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Effect of Thyroxine on the Content of Cardiolipin

Benlan Ye, Qi Hou, Fan Guo, Ting Sun, Hailong Jiang, Wei Jiang

***Abstract*— Hyperthyroidism is a common disease. It has been well recognized that once thyroid hormones enter cells through membrane transporter proteins it binds its receptor and interacts with specific sequences of DNA in the promoters of responsive genes, modulating gene expression. However, it is not clear if phospholipids play any part in the procedure of hyperthyroidism. Some reports showed that cardiolipin has close relationship with energy metabolism. And, hyperthyroidism is a high metabolic disorder. Therefore, in this paper we studied the relationship between thyroxine and the content of cardiolipin. The results showed that thyroxine increases the content of cardiolipin both in vivo and in vitro. It suggested that increase cardiolipin in hyperthyroidism is a direct effect of thyroxine and not mediated by other neurohumoral factor.**

***Index Terms*— Cardiolipin, Thyroxine, Cardiomyocyte**

I. INTRODUCTION

Hyperthyroidism is a high metabolic disorder caused by high serum level of thyroxine (T4) and/or triiodothyronine (T3). It has been well recognized that once thyroid hormones enter cells through membrane transporter proteins it binds its receptor and interacts with specific sequences of DNA in the promoters of responsive genes, modulating gene expression [1-2]. It is also found that the second way of thyroid hormones effect is through the non-genomic effects on plasma membrane, mitochondria, and sarcoplasmic reticulum [3]. Although both T3 and T4 pass easily through the cytoplasmic membrane of target organs because of their lipophilic nature, the majority of thyrine is T4. The lipophilic T3 subsequently enters the nucleus, where it binds to thyroid hormone receptors. T3 is the thyroid hormone that is active at the molecular level and has genetic and extragenic effects on target cells [4]. T4 converts to T3 to exert its biological influence.

What we are interested in is if phospholipids have any relationship with the procedure of hyperthyroidism, since the non-genomic effects of T3/T4 is through the effect on the plasma membrane [3], and phospholipids are the main components of these membranes. Some reports showed that cardiolipin has close relationship with energy metabolism [5] and hyperthyroidism is a high metabolic disorder [1-2]. Therefore, in this paper we studied the relationship between thyroxine and the content of cardiolipin both in vivo and in vitro, tried to answer the question whether T4 influence the content of cardiolipin or not, and whether it is a direct effect of thyroid hormone or it is an indirect effect of T4 mediated by any other neurohumoral factor involved if it does.

Benlan Ye, corresponding author, Department of Physiology, School of Medical, Xiamen University, 361005,China. Email: benlanye@xmu.edu.cn
Qi Hou, Department of Physiology, School of Medical, Xiamen University, 361005,China
Fan Guo, Department of Physiology, School of Medical, Xiamen University, 361005,China
Ting Sun, Department of Physiology, School of Medical, Xiamen University, 361005,China
Hailong Jiang, Department of Physiology, School of Medical, Xiamen University, 361005,China
Wei Jiang, Department of Physiology, School of Medical, Xiamen University, 361005,China This study was supported by grants from National Natural Science Foundation of China (81170727).

II. MATERIALS AND METHODS

A. Reagents

Levothyroxine (T4) were purchased from Sigma-Aldrich Co. LLC. Dulbecco's Modified Eagle's Medium (DMEM). Fetal bovine serum (FBS) and trypsase were purchased from GIBCOTM Invitrogen Corporation. Penicillin and Streptomycin was purchased from HyClone™. Nonyl Acridine Orange (Acridine Orange 10-Nonyl Bromide, NAO) was purchased from Molecular Probes®.

B. Animals care

To assay the effect of T4 on cardiolipin, male SPF Kunming mice aged 10 weeks were randomly sampled and divided into sham group and hyperthyroidism group. Animals were housed in the medical school animal center with Animal Qualification Certificate No. SCXK（Min）2014-0001.

To induce hyperthyroidism, 10 weeks old mice were treated with levothyroxine (Sigma CAS 51-48-9) of 10μg/10g body weight daily for 14 days by intraperitoneal injection [6]. Levothyroxine was in the vehicle of 0.01N NaOH and 0.9% NaCl. The mice of control animals were injected with the vehicle for the same duration. All the mice were maintained in the same environment with free access to food and water.

