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Original Article

Effects of myocardial mir-124 on the expression of insulin-like growth factor-1 (IGF-1) in hypothyroid rats

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Abstract: Hypothyroidism is a common disorder of the endocrine system caused by reduced thyroid hormone synthesis in the thyroid gland. This study aimed to investigate the myocardial pathologic change and the interaction between mir-124 and IGF-1 expression in hypothyroid rats. Sixty SPF male Westar rats were randomly divided into 3 groups: hypothyroidism, L-T4 treatment and control group. After 12 weeks, serum levels of thyroid-stimulating hormone (TSH) and total thyroxin (TT4) were measured by immunofluorescence. The morphological change of the myocardial tissue was observed by HIM staining. The expression of mir-124 and IGF-1 mRNA was determined by real-time quantitative PCR. The expression of IGF-1 protein was quantified by Western blot analysis. At week 2, the TSH level in hypothyroidism and L-T4 treatment groups was significantly higher compared with control group ($P < 0.01$), whereas the TT4 level in both groups was significantly lower ($P < 0.01$). At week 12, the TSH level in hypothyroidism group was significantly higher compared with L-T4 treatment and control group ($P < 0.01$), but the TT4 level in hypothyroidism group was significantly lower ($P < 0.01$). The mir-124 expression in hypothyroidism group was significantly higher compared with both L-T4 treatment and control group ($P < 0.05$). The expression of IGF-1 protein in hypothyroidism group was significantly lower compared with L-T4 treatment and control group ($P < 0.05$). The level of thyroid hormone was reduced in the thyroid gland in hypothyroid rats. IGF-1 expression was inhibited through increasing the myocardial mir-124 level.

Keywords: Hypothyroidism, myocardium, mir-124, IGF-1

Introduction

Hypothyroidism is one of the most common diseases of the endocrine system caused by reduced thyroid hormone level in the thyroid gland. It can lead to abnormal functioning of numerous systems in the body, especially the metabolic system, and even multiple organ dysfunction syndromes (MODS) [1]. The heart has been known as the most commonly affected organ in hypothyroid patients. A high incidence rate of heart diseases such as cardiac dysfunction, cardiac hypertrophy and aortic diseases has been reported [2].

MicroRNAs (miRNA), a class of small (21-25 nucleotides in length) non-coding RNAs, are common in the eukaryotic nucleus. miRNAs play important regulatory roles in eukaryotes by targeting mRNAs for cleavage or translational repression. Of the vast number of RNAs in the myocardium, mir-124 is a cardiac-specific miRNA. It has been shown that mir-124 level was increased in hypothyroidism cases [3].

Insulin-like growth factor 1 (IGF-1) is a single-chain polypeptide that is structurally homologous to proinsulin, and an important mediator of cell growth, differentiation. Moreover, IGF-1 can dilate blood vessels, improve ischemic myocardial function, and increase myocardial contractility [3]. Recent studies have suggested the low IGF-1 expression as an independent risk factor for cardiovascular diseases, especially myocardial infarction and hypertension [4]. However, IGF-1 expression in the myocardium in hypothyroidism has not been previously reported [5]. The IGF-1 gene has been known as a potential target for mir-124 [6]. In this study, we aimed to investigate the myocardial patho-

logic change and the expression of mir-124 and IGF-1 in hypothyroid rats in order to understand their interaction in the myocardium of these rats. The current study may provide insights on the pathogenesis of hypothyroidism.

Material and methods

Animals

A total of 60 specific pathogen free (SPF) male Wistar rats, weighing approximately 210 to 240 g, were purchased from Taishan Medical University (Tai'an, China; license number: SCXK-Tai'an 2015-0007) and housed in separate cages with a relative humidity of 50-60% at 20-25°C. Rats were subjected to subsequent experiments after 1 week of adaptive feeding. The animal experiment was approved by the Ethics Committee of The Affiliated Hospital of Taishan Medical University and was performed in strict accordance of animal ethics standards.

