HYPOTHYROIDISM INCREASES EXPRESSION OF STERILE INFLAMMATION PROTEINS IN RAT HEART TISSUE

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Abstract

Purpose. In this study, we aimed to investigate the relationship between hypothyroidism and sterile inflammation in rat heart tissue.

Methods. Groups; control group (fed with standard rat chow diet and tap water) and the hypothyroid group (fed with a standard rat chow diet and tap water containing 0.05% 6-n-propyl-2-thiouracil for 6-weeks). At the end of the experiment, histopathologic examination was performed. The T3, T4, TSH and myocardial malondialdehyde (MDA) measurements were performed with an ELISA kit. TUNEL assay was performed to demonstrate apoptosis. Sterile inflammation markers, caspase-1 and NLRP3, were investigated by immunohistochemistry and western blot.

Results. In histopathological examination, we observed leukocyte infiltration, myocardial atrophy, pyknotic nucleated cells and cytoplasmic vacuolization in hypothyroid group whereas the control group showed normal structure. MDA levels in myocardial tissue were significantly high in hypothyroid group when compared to the control group ($P < 0.05$). Myocardial apoptosis increased in hypothyroid group when compared to the control group. NLRP3 and caspase-1 immunoreactivity was higher in the hypothyroid group. In ELISA results, we found significantly higher level of TSH and lower levels of T3 and T4 in hypothyroid group when compared to the control group.

Conclusion. Hypothyroidism increased oxidative stress, and caused inflammatory alterations in cardiac tissue. In addition, our study also suggested that thyroid hormone deficiency would increase the amounts of cardiac NLRP3 and caspase-1 protein, which indicates that hypothyroidism exerts its destructive effects through sterile inflammation. Elucidation of sterile inflammation-associated pathways may produce promising results in the treatment of hypothyroidism-induced cardiac damage.

Key words: Hypothyroidism, Oxidative stress, NLRP3, Caspase 1, Sterile inflammation.

INTRODUCTION

The thyroid gland produces thyroxine (T4) and triiodothyronine (T3). The main part of T3 is derived from T4 in extra thyroidal tissues after deiodination of a single iodine atom catalyzed by deiodinases (DIO1, DIO2 and DIO3). The heart tissue mostly has DIO2 (1). Thyroid hormones (TH) greatly impact energy homeostasis in the heart, and excess thyroid hormone leads to a hypermetabolic state. It performs these important functions by binding to its receptors in the nuclei of cardiomyocytes (2). Either congenital hypothyroidism or hyperthyroidism causes structural and functional disorders in the heart tissue. Thyroid dysfunction changes cardiac contraction and electrophysiological states (3, 4). The cardiomyocytes contain contraction proteins in their cytoplasm. It has been shown that thyroid hormone regulates the activation of many genes in contraction proteins, particularly myosin (5). TH controls expression of both structural and functional genes involved in the maintenance of cardiac function, including alpha myosin heavy chain (α -MHC, Myh6) and sarcoplasmic/endoplasmic reticulum calcium ATPase 2a (Serca2a) (6).

Untreated hypothyroid patients have low cardiac output, decreased stroke and vascular volume and increased systemic vascular resistance (4, 7). Diastolic dysfunction is a common abnormality reported in hypothyroidism (8). There are studies in the literature showing that especially in women hypothyroidism causes Takotsubo Cardiomyopathy (8, 9). Takotsubo Cardiomyopathy is a cardiac syndrome characterized by transient LV dysfunction, electrocardiographic changes that can mimic acute myocardial infarction (MI), and minimal release of myocardial enzymes in the absence of obstructive coronary artery disease (10).

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Hypothyroidism, besides physiological impairments, also shows histopathological changes in the heart. Long-term hypothyroidism has been shown to induce fibrosis due to neutrophil infiltration in the myocardium and ventricular dilatations. Thyroid hormone deficiencies decrease salusin-alpha (11) and increase blood inflammation markers such as C-reactive protein, tumor necrosis factor alpha, interleukin-6 and pro-fibrotic transforming growth factor beta 1 (12).

