International Immunopharmacology 33 (2016) 119–127

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Astragaloside IV improves the isoproterenol-induced vascular dysfunction via attenuating eNOS uncoupling-mediated oxidative stress and inhibiting ROS-NF-κB pathways

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ARTICLE INFO

Article history:
Received 28 August 2015
Received in revised form 24 December 2015
Accepted 8 February 2016
Available online 21 February 2016

Keywords:
Astragaloside IV
Isoproterenol
eNOS uncoupling
NF-κB
Tetrahydrobiopterin

ABSTRACT

Objective: Oxidative stress and inflammation are regarded as two important triggers of endothelial dysfunction and play pivotal role in progression of vascular damage associated with cardiac hypertrophy. Our previous studies demonstrated that astragaloside IV (AsIV) could protect against cardiac hypertrophy in rats induced by isoproterenol (Iso), but its effects on the aorta are not known. In present study, we aimed to assess the effects of AsIV on Isoinduced vascular dysfunction.

Methods: Sprague-Dawley (SD) rats were treated with Iso (10 mg/kg/d) alone or in combination with AsIV (50 mg/kg/d).

Results: Compared with Isotreated alone, AsIV significantly reduced the ratios of heart weight/body weight and left ventricular weight/body weight. AsIV ameliorated the increased vasoconstriction response to phenylephrine induced by Iso and suppressed superoxide anion generation in rat aorta, increased endothelial nitric oxide synthase (eNOS) dimer/monomer ratio and its critical cofactor tetrahydrobiopterin (BH_4) content in aorta as well as the NO production in the serum, reduced the plasmatic peroxynitrite (ONOO–). Moreover, in contrast with Isotreatment alone, AsIV decreased the ratio of nuclear-to-cytosolic protein expression of the NF-κB p65 subunit while enhanced its inhibited protein expression of IκB-α, down-regulated mRNA expression of IL-1β, IL-6 and TNF-α of the aorta.

Conclusions: The present study suggested that AsIV protects against Isoinduced vascular dysfunction probably via attenuating eNOS uncoupling-mediated oxidative stress and inhibiting ROS-NF-κB pathways.

1. Introduction

Persistent β-adrenergic stimulation with isoproterenol (Iso) could induce ventricular hypertrophy [1], increase ventricular collagen content [2]. Overexpressing β_2-AR showed enhanced superoxide anion production and contributed to cardiac remodeling and failure [3]. In addition, evidence has shown that Isoinduced β_2-AR overactivation leads to vascular oxidative stress and endothelial dysfunction via a Gia-coupled β_2-adrenoceptor signaling pathway, which was associated with endothelial nitric oxide synthase (eNOS) uncoupling [4,5]. Endothelial nitric-oxide synthase (eNOS) plays a critical role in nitric oxide (NO) release which critically determines vascular tone as well as vascular wall homeostasis [6]. Reduced availability of NO due to enhanced degradation by superoxide anion or impaired synthesis function has been demonstrated to be a major cause of endothelial dysfunction in numerous cardiovascular diseases [7–9]. Tetrahydrobiopterin (BH_4) is a critical cofactor of eNOS for the NO synthesis, and eNOS becomes uncoupled for absence of BH_4, producing superoxide (O^{2-}) instead of NO [10–12]. It has been demonstrated that Iso treatment increased superoxide anion production in the rat aorta [5,13] and superoxide anion could avidly react with eNOS-derived NO to form peroxynitrite (ONOO–). BH_4 is extremely sensitive to be oxidized by this ONOO– [14]. Both ONOO– and O^{2-} can oxidize the endothelial cell BH_4 to BH_3, which is further oxidized to BH_2 [15–19]. BH_2 can compete with BH_4 for binding to the oxygenase domain of eNOS [20], which finally results in eNOS uncoupling. In turn, eNOS uncoupling enhances vascular oxidative stress [21], consequently leading to the vascular dysfunction. Previous study have demonstrated that BH_4 levels declined in pressure-overload hypertrophy, subsequently contributing to NOS uncoupling [22], but it has not been studied in Isoinduced hypertrophy.

