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Mechanisms underlying the vascular and hypotensive actions of the labdane *ent*-3-acetoxy-labda-8(17),13-dien-15-oic acid

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ABSTRACT

We investigated the mechanisms underlying the vasorelaxant and hypotensive actions of the labdane-type diterpene *ent*-3-acetoxy-labda-8(17),13-dien-15-oic acid (labda-15-oic acid). Vascular reactivity experiments were performed in aortic rings isolated from male Wistar rats. cAMP and cGMP were measured by enzyme immunoassay (EIA) whereas nitrate measurement was performed by chemiluminescence. Nitric oxide (NO) concentration ([NO]c) was measured in endothelial cells by flow cytometry. The cytosolic calcium concentration ($[Ca^{2+}]c$) in vascular smooth muscle cells (VSMC) was measured by confocal microscopy. Blood pressure measurements were performed in conscious rats. Labda-15-oic acid inhibited the contraction induced by phenylephrine and serotonin in either endothelium-intact or endothelium-denuded rat aortic rings. The labdane significantly reduced $CaCl_2$-induced contraction in a Ca^{2+}-free solution containing KCl or phenylephrine. Labda-15-oic acid (0.1–300 μmol/l) concentration-dependently relaxed endothelium-intact and endothelium-denuded aortas pre-contracted with either phenylephrine or KCl. In endothelium-intact rings, the relaxation induced by labda-15-oic acid was affected by L-NAME, 7-nitroindazole, ODQ, hemoglobin, Rp-8-Br-Pet-cGMPS and thapsigargin. Blockade of K^+ channels with 4-aminopyridine, apamin, charybdotoxin and glibenclamide affected the relaxation induced by labda-15-oic acid. The labdane increased cGMP and nitrate levels but did not affect cAMP levels in endothelium-intact aortas. Labda-15-oic acid increased [NO]c in endothelial cells and decreased $[Ca^{2+}]c$ in VSMC. The hypotension induced by intravenous administration of labda-15-oic acid (0.3–3 mg/kg) was partially reduced by L-NAME. In conclusion, the mechanisms underlying the cardiovascular actions of the labdane involve the activation of the endothelial NO-cGMP pathway, the opening of K^+ channels and the alteration on Ca^{2+} mobilization.

1. Introduction

Diterpenoids form a large class of plant-derived secondary metabolites and these compounds exert significant cardiovascular effects such as vascular relaxation and hypotension (Tirapelli et al., 2004, 2010; Hipólito et al., 2009). For this reason, a great number of studies have focused on the cardiovascular properties of these compounds.

Forskolin (7 beta-acetoxy-8, 13-epoxy-1 alpha,6 beta, 9 alpha-trihydroxy-labd-14-ene-11-one) is a well-known labdane-type diterpene, which is described to lower blood pressure via relaxation of vascular smooth muscle (Lindner et al., 1978; Dubey et al., 1981; Kramer et al., 1987; Schlepper et al., 1989). In the vasculature, forskolin-induced relaxation involves the activation of adenylyl cyclase, producing an increase in cAMP and cAMP-dependent protein kinase (PKA) activation (Lincoln and Fisher-Simpson, 1984). Hyperpolarization of smooth muscle and Ca^{2+} extrusion across the plasma membrane are also described as mechanisms underlying forskolin-induced relaxation (Den Hertog et al., 1984). In humans, intravenous administration of forskolin decreased vascular resistance, reduced diastolic blood pressure and improved left ventricular function in patients with cardiomyopathy (Kramer et al., 1987; Schlepper et al., 1989).

In addition to forskolin, other labdane-type diterpenes were also described to exert cardiovascular effects. In this line, marrubenol was shown to induce vascular relaxation via inhibition of extracellular Ca^{2+} influx through L-type Ca^{2+} channels in the

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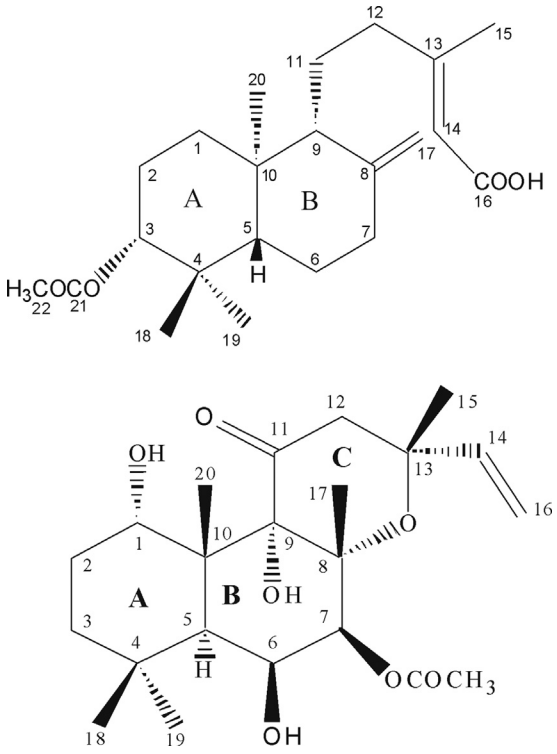

Fig. 1. Chemical structure of *ent*-3-acetoxy-labda-8(17),13-dien-15-oic acid (labda-15-oic acid; top) and 7 beta-acetoxy-8, 13-epoxy-1 alpha,6 beta,9 alpha-trihydroxy-labd-14-ene-11-one (forskolin, bottom).

vascular smooth muscle (El Bardai et al., 2003). The hypotensive and relaxant actions of the labdane 8(17), 12E, 14-labdatrien-18-oic acid in normotensive rats involve the activation of the nitric oxide (NO)-cGMP pathway (De Oliveira et al., 2006). The labdane-type diterpene labd-8 (17)-en-15-oic acid was shown to induce endothelium-dependent relaxation in isolated aortas via the NO-cGMP pathway and intravenous administration of this diterpene decreased blood pressure in normotensive rats (Lahlou et al., 2007).

