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Low-salt diet increases NO bioavailability and COX-2 vasoconstrictor prostanoid production in spontaneously hypertensive rats

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ABSTRACT

Aims: The ability of dietary sodium restriction to reduce the incidence of cardiovascular mortality and improve vascular function in hypertension still remains poorly understood. The aim of this study was to observe the effects of a long period of salt restriction on the vascular reactivity of mesenteric resistance arteries of SHRs.
Methods: Male SHRs received either standard-salt diet (0.3% NaCl) or low-salt diet (0.03% NaCl) for 28 weeks. Vascular reactivity was studied in mesenteric artery segments and the influence of cyclooxygenase-2 (COX-2), reactive oxygen species (ROS) and participation of the renin-angiotensin system were analyzed.
Key findings: Decreased salt intake did not affect phenylephrine-induced vasoconstriction but increased acetylcholine-induced vasodilatation and also increased the response to phenylephrine after inhibition of NO synthase by L-NAME (100 μM) and iNOS protein expression was elevated. Cyclooxygenase inhibitor indomethacin (10 μM) and COX-2 inhibitor NS 398 (1 μM) decreased the reactivity to phenylephrine in low-salt-treated group, and COX-2 protein expression was elevated in low-salt group. The effects of apocynin (10 μM); superoxide anion scavenger, tiron (1 mM); hydrogen peroxide scavenger, catalase (1000 U mL^{-1}); and ACE and AT1 receptor blockers, enalapril (10 μM) and losartan (10 μM) on vascular reactivity were not different between two groups. The levels of AT1 protein expression were similar in both groups.
Significance: Low-salt diet modulates mesenteric vascular responses via increased NO bioavailability suggested by increased iNOS protein expression and vasoconstrictor prostanoid production via COX-2 pathway, in SHRs. Neither ROS nor the local renin-angiotensin system is involved in these responses.

1. Introduction

Cardiovascular disease (CVD) remains the leading cause of death in developed countries and also remains a tremendous burden for the health care system [1]. Hypertension is a major risk factor for CVD in the general population and is associated with vascular endothelial dysfunction [2–5], which is reflected by impaired endothelium-dependent dilation of conduit arteries and resistance vessels [6]. The vascular endothelial dysfunction may precede the appearance of symptoms of clinical CVD and lead to organ damage like kidney injury, stroke and myocardial infarction [2–6].

The role of dietary sodium intake in CVD, particularly hypertension, has been a matter of debate [7]. High salt intake has been considered an important factor contributing to hypertension development and its complications, including vascular, heart and kidney damage. The results of many studies involving both humans and laboratory animals have demonstrated a clear relationship between high salt intake and the development of vascular endothelial dysfunction and hypertension [4,8]. High sodium diet has been associated with vascular dysfunction characterized by increased peripheral vascular resistance [9,10], as well as increased reactive oxygen species production [11] and decreased nitric oxide synthase (NOS) subtype production [12]. Additionally, increased sodium intake results in impaired endothelium dependent relaxation in spontaneously hypertensive rats (SHR) [13].

As the high sodium intake has been considered a major risk factor for cardiovascular disease, the reduction of sodium intake has been recommended as a non-pharmacological treatment. However, there are doubts whether such a measure would only bring benefits. Recently, an association between elevated systolic blood pressure and sodium intake was observed, but without increasing the risk of cardiovascular events. Contrary to expectations, the group with the lowest sodium intake had a high mortality rate and cardiovascular events [14].

Currently, although a low-salt diet either is indicated for the treatment and prevention of cardiovascular disease, the mechanisms underlying its potential benefits remain unknown [4,5]. Even if, low sodium intake has been associated with reduced arterial stiffness in normotensive adults, some reports have demonstrated that it can present some

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adverse events [5]. Indeed, sodium restriction results in several physiological effects, some of which may adversely influence health outcomes and have been associated with changes in the metabolism of lipids and glucose, raising doubts regarding the efficacy of this diet with respect to the prevention of coronary artery disease and the subsequent reduction of mortality associated with side effects [15,16]. However, it is unknown whether dietary sodium restriction reverses endothelial dysfunction in the setting of elevated SBP. The physiological mechanisms by which dietary sodium restriction improves human vascular endothelial function are also unknown.

Since, the effects of low-salt diet on vascular reactivity remain poorly understood and vascular dysfunction is an important factor related to adverse outcome in hypertension and cardiovascular disease, the goal of the present study was to investigate the effects of a long period of salt restriction at the vascular function of mesenteric arteries from SHRs.

2. Methods

2.1. Animals and experimental groups

Male SHRs were provided by the animal facility at Federal University of Espirito Santo. Laboratory animals were cared for and utilized in accordance with the Guide for the Care and Use of Laboratory Animals, and protocols were approved by the Ethics Committee of the Federal University of Espirito Santo (053/2012 CEUA-UFES). During treatment, animals had free access to both food and drinking water. Rats were randomly assigned to receive either a standard-salt diet (0.3% NaCl) or a low-salt diet (0.03% NaCl) for 28 weeks. The rats were housed at a constant temperature and humidity, under a regular light cycle (12:12 h light–dark). Twenty-four hour food and water intake were measured at the end of the protocol.

