVOO phenols was obtained in the presence of the specific inhibitor of BK_{Ca} channels, Paxillin (10 μM) Figure 2. Pre-incubation with Paxillin shifted the dose-response curve to the right, with a significant increasing of EC_{50} (1 ± 0.08 μM control vs. 5 ± 0.09 μM Paxillin, $p < 0.01$), and reducing of E_{max} ($98.0 \pm 5.7\%$ control vs. $52.4 \pm 13.6\%$ Paxillin, $p < 0.05$). Interestingly, Paxillin completely abolished EVOO phenol-mediated vasodilation in the dose range of 0.1 μM \div 2 μM.

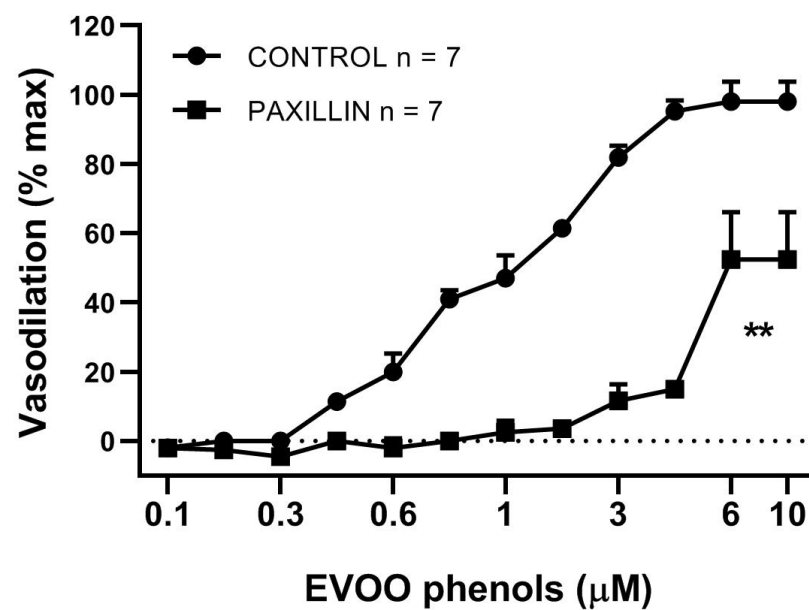


Figure 2. Large-conductance calcium-activated potassium channel blocker reduces EVOO phenol-vasodilation. EVOO phenols were tested on mesenteric resistance artery in the absence (control) and in the presence of the selective inhibitor of the large-conductance calcium-activated potassium (BKca) channels, Paxillin (10 µM). Data are presented as means \pm SEM, n (experimental number), ** $p < 0.01$ refers to the area under the curve.

To establish whether EVOO phenol-mediated vasodilation was associated with extracellular Ca^{2+} influx, MA were pre-incubated with the L-type calcium channel blocker, Verapamil (10 µM) Figure 3. By blocking extracellular Ca^{2+} influx, Verapamil induced a rightward shift of the dose-response curve of EVOO phenols, with a significant increase of EC_{50} (1 ± 0.07 µM control vs. 4 ± 0.24 µM verapamil, $p < 0.05$), and a significant attenuation of E_{max} ($97.9 \pm 5.0\%$ control vs. $64.5 \pm 12.3\%$ verapamil, $p < 0.05$).

