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In renal hypertension, *Cirsium japonicum* strengthens cardiac function via the intermedin/nitric oxide pathway

Xiaoling Yang[a,1], Hui Shao[b,1], Yong Chen[c,1], Ning Ding[a], Anning Yang[a], Jue Tian[a], Yuanxu Jiang[a], Guizhong Li[a], Yideng Jiang[a,*]

[a] Ningxia Key Laboratory of Vascular Injury and Repair Research, School of Basic Medical Science, Ningxia Medical University, Yinchuan, Ningxia, 750004, China
[b] Otorhinolaryngology, General Hospital of Ningxia Medical University, Yinchuan, Ningxia, 750004, China
[c] Cardiology, The Second People's Hospital of Yinchuan City, Yinchuan, Ningxia, 750004, China

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ABSTRACT

Cirsium japonicum, a constituent of traditional Chinese medicine, has been shown to exert inflammatory effects as well as to improve the circulation and thus to counteract hematologic stasis. Studies have demonstrated that intermedin (IMD) has protective effects on hypertension in rats by regulating the Ang/NO metabolic pathway. In this study, we investigated whether by regulating the expression of IMD, *Cirsium japonicum* could improve cardiac function in rats with 2k1c-induced renal hypertension. Renal hypertension was induced in Sprague-Dawley rats by occluding the renal artery. The rats were maintained on a normal diet and randomly divided into four groups: sham, 2k1c, 2k1c with *Cirsium japonicum* (1.8 g/kg per day) and 2k1c with IMD (n = 10 in each group). Cardiac function, plasma angiotensin II (Ang II), IMD, serum nitric oxide (NO) and nitric oxide synthase (NOS), as well as the expression of IMD and adrenomedullin (ADM) in the aorta and left ventricle were analyzed. Administration of *Cirsium japonicum* or IMD significantly strengthened cardiac function in 2k1c-induced rats, increased serum NO and NOS levels, reduced plasma Ang II, and upregulated IMD expression in the aorta and left ventricle. These results demonstrate that *Cirsium japonicum* has cardioprotective effects on 2k1c-induced renal hypertension in rats via the IMD/NO pathway.

1. Introduction

Hypertension is a common condition that can cause fatal cardiovascular events, such as myocardial infarction, stroke, sudden cardiac death and heart failure. Peripheral resistance is an important aspect of high blood pressure. When persistent peripheral resistance increases, cardiac afterload also rises, and myocardial contractility strengthens, leading to increased blood pressure. As a common clinical disease, hypertension can induce multiple organ dysfunction, such as diseases affecting the heart, kidneys, and aorta [1]. The heart is most frequently involved; therefore, stroke and myocardial infarction may result [2]. Therefore, the alleviation of impaired cardiac function due to hypertension may serve to improve patients' survival and quality of life.

Cirsium japonicum regarded as the most important memberof the cardoon plant family, is a constituent of traditional Chinese medicine [3–5]. It has multiple pharmacologic effects on the cardiovascular, renal and immune system, among others [6–8]. Studies have shown decreased blood pressure in 95% of patients with primary hypertension after treatment with *Cirsium japonicum* [7]. Another study reported that *Cirsium japonicum* can not only lower blood pressure in mice with stress-induced hypertension, but also relieve cardiac and kidney disease [8]. These data indicate that *Cirsium japonicum* has strong cardiovascular and renal protective effects in addition to its effect on blood pressure. However, the mechanisms behind these effects remain unclear.

Intermedin (IMD), also known as ADM 2, was discovered in 2004. It is a vasoactive peptide of the calcitonin gene-related peptide (CGRP) family and is widely expressed in many tissues, including those of the cardiovascular system, gastrointestinal tract, pancreas, lung, and central nervous system [9–11]. IMD can bind to both CGRP and ADM receptors and, like ADM, plays a vital role in maintaining cardiovascular function [12,13]. IMD has been shown to have pathophysiologic effects in multiple diseases of the cardiovascular and renal systems [14]. Studies have shown that IMD inhibits vascular calcification in rats and reduces atherosclerotic lesions in ApoE-/- mice [15,16]. In mice, intravenous or intraperitoneal administration of IMD caused hypotension. However, IMD gene transfer prevented endothelial cell loss, kidney

* Corresponding author at: 1160 Shengli Street, Yinchuan, Ningxia, 750004 China.
E-mail address: yangxl@nxmu.edu.cn (Y. Jiang).
[1] These authors contributed equally to this work.

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damage, inflammation, and fibrosis in salt-induced hypertensive rats [17]. Continuous IMD infusion reduced blood pressure and improved hemodynamic function in spontaneously hypertensive rats [18,19]. These data suggest that IMD may be a potent endogenous cardioprotective substance that plays multiple roles in blood pressure regulation by many drugs. Administration of IMD to diabetic rats inhibited the generation of oxidative stress products, inhibited apoptosis, and reduced infarct size [20]. However, whether IMD is involved in the lowering of blood pressure and protection of the cardiovascular system by *Cirsium japonicum* requires further demonstration.

Hypertension is a common clinical problem involving significant morbidity and mortality. *Cirsium japonicum* may have promising potential for the treatment of this condition. In this study, we aimed to observe whether *Cirsium japonicum* can protect the cardiovascular system of rats from renovascular hypertension, and to explore the possible mechanism of *Cirsium japonicum* in cardiovascular protection.

2. Materials and methods

2.1. Establishment of the animal model

Sixty 8-week old male Sprague-Dawley rats were obtained from the Laboratory Animal Center of Ningxia Medical University, (Ningxia, China) and maintained under normal conditions (temperature at 22°–24 °C, humidity 40%–60%) with free access to rat chow and water. All protocols were in compliance with the principles of the Animal Management Rule of the Ministry of Health, People's Republic of China and were approved by the Ningxia Medical University Animal Care Committee.

