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Treatment with salvianolic acid B restores endothelial function in angiotensin II-induced hypertensive mice

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ABSTRACT

Salvianolic acid B (Sal B) is one of the most abundant phenolic acids derived from the root of Danshen with potent anti-oxidative properties. The present study examined the vasoprotective effect of Sal B in hypertensive mice induced by angiotensin II (Ang II). Sal B (25 mg/kg/day) was administered via oral gavage for 11 days to Ang II (1.2 mg/kg/day)-infused C57BL/6J mice (8–10 weeks old). The vascular reactivity (both endothelium-dependent relaxations and contractions) in mouse arteries was examined by wire myography. The production of reactive oxygen species (ROS), protein level and localization of angiotensin AT_1 receptors and the proteins involved in ROS formation were evaluated using dihydroethidium (DHE) fluorescence, lucigenin-enhanced chemiluminescence, immunohistochemistry and Western blotting, respectively. The changes of ROS generating proteins were also assessed *in vitro* in human umbilical vein endothelial cells (HUVECs) exposed to Ang II with and without co-treatment with Sal B (0.1–10 nM). Oral administration of Sal B reversed the Ang II-induced elevation of arterial systolic blood pressure in mice, augmented the impaired endothelium-dependent relaxations and attenuated the exaggerated endothelium-dependent contractions in both aortas and renal arteries of Ang II-infused mice. In addition, Sal B treatment normalized the elevated levels of AT_1 receptors, NADPH oxidase subunits (NOx-2 and NOx-4) and nitrotyrosine in arteries of Ang II-infused mice or in Ang II-treated HUVECs. In summary, the present study provided additional evidence demonstrating that Sal B treatment for 11 days reverses the impaired endothelial function and with a marked inhibition of AT_1 receptor-dependent vascular oxidative stress. This vasoprotective and anti-oxidative action of Sal B most likely contributes to the anti-hypertensive action of the plant-derived compound.

1. Introduction

Hypertension, arteriosclerosis, and chronic heart failure are collectively associated with endothelial dysfunction [1], a condition described by impaired endothelium-dependent relaxations mainly due to diminished nitric oxide (NO)-cyclic GMP signaling [2]. Elevated accumulation of reactive oxygen species (ROS) in the vascular wall is one of the key initiators of endothelial dysfunction following inactivation of endothelium-derived NO [2,3]. ROS, in particular superoxide anions react with NO to form peroxynitrite; the latter, uncouples endothelial nitric oxide synthase (eNOS), causing the enzyme to release additional superoxide anions which further reduces the bioavailability of NO. This continuous cascade eventually leads to endothelial injury [1,4]. The main source of superoxide anions in the vasculature is the NADPH oxidase, a multi-subunit enzymatic complex [5].

Angiotensin II (Ang II) a major component of the renin-angiotensin system participates in cardiovascular dysfunction by promoting vasoconstriction, oxidative stress, inflammation, salt and water reabsorption [6,7]. These harmful actions are mediated primarily by angiotensin type 1 (AT_1) receptors [8]. The expression of AT_1 receptor is elevated in the vasculatures during cardiovascular pathogenesis [9–11]. Activation of AT_1 receptors is closely associated with increased oxidative stress and the activation of NADPH oxidases [12,13]. Indeed, exposure to Ang II causes endothelial dysfunction through NADPH-derived ROS production [14,15].

Radix Salvia miltiorrhiza also known as Danshen in China is a herbal medicine commonly used to invigorate the blood and reduce clotting in oriental countries [16]. The plant is widely used to treat patients with cardiovascular and cerebrovascular diseases

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especially angina pectoris, hyperlipidemia and acute ischemic stroke in many parts of China [17] and is presently gaining increasing popularity in both eastern and western countries, including the United States and European nations. Dantonic® (T89 or Cardiotonic pill) is one of the most commonly used Chinese herbal formulations based on Danshen for the treatment of heart diseases in China. Salvianolic acid is the major water soluble agent compound and salvianolic acid B (Sal B, Fig. 1A) as the most abundant bioactive constituent in Danshen [18].

Sal B has strong antioxidant properties [19–21], protects endothelial cells against oxidative stress and inflammation [22–24]. In the heart, Sal B is protective against ischemia-reperfusion injury, myocardial infarction and cardiac remodeling [25]. Although Sal B has several beneficial effects in the prevention and treatment of heart diseases, information is still lacking concerning its mechanistic effect against hypertension, an important risk factor in the development of cardiac diseases. Thus, the aim of the present study is to investigate the vasoprotective effect of Sal B in Ang II-induced hypertension in mice.

2. Materials and methods

2.1. Animals and experimental protocols

Male C57BL/6J mice (8–10 weeks old) were obtained from the Chinese University of Hong Kong (CUHK) Laboratory Animal Service Center. The animal care and investigation was approved by the CUHK Animal Experimentation Ethics Committee and conformed to the ARRIVE guidelines for experiments involving animals [26]. Mice were housed in a well-ventilated holding room at a controlled temperature (24 ± 1 °C) and lighting condition (12-h light/dark cycle) with free access to standard chow and water. Ang II-(1.2 mg/kg/day) or phosphate-buffered saline (PBS)-loaded Alzet osmotic minipumps (Model 1002; DURECT Corporation, Cupertino, CA, USA) were implanted subcutaneously in mice under ketamine/xylazine anesthesia (75 and 6 mg/kg body weight; intraperitoneal injection) [27]. Mice then received administration of either Sal B (25 mg/kg/day) or vehicle (distilled water) by oral gavage for 11 days. The dosage of Sal B (25 mg/kg/day) was chosen based on similar dosage reported in previous studies on mice [28,29] and is below the toxicity dose range [30,31]. At the end of the treatment, mice were sacrificed by CO_2 inhalation.

