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Therapeutic effect of astragaloside-IV on bradycardia is involved in up-regulating klotho expression

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ABSTRACT

Aims: In order to determine whether klotho is involved in the therapeutic effects of Astragaloside-IV on bradycardia, we evaluated the effect of ASG-IV on klotho and the effect of klotho on HCN4 and I_f.
Main methods: Administrating isoproterenol (5mg/kg) for 15 days to establish a rat bradycardia model randomized SD rats into control, model (ISO) and ASG-IV (5 mg/kg/day) groups to explore the effect of ASG-IV on klotho. Rats were sacrificed on day15 after heart rate and heart function were measured; SAN tissues were collected to measure the expression of klotho and HCN4. In vitro, neonatal rat myocardial cells were incubated with LPS for 24 h to inhibit the expression of HCN4 and incubated with LPS + klotho to explore the effect of klotho on HCN4 expression. We also adopted full-patch-clamp technique to explore the effect of klotho on I_f.
Key findings: Heart rate in model group was significantly decreased (356.6 ± 19.7 vs. 428.9 ± 19.9 in control group, $P < 0.01$) and ASG-IV can increase heart rate (401.4 ± 12.0 vs. 356.6 ± 19.7 in model group, $P < 0.01$). The expression of klotho was also up-regulated ($P < 0.05$). In vitro, after incubation with LPS for 24 h, HCN4 expression was significantly decreased in neonatal rat myocardial cells (0.6 ± 0.07 vs. 1.0, $P < 0.01$) and I_f was significantly declined. Exogenous klotho showed protective effect on HCN4 expression (1.58 ± 0.16 in ASG-IV group vs. 0.6 ± 0.07 in LPS group, $P < 0.05$) and I_f.
Significance: Klotho is involved in the treatment mechanism of ASG-IV.

1. Introduction

In general, bradycardia are heart rhythm disorders characterized by a reduction in heart rate with a consequent reduction in cardiac output, These often occur in the elderly population or with diseased hearts [1].

Traditional Chinese medical treatments such as Buyang Huanwu decoction and Buzhong Yiqi decoction are used in treatment of bradycardia. These decoctions have the same key ingredient called Astragalus Membranaceus Bunge in which the main active compound is Astragaloside IV (AGS-IV). Experimental studies have provided abundant evidence demonstrating the explicit cardiovascular-protective effects of AGS-IV [2–4]. ASG-IV has been reported to decrease the level of malondialdehyde which is an indicator of lipid peroxidation, and increase the levels of the antioxidant enzymes glutathione peroxidase, superoxide dismutase [5,6]. However, the mechanism of bradycardia treatment of AGS-IV is not clear.

Klotho was identified in 1997 as an anti-aging gene [7]. Genetic mutation of klotho causes extensive premature aging phenotypes and drastically shortens life span [8]. As a circulating hormone, klotho regulates several signaling pathways such as insulin/insulin-like growth factor 1(IGF-1) [9], Wnt [10], PKC [11], cAMP [12,13], Na+/K+ ATPase [14] signaling pathways. The protection from oxidative stress by klotho may play a role in the various aging-related changes. It has been suggested that oxidative stress decreases klotho expression in a mouse kidney cell line [15].

In the heart, klotho is expressed solely at the sinoatrial node and is essential for the sinoatrial node to function as a dependable pacemaker under conditions of stress [16]. Mice with a defect of klotho gene expression (kl/kl) showed sinoatrial node dysfunctions which bear certain similarities to sinus node dysfunction in humans with senescence [17]. Furthermore, the klotho level decreases with age [18], whereas the prevalence of bradyarrhythmias increases with age [19], suggesting an essential role of klotho in bradyarrhythmias.

Whether klotho participate in the treatment mechanism of ASG-IV on bradycardia has not been reported, in this study, we employed the ISO-induced bradycardia model to observe the effect of ASG-IV on bradycardia and study whether klotho participate in the treatment mechanism of ASG-IV on bradycardia.