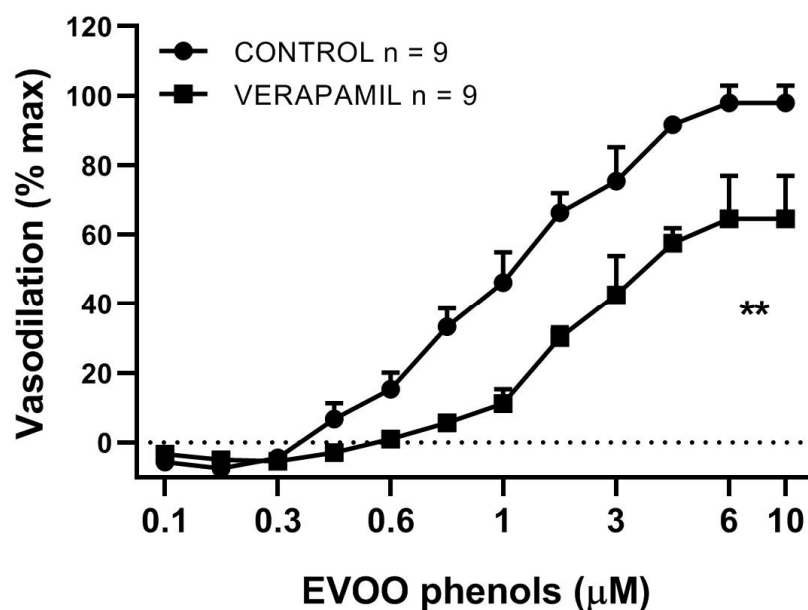


Figure 3. L-type calcium channel blocker attenuates EVOO phenol-vasodilation. EVOO phenols were tested on mesenteric resistance artery in the absence (control) and in the presence of the selective blocker of L-type calcium channel, Verapamil (10 µM). Data are presented as means \pm SEM, n (experimental number), ** $p < 0.01$ refers to the area under the curve.

To assess whether the release of Ca^{2+} from intracellular stores was also involved in the vasodilation, a set of experiments was performed using two different blockers of intracellular Ca^{2+} release: Ryanodine ($10 \mu\text{M}$), a potent inhibitor of RyR, and 2-APB ($3 \times 10^3 \mu\text{M}$) an inhibitor of IP3R. Ryanodine decreased E_{max} ($98.1 \pm 1.53\%$ control vs. $83.3 \pm 10.9\%$ ryanodyne, $p > 0.05$), and caused a significant increase of EC_{50} ($1 \pm 0.05 \mu\text{M}$ control vs. $3 \pm 0.11 \mu\text{M}$ Ryanodine, $p < 0.05$), Figure 4A. 2-APB did not significantly affect E_{max} ($98.7 \pm 1.2\%$ control vs. $87.3 \pm 8.3\%$ 2-APB, $p > 0.05$), but significantly potentiated EC_{50} ($1 \pm 0.08 \mu\text{M}$ control vs. $4 \pm 0.04 \mu\text{M}$ 2-APB, $p < 0.01$), Figure 4B.