Materials

Levothyroxine (L-T4) was purchased from available from Zhonglian Pharmaceutical Group Co., Ltd. (Shenzhen, China; SFDA approval No. H41021247). Chloral hydrate was purchased from Jinghua Pharmaceutical Co., Ltd. (Zhejiang, China; SFDA approval No. H32020869). Trizol total RNA extraction kit was purchased from Bode Biotech (Shanghai, China). Protein extraction kit and BCA protein assay kit were purchased from Kairui Biotech (Nanjing, China). rno-miR-124 primer mix kit was purchased from Kemin Biotech (Shanghai, China). IGF-1 ELISA kit was purchased from Abcam (Cambridge, MA, USA). IGF-1 primary and secondary antibodies were purchased from Ruisheng Biotech (Nanjing, China). ECL detection kit was purchased from Jinqi Biotech (Dalian, China). The quantitative fluorescence PCR instrument was purchased from Wanman Biotech (Shanghai, China) and the microplate reader was purchased from Lida Biotech (Shanghai, China).

Establishment of the animal model

The hypothyroid rat model was constructed as follows [7]: a rat was given an intraperitoneal injection of 10% chloral hydrate (3 cml/kg weight). The anesthetized rat was placed on an animal surgery board. The skin around the neck was disinfected, and a 2-cm incision was made in the neck. The lobus glandulae thyroideae on both sides were exposed by blunt dissection of surrounding tissues. The thyroid glands and isthmus were completely and carefully removed by blunt tweezers to avoid any injury on the recurrent laryngeal nerve. Four to five drops of penicillin sodium was injected in the wound to prevent infection. The incision was sutured, and the rat was kept warm until it woke up from the surgery. The rat was fed normally and closely monitored for changes in hair color, weight, breathing and other vital signs. The construction of hypothyroid rat model was confirmed by increased TSH level and decreased TT4 level measured at 2 weeks after the surgery.

Grouping

The 60 SPF male Wistar rats were randomly divided into 3 groups: a normal control group (n=20) that was given normal ordinary rat chow and deionized water for a feeding period of 12 weeks, a hypothyroidism group (n=20) that was constructed as described in section 1.3.1, and given normal ordinary rat chow and deionized water for 12 weeks, and an L-T4 treatment group (n=20) that was given a subcutaneous injection of L-T4 (1.25 μg • 100 g-1 • d-1) for 4 weeks starting from 8 weeks after the surgery.

Measurement of TSH and TT4

Orbital venous sinus blood and heart blood was obtained from each rat at 2 and 12 week after the surgery, respectively. The serum level of TSH and TT4 was measured by immunofluorescence as previously described [8].

Myocardial pathological examination

At week 12, the myocardial tissue of each rat was subjected to HE staining [9] and observed under an optical microscope to detect the morphological changes.

Determination of mir-124 and IGF-1 expression

At week 12, total RNA was extracted from the myocardial tissues in each group. The RNA sample was diluted 100 times in RNase-free water, and the absorbance was measured at 260 and 280 nm, respectively. The target ratio

Table 1. Comparison of serum TSH and TT4 level in all groups (x±s)

Group	No. of cases (n)	Week 2		Week 12	
		TSH (m IU/L)	TT4 (g/dl)	TSH (m IU/L)	TT4 (g/dl)
Control	20	0.075±0.021	4.512±0.342	0.098±0.031	4.104±0.310
Hypothyroidism	20	5.213±1.321*	1.987±0.321*	5.543±1.341*	1.524±0.213*
L-T4 treatment	20	4.221±1.110*	1.889±0.341*	0.321±0.124$^{\Delta}$	3.973±0.323$^{\Delta}$

*P < 0.01 compared with the control group. $^{\Delta}$P < 0.01 compared with hypothyroidism group.