2.2. Blood pressure measurements

Systolic blood pressure was measured weekly in conscious rats via noninvasive tail-cuff plethysmography (IITC Life Science, Inc.). The rats were restrained for 5–10 min in a warm and quiet room and conditioned to cuff inflation-deflation cycles prior to the recordings. Systolic blood pressure was measured, and the mean of three measurements was recorded for each animal as previously described [17].

2.3. Vascular reactivity measurements of the mesenteric arteries

Following 28 weeks of treatment, rats were anesthetized with ketamine (70 $mg \cdot kg^{-1}$, i.p., Agener — União Brasil) and xylazine (10 $mg \cdot kg^{-1}$, i.p., Bayer, Brasil). The mesenteric arteries were carefully dissected, and the connective tissue was removed. To investigate the expression of iNOS, COX-2 and AT1, mesenteric arteries were frozen at −80 °C until needed for analysis. Regarding the vascular reactivity experiments, the third branch of the mesenteric artery was isolated, and segments of 2 mm in length were mounted in a small vessel chamber myograph for isometric tension measurements in accordance with the method described by Mulvany and Halpern [18]. Following a 30 min equilibration period in oxygenated Krebs–Henseleit solution (KHS, in mM: 115 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4 \cdot 7H_2O$, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 11.1 glucose and 0.01 Na_2EDTA), the buffer was equilibrated with a gas mixture of 95% O_2 and 5% CO_2 to pH 7.4 at 37 °C. The vessel segments were stretched to their optimal luminal diameter for the active tension measurements, washed with KHS and left to equilibrate for 30 min. The contractility of segments was subsequently tested via exposure to a high-K^+ solution (120 mM K^+-KHS), which was identical to the KHS solution, although the NaCl was replaced by an equal molar amount of KCl. The presence of the endothelium was determined based on the ability of 10 μM ACh to induce relaxation in small mesenteric arteries pre-contracted with phenylephrine at a concentration that produces approximately 50% of the contraction induced by K^+-KHS. All experiments were performed in segments using intact endothelium. Following a washout period of 30 min, concentration–response curves to both phenylephrine and ACh were generated. A parallel study was performed to determine the effects of the nitric oxide synthase (NOS) inhibitor N-nitro-L-arginine methyl ester (L-NAME, 100 mM), a non-selective cyclooxygenase inhibitor (indomethacin, 10 μM), a COX-2 inhibitor (NS 398, 1 μM), a ROS scavenger (apocynin, 10 μM), a superoxide anion scavenger (tiron, 1 mM), a hydrogen peroxide scavenger (catalase, 1000 U/mL), an angiotensin-converting enzyme (ECA) inhibitor (enalapril, 10 mM), and an angiotensin II type 1 (AT1) receptor antagonist (losartan, 10 mM). These drugs were added 30 min prior to the generation of the phenylephrine concentration-response curves.

2.4. Western blot analysis

For the Western blot analyses, the mesenteric arteries were carefully dissected, and connective tissue, removed. To analyze the expression of iNOS and COX-2 and AT1 receptors, the mesenteric arteries were frozen at −80 °C until needed for analysis.

iNOS, COX-2 and AT1 protein expression were detected in the homogenates of mesenteric segments in both standard and low-salt-treated rats. The proteins were separated via 10% SDS-PAGE and subsequently transferred to nitrocellulose membranes before being incubated with mouse monoclonal antibodies for either iNOS (1:250, Transductions Laboratories KY), COX-2 (1:200; Cayman Chemical; Ann Arbor, MI, USA) or AT1 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Following washing, the membranes were incubated with an anti-mouse immunoglobulin antibody conjugated to horseradish peroxidase (1:5000, StressGen, Victoria, Canada). Following a thorough washing, the immunocomplexes were detected using an enhanced horseradish peroxidase/luminal chemiluminescence system (ECL Plus, Amersham International, Little Chalfont, UK) and film (Hyperfilm ECL — International). The signals were quantified using the National Institutes of Health Image V1.56 software. The same membrane was used to detect α-actin expression using a mouse monoclonal antibody (1:5000, Sigma, USA).

2.5. Data analysis and statistics

The vasoconstrictor responses induced by PHE were expressed as percentages of the tension generated by 120 mM KCl administration. The vasodilatory responses were expressed as percentages of the previous contraction. To compare the effects of the drugs on the response to PHE in the mesenteric segments subjected to both treatments, some of the results were expressed as differences in the area under the concentration–response curves (dAUC) between the standard and low-salt-diet groups. The AUCs were calculated using individual concentration–response curve plots; the differences were expressed as percentages of the AUC of the control situation. All values were expressed as the mean ± SEM of the number of animals used in each experiment. The results were analyzed using Student's *t*-test or a completely randomized two-way ANOVA when comparisons between groups were indicated. When the ANOVA demonstrated a significant treatment effect, Bonferroni's *post hoc* test was used to compare individual means. Differences were considered significant at P-values <0.05.

2.6. Drugs and reagents

L-NAME, 1-phenylephrine hydrochloride, acetylcholine chloride, indomethacin, NS 398, apocynin, tiron, catalase, enalapril and losartan were purchased from Sigma-Aldrich (St. Louis, USA). All salts and reagents were of analytical grade and were obtained from either Sigma-Aldrich or Merck (Darmstadt, Germany).