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Endothelium- and smooth muscle-dependent vasodilator effects of *Citrus aurantium* L. var. *amara*: Focus on Ca^{2+} modulation

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ABSTRACT

Neroli, the essential oil of *Citrus aurantium* L. var. *amara*, is a well-characterized alleviative agent used to treat cardiovascular symptoms. However, because it has been found to have multiple effects, its mechanism of action requires further exploration. We sought to clarify the mechanism underlying the actions of neroli in mouse aorta. In aortic rings from mice precontracted with prostaglandin F_2 alpha, neroli induced vasodilation. However, relaxation effect of neroli was decreased in endothelium-denuded ring or pre-incubation with the nitric oxide synthase inhibitor NG-Nitro-L-arginine-methyl ester (L-NAME). And also, neroli-induced relaxation was also partially reversed by 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ), a soluble guanylyl cyclase (sGC) inhibitor. In addition, neroli inhibited extracellular Ca^{2+}-dependent, depolarization-induced contraction, an effect that was concentration dependent. Pretreatment with the non-selective cation channel blocker, Ni^{2+}, attenuated neroli-induced relaxation, whereas the K^+ channel blocker, tetraethylammonium chloride, had no effect. In the presence of verapamil, added to prevent Ca^{2+} influx *via* smooth muscle voltage-gated Ca^{2+} channels, neroli-induced relaxation was reduced by the ryanodine receptor (RyR) inhibitor ruthenium red. Our findings further indicate that the endothelial component of neroli-induced vasodilation is partly mediated by the NO-sGC pathway, whereas the smooth muscle component involves modulation of intracellular Ca^{2+} concentration through inhibition of cation channel-mediated extracellular Ca^{2+} influx and store-operated Ca^{2+} release mediated by the RyR signaling pathway.

1. Introduction

Endothelial and vascular smooth muscle cells have fundamental roles in controlling vascular contractility, which in turn determines blood flow and, consequently, blood pressure [1]. In these vascular cells, the concentration of intracellular Ca^{2+} modulates vascular resistance by promoting the release of vasodilators or vasoconstrictors [2]. In addition, dynamic changes in intracellular Ca^{2+} levels directly modulate pressure-induced constriction (myogenic tone) in vascular smooth muscle cells [3]. Modification or inappropriate operation of these mechanisms can promote a hypercontractile state and cardiovascular diseases [2]. Therefore, evaluation of Ca^{2+} dynamics is a crucial issue in the experimental assessment of vascular contractility.

Neroli, the essential oil of *Citrus aurantium* L. var. *amara* and other *Citrus* species, is rich in limonene and linalool [4]. It has been widely used in humans for managing anxiety and stress because of its relaxing effect, for instance during labor [5]. A recent cardiovascular study also reported that neroli essential oil reduced blood pressure in humans [6]. Consistent with this, a case report showed that neroli causes bradycardia and hypotension in adolescent patients [7], and limonene, the major component of neroli essential oil, has been reported to decrease blood pressure and heart rate in rats fed a high-fat diet [8]. These observations predict that neroli acts by affecting the autonomic nervous system or cardiovascular system.

An accumulating body of evidence describes the effects of neroli and its components at the cellular or tissue level. Limonene, which constitutes a large fraction of neroli essential oil, is reported to increase cytosolic cyclic AMP (cAMP) and Ca^{2+} levels in Chinese hamster ovary cells [9]. In addition, bergamot essential oil, another *Citrus* species essential oil, reduces vascular contractility by modulating Ca^{2+} influx [10]. These previous studies provide evidence for an effect of neroli on the vascular system; however,

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the direct effects of neroli on a vascular bed have not been investigated. Thus, in this study, we evaluated the relaxation-inducing effects of neroli on mouse aorta and explored possible mechanisms of action, focusing on Ca^{2+} dynamics.

2. Materials and methods

2.1. Chemicals

Prostaglandin F_2 alpha ($PGF_2\alpha$), phenylephrine (PE), 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ), 2-aminoethoxydiphenyl borane (2-APB), tetraethylammonium chloride (TEA), 2,5-di-t-butyl-1,4-benzohydroquinone (BHQ), Hydralazine hydrochloride, Ni^{2+}, verapamil, acetylcholine (ACh), and ruthenium red were from Sigma (St. Louis, MO). N^G-Nitro-L-arginine-methyl ester (L-NAME) was obtained from Alexis Biochemicals (San Diego, CA).

Neroli essential oil, purchased from Aromarant Co. Ltd. (batch no. 131223; Röttingen, Germany), was diluted in dimethyl sulfoxide (DMSO). The final concentration of DMSO in solution was less than 0.1%. All other chemicals were dissolved in distilled water.

2.2. Gas chromatography-mass spectrometry analysis

Compounds of Citrus *aurantium* L. var. *amara* were analyzed by gas chromatography-mass spectrometry (GC–MS), according to the established protocol [11]. Agilent 7890 gas chromatograph used with a 5975 inert mass spectrometer (USA), an analytical capillary column (DBWax; 60 mm × 0.25 mm i.d., 0.25 μm film thicknessPalo Alto, CA, USA), and helium at a controlled flowrate of 1.0 mL/min. The oven temperature was increased to 240 °C. The column temperature was held at 40 °C for 3 min, ramped to 240 °C at 3 °C/min, and maintained at 240 °C for 20 min. Samples (1.0 μL) were inserted at a split ratio of 80:1. The temperatures of the ion source and transfer line were set at 230 °C and 250 °C, respectively. Mass spectra were acquired at 70 eV ionizing energy.