In addition, nuclear factor κB (NF-κB) is a nuclear transcription factor that regulates expression of a large number of genes. In its inactive form, NF-κB is bounded by inhibitor proteins of the IκB family in the cytoplasm. Once being activated, subunit of NF-κB translocates into nuclear from cytoplasm which further regulates the targeted genes. Previous studies have demonstrated that vascular oxidative stress induced by Iso

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http://dx.doi.org/10.1016/j.intimp.2016.02.009
1567-5769/

treatment can activate NF-κB and consequently increase pro-inflammatory cytokine release resulting in altered vascular function [13]. So NF-κB is a convergent signal of proinflammatory cytokines and oxidative stress signaling pathways.

Astragaloside IV (AsIV), which is found in Astragalus membranaceus (Fisch.) Bge, has been proved to have potential protective effect on cardiac hypertrophy and myocardial ischemia/reperfusion [23–26]. AsIV has effect of *anti*-oxidation, *anti*-inflammation. Our previous studies proved that AsIV can suppress Isoinduced myocardial hypertrophy via regulating TLR4/NF-κB signaling pathway and NF-κB/PGC-1α signaling [23,25]. Evidence suggests that AsIV has a potential effect on vascular-related pathways to exert its effects on vascular function and could inhibit vessel contraction through endothelium dependent NO/cGMP pathway [27]. But the effects of AsIV on the vascular endothelial dysfunction in cardiac hypertrophy model induced by Iso and the underlying mechanisms are not clear. Thus, the purposes of our present study are to investigate the potential protective effects of AsIV against vascular endothelial dysfunction induced by Iso in myocardial hypertrophy rats. Furthermore, the underlying mechanisms for AsIV-induced protection were investigated.

2. Materials and methods

2.1. Materials

AsIV (purity > 98%) was purchased from Chengdu Conbon Bio-Tech Co., Ltd. (Chengdu, China). Isoproterenol hydrochloride (MW 247.72, CAS N5984–95–2), propranolol (Pro), (6*R*)-5,6,7,8-tetrahydrobiopterin dihydrochloride (MW 314.17, CAS 69056-38-8), *N*-nitro-L-arginine methyl-ester (L-NAME)were purchased from Sigma (St, Louio, USA). Hydroethidine was purchased from AAT Bioquest, Inc. (formerly ABD Bioquest, Inc.). Antibodies against IκB-α, eNOS, lamin B were from abcam (Cambridge, MA, USA). p65, β-actin were from Proteintech Group (Wuhan, China). NO assay kits were from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Enzyme-linked immunosorbent assay (ELISA) kits for 3-nitrotyrosine were from R&D Systems (Minneapolis, MN, USA). qRT-PCR SYBR® Green Kit (Vazyme, Nanjing, China).

2.2. Animal experiment

All experimental protocols were approved by the Ethics Committee on Animal Use of the Liaoning Medical University (Permit Number: LMU-2013-368) and conformed to the ethical principles for animal experimentation of Laboratory Animal Science. Forty six-week-old male Sprague-Dawley rats (200–250 g) were obtained from Animal Centre of the Liaoning Medical University (license number: SCXK 2009–0004) and housed in a constant room temperature and light cycle (12:12-h light-dark cycle). The rats were allowed to take food and water freely. Rats were divided randomly into four groups ($n = 10$): (1) the control (Con), rats received vehicle (1% sodium carboxymethyl cellulose (CMC), 1 mL, i.g) and physiological saline injections (i.p); (2) Iso group (Iso), rats received daily injection of Iso (10 mg/kg/day, i.p.) and the same volume of CMC (i.g) with Con group; (3) Iso plus propranolol (Pro,40 mg/kg/day, i.g) (4) Iso plus AsIV (AsIV, 50 mg/kg/day, i.g). AsIV and Pro were administrated 2 weeks before Iso injection and lasted 4 weeks. AsIV were dissolved in 1% CMC solution.