C. Frozen sample preparation

Mice were killed by cervical vertebra dislocation and myocardial tissue frozen sections were made for cardiolipin assay. Fresh myocardial tissues were enbeded with frozen tissue matrix OCT and kept to frozen with liquid nitrogen. Before cutting sections, the block temperature was equilibrated to -20°C. The myocardial tissues were cut of 5μm. The frozen section slides were stored at -80°C before assayed.

D. Cell culture

In the study in vitro, cardiac myoblast cell line H9C2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum. H9C2 cells were purchased from the Cell Bank of Jiangyin Qi's Biological Technology Co., Ltd., China. The cells were maintained in a humidified 37°C incubator with 5% CO2,

supplied with fresh medium every 3 days, and subcultured before reaching confluence.

To build hyperthyroid cell model in vitro, H9C2 cells were cultured with a final concentration of 1 μmol levothyroxine for 8 days [7]. Levothyroxine was in the vehicle of 0.01N NaOH and 0.9% NaCl. Cells of control group were added with the equal vehicle for the same duration.

E. Cardiolipin Assay

Cardiolipin content was assayed by analyzing the fluorescence intensity of labeled NAO [8]. NAO has a very large head and small tail structure which can compensate with cardiolipin's small head large tail structure in a highly ordered way [9]. This structural characteristics of NAO makes it widely used as a simple and feasible fluorescent dyes to detect cardiolipin.

The content of cardiolipin in hyperthyroidism mice was assayed with myocardial tissue frozen sections. The frozen sections were stained by 10μmol NAO for 20 minutes in dark enviroment prior to confocal laser scanning microscope (CLSM, Olympus FV100) observation. The content of cardiolipin in hyperthyroid cell model in vitro was assayed by flow cytometry (Partec CyFlow Cube 6). Cells were stained with 1μM NAO for 20 minites then washed and analyzed by flow cytometry. Dead cells and debris were gated out by forward scatter (FSC) and side scatter (SSC) measurements.

F. Statistical Analysis

Data were presented as Mean ± Std. Deviation. The statistical analysis applied were two-tailed Student's t-test and independent-samples T test. A P-value < 0.05 was accepted as statistically significant .

III. RESULTS

A. Effect of T4 on Cardiolipin in vivo

Content of cardiolipin in frozen myocardial tissues from hyperthyroidism mice was indicated by the fluorescence intensity of labeled NAO. In Figure 1(C), T4-treated group shows higher fluorescence intensity of labeled NAO than the control group. From the comparison of graphs (A) and (B), we can tell that in T4-treated mice the increase of cardiolipin content accompanies with myocardial hypertrophy. This indicates that T4 induced myocardial hypertrophy and increased the content of caridiolipin as well .

B. Effect of T4 on Cardiolipin in vitro

To investigate whether the increase of cardiolipin described above was a direct effect of T4 on cells or an indirect effect of T4 mediated by other factors in vivo, we cultured H9C2 cells with T4 in vitro, and assayed the content of cardiolipin in H9C2 cells. See Fig. 2. The result shows that the content of cardiolipin incereased significantly in H9C2 cultured with T4 when compared with the controlled normal H9C2 cell group. That is, the effect of T4 on cardiolipin in vitro was concordant with that in vivo.

