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Original Article

Decreased gene expression of K_{ACh} and K_{ATP} channels in hyperthyroid rabbit atria

Feng Zhang[1], Guang Hai Zhou[1], Qi An[2], Jie Yang[2], Yu Hao Wang[2], Jia Quan Zhu[2], Song Nan Jin[2], Jin Fu Wen[1]

[1]School of Basic Medical Sciences, The Second Affiliated Hospital of Shandong First Medical University & Shandong Academy of Medical Science, Jinan 250117, Shandong, China; [2]School of Pharmacy, Shandong First Medical University & Shandong Academy of Medical Sciences, Taian 271000, Shandong, China

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Abstract: Cardiac hypertrophy is a common myocardial structural abnormality which may cause heart failure. Many studies have shown that cardiac hypertrophy can be induced by hyperthyroidism. Ligand-gated potassium channels have been reported to be involved in various biological processes in the cardiovascular system, such as GPCR coupled K_{ACh} and metabolism sensor K_{ATP} channel. It is unclear whether the gene expression of K_{ACh} and K_{ATP} was altered in hyperthyroid rabbit atria. We aimed to investigate the expression of K_{ACh} and K_{ATP} genes in rabbit atria in our experimental model. We established an effective hyperthyroidism-induced cardiac hypertrophy animal model through an injection of T4. H&E staining and RT-PCR were used to observe the histomorphological damages and alteration of gene expression. The results showed that the heart weight, heart rate significantly increased in T4-treated rabbits. The systolic pressure increased from 115.60 mmHg to 152.6 mmHg in T4-treated rabbits. The expression of K_{ACh} and K_{ATP} genes was decreased in the atria of hyperthyroidism-induced cardiac hypertrophied rabbits. These findings indicated that the decreased gene expression of K_{ACh} and K_{ATP} may be related to hyperthyroidism-induced cardiac hypertrophy and atrial fibrillation.

Keywords: Hyperthyroidism, cardiac hypertrophy, K_{ACh} channel, K_{ATP} channel, gene expression

Introduction

Thyroid hormone plays important roles in development, metabolism, thermoregulation, and growth. The level of thyroid hormone increases and reaches a state of hyperthyroidism under some pathological conditions, such as thyroid cancer and pituitary gland abnormality [1-3]. Hyperthyroidism may cause different diseases including cardiac hypertrophy, heart failure, increased risk of heart attack, oxidative damage of liver, and osteoporosis [4]. Cardiac hypertrophy is one of the adaptive responses induced by hyperthyroidism, which may evolve to heart failure [5].

Cardiac potassium channels determine the heart rate, the resting membrane potential, and the repolarization of the action potential. They are vital targets for hormones, neurotransmitters, and drugs to modulate cardiac function [6]. In mammalian cardiac cells, potassium channels can be categorized as ligand-gated and voltage-gated channels. The ligand-gated potassium channels include acetylcholine activated potassium channel (K_{ACh}) and a decrease in the intracellular level of adenosine triphosphate activated potassium channel (K_{ATP}) [5, 7]. K_{ACh} is one of the most well-characterized G protein-regulated ion channels, exhibiting potent activation by $G_{\beta\gamma}$ subunits [8]. The heterotetrameric assembly of Kir3.1 and Kir3.4 of K_{ACh} has been confirmed by former biochemical experiments and electrophysiological analyses, which controls cardiac rate and conduction [7, 9]. Previous studies have suggested that K_{ACh} channel-related atrial natriuretic peptide (ANP) release increase in atrial myocytes may be responsible for the increased levels of plasma ANP observed in patients with atrial fibrillation (AF) [9]. Kirstine Calloe found a glycine to arginine substitution in codon 247 of Kir3.4 in a patient with a single episode of AF [10, 11]. These findings demonstrated that mutation of

K_{ACh} was predisposing to AF. K_{ATP} channels were first identified in cardiac myocytes. They are also found in pancreatic β-cells, skeletal muscle, vascular smooth muscle, and neurons [9]. The K_{ATP} consists of inward rectifier potassium channel subunits (Kir6s) and regulative sulfonylurea receptors (SURs). Cardiac K_{ATP} is composed of SUR2A/Kir6.2. Vascular smooth muscle K_{ATP} is composed of SUR2B/Kir6.1 or SUR2B/Kir6.2. K_{ATP} activated by intracellular concentration of ATP decrease can cut off potassium ion permeation upon increased intracellular ATP levels to promote membrane excitability [10]. The openers of K_{ATP} have antihypertensive effects [12, 13]. Gao et al. found that the activation of K_{ATP} counteracted cardiac hypertrophy and prevented the progression of cardiac hypertrophy to failure induced by pressure overload [14]. Ion channel related potassium channel electrical and structural remodelling in atria was the key element in atrial fibrillation [15].