Inflammation is not only originated from microorganisms, but it can also be originated from ischemia-reperfusion, trauma and chemical substances. This type of inflammation is called sterile inflammation. Sterile inflammation leads to many diseases such as chronic obstructive pulmonary disease, silicosis, obesity, gout, pseudogout, arthritis, type 2 diabetes, myocardial infarction, Alzheimer's disease and acute pancreatitis (13). Although the exact molecular mechanism of sterile inflammation has not been discovered, the main pathways are known (14, 15). The formation of the inflammasome complex is crucial in sterile inflammation. Although there are many inflammasome complexes, NLRP3 (nucleotide-binding domain and leucine-rich repeat containing proteins 3) inflammasome complex is the most important and well known. NLRP3 inflammasome consists of NLRP3, ASC and pro-caspase 1 proteins. With NLRP3 activation, pro-caspase 1 converts into caspase 1, which leads to release proinflammatory cytokines like IL-1 α , IL-1 β , IL-18 and TNF- α (16). In addition to many cellular activities, these cytokines are primarily involved in inflammation (17). Increased NLRP3 complex causes inflammation to increase and spread in cells and tissues (18). Recent studies show an obvious relationship between hypothyroidism and sterile inflammation (12). The suppression of the NLRP3 inflammasome complex formation appears to be very promising in preventing pathological conditions caused by hypothyroidism.

There are a number of studies showing that thyroid hormone deficiency impairs cardiac function and increases inflammation markers, but there are insufficient numbers of studies about hypothyroidism-induced sterile inflammation. Therefore, we aimed to investigate the relationship between hypothyroidism and sterile inflammation in the heart tissue.

MATERIALS AND METHODS

The study was carried out on male Wistar albino rats, aged 7-8 months, weighing 280–300g that were

bred and kept at Medical and Surgical Experimental Research Center, Eskisehir Osmangazi University (ESOGU) and all experimental protocols were carried out in accordance with the guidelines supplied by the Local Institutional Committee for the Ethical Use of Animals of ESOGU, Turkey. All animals were housed in separate polycarbonate cages in a temperature controlled room (21–24°C) and humidity of 30–40% under 12 h light-12 h dark cycle. Animals were fed with standard diet and water ad libitum.

The rats were anyhow divided into two experimental groups (7 animals in each group). Control group was fed with standard rat chow diet and tap water, and the hypothyroid group was fed with a standard rat chow diet and tap water containing 0.05% 6 n-propyl-2-thiouracil for 6 weeks (19).

After a 6-week treatment, the rats were intraperitoneally injected with a mixture of 2% xylazine and 2.5% ketamine hydrochloride for anesthesia. Blood samples were collected from the animal hearts for measuring TSH, fT3 and fT4 parameters. After blood puncture from the heart, serum was collected through centrifugation. While the animals were under anesthesia, their hearts were quickly removed. Their hearts were cut in two pieces. One-half was placed in a 10% formaldehyde solution for immunohistochemistry (IHC), histopathology, and apoptotic examinations. The other half of the heart was stored at -80 °C for the western blot examinations. We used both heart atrial and ventricular parts for analyses.

Histochemical and immunohistochemical analysis

Histochemical analysis: tissue samples of each group were fixed in formalin for 24 hours and embedded in paraffin. Sections of 5 μ m thickness were placed onto microscope slides. Sections were stained with hematoxylin-eosin for histopathological examination.

IHC analysis of NLRP3 and Caspase-1: for antigen retrieval, we heat the sections in citrate buffer (0.01M) for 5 min in the microwave oven. We blocked the peroxidases in the cell cytoplasm with 3% hydrogen peroxide. Blocking solution was applied to the sections for 10 minutes. Primary antibodies (caspase-1: sc-56036 and NLRP3: sc-66846, Santa Cruz, USA) were applied to the sections at 4°C for overnight. Then the secondary antibody (Lab Vision, Biotinylated Goat anti-Polyvalent, TP-125-BN, Fremont, USA) was applied to the sections for 10 minutes. The reaction was made visible with the chromogen (AEC: 3 amino

9 ethylcarbazole, Lab Vision, TA-125-SA, Fremont, USA). Hematoxylin was used for counterstaining and slides were mounted with water-based media. Then the slides were examined with a photomicroscope.

Lipid peroxidation assay

Malondialdehyde (MDA) was measured in cardiac tissue. For cardiac MDA determination, 25 mg of tissue was weighed and 250 μ L of RIPA buffer was added. The samples were homogenized with homogenizer and centrifuged at 1600 $\times$ g for 15 minutes at 4°C. The supernatant was used for further analysis. MDA was quantified using the thiobarbituric acid (TBA) reaction. MDA levels were measured using Cayman's TBARS Assay Kit. According to instructions primarily, 100 μ L of sample supernatants and standards were placed in 5 mL tubes. 100 μ L of SDS solution added to tubes and mixed. 4 mL of color reagent was added to tubes and mixed. We boiled tubes in a water bath for an hour. After one hour we placed tubes in an ice bath for 10 min to stop the reaction. After 10 min the tubes were centrifuged at 1600g, 4°C for 10 min and the absorbance of samples was measured at 535 nm with a microplate reader. MDA levels were expressed in micro molar.