The pacemaker current (I_f) of sinoatrial-node myocardium determines the slope of the diastolic depolarization of pacemaker cells and thus has a key role in the generation and autonomic regulation of sinus rhythm and rate [20]. The I_f-channels are encoded by the

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hyperpolarization-activated, cyclic nucleotide–gated (HCN) channel gene family [21].

The HCN channels belong to the superfamily of pore-loop cation channels. In mammals, the HCN channel family comprises 4 members (HCN1–4) that are expressed in heart and nervous system [22]. All 4 HCN channel isoforms, particularly HCN4 and to a lesser extent HCN2, are expressed in the heart [23]. The SA node contains 25 times the total HCN message of Purkinje fibers and 140 times the total HCN message of ventricle [24]. In the rabbit SA node, the dominant HCN transcript is HCN4, representing >81% of the total HCN message. In the rabbit SAN, HCN4 proteins are major constituents of native f-channels, and their distribution matches closely the SAN as defined morphologically and electrophysiologically.

Recent study reports that in an inducible and cardiac-specific HCN4 knockout (ciHCN4-KO) mouse model, ablation of HCN4 consistently leads to progressive development of severe bradycardia (~50% reduction of original rate) and AV block, eventually leading to heart arrest and death. This indicates that cardiac HCN4 channels are essential for normal heart impulse generation and conduction in adult mice. Dysfunctional HCN4 channels can be a direct cause of rhythm disorders [25].

HCN4 gene mutation has been found in bradycardia and sick sinus syndrome patients, and a marked down-regulated transcriptions of HCN4 were observed in the sinoatrial node from young to adult and further to aged rats [26].

We propose that ASG-IV might have a protective effect on klotho by inhibiting oxidative stress, and klotho, which is essential in sinoatrial node function might have a regulating effect on HCN4.

This study was conducted to explore the protective effect of AGS-IV against sinus node dysfunction and the mechanism behind these effects. We focused on the effect of ASG-IV on klotho in ISO treated rats, and the regulation effect of klotho on HCN4.

2. Materials and methods

2.1. Materials

All animals were purchased from Suzhou Industrial Park El Matt Technology Co. Ltd., with the license number scxk (Su) 2009-0001. ASG-IV with a purity of 98% was purchased from Zelang Group. 98% Isoprenaline (ISO) was from Sigma-Aldrich Co. LLC. Primers for PCR were from Sangon Biotech, Shanghai, Klotho anti-body was from Thermo Fisher Scientific and HCN4 anti-body was form Biosynthesis Biotechnology CO. LTD. ECG parameters were measured by DL-420S (Chengdu Taimeng Software Co. LTD). All other materials used were commercial products of the highest grade.

2.2. Bradycardia model

This study use male SD rats weighing 200 to 250 g (7–8 weeks old), with free access to standard food and water, cared for in accordance with the Institutional Animal Research Committee Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication number 85–23, revised 1996) to conduct the experiments. Cardiac remodeling induced by ISO injection is a well-established model [27]. And this model is often used in our lab to establish arrhythmia model [28], we found that high doses of ISO could induce bradycardia in rats. We conducted a series of experiments and explored that 5 mg/kg ISO administrated for 15 days could induce bradycardia model. SD rats were randomly divided into 3 groups, 8 rats were used in each group: model (ISO 5 mg/kg/day), ISO + ASG-IV (ASG-IV, 5 mg/kg/day) and control groups. ISO was administered by subcutaneous injection (s.c) for 15 days. ASG-IV was suspended in 0.5% CMC-Na and administered orally from the sixth day. Control rats were subcutaneous injection saline for 15 days. Electrocardiogram parameters were recorded at day 0, 5 and 15 respectively, plasma and SAN tissues were harvested on the fifteenth day. We use three methods to verify the tissues we got were SAN tissues: a, choose the right position: a, SAN locates in the conjunction of superior vena cava and crista terminalis of right atrium [29], after remove the right atrium, we clipping in the corresponding parts according to the position of the superior vena cava, b: According to the characteristics of SAN cells, we put the tissues we got in Tyrode's solution containing 1.8 mM calcium in 37 °C, tissues under the microscope can see persistent spontaneous pulsation, c, HCN4 specifically expressed in sinoatrial node, we tested HCN4 expression in the tissues we got. In order to observe the change during the model establish, we collected plasma and tissues on the fifth, tenth and fifteenth day respectively for the model group.