K_{ACh} and K_{ATP} play important roles in cardiac diseases, but their detailed expression alteration in hyperthyroid rabbit atria is not clearly determined. Thus, we examined K_{ACh} and K_{ATP} gene expression in hyperthyroid rabbit atria.

Materials and methods

Establishment of the animal model

Thirty-six male New Zealand white rabbits (weighing 2.00±0.20 kg) were used in accordance with protocols approved by the laboratory animal ethics committee of Shandong First Medical University (Approve No. W2021030-30103). Prior to the experiment, the animals were contained in separate cages and handled by laboratory workers for seven days to reduce stress. Thirty-six animals were randomly divided into three groups: a sham-operated control group, a T4 (L-thyroxine, Sigma-Aldrich, USA) injected for 3 days group (0.5 mg/kg/day), and a T4 injected for 12 days group (0.5 mg/kg/day) [6, 16]. The experiments were carried out after 24 hours of the last injection.

Carotid cannula to detect blood pressure

The rabbits were anaesthetized with 1% pentobarbital sodium (Sigma-Aldrich, USA) solution (30 mg/kg, iv). A longitudinal incision of 7 cm was opened on the neck skin of the supine fixed rabbits to carry on tracheal intubation. The carotid artery was separated, and the artery intubation filled with heparin was inserted into this artery. The blood pressure of rabbits was then input into a computer with the BL-420E system by pressure transducer [11].

Cardiac muscle histology

Formalin-fixed tissues were paraffin-embedded and cut into 4-μm thick sections for Hematoxylin and Eosin (H&E) staining. The pathological changes of cardiac muscles were observed under an optical microscope.

Extraction of total RNA

Total RNAs were extracted with Trizol reagent (Invitrogen). The content and purity of total RNAs were measured by a UV spectrophotometer. The A_{260}/A_{280} ratio should be between 1.8 and 2.0. RNA integrity was detected by agarose gel electrophoresis.

The detection of gene expression by RT-PCR

The cDNA sequences of the objective genes were retrieved from Gene Bank. The primers were designed by primer design software (Primer5). The primer sequences were as follows: GAPDH, (F) AGGTCATCCACGACCACTTC, (R) GTGAGTTTCCCGTTCAGCTC, 202 bp; Kir6.2, (F) TTTTCTCCATCGAGGTCCAG, (R) ATGACGTGGTGGATGATGAG, 418 bp; SUR2, (F) GGAACTGACGGACACAAACAAC, (R) TGGAAGACCCGCTAATGGA, 134 bp; Kir3.4, (F) CACCCTGGTGGACCTCAAGTGGCGC, (R) AGCTCCGGGCTTGGCAGGTCATGC, 725 bp; and Kir3.1, (F) CTCTCGGACCTCTTCACCAC, (R) GAAAAGCGGAGGGGAAGTTA, 202 bp.

GAPDH was used as an internal control. A 25 μL of reaction mixture containing 1 μg total RNA, 0.5 μg random primer, 5 μL 5 × RT buffer, 5 μL dNTP (2.5 mmol/L), 40 U RNase inhibitor, and 200 U MMLV reverse transcriptase was incubated at 37°C for 60 minutes and then 70°C for 10 minutes.

PCR was carried out in a 50 μL reaction mixture with 2 μL cDNA, 5 μL 10 × PCR buffer (without Mg^{2+}), 4.0 μL dNTP (2.5 mmol/L), different volumes of $MgCl_2$ (25 mmol/L) (3 μL for GAPDH, 7 μL for Kir3.1, 5 μL for Kir3.4, 7 μL for Kir6.2 and 7 μL for SUR2), 1 μL each primer (20 μM) of

Table 1. Characteristic changes in heart weight in T4-treated rabbits

Groups	Body weight (kg)	Heart weight (g)	Heart weight/Body weight (g/kg)
sham	2.14±0.03	4.43±0.02	2.07±0.03
T4-treated 3 d	2.01±0.02**	4.64±0.03**	2.31±0.04**
T4-treated 12 d	1.74±0.02**	5.45±0.04**	3.13±0.04**

