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Investigation of terpinen-4-ol effects on vascular smooth muscle relaxation

Rebeca Peres Moreno Maia-Joca [a], Humberto Cavalcante Joca [b], Francisca Jéssica Penha Ribeiro [a], Renata Vieira do Nascimento [a], Kerly Shamyra Silva-Alves [a], Jader S. Cruz [b,*], Andrelina Noronha Coelho-de-Souza [a], José Henrique Leal-Cardoso [a,**]

[a] Superior Institute of Biomedical Sciences, Universidade Estadual do Ceará, Fortaleza, CE, Brazil
[b] Department of Biochemistry and Immunology, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

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ABSTRACT

Aims: This study investigated the mechanisms underlying the vascular effects of terpinen-4-ol in isolated rat aortic ring preparations.
Main methods: The thoracic aortae of healthy rats were submitted to isometric tension recording. Membrane resting potential and input membrane resistance were measured by conventional microelectrode technique.
Key findings: Terpinen-4-ol reversibly relaxed endothelium-containing preparations pre-contracted with high K^+ and phenylephrine with IC_{50} values of 421.43 μM and 802.50 μM, respectively. These effects were significantly reduced by vascular endothelium removal. In Ca^{2+}-free and high K^+ (80 mM) medium, the contractions produced by Ba^{2+} were reduced by terpinen-4-ol (100–1000 μM) in a concentration-dependent manner. In aortic rings maintained under Ca^{2+}-free conditions, terpinen-4-ol significantly reduced the contractions induced by either phenylephrine (1 μM) or phorbol 12,13-dibutyrate (1 μM). Terpinen-4-ol (10–1000 μM) also relaxed the contractions evoked by BAYK-8644 (3 μM) with an IC_{50} of 454.23 μM. Neither membrane resting potential nor input resistance of smooth muscle cells was altered by terpinen-4-ol exposure.
Significance: The present results suggest that terpinen-4-ol induced vascular smooth muscle relaxation that was preferentially due to the inhibition of electromechanical pathways related to calcium influx through voltage-operated calcium channels.

Introduction

Plants are sources of potential biologically active (Leal-Cardoso and Fonteles, 1999) substances which are mostly used in drug manufacturing. One class of these compounds is the monoterpenoids that are present in abundance in all aromatic plants and are well characterized. The fact that these compounds showed a rather low toxicity and beneficial effects on the cardiovascular system is relevant (Santos et al., 2011b).

Terpinen-4-ol is a monoterpenoid that is largely found in essential oils obtained from a large number of plant species (Bezerra et al., 2000; Hart et al., 2000). Terpinen-4-ol has been reported to have several pharmacological activities (Calcabrini et al., 2004; Hart et al., 2000; Loughlin et al., 2008; Nascimento et al., 2005).

Alpinia zerumbet, popularly known as "colônia", is found in some regions of northeast Brazil, where it has been broadly used in folk medicine as infusions or decoctions of its leaves to treat hypertension (Matos, 2007). *A. zerumbet* has a large content of terpinen-4-ol in its essential oil composition (Bezerra et al., 2000). Recently, it was reported that chronic treatment of spontaneously hypertensive rats with the essential oil of *A. zerumbet* (EOAZ) resulted in a significant reduction of both baseline blood pressure values and cardiac hypertrophy (Barcelos et al., 2010). Importantly, studies on the toxicity of essential oils with high terpinen-4-ol content have shown a very low acute toxicity (LD_{50}: 1457.8 mg/mL; Yang et al., 2011).