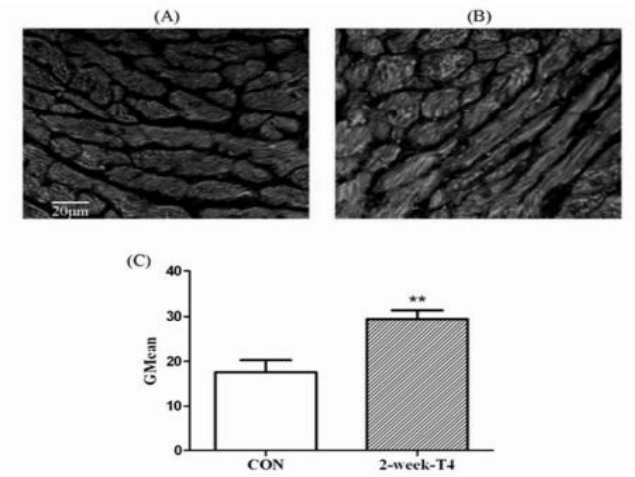

Fig. 1. Content of cardiolipin in frozen myocardial section from mice. The content of cardiolipin was ?assayed by the fluorescence intensity of labeled nonyl acridine orange (NAO). Frozen sections of mice myocardial tissue were stained with 10μM NAO for 20 minutes in dark environment/darkness prior to confocal laser scanning microscope observation. These representative fluorescent images show the content of cardiac cardiolipinin in control and 2-week T4-treated mice. (A) Control mice and (B) 2-week T4-treated mice. ?Scale bar, 20μm. (C) Bar plot shows (, from left to right,) the change of geometric mean of fluorescence intensity in control and 2-week T4-treated mice. Values are means ± SD, n = 7 mice per group. **P < 0.01 vs. control.

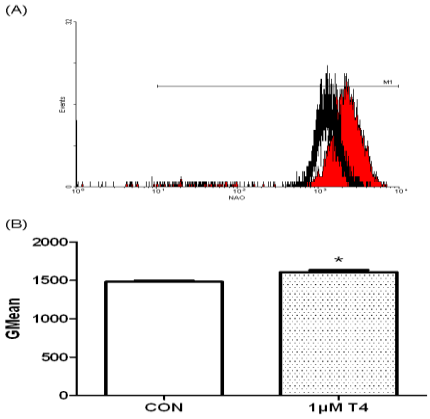

Fig. 2. Content of cardiolipin in H9C2 cells. The content of cardiolipin was ?assayed by the fluorescence intensity of labeled NAO by flow cytometry. H9C2 cells were preincubated with (?) and withoutT4 for 8 days. Cells were stained with 1μM NAO for 20 minites then washed and analyzed by flow cytometry. Dead cells and (other) debris were gated out by forward (FSC) and side (SSC) scatter measurements. (A) The content of cardiolipin in H9C2 cell was assayed using the nonyl acridine orange (NAO). The open curve represents the control (controlled?) cells, and the shaded curve corresponds to the T4-treated cells. (In plot A,) The x axis shows log FL-1 fluorescence intensity; the y axis indicates cell number (events). Cells with high cardiolipin were gated as M1. (Plot) (B) shows the geometric mean of fluorescence intensity in control and T4-treated cells. Values are means ± SD, n = 7 mice per group. **P < 0.01, *P < 0.05 vs. Control.

IV. DISCUSSION

Mary Pangborn first reported the isolation and purification of cardiolipin from bovine heart of in 1942 [10]. Then it was found that cardiolipin existed widely in both prokaryotic cells and eukaryotic cells as a major membrane glycerophospholipid [11]. It is characteristically located in mitochondria, especially in the inner mitochondrial membrane [12, 13]. Cardiolipin is a key mitochondrial phospholipid required for the activity of the electron transport chain [5]. Some reports showed that cardiolipin is involved in different stages of the mitochondrial apoptotic process and in mitochondrial membrane dynamics [14].

Cardiolipin is a kind of diphosphatidylglycerol lipid, there are two phosphates in cardiolipin molecule. The hydroxyl groups (–OH and –O-) on phosphate would form a stable intramolecular hydrogen bond with the centered glycerol's hydroxyl group, thus forming a bicyclic resonance structure. As the head group forms such compact bicycle structure, the head group area is relatively smaller than the big tail region consisting of 4 acyl chains. On the other hand, nonyl acridine orange (NAO) has a very large head and small tail structure which can compensate with cardiolipin's small head large tail structure, and arrange in a highly ordered way [15]. Based on this special structure, NAO was introduced to be the fluorescent indicator to assay the content of cardiolipin since 1982 [16], and it still represents a simple and sensitive method of assessing cardiolipin content. Here in our research, we used NAO as the fluorescent indicator to assay the content of cardiolipin in both the in vivo and in vitro experiment models of hyperthyroidism. The result of in vivo experiment showed that T4 increased the content of caridiolipin, which is consistent with the previous report [17]. The result of in vitro experiment, which excluded the effect of other neurohumoral factors, showed that T4 increased the content of caridiolipin in cultured H9C2 cell as well. Thus, we can conclude that T4 increase the content of cardiolipin directly and the process is not mediated by other factors.

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