**P<0.01 versus the Sham group.

Table 2. The levels of systolic blood pressure and heart rate in T4-treated rabbits

Groups	Systolic blood pressure (mmHg)	Heart rate (beats/min)
Sham	115.60±0.60	248.94±3.96
T4-treated 3 d	133.23±0.56**	342.18±9.01**
T4-treated 12 d	152.60±0.78**	407.96±5.12**

**P<0.01 versus the Sham group.

target genes, and 1 U of Taq DNA polymerase (TaKaRa). Thermal cycler conditions were as following: initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 60°C (GAPDH, Kir3.1, and Kir3.4) or 55°C (Kir6.2, SUR2) for 30 seconds, and 72°C for 30 seconds (GAPDH, Kir3.1, Kir6.2, SUR2), or one minute (Kir3.4) followed by a 5 minutes extension at 72°C.

PCR products (8 μL each hole) were separated on 2% agarose gel, then visualized and photographed under UV light after being stained with ethidium bromide. The optical density of bands were measured by the automatic image analysis system Quantity One. The relative expression level of target gene was calculated using GAPDH as internal control.

Statistical analysis

The statistical analysis was performed using SPSS12.0. The experimental results were expressed as mean ± SE. Student's t-test and ANOVA was used to compare differences among the groups, and P<0.01 was considered statistically significant.

Results

Effects of thyroid hormone on heart weight, heart rate, and systolic blood pressure in rabbits

To verify the roles of K_{ACh} and K_{ATP} channels in cardiac hypertrophy, we established a hyperthyroidism-induced cardiac hypertrophy model. After an injection of T4 for 3 days or 12 days, the rabbits became excited and fiery. Compared with the sham-operated control group, T4 injection rabbits exhibited an increase in heart weight and indexes, heart rate, and systolic blood pressure with statistical significance (P<0.01) (**Tables 1** and **2**). The results indicated that excessive thyroid hormone could lead to cardiac hypertrophy.

Effects of thyroid hormone on cardiac morphology in rabbits

To determine the damage of excessive thyroid hormone to cardiac muscles, Hematoxylin Eosin (H&E) staining was performed in sham-operated control and T4-treated rabbits. The red part represented muscle cytoplasm and the blue part demonstrated the nucleus. Compared with the sham-operated control group, the cardiac muscles in the T4-treated group were dissolved and infiltrated by inflammatory cells (**Figure 1A2**). The proliferation of fibrous tissue in blood vessel and scar tissue was also found in the cardiac muscle in the T4-treated group (**Figure 1A2** and **1C2**). These results indicated that excessive thyroid hormones could lead to severe morphological damage of myocardium.

Gene expression changes of K_{ATP} and K_{Ach} channels in hyperthyroid rabbit atria

To examine the alteration of potassium gene expression in T4-induced cardiac hypertrophy, the expressions of K_{ACh} (Kir3.1/Kir3.4) and K_{ATP} (SUR2/Kir6.2) genes in rabbit atria were detected by RT-PCR. The lengths of Kir3.1, Kir3.4, SUR2, Kir6.2, and GAPDH amplified fragments were 202 bp, 725 bp, 134 bp, 418 bp, and 202 bp, respectively. The level of GAPDH served as an internal control. Compared with the sham-operated control group, the gene expressions of K_{ATP} and K_{ACh} were decreased in both T4 injected for 3 days and T4 injected for 12 days groups with statistical significance (P<0.01) (**Figures 2** and **3**). The results also displayed a T4-treated time dependent decrease of K_{ATP} and K_{ACh} genes in rabbit atria.

Discussion

Excessive thyroid hormone could induce cardiac hypertrophy. Our results showed that the