Previous studies of our group showed that intravenous administration of EOAZ induced dose-dependent hypotensive effects in rats, despite the presence of an operational sympathetic nerve drive to the vascular system (Lahlou et al., 2002). Interestingly, terpinen-4-ol also caused hypotension in the same experimental model. Using DOCA-salt hypertensive conscious rats, which display an increased basal sympathetic activity as a model to study hypertension, EOAZ and terpinen-4-ol evoked dose-dependent hypotensive effects, but the magnitude and

* Correspondence to: J. dos Santos Cruz, Laboratory of Excitable Membranes, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av Antônio Carlos, 6627, Pampulha, 31270-901, Belo Horizonte, MG, Brazil. Tel.: +55 31 3409 2668.
** Correspondence to: J.H. Leal-Cardoso, Laboratory of Electrophysiology, Superior Institute of Biomedical Sciences, State University of Ceará, Av. Paranjana, 1700, Campus of Itaperi, 60740-903, Fortaleza, CE, Brazil. Tel.: +55 85 3101 9814; fax: +55 85 3101 9810.
E-mail addresses: jcruz@icb.ufmg.br (J.S. Cruz), lealcard@gmail.com (J.H. Leal-Cardoso).

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duration were significantly enhanced when compared to normotensive rats (Lahlou et al., 2003). Such effects was mostly independent of the degree of sympathetic vascular tonus as it was unaltered by blockade of ganglionic transmission with hexamethonium and seemed mainly related to an increase in EOAZ- and terpinen-4-ol-induced vascular smooth muscle relaxation (Lahlou et al., 2003).

To the best of our knowledge, no information is yet available concerning the mechanisms by which terpinen-4-ol causes vasorelaxation. Therefore, the present study was undertaken to determine the mechanisms underlying the vascular effects of terpinen-4-ol using isolated rat aortic ring preparations.

Materials and methods

Animals

Wistar healthy rats, 10–14 weeks old, weighing 200–300 g, of both sexes, were kept under conditions of constant temperature (22 ± 2 °C) with a 12 h light/12 h dark cycle and free access to food and water. All animals were handled in compliance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication 85-23, revised 1996; http://www.nap.edu/readingroom/books/labrats/index.html) and all efforts were made to minimize animal suffering. All procedures described herein were first reviewed and approved by the local animal ethics committee (process number 10724456-0/07).

Solutions and drugs

Terpinen-4-ol was prepared daily and dissolved in Tween 80 (0.05% v/v) and vigorously agitated just before use. Krebs–Henseleit's solution (KHS) was used as perfusion medium with the following composition (in mM): 118 NaCl, 4.7 KCl, 25 $NaHCO_3$, 2.5 $CaCl_2 \cdot 2H_2O$, 1.2 KH_2PO_4, 1.2 $MgSO_4 \cdot 7H_2O$, 11 glucose, 0.01 EDTA (ethylenediamine-tetraacetic acid), and pH 7.4. In some experiments in which Ca^{2+} free solutions were needed, equimolar substitution of $MgCl_2$ for $CaCl_2$ and addition of EGTA (ethyleneglycol-tetraacetic acid, 1 mM) was carried out. Solutions with high extracellular [K^+] (80 mM) were prepared by hypertonic addition of KCl to the KHS. For electrophysiological measurements Tyrode's solution (pH 7.4) was used with the following composition (mM): NaCl 136, KCl 5, $MgCl_2$ 0.98, $CaCl_2$ 2, NaH_2PO_4 0.36, $NaHCO_3$ 11.9 and glucose 5.5. Phenylephrine (PHE) hydrochloride, N^G-nitro-L-arginine methyl ester (L-NAME), and acetylcholine (ACh) chloride were first dissolved in distilled water to prepare stock solutions. Phorbol 12,13-dibutyrate (PDB) and BAYK-8644 were dissolved in dimethyl sulfoxide (DMSO) while nifedipine and indomethacin (INDO) were dissolved in ethanol and sodium bicarbonate (at 5%), respectively. All reagents were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Tissue preparation and experimental protocols

The tissue preparation and the isometric tension recordings were made as previously reported (Peixoto-Neves et al., 2010). Briefly, rats were sacrificed and the thoracic aortae were removed and cleaned of adhering fat and connective tissue. The aorta was cut transversally and the segments (2.0–3.0 mm) were mounted on a myograph at 37 °C and continuously gassed with 95% O_2 and 5% CO_2 under a resting tension of 1 g. Mechanical activity was recorded by an isometric force transducer (Grass Model FTO3, Quincy, MA, USA) connected to a PC-based data acquisition system (PM-1000, CWE Inc., Akron, OH, USA). The presence of functional endothelium was assessed by application of ACh (1 μM) to induce more than 70% relaxation. In some experiments we removed the endothelial layer by rubbing the intimal surface. We considered complete removal if the ability of ACh to provoke relaxation was less than 5%.