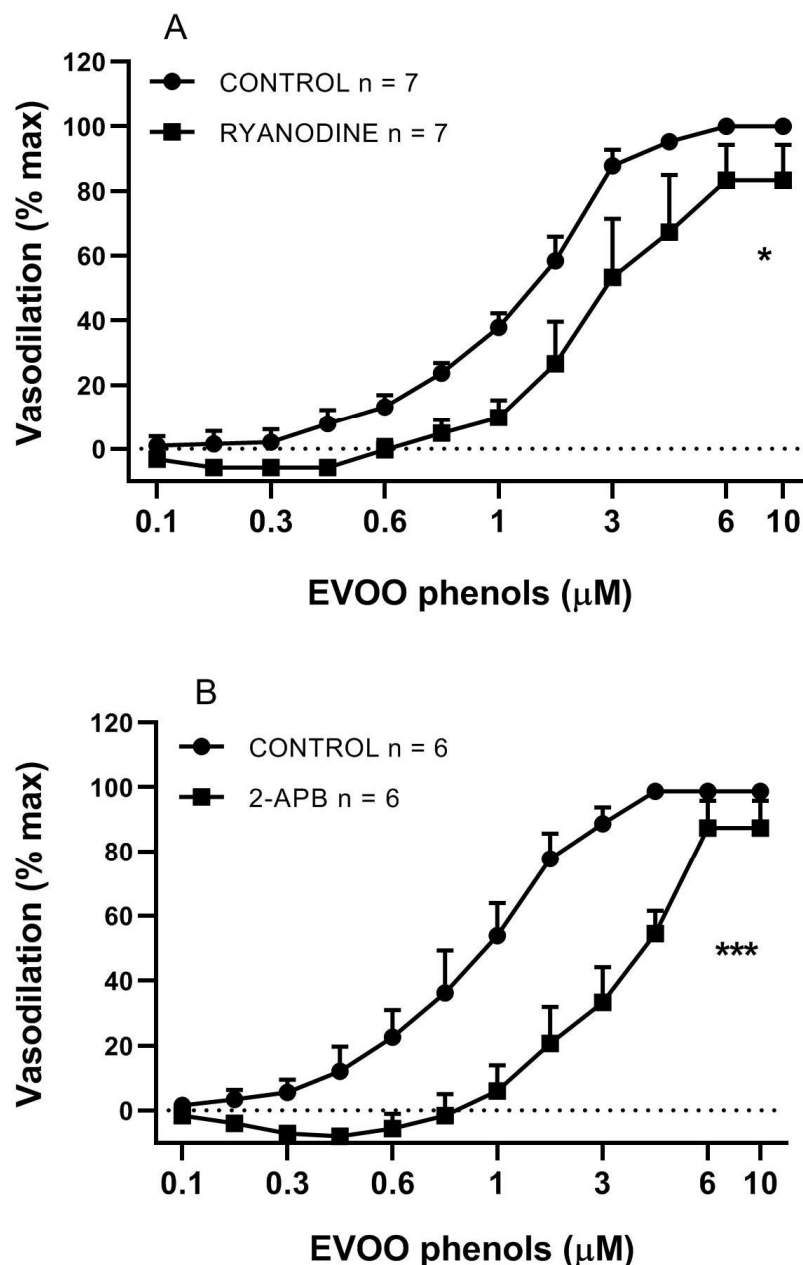


Figure 4. Blockers of intracellular calcium release reduce EVOO phenol-vasodilation. EVOO phenols were tested on mesenteric resistance artery in the absence (control) and in the presence of specific inhibitors of Ryanodine (Ryanodine, $10 \mu\text{M}$, **A**) and inositol triphosphate receptors (2-APB, $3 \times 10^3 \mu\text{M}$, **B**); Data are reported as means \pm SEM, n (experimental number), * $p < 0.05$, *** $p < 0.001$ refer to the area under the curve.

Furthermore, to confirm the contribution of PLC-initiated in the vasodilatory effect of EVOO phenols, a dose-response curve to EVOO phenols was tested in the presence of the PLC inhibitor U73122 (10 μ M), which reduced E_{max} ($98.8 \pm 1.1\%$ control vs. $27.7 \pm 5.1\%$ U73122, $p < 0.001$) and increased EC_{50} ($0.5 \pm 0.06 \mu$ M control vs. $1 \pm 0.07 \mu$ M U73122, $p < 0.01$), Figure 5.

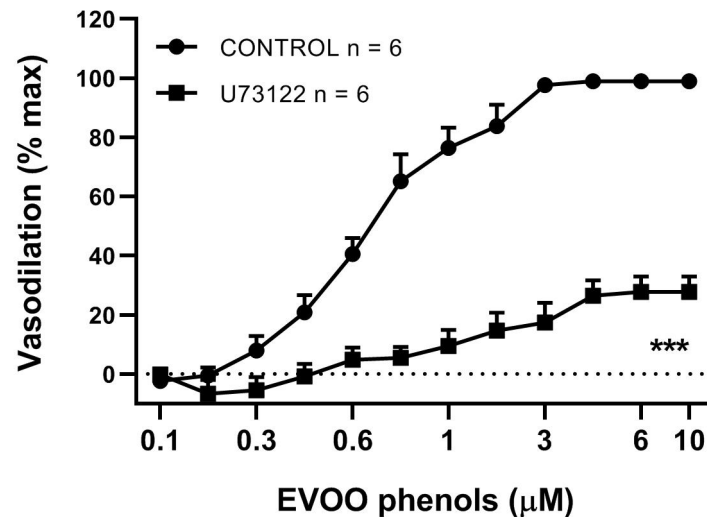


Figure 5. Phospholipase C blocker inhibits EVOO phenol-vasodilation. EVOO phenols were tested on mesenteric artery in the absence (control) and in the presence of the specific inhibitor of phospholipase C, U73122 (10 μ M). Data are reported as means \pm SEM, n (experimental number), *** $p < 0.001$ refers to the area under the curve.

We investigated also whether activation of GPCR is involved in the EVOO phenol-induced vasodilation. Our data showed that inhibition of the subunit $G_{\alpha i}$ of GCPR with Pertussis Toxin (10 μ M), significantly decreased E_{max} ($97.6 \pm 1.2\%$ control vs. $63.4 \pm 9.0\%$ Pertussis Toxin, $p < 0.05$), but did not alter EC_{50} ($1 \pm 0.06 \mu$ M control vs. $2 \pm 0.12 \mu$ M Pertussis Toxin, $p > 0.05$), Figure 6.