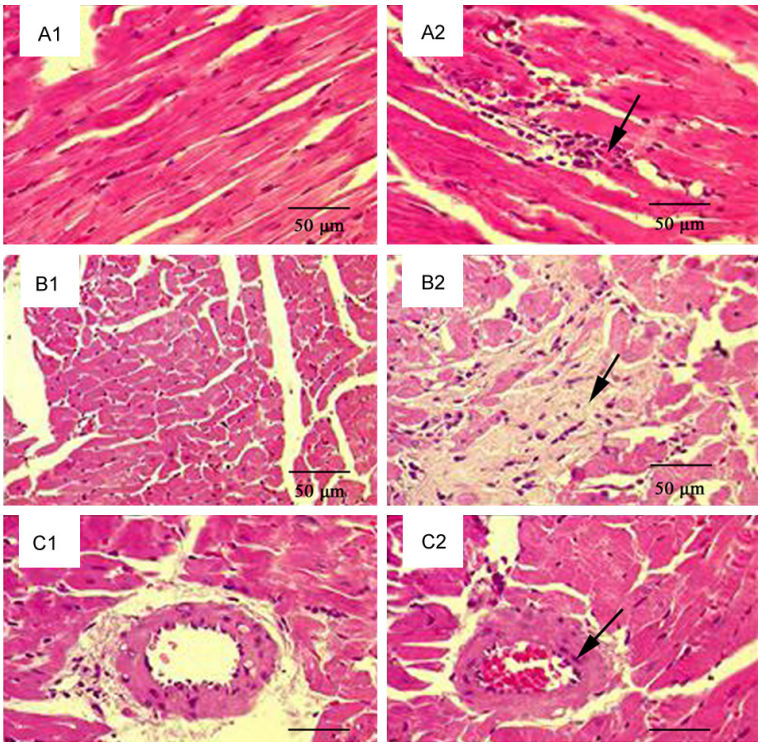

Figure 1. Morphological alteration of cardiac muscle. (H&E staining, A1, A2, B1, B2. × 200; C1, C2. × 400). (A1, B1, C1) Cardiac tissue of control group; (A2, B2, C2) Cardiac tissue of T4 injected for 12 days rabbits.

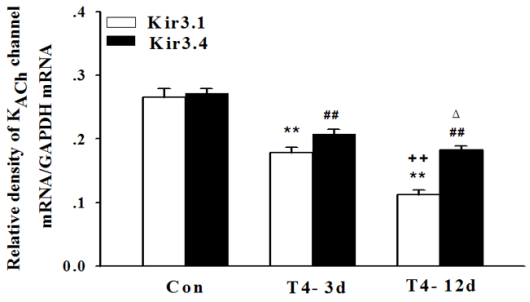

Figure 2. K_{ACh} gene expression in the left atria from T4-treated rabbits. **P<0.05 versus control; ##P<0.01 versus control; ++P<0.01 T4-12 d versus T4-3 d; ΔP<0.05 T4-12 d versus T4-3 d.

heart weight, heart rate, and systolic blood pressure were increased significantly, compared with the sham-operated control group in T4-treated rabbits. From cardiac morphology detected by H&E staining, we found that the cardiac muscle and vessel were seriously damaged by T4 injection. These indexes indicated that hyperthyroidism successfully induced cardiac hypertrophy.

The cardiac muscles in the T4-treated group were dissolved and infiltrated by inflammatory cells. Takeshi Yamashita et al. also reported the recruitment of immune cells-macrophages across atrial endocardium in hyperthyroidism related cardiovascular disease-atrial fibrillation [17]. They found that enlargement of the left atrium was associated with increased infiltration of immune cells in patients with atrial fibrillation who had undergone surgery [18]. One previous study showed that the potassium channels expressed in immune cells were mainly the inward rectifier potassium channel subfamily (Kir2 channels) [19]. The potassium channel genes in immune cells in atria of rabbits would not affect the expression levels of potassium channel subfamilies Kir3 and Kir6.

Based on the experimental model, K_{ACh} genes Kir3.1, Kir3.4, K_{ATP} genes, Kir6.2, and SUR2 in rabbit atria were detected to be decreased compared to the sham-operated control group. The gene expression levels of K_{ACh} and K_{ATP} proved to be decreased in hyperthyroidism-induced cardiac hypertrophy. In cardiac atrial myocytes, acetylcholine can bind to muscarinic receptors and activate muscarinic-gated potassium ion channels through a pertussis toxin-sensitive G-protein [4]. Dissociated $G_{\beta\gamma}$ subunits directly activate K_{ACh} channel, which is a heterotetrameric complex formed by the homologous Kir3.1 and Kir3.4 subunits, and the resultant current hyperpolarize the cardiac membrane [4, 7]. The homologous Kir3.1 and Kir3.4 subunits are mainly expressed in atrial myocardium, atrioventricular nodes, and sinus, but they do not exist in ventricle basically [4]. The Kir3.4 knock-out mice lacked cardiac K_{ACh} and exhibited slow heart rate decreases in response to indirect vagal stimulation [2, 20]. It was also showed that Kir3.4 knock-out mice were unable to significantly vary heart rate on a beat-to-beat time scale and were resistant to atrial fibrillation caused by vagal stimulation [2, 21, 22]. K_{ACh} activity can be stimulated by PIP2, ETA endothelin, and μ opioid [23, 24]. Noriaki Yamada, et al. generated