2.3. RT-PCR

SAN tissues were dissected out in cold isotonic NaCl solution and then immediately frozen in liquid nitrogen. Total RNA was extracted from SAN with TRIzol Reagent (Life Science Products & Serives). The first-strand cDNA was synthesized from 1 μg of total RNA using a AMV First Strand cDNA Synthesis Kit (Bio Basic Inc., Canada). Gene expression was assessed by quantitative RT-PCR using Maxima SYBR Green Qpcr Master Mix (2X) (Thermo Scientific, USA) and Eppendorf AG 22331 Hamburg real-time PCR system (Eppendorf, Germany) according to the manufacturer's instructions. PCRs were carried out in 96-well plates in a total volume of 25 μl, including 1 μl of cDNA and 1 μl of primers (Table 1) (Sangon Biotech, Shanghai, China). The PCR conditions were: pre-denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s, annealing at 60°C for 30 s, and extension at 70 °C for 30s. The mRNA levels were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. All reactions were carried out in duplicate. RT-PCR data was expressed as Ct (cycle threshold) and ΔCt ($Ct_{\text{gene of interest}} - Ct_{\text{GAPDH}}$). The fold changes of PAR mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = \Delta Ct_{\text{experimental sample}} - \Delta Ct_{\text{control}}$.

2.4. Western blot

Parts of Right atrial which contents SAN tissues were directly removed, frozen in liquid nitrogen and stored at −80 °C until analysis. Then they were lysed in 500 μl lysis buffers (Sangon Biotech, Shanghai, China). Lysates were centrifuged 14,000 rpm for 15 min at 4 °C to remove nuclei and cells debris. The supernatants were mixed with SDS sampling buffer (63.5 mM TrisHCl pH 6.8, 2% SDS, 10% glycerol, 0.001% BPB and 13.3 mM DTT) and boiled for 5 min. Samples were applied to 4% gradient SDS-polyacrylamide gels and blotted on PVDF membranes (Millipore, MA) for Western blotting. Each blot was incubated overnight with a primary antibody diluted in TBST (0.1% Tween 20, 25 mM TrisHCl pH 7.4, 150 mM NaCl and 5 mM KCl) at 4 °C after blocked in blocking buffer (5% skim milk in TBST for 1 h). A rabbit polyclonal anti-klotho antibody (1:500, Thermo Fisher Scientific, USA) and a rabbit anti-HCN4 antibody (1: 500, Biosynthesis Biotechnology CO. LTD) were used to detect klotho and HCN4 respectively. Anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (1:1000, Biouniquer) antibody were used to verify equal loading of protein. All washes were conducted in TBST. The membrane was incubated with HRP-conjugated secondary antibodies and assessed by enhanced chemiluminescence detection

Table 1
Primer sequences for RT-PCR.

Target	Primer sequence (5′–3′)	GenBank no.
klotho	F:GGCGACTACCCAAAGAGTATGA R:CCGAAGGAGAGAGCAAAGAAGT	NM_031336.1
HCN4	F:GCATCCACGACTACTACGAACA R:GAAAGACCTCAAAACGCAACTT	NM_021658.1
GAPDH	F:ACAGCAACAGGGTGGTGGAC R:TTTGAGGGTGCAGCGAACTT	NM_017008.4