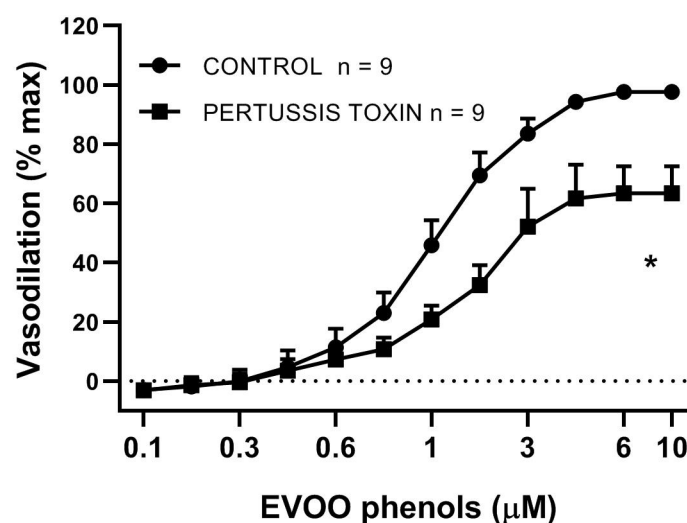


Figure 6. Inactivation of G protein attenuates EVOO phenol-vasodilation. EVOO phenols were tested on mesenteric artery in the absence (control) and in the presence of the specific inhibitor of the GPCR subunit, $G_{\alpha i}$, Pertussis Toxin (10 μ M). Data are reported as means \pm SEM, n (experimental number), * $p < 0.05$ refers to the area under the curve.

4. Discussion

Our study shows that EVOO phenols dilate resistance MA in a dose-dependent manner. The vasodilation is induced by activation of BK_{Ca} channels through an increase of local intracellular Ca²⁺ level (Ca²⁺ microdomains) as result of influx across the plasma membrane (extracellular Ca²⁺) and release from sarcoplasmic reticulum (SR) Ca²⁺ store. Furthermore, we showed that EVOO phenols activated PLC, which plays a significant role in the regulation of the Ca²⁺ microdomains.

Our results confirm the vasodilation that we showed in our previous study in the same type of artery [20]. A similar effect was reported in rat aortic rings [29,30], suggesting that EVOO phenols are vasodilator of both large (conductive) and small (resistance) vessels. Despite recent studies demonstrated the vasodilatory properties of EVOO phenols, the underlying molecular mechanism(s) of vasodilation are not fully known. A canonical mechanism for the vasodilation is the hyperpolarization mediated by potassium channels. We showed that activation of BK_{Ca} channels is involved in the EVOO phenol-induced vasodilation of MA. In addition, here we demonstrated that both the release of intracellular Ca²⁺ from the SR by IP3R and RyR and extracellular Ca²⁺ influx through the L-type Ca²⁺ channels are implicated in the EVOO phenols vasodilation. Taken together, our results suggest that EVOO phenols activate BK_{Ca} channels potentially through an increase in intracellular localized Ca²⁺ transients. In agreement with other studies, our data suggest that EVOO phenols may lead to the increase of local Ca²⁺ microdomains which, in turn, are responsible for the vasodilatory effect on mesenteric resistance arteries. In muscle cells, the release of Ca²⁺, in the form of Ca²⁺ sparks, induced by RyR in the sub-plasma membrane of the SR, controls the activity of the nearby BK_{Ca} channels in many resistance arteries [31–34]. On the other hand, Ca²⁺ influx through the L-type Ca²⁺ channels has been shown to activate BK_{Ca} channels in different types of vessels, including hamster and mouse cremaster arterioles [35,36], rat mesenteric arteries [37] and mouse mesenteric arteries [38]. In striated muscle resistance arteries, both Ca²⁺ influx through L-type channels and RyR-based Ca²⁺ sparks, contribute to activation of BK_{Ca} channels [35,36]. Studies in VSMCs from mouse MA [38] have shown that Ca²⁺ influx through L-type Ca²⁺ channels can directly activate BK_{Ca} channels. Furthermore, it has been demonstrated that, in vascular smooth muscle cells, BK_{Ca} and L-type Ca²⁺ channels can directly interact forming complexes that are accumulated in caveolae by the interaction of caveolin-1, thereby regulating spatiotemporal Ca²⁺ dynamics including the negative feedback, to control the arterial excitability and contractility [38].