2.2. Systolic blood pressure measurement

Prior to the commencement of Sal B treatment, the systolic blood pressure of mice was measured by tail-cuff method (NIBP monitoring system, IITC Inc, Woodland Hills, CA, USA), and thereafter recorded every three days after the treatment started. All mice were acclimated before the actual measurement was made. During measurement, mice were immobilized in a pre-warmed chamber at around 28–30 °C for at least 30 min before the readings were recorded. At least six to seven successive measurements were taken and the averaged values were then calculated [32].

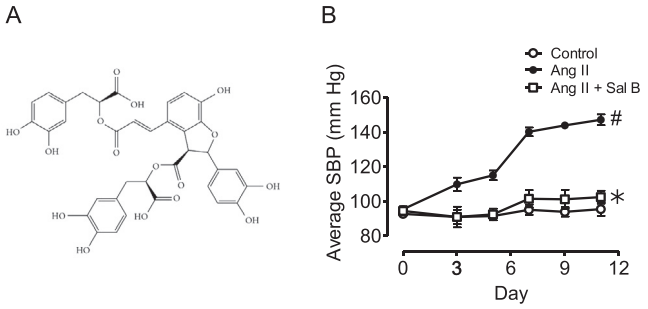

Fig. 1. Chemical structure of salvianolic acid B (A) and measurement of average systolic blood pressure (SBP) by tail-cuff method (B) following 11 days of treatment with salvianolic acid B (Sal B; 25 mg/kg/day) in Ang II (1.2 mg/kg/day)-infused hypertensive mice. Data are mean ± SEM (n = 6–7). $^{\#}p < 0.05$ compared to Control; $^{*}p < 0.05$ compared to Ang II.

2.3. Artery preparation

Mouse thoracic aorta and renal arteries were isolated and placed in ice-cold Krebs solution (in mM: NaCl 118.93, $NaHCO_3$ 25.00, $MgSO_4$ 1.18, KCl 4.69, KH_2PO_4 1.03, Glucose 11.10, $CaCl_2$ 2.38). Each artery was cleaned of adhering connective tissues and cut into ring segments (each 2 mm in length). Some arteries were snap-frozen in liquid nitrogen and stored in −80 °C for later process. The rings were suspended in a myograph (Danish Myo Technology, Aarhus, Denmark), and continuously oxygenated by 95% O_2 and 5% CO_2 throughout the experiment. Changes in the isometric tension were recorded using a PowerLab recording system (AD Instruments, Bella Vista, NSW, Australia).

2.4. Vascular reactivity

After 30 min equilibrium period, each ring was contracted by 80 mM KCl to test its viability and washed three times in Krebs solution. The functional integrity of the endothelial cells was verified by more than 80% relaxation induced by acetylcholine (ACh, 10 μM) in phenylephrine pre-contracted rings. Thereafter, rings were rinsed and contracted again by phenylephrine before cumulative addition of ACh (3 nM–10 μM) to evoke endothelium-dependent relaxations (EDRs). Cumulative concentration-responses to the endothelium-independent vasodilator sodium nitroprusside (SNP, 1 nM–10 μM) were also obtained. To visualize the endothelium-dependent contractions (EDCs), rings were exposed to 100 μM Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME, a non-selective NOS inhibitor) for 30 min in order to eliminate the interference of basal endothelium-derived NO before they were exposed to increasing concentrations of ACh (0.3–300 μM). The EDCs were expressed as the percentage of 80 mM KCl-induced tension in the arteries. L-NAME in the concentration used did not affect the resting tension of the vascular rings, but it abolished ACh-induced relaxation [33].

2.5. Cell culture

Human umbilical vein endothelial cells (HUVECs, American Type Culture Collection, Manassas, USA) were cultured in EGM-2 medium (EGM-2, Single Quots, Lonza) containing 20% fetal bovine serum in 6-well plates. When cells reached a confluency of 80–90%, they were treated with Ang II (1 μM) for 24 h with and without co-incubation with Sal B (0.1–10 nM) or losartan (AT_1 receptor antagonist, 10 μM as positive control).

2.6. Detection of ROS formation in cryostat sections of arteries

Intracellular ROS production was determined using dihydroethidium (DHE)-mediated fluorescence microscopy. Isolated mouse aortas and renal arteries were embedded in OCT compound (Sakura Finetik, AJ Alphen aan den Rijn, Netherlands) until being frozen. The frozen segments were cut into 5 μm cryostat sections which were incubated for 15 min in normal physiological saline solution [in mM: NaCl 140, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, glucose 10 and HEPES 5] containing 5 μM DHE (Invitrogen, Carlsbad, CA, USA) at 37 °C. In another set of experiment, the cryostat sections of aorta and renal artery from Ang II-infused hypertensive mice were incubated with Sal B (10 nM), superoxide dismutase (SOD;