2.3. Method

2.3.1. Determination of the parameters of heart weight

At the end of treatment, rats were anaesthetised with sodium pentobarbital (20 mg/kg, i.p.), Then the rat thoracic cavity were opened quickly, 2 mL blood was taken from the abdominal aorta into anticoagulative tubes and ordinary tubes respectively. Plasma and serum were obtained and stored at −80 °C. The hearts and thoracic aortas were removed. The whole hearts and left ventricles were weighed, and normalized to body weight (mg/kg) respectively. The ratios were used as the index of ventricular hypertrophy. Then thoracic aortas were dissected and stripped the connective tissue from the aortas carefully. For the analysis of superoxide anion production, thoracic aortic segments were first immersed in an embedding medium (OCT Tissue Freezing Medium) and then frozen and kept at −80 °C until superoxide anion was measured. For reactivity experiments, the aortas were cut into 4 mm in length. For the other experiments, the aortas were frozen at −80 °C until the day of analysis.

2.3.2. Vascular reactivity experiments

Segments of thoracic aorta (4 mm in length), free of fat and connective tissue, were mounted in an isolated tissue chamber containing Krebs–Henseleit solution (in mM: NaCl 118; KCl 4.7; $NaHCO_3$ 25; $CaCl_2$-$2H_2O$ 2.5; KH_2PO_4 1.2; $MgSO_4$–$7H_2O$ 1.2; glucose 11 and ethylenediamine-tetraacetic acid (EDTA) 0.01), gassed with 95% O_2 and 5% CO_2, and maintained at a resting tension of 14 mN at 37 °C. Isometric tension was recorded using DMT620 Myograph (Danish Myo Technology A/S, Aarhus N, Denmark). After a 60 min equilibration period (Krebs-Henseleit solution were changed every 20 min during the period), all aortic rings were successively exposed to 125 mM K-PSS(in mM: NaCl 74.7; KCl 125; $NaHCO_3$ 14.9; $CaCl_2$-$2H_2O$ 2.5; KH_2PO_4 1.18; $MgSO_4$–$7H_2O$ 1.17; glucose 5.5 and ethylenediamine-tetraacetic acid (EDTA) 0.026) three times until a plateau was reached. After washout period, endothelial integrity was tested by acetylcholine-induced relaxation (10 μM) in aortic rings that were contracted with phenylephrine (~0.1 μM). A relaxation response to acetylcholine larger than 50% was considered to be functional integrity of the endothelium. After a washout period, concentration-response curves to the a_1-adrenoceptor agonist phenylephrine (0.1 nM ~ 10 μM) were obtained. To evaluate the role of NO in the vasoconstrictor response to phenylephrine, some aortic rings were pre-incubated for 30 min with the nonselective nitric oxide synthase (NOS) inhibitor *N*-nitro-L-arginine methyl-ester (L-NAME, 100 μM) before the concentration-response curves to phenylephrine were assessed. Vasoconstrictor responses to phenylephrine were expressed as a percentage of the contraction produced by 125 mM K-PSS.

2.3.3. Measurement of nitrite/nitrate

Oxidation products of NO (nitrite and nitrate) were assayed in rat serum as a measure of the total NO synthesis [28]. Nitric oxide in serum was measured using a commercially available nitrite/nitrate assay kit according to manufacturer's instructions. In briefly, we added prepared samples and R1,R2 mixed reagents and then heated for 60 min in water bath at 37 °C.·Then R3,R4 were added into the mixture, the mixture was kept standing for 40 min, followed by centrifugation at 2500 × g for 10 min at 4 °C. The supernatant (0.5 mL) were taken out into another tubes containing chromogenic agent, reacting 10 min at 37 °C. The OD values were measured in 550 nm by ultraviolet spectrophotometer (Mapada Instruments, Shanghai, China).

2.3.4. Measurement of total plasma ONOO– levels by ELISA

NO could react with superxide anion following the generation of the highly reactive ONOO–. This free radical then can nitrate tyrosine residues to 3-nitrotyrosine (3-NT) in proteins, a condition known as nitrosative stress [22]. So the $ONOO^-$ content of plasma were detected by measuring 3-NT levels from tyrosine as an indicator of $ONOO^-$ production using enzymelinked immunosorbent assay (ELISA) kits in accordance with the manufacturer's protocol [29–31]. Absorbance was measured at 450 nm by a microplate reader (Perlong Medical, Beijing, China).

2.3.5. Superoxide anion measurement in aorta

Superoxide anion of the aortas was measured by dihydroethidine (DHE) fluorescent microtopography as previously described [32,33]. Briefly, longitudinal aortic sections (14 μm) were obtained on a cryostat