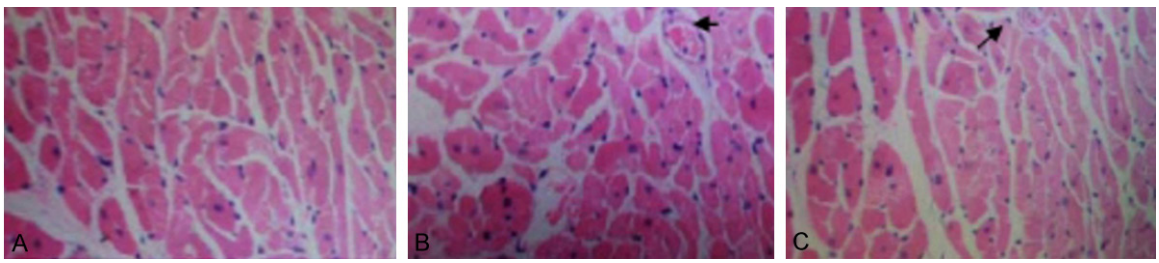

Figure 1. Myocardial pathological changes. A. Myocardial tissue in the control group (× 200); B. Myocardial tissue in hypothyroidism group (× 200); C. Myocardial tissue in L-T4 treatment group (× 200).

Table 2. Comparison of myocardial expression of mir-124, IGF-1 mRNA and protein in all groups

Group	No. of cases (n)	mir-124	IGF-1 m RNA	IGF-1 protein
Control	20	1.036±0.231	1.412±0.432	0.187±0.004
Hypothyroidism	20	3.768±1.432*	1.517±0.542	0.104±0.002*
L-T4 treatment	20	1.214±0.546$^{\Delta}$	1.432±0.431	0.186±0.004$^{\Delta}$

*P < 0.05 compared with the control group. $^{\Delta}$P < 0.05 compared with hypothyroidism group.

of OD260/OD280 was between 1.5 to 2.2. The mir-124 and IGF-1 mRNA level was quantified using the real-time quantitative PCR kit as described [10]. The expression of IGF-1 protein was quantified by Western blot analysis. Briefly, 100 mg of myocardial tissues was mixed with 100 μl of extraction buffer and grounded for 30 min. The mixture was centrifuged at 8000 g for 15 min. The supernatant was transferred to a new tube and quantified using a BCA protein assay kit according to the manufacture's instruction. A total of 50 μl of protein was mixed with loading buffer (5 ×) and denatured in a boiling water bath for 5 min. The sample was separated by 13% SDS-PAGE and wet transferred for 1 h. The membrane was washed once with TBST and incubated with rat primary antibody (1:400 dilution) at 25°C for 1 h with gentle shaking. The membrane was then washed and incubated with goat anti-rat secondary antibody (1:1000 dilution) at 25°C for 1 h. the membrane was washed 3 times (5 min each time) with TBST and treated with ECL for color development. The intensity of bands was detected by a Molecular Imager® ChemiDocTM XRS System (Bio-Rad Laboratories). The gray value of bands was analyzed by Image Lab 2.0 software (Bio-Rad Laboratories Inc).

Statistical analysis

Measurement data were expressed as mean ± standard deviation. Statistical analyses were performed using the SPSS18.0 (SPSS Inc., Chicago, IL, USA). Difference between any two groups was compared by t tests. P < 0.05 was considered statistically significant.

Results

Changes in the thyroid function

As shown in **Table 1**, at 2 weeks after the modeling, the TSH level in hypothyroidism and L-T4 treatment group was significant higher compared with the control group (P < 0.01), whereas the TSH level in both groups was significant lower than that in the control group (P < 0.01), indicating the successful construction of the hypothyroidism rat model. At week 12, the TSH level in hypothyroidism group remained higher than that in the control group (P < 0.01), where-

as the TSH level in L-T4 treatment group was significantly lower compared with hypothyroidism group ($P < 0.01$). While the TT4 level in hypothyroidism group was significantly lower compared with the control group ($P < 0.01$), the TT4 level in L-T4 treatment group was significantly higher compared with hypothyroidism group ($P < 0.01$). There was no significant difference in the TSH or TT4 level between L-T4 treatment and the control group. These results suggested that L-T4 was effective for the treatment of hypothyroidism.