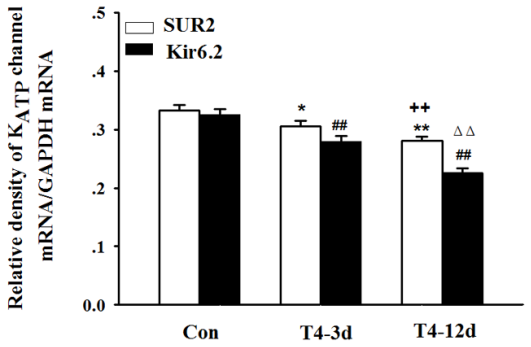

Figure 3. K_{ATP} gene expression in the left atria from T4-treated rabbits. *P<0.05 versus control; **P<0.01 versus control; ##P<0.01 versus control; ++P<0.01 T4-12 d versus T4-3 d; ΔΔP<0.01 T4-12 d versus T4-3 d.

transgenic zebrafish overexpressed either human Kir3.1-WT or Kir3.1-N83H along with Kir3.4 in the atrium. They found that all Kir3.1-N83H zebrafish exhibited bradycardia accompanied by sinus bradycardia, sinus arrest, and sinoatrial block. The sinus rate of Kir3.1-WT transgenic zebrafish was within the physiological range. It was an overexpression model [25]. The parasympathetic could mediate the heart rate decrease by acetylcholine [26]. The decreased expression of acetylcholine sensitive potassium channel gene could lead to the diminished function of this channel. Its heart rate decrease function could not work efficiently. This variation may be related to arrhythmia, cardiac hypertrophy, heart failure, ventricular fibrillation, or atrial fibrillation. The increased heart rate in cardiac hypertrophy rabbits induced by experimental hyperthyroidism may be related to the decreased gene expression of Kir3.1 and Kir3.4.

K_{ATP} channels are highly expressed in cardiomyocytes and play vital roles in the metabolic sensor [8, 27, 28]. K_{ATP} channels sense intracellular concentrations of ATP or ADP and regulate the membrane potential of cells. Turning on potassium conductance under low-energy supply reduces the excitability of cardiomyocytes and shortens the duration of action potential [8]. K_{ATP} channels were also shown to be involved in physiological stress situations, such as vigorous exercise. It was demonstrated that mice with reduced cardiac K_{ATP} gene expression only tolerate half the level of the stress of the wild-type [14, 29]. Hu et al. found disruption of Sarcolemma K_{ATP} activity impaired the cardiac response to systolic overload [30]. This was consistent with our discovery that the decreased gene expression of K_{ATP} in the rabbit atria may be related to increased systolic pressure in the experimental model. PKC activation can enhance K_{ATP}. The effects of PKC on K_{ATP} activity are related to phosphorylation at T180 in the Kir6.2 subunit [31]. PKA and other protein kinase-coupled receptors can also indirectly activate K_{ATP} through phosphorylation of channel proteins or by depleting cellular ATP levels [32]. In our study, we showed the decreased expression of Kir6.2 and SUR2 in cardiac hypertrophy rabbits atria induced by experimental hyperthyroidism. In the future, antibody development for rabbit potassium channel will help to understand the roles of those channels in our model.

As cardiac hypertrophy has become a worldwide problem, it is urgent to explore new targets and therapeutic drugs for cardiac hypertrophy. Our results indicated the relationship between potassium channels and cardiac hypertrophy induced by experimental hyperthyroidism. The K_{ACh} and K_{ATP} may serve as targets for hyperthyroidism-induced cardiac hypertrophy and atrial fibrillation treatment.

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Disclosure of conflict of interest

None.

Address correspondence to: Jin Fu Wen, Shandong First Medical University & Shandong Academy of Medical Sciences, 2 Yingsheng Road, Taishan District, Taian 271000, Shandong, China. Tel: +86-538-6223006; E-mail: jfwen0603@aliyun.com; Song Nan Jin, School of Pharmacy, Shandong First

Medical University & Shandong Academy of Medical Sciences, Taian 271000, Shandong, China. E-mail: snjin0504@aliyun.com

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