The mechanisms underlying the cardiovascular actions of labdane-type diterpenes involve multiple actions on various targets. Structural differences among labdanes could explain these diverse mechanisms of action and structural alterations could improve the cardiovascular activity of labdane-type diterpenes (Khandelwal et al., 1988). A comparison of the chemical structure of forskolin and the labdane *ent*-3-acetoxy-labda-8(17),13-dien-15-oic acid (labda-15-oic acid) shows that they share common points (Fig. 1). Thus, based on the knowledge that labdane-type diterpenes exert cardiovascular actions and that the chemical structure of these compounds is important for such effects, we hypothesized that the labdane labda-15-oic acid would induced vascular relaxation and hypotension. In the present study we aimed to investigate the mechanism(s) involved in the vasorelaxant and hypotensive action of the diterpene labda-15-oic acid.

2. Material and methods

2.1. Isolation of labda-15-oic acid

The isolation of labda-15-oic acid was performed as previously described (Souza et al., 2011). One hundred grams of oleoresin was chromatographed over silica gel 60H (Merck, art. 7736) using vacuum liquid chromatography (VLC) with increasing amounts of ethyl acetate (EtOAc) in *n*-hexane as eluent. This procedure furnished six fractions (2000 ml each) that were named F1 (34.7 g; *n*-hexane), F2 (13.5 g; 20% EtOAc), F3 (11.4 g; 40% EtOAc), F4 (9.7 g; 60% EtOAc), F5 (7.6 g; 80% EtOAc), and F6 (17.8 g; EtOAc) after solvent evaporation. Fraction F4 was initially chromatographed by VLC over silica gel 60 H (Merck, art. 7736) as described above, to give additional fractions (F4.1–F4.5). Labda-15-oic acid (1132.0 mg) was obtained from F4.3 through medium pressure chromatography (flash chromatography) using silica gel 60 (Merck, art. 9385), isocratic *n*-hexane: EtOAc:$CHCl_3$ (5:2:3) as mobile phase, and a flow rate of 5 ml/min (Still et al., 1978). The purity of (−)-acetoxycopalic acid (98%) was estimated by HPLC, mass spectrometric analysis and ^1H and ^{13}C NMR spectral data.

2.2. Vessel ring preparation

Male Wistar rats weighting between 200 and 250 g (50–60 days old) were anesthetized with tribromoethanol (250 mg/kg, i.p.) and killed by aortic exsanguination in accordance with the Ethical Animal Committee of the Campus of Ribeirão Preto – University of São Paulo (Protocol number 09.1.1007.53.0). The thoracic aorta was quickly removed, cleaned of adherent connective tissues and cut into rings (5–6 mm in length). Two stainless-steel stirrups were passed through the lumen of each ring. One stirrup was connected to an isometric force transducer (TRI201; Panlab, Spain) to measure tension in the vessels. The rings were placed in a 5 ml organ chamber that contained Krebs solution, gassed with 95% O_2/5% CO_2 maintained at 37 °C. The composition of Krebs solution was as follows (mmol/l): NaCl, 118.0; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 15.0; Glucose, 5.5; $CaCl_2$, 2.5. The rings were stretched until they reached a basal tension of 15 mN, which was determined by length–tension relationship experiments and were then allowed to equilibrate for 60 min; during this time, the bath fluid was changed every 15–20 min. For some rings, the endothelium was removed mechanically by gently rolling the lumen vessel on a thin wire. Endothelial integrity was assessed qualitatively by the degree of relaxation caused by acetylcholine (1 μmol/l) in the presence of contractile tone induced by phenylephrine (0.1 μmol/l). For studies of endothelium-intact vessels, a ring was discarded if relaxation with acetylcholine was not 80% or greater. For studies of endothelium-denuded vessels, a ring was discarded if there was any degree of relaxation. Agonist concentration–response curves were fitted using a nonlinear interactive fitting program (Graph Pad Prism 3.0; GraphPad Software Inc., San Diego, CA). Agonist potencies and maximal responses were expressed as pD_2 (−logEC50) and Emax (maximum effect elicited by the agonist), respectively.

2.3. Determination of the time-sensitive effects of labda-15-oic acid

Endothelium-intact rings were contracted with KCl at 30 mmol/l (control) and then washed out and pre-incubated for 30 or 60 min with labda-15-oic acid at 10, 50 or 100 μmol/l. Subsequently, a new stimulation was performed with KCl at 30 mmol/l on the same ring; each ring served as its own control. Vessel rings from the same animal that were not exposed to the diterpene served as time controls.

2.4. Evaluation of the reversibility of the inhibitory effect displayed by labda-15-oic acid

In order to verify whether the inhibitory effect of labda-15-oic acid was reversible, endothelium-intact and endothelium-denuded rings were contracted with KCl at 30 mmol/l before (control) or after incubation with labda-15-oic acid at 100 μmol/l for 30 min. Subsequently, the rings were washed out and new stimulations with KCl (30 mmol/l) were performed on the same ring each 30 min until 120 min.