Myocardial pathological changes

At week 12, the myocardial tissue of each group was subjected to HE staining to detect the myocardial pathological changes. As shown in **Figure 1A**, the myocardial fibers in the control group were orderly and tightly arranged. The chromatin was evenly distributed in the nucleus that was located right in the middle of myocardial cells. Atrophied myocardial cells in hypothyroidism group were disorderly organized with increased intercellular space (**Figure 1B**). The myocardial cells in L-T4 treatment group was in much better organization compared with hypothyroidism group. A much lower degree of atrophy was observed (**Figure 1C**).

Myocardial expression of mir-124, IGF-1 mRNA and protein

At week 12, the level of myocardial mir-124 expression in hypothyroidism group was significantly higher compared with both L-T4 treatment and control group ($P < 0.05$), whereas there was no significant difference in the mir-124 level between L-T4 treatment and the control group. No significant difference in IGF-1 m RNA expression was detected among the three groups. While the expression of IGF-1 protein in hypothyroidism group was significantly lower compared with L-T4 treatment and control group ($P < 0.05$), there was no significant difference in the mir-124 level between L-T4 treatment and the control group (**Table 2**).

Discussion

Hypothyroidism has been seriously threatening the human health. The heart has been known as the most commonly affected organ in hypothyroid patients. Myocardial injury associated with hypothyroidism is primarily induced by decreased myosin ATPase activity due to reduced thyroid level [11]. Clinical studies have shown that hypothalamus growth hormone (GH)-IGF axis affects the body's growth and development through interference with the endocrine system, indicating that TH is related to GH-IGF axis and abnormal secretion of TH induced a reduced regulation of the GH-IGF axis [12].

IGF-1 is a member of the insulin-like growth factor family and is present in most types of tissues in the body. It can promote the proliferation and differentiation of cells, and stimulate the growth and metabolic activity of the body in an autocrine or paracrine manner [13]. IGF-1 is secreted in both endothelial cells and fibroblasts in the normal myocardium, and can improve the blood supply to the outer periphery of the heart by regulating the differentiation and increasing the volume of these cells. It has been shown that IGF-1 can accelerate the differentiation of thyroid cells by regulating the DNA synthesis in these cells, and thus enhance the regulatory function of the thyroid. Studies have found that the level of IGF-1 was reduced in patients who underwent total thyroidectomy, whereas the serum IGF-1 level returned to the normal level after receiving a post-operative supplement of TH, indicating an inverse correlation between serum TH and IGF-1 levels [14, 15]. Moreover, it has been known that the apoptosis of myocardial cells in hypothyroidism patients is affected by the TH level [16]. Consistent with the previous result, out study showed that the expression of IGF-1 protein was decreased compared with L-T4 treatment and control groups, whereas there was no significant difference in IGF-1 mRNA or protein expression between the latter two groups, suggesting that the change in TH level affected the expression of IGF-1.

miRNAs are a class of small (21-25 nucleotides in length) non-coding RNAs that inhibit the expression of target gene through binding to the 3-UTR of mRNA [17]. The expression of mRNAs in the liver of methimazole-induced transient hypothyroidism mice model was significantly changed [18]. The most pronounce change was observed in the mRNA level of two target genes of mir-124, indicating that TH regulates the expression of proteins through interaction with miRNAs [19, 20]. In this study, mir-124 was highly expressed in the damaged myocardium in hypothyroid mice, but expressed

in low levels in L-T4 treatment group, which suggested that TH regulated the myocardial expression of miRNA. Furthermore, IGF-1 gene has been previously known as a potential target for mir-124. Our results showed that IGF-1 protein expression was significantly lower compared with L-T4 treatment and treatment group although there was no substantial difference in IGF-1 mRNA expression among the three groups, indicating that the IGF-1 expression was regulated by mir-124 at the protein level.

In summary, the high expression of mir-124 in the myocardium of hypothyroid rats prohibited the expression of IGF-1 protein in myocardial cells, leading to a decreased number of myocardial cells and reduced cell growth. Consequently, the contractility of myocardial tissues were reduced, resulting in a decrease in both the oxygen supply of myocardial cells and the blood flow to the heart, and ultimately irreversible damage to myocardial cells.

Disclosure of conflict of interest

None.

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