Furthermore, we demonstrated that EVOO phenols indirectly activated PLC which plays a crucial role in the generation of inositol triphosphate (IP₃) a specific agonist of the abundant SR Ca²⁺ channels IP₃R. Our results showed that EVOO phenols-PLC activation occurred through the subunit G α i of GPCR since its inhibition by the specific inhibitor pertussin toxin decreased EVOO phenols vasodilation. This can be explained by the fact that G α i activation inhibits cAMP-PKA signaling which exerts its inhibitory effects by phosphorylation of both PLC and IP₃R, thus limiting the Ca²⁺ mobilization [39].

The involvement of the individual factors PLC, IP₃R, RyR and G protein has been reported also in other studies of vasodilation mechanism [40–45].

In conclusion, the present study contributes to understand the molecular mechanisms of the EVOO phenol-induced vasodilation of MA as summarized in Figure 7. Moreover, it shows, for the first time, that EVOO phenols lower vascular resistance through the activation of the subcellular Ca²⁺ microdomains-BK_{Ca} signal.

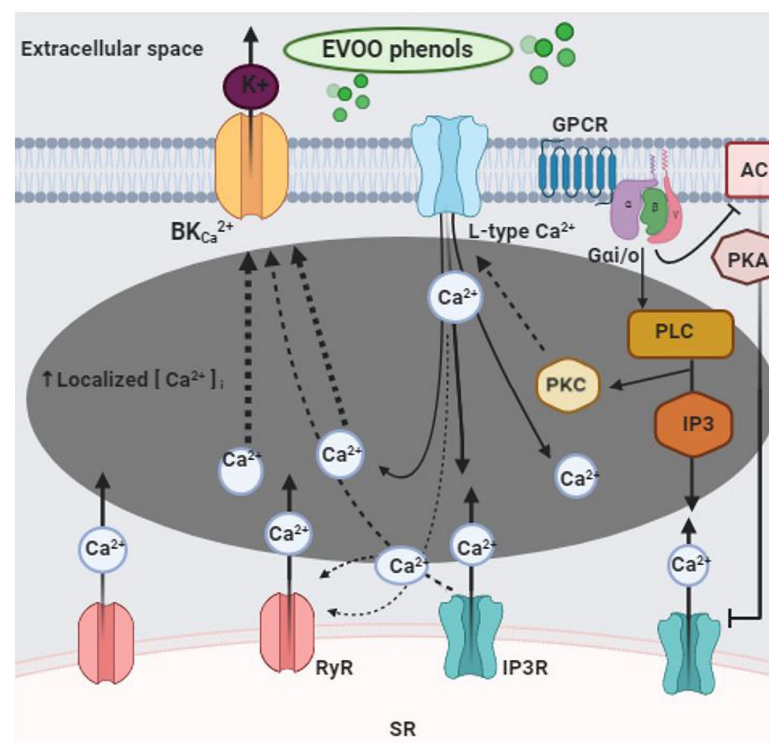


Figure 7. Mechanisms of action of EVOO phenols on vascular smooth muscle cells in mesenteric resistance arteries. EVOO phenols act on the smooth muscle cells of mesenteric resistance artery and trigger intracellular Ca^{2+} signaling. In detail, EVOO phenols activate L-type Ca^{2+} channels and GPCR- $\text{G}\alpha\text{i}$ -mediated-PLC pathways, which in turn activate the sarcoplasmic reticulum channels, RyR and IP3R. The overall result of these signaling cascade is the production of localized sites with high concentrations of Ca^{2+} , named Ca^{2+} microdomains, which determine the activation of the BKCa channels. Calcium Channels L-type (L-type Ca^{2+}), large-conductance calcium-activated potassium channels (BKCa), inositol 1,4,5-triphosphate receptor (IP3R), phospholipase C (PLC), Ryanodine receptor (RyR), $\text{G}\alpha\text{i}$ subunit of G protein-coupled receptor (GPCR- $\text{G}\alpha\text{i}$), protein kinase C (PKC), adenylate cyclase (AC), protein kinase A (PKA), sarcoplasmic reticulum (SR).

Author Contributions: Conceptualization, M.M. and R.D.; Methodology, R.D.; Formal Analysis, R.D. and L.B.; Investigation, R.D., L.B., M.G.; Resources, M.M.; Data Curation, R.D.; Writing R.D. and M.M.—Original Draft Preparation, R.D. and M.M.; Writing—Review & Editing, T.T. and M.D.L.; Supervision, M.M. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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