2.3. Experimental animals

This study was approved by the Institutional Animal Research Ethics Committee of Korea University (approval no. KUIACUC-2012-181) and was conducted in accordance with guidelines governing the use of experimental animals. Male C57BL/6 mice (weight 17–21 g) approximately 4–5 weeks old (Samtaco Inc., Osan City, Korea) were used for experiments. The animals were maintained at 22–23 °C under a 12-h light/dark cycle and were provided standard mouse chow and drinking water *ad libitum*.

2.4. Preparation of mouse thoracic aorta

Contraction and relaxation in arterial blood vessels were measured using mouse thoracic aortic rings in organ baths. After anesthetizing with Zoletil and Rompun (3:2 v/v; 0.05 mg/g, intraperitoneal), mice were sacrificed by cervical dislocation. The thoracic aorta was excised and immersed in Krebs solution (118.3 mM NaCl, 4.78 mM KCl, 25 mM $NaHCO_3$, 1.22 mM KH_2PO_4, 11.1 mM glucose, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$). Connective tissue was then removed and aortas were cut into 3-mm rings. When required, the endothelium was removed by rubbing the intimal surface of the vessel. Prepared rings were transferred to an organ bath containing Krebs solution and fixed onto a mechanotransducer (Myo-Interface model 620 M; DMT, Aarhus, Denmark) using two tungsten wires. The organ bath was maintained at 37 °C in a 95% O_2/5% CO_2 atmosphere. Each aortic ring was equilibrated for 30 min at an optimal resting tension of 0.7–0.8 g prior to initiating experiments. The function of arterial endothelium was confirmed by incubation with ACh. Ca^{2+}-free Krebs solution, which contains 0.5 mM ethylene glycol tetraacetic acid (EGTA) in place of Ca^{2+}, was used in experiments involving manipulation of extracellular Ca^{2+}.

2.5. Statistical analysis

Data are expressed means ± standard error of measurement (SEM). Differences between groups were analyzed by Student's *t*-test or one-way analysis of variance (ANOVA) with Duncan's post *hoc* test using SPSS 21.0 software. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Compounds of neroli essential oil

A total of 125 compounds were identified Table 1. The major compounds of neroli essential oil were linalool (23.21%), β-pinene (9.59%), limonene (8.54%), α-terpineol (7.63%), nerolidol (6.08%), geranyl acetate (5.67%), (2E,6E)-farnesol (4.74%), and geraniol (4.17%).

3.2. Vasodilator effect of neroli on mouse aorta

After a stabilization period to establish baseline tension for 30 min, neroli was tested on aortic rings precontracted with 3 μM $PGF_2\alpha$ for 15 min. Neroli was added for 15 min, with endothelium. Addition of neroli produced a concentration-dependent relaxation, dilating aortic rings by 5.1 ± 1.64%, 19.6 ± 2.96%, 68.7 ± 5.78%, 81.2 ± 4.59% and 111.3 ± 9.99% at concentrations of 0.001%, 0.01%, 0.05%, 0.1% and 0.2% (Fig. 1). The EC_{50} of neroli was calculated to be 0.051%; in all subsequent experiments, a neroli concentration of 0.05% was used.

Then neroli also exposed endothelium-denuded aorta. To evaluate the endothelial functionality, the effect of ACh was examined. Neroli reduced vascular contraction induced by $PGF_2\alpha$ on mouse endothelium-denuded aortic rings by 34.3 ± 3.72% (Fig. 2B). And, in the presence of L-NAME, 0.05% neroli dilated aortic rings by 47.1 ± 4.17% (Fig. 2C). Vasodilative effects of neroil in

Table 1
Compounds extracted from *Citrus aurantium* L. var. *amara*.

[a]TR (min)	[b]RI	Compound	[c]Area%
34.483	1541	Linalool	23.205
14.813	1098	β-Pinene	9.593
18.907	1189	Limonene	8.535
40.232	1685	α-Terpineol	7.633
52.095	2015	Nerolidol	6.084
42.328	1741	Geranyl acetate	5.668
61.715	2266	(2E,6E)-farnesol	4.739
45.550	1829	Geraniol	4.168
21.184	1240	β-Ocimene	3.861
41.143	1708	Neryl acetate	3.452
17.123	1152	β-Myrcene	2.281
43.852	1782	Nerol	2.057
34.516	1542	Linalyl acetate	1.987
15.230	1108	Sabinene	1.423
36.536	1591	4-Terpineol	1.281
11.132	1008	α-Pinene	1.171
36.581	1592	*trans*-Caryophyllene	1.042
20.317	1220	2,6-Dimethyl-1,6-octadiene,	0.926
58.190	2164	Methyl anthranilate	0.616
64.063	2345	Indole	0.460
21.946	1257	ρ-Cymene	0.453
Total			90.635%

[a] TR: retention time.
[b] RI: retention indices.
[c] Area: Calculated from the peak area on GC/MS.