For the first experiment, 30 rats were randomly divided into 3 groups: (1) 2-kidney 1-clip (2k1c) animals receiving 0.9% saline, (2) sham animals receiving 0.9% saline, and (3) 2k1c animals that receiving *Cirsium japonicum* at 1.8 g/kg IG per day. Hypertension was induced in the 2k1c rats as previously described [21]. Briefly, the rats were anesthetized by exposure to pentobarbital (40 mg/kg IP). A 2.5-cm retroperitoneal flank incision was made under sterile conditions. The left kidney was exposed and the renal artery carefully separated from the renal vein. The renal artery was partially occluded by placing a silver clip (0.2 mm) on the vessel. The wound was then closed with a running 3-0 silk suture. Sham-operated rats underwent the same surgical procedure except for the clip placement. Six weeks later, tail systolic blood pressure (SBP) was assessed weekly with the BL-420F Biological Functional Experimental System (Chengdu Taimeng, Chengdu, China). For the second experiment, 30 rats were randomly divided into 2k1c, sham, and IMD groups. IMD animals received IMD (Phoenix Pharmaceutical Inc., Belmont, CA, USA) at 1.5 mg/kg IG per day, while sham and 2k1c rats were treated as in experiment 1.

2.2. Measurement of hemodynamic parameters, plasma Ang II and IMD concentration

Six weeks after renal artery clipping, rats were anesthetized with 1%pentobarbital sodium (40 mg/kg IP) for the implantation of catheters to facilitatedirect hemodynamic measurements. Polyethylene catheters with full heparin were inserted into the left internal carotid artery of each rat. Mean carotid arterial pressure (mCAP), left ventricular end-diastolic pressure (LVEDP), maximum rate of left ventricular pressure (LV ± dp/dtmax), and heart rate were measured via a BL-420F Biological Functional Experimental System. Ang II and IMD concentrations in plasma were examined using a radioimmunoassay kit (Beijing Chemclin Biotech Co. Ltd., Beijing, China).

2.3. RNA purification and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from hearts and aortas using RNA isolation kit (Invitrogen) according to the manufacturer's protocol. The isolated RNA was immediately converted into cDNA using areverse transcription kit (Takara, Dalian, China). The cDNA was amplified by PCR in a real time cycler using FastStart SYBR Green master mix (MBI, Vilnius, Lithuania) and primers for IMD, ADM and β-actin (Shenggong, Shanghai, China). Rat IMD primers: forward: 5′-CCTCACTTCGGCCTG TAGTT-3′, reverse: 5′-ACCCACCTCAGCCATAACTT-3′. Rats ADM primers: forward: 5′- GAGCGGACTGAGACAATC -3′, reverse: 5′- GTAAG TAATGAGGCGTATGC -3′. Rats β-actin primer: forward: 5′- GTCAGGT CATCACTATCGGCAAT -3′, reverse: 5′- AGAGGTCTTTACGGATGTCAA CGT-3′. The SYBR Green PCR cycle was run as follows: initial denaturation for 5 min at 95 °C, 45 cycles consisting of 30 s of denaturation at 95 °C, 30 s annealing at primer-specific temperatures, and extension at 72 °C. All the experiments were performed in triplicate, the data were normalized using β-actin as the housekeeping gene, and expressed as mean ± SD.

2.4. Western blot

Hearts and aortas were lysed in a lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with protease inhibitor (Sigma Fast; Sigma, St. Louis, MO). Equal amounts of protein (30 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15% for IMD) and transferred to PVDF membranes. After blocking with 5% dry nonfat milk, the membranes were incubated with primary antibodies (1:2000, Sigma, St Louis, USA) overnight at 4 °C. After washing, membranes were incubated with HRP conjugated secondary antibody (anti-goat IgG, 1:5000, Zhongshan Biotec, Beijing, China) for 1 h at room temperature. Target proteins were detected using an enhanced chemiluminescence detection method (ECL Plus, Biyuntian, China). Protein expression was analyzed using BioRad Gel Doc Imaging System (BioRad, Hercules, CA, USA) and normalized by ß-actin (1:5000; Sigma Chemical, Co., USA).

2.5. Detection of NO concentration and NOS activity in serum

The concentration of NO in serum and NOS activity were determined using a NO and NOS assay kit (Nanjing Jiancheng Institute of Biological Engineering, Nanjing, China) according to the manufacturer's instructions.

2.6. Statistical analysis

Values were expressed as the mean ± standard (SD). Statistical comparisons between more than 2 groups were performed using 1-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. The statistical analyses were performed using Prism software (Prism 5, GraphPad Software, Inc., San Diego, CA, USA). A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Hemodynamic changes and the ratio of LVMW/BW and RK/LK in rats

The use of 2k1c is a classic method for establishing a model of renal hypertension. In our rats, as expected, mCAP, LVEDP, the ratio of left ventricular weight to body weight (LVMW/BW), and left kidney weight to right kidney weight (RK/LK) in 2k1c rats increased by 48.23%, 340%, 26.35%, and 36.77% respectively, while LV + dp/dt_{max} and LV - dp/dt_{max} decreased 51.26% and 24.65% ($P < 0.01$) as compared with the sham group. When these animals were treated with IMD or *Cirsium japonicum*, their hemodynamic condition improved, as characterized by decreased mCAP and LVEDP, LVMW/BW, and RK/LK and increased LV + dp/dt_{max} and LV - dp/dt_{max} ($P < 0.01$). These results indicate that our renal hypertension model was established successfully and that IMD and *Cirsium japonicum* might alleviate hemodynamic changes due to