To facilitate the comprehension we decided to group all experiments as follows:

Experimental series #1. In this series of experiments, the effects of cumulative increasing concentrations (1–6000 μM) of terpinen-4-ol on the sustained contractile responses to high [K^+] (80 mM) or PHE (1 μM) were studied in either endothelium-containing or endothelium-denuded aortic ring preparations maintained in KHS. In order to assess the role of nitric oxide (NO) and cyclooxygenase products on the vascular effects of terpinen-4-ol in PHE- or high [K^+]-stimulated preparations, experiments were performed in endothelium-containing aortic rings incubated for 20 min with L-NAME (100 μM) and/or INDO (10 μM).

Experimental series #2. This series of experiments was carried out to assess the inhibitory effects of terpinen-4-ol on the contractions induced by addition of Ba^{2+} in aortic ring preparations without intact endothelium. After determining tissue responsiveness, the preparation was maintained in Ca^{2+}-free medium and then after 5 min 80 mM K^+ was added to the bath. Thereafter, terpinen-4-ol (100–1000 μM), vehicle (control) or nifedipine used as positive control (1 μM) was added to the preparation for 5 min, and then cumulative concentration–response curves for Ba^{2+} (0.1–30 mM) were obtained. The contractile responses obtained were expressed as percentage of high K^+-induced contractions.

Experimental series #3. In these experiments, inhibitory effects of increasing concentrations (10–3000 μM) of terpinen-4-ol on the sustained contractile response to BAYK-8644 (3 μM) were studied in endothelium-denuded aortic rings that had been sensitized with 10 mM extracellular K^+ and maintained in regular KHS.

Experimental series #4. The effects of terpinen-4-ol on PHE-induced contractions in Ca^{2+}-free medium were investigated. After the usual stabilization time, endothelium-denuded aortic rings were washed with Ca^{2+}-free solutions for 15 min followed by the addition of terpinen-4-ol (1000, 3000 and 6000 μM) or vehicle (control). PHE (1 μM) was then added to elicit a transient contraction.

Experimental series #5. In this next series of experiments, inhibitory effects of increasing concentrations (600, 1000, 3000 and 6000 μM) of terpinen-4-ol on the contraction elicited by phorbol 12,13-dibutyrate (PDB, 1 μM) were studied in endothelium-denuded aortic ring preparations incubated in Ca^{2+}-free solution.

Membrane resting potential and input membrane resistance measurements

Membrane resting potential and input resistance were measured as previously described (Peixoto-Neves et al., 2010). The aorta was cut transversally into cylindrical ring-like pieces which were devoid of functional endothelium. The tissue was then transferred and fixed into an acrylic chamber and visualized by a stereoscopic microscope (model College Stereo, MLW Intermed, Schöneiche, Germany). Microelectrodes were prepared from aluminum silicate glass (1.0 mm o.d., 0.68 mm i.d., WPI Corp., New Haven, CT, USA) filled with 3.0 M KCl solution with tip resistances ranging from 25 to 50 MΩ and were connected via an Ag–AgCl wire to an Axoclamp-2B amplifier (Axon Instruments, Burlingame, CA, USA). Voltage outputs were displayed on-line on the oscilloscope screen for monitoring the signals and analysis of the digitized data was performed with pClamp6 software (Axon Instruments, Burlingame, CA, USA).

The superfusion, with modified Tyrode's solution, was maintained by gravity and adjusted to give 1.0–1.5 mL/min. Reservoirs containing superfusate solution with or without terpinen-4-ol were then connected to the test chamber. In each cell studied, membrane resting potential was recorded in the absence (3–5 min) and in the presence (5 min) of terpinen-4-ol at 1000 μM.