The apoptosis (TUNEL) assay

To determine the number of apoptotic cells, sections were stained with *in situ* cell death detection kit (Apop Tag plus Peroxidase in Situ Apoptosis Detection Kit, Chemicon, Temecula, CA, USA). Sections were incubated with 20 μ g/mL proteinase K. Endogenous peroxidase activity was blocked with 3% H₂O₂. After washing with PBS, sections were incubated with equilibration buffer and TdT enzyme (77 μ L reaction buffer + 33 μ L TdT enzyme mix, 1 μ L TdT enzyme) at 37°C. Stop/wash buffer was applied at room temperature and sections were incubated with anti-digoxigenin conjugate for 30 min. After washing with PBS, the sections were stained with 3'3 diaminobenzidine components to detect TUNEL positive cells, then counter-stained with methyl-green and examined with a photomicroscope.

Measurement of T3, T4 and TSH levels in blood samples

The TSH, fT3 and fT4 levels were determined by ELISA method. The TSH, fT3 and fT4 levels of samples were detected with ELISA kit (Elapsience, PRC) according to the manufacturer's instructions. Then the absorbance of each well was measured at

450 nm with a microplate reader.

Western Blot Analysis of Caspase-1 and NLRP3

The heart tissue homogenization was performed with bead homogenizer (Bead Blaster Homogenizer, Benchmark Scientific, USA). Approximately 25 mg of tissue pieces were placed in homogenization tubes with ice cold RIPA. Homogenization was carried out for 2 min. Then tubes were centrifuged (for 10 min at 1600g and 4°C) and stored at -80°C.

The total protein concentration of samples was detected with Qubit fluorometer (Invitrogen, USA) according to the manufacturer's instructions. In this assay, we used commercial assay kit (Thermo Fisher Scientific, Qubit Protein Assay Kit, Q33211, Waltham, USA). Firstly, the samples were diluted with distilled water. Fluorescent dye capable of binding to proteins in the kit was diluted 1 to 200 with its own buffer. The total protein and fluorescent working solution were mixed with a 1:10 ratio. The mixture was incubated in the dark for 15 min and measured with a fluorometer. The total proteins in the samples were specified as μ g/mL.

The proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes. The membranes were blocked by 5% BSA in TBST (0.05% Tween 20 in Tris-buffered saline, TBS) to prevent nonspecific binding, and incubated overnight at 4°C with primary antibodies (Caspase-1: sc-56036 and NLRP3: sc-66846, Santa Cruz, USA). After overnight incubation, the membranes were washed in TBST and incubated with HRP-conjugated secondary antibodies (Santa Cruz, USA, sc-2030 and sc-2005) for 60 min at 24°C. Then, the membranes were washed three times in TBST and developed with the Bio-rad western ECL Blotting Substrates (Hercules, California, USA). Finally, C-DiGit Blot Scanner (LI-COR, Lincoln, USA) was used for image analyzing.

Statistical analysis

Data were analyzed with statistical package program SPSS for Windows, version 18.0. (Chicago, Illinois, USA). Results were expressed as mean $\pm$ SEM. $p < 0.05$ was considered statistically significant. Shapiro-Wilk test was used to determine whether the parameters were normally distributed. Parametrical test (One-way ANOVA) was used for parameters with normal distribution. Tukey's test was performed for multiple comparisons.

Figure 1. Hematoxylin and eosin staining of heart tissue. A: Control group shows normal heart histological structure. B-E: Hypothyroid group shows leukocyte infiltration (★), myocardial atrophy (→), pyknotic nucleated cells (▲) and cytoplasmic vacuolization (→). All bars are 50 μ M.

Figure 2. Immunohistochemical staining of heart tissue. A and C: Control group shows no immunostaining of NLRP3 and caspase 1. Hypothyroid group shows high NLRP3 (Fig. 2B) and caspase 1 (Fig. 2D) immunostaining (→). All bars are 50 μ M.

Figure 3. Lipid peroxidation results. The MDA levels were significantly higher in hypothyroid group than the control group. *: ($p < 0.05$).

RESULTS

Histochemical and immunohistochemical results

Histochemical results

We did not observe any pathologic alterations in the heart tissue of the control rats. However, we observed several pathological changes in the hypothyroid group such as leukocyte infiltration, myocardial atrophy, pyknotic nucleated cells and cytoplasmic vacuolization (Fig. 1).

Immunohistochemical results

NLRP3 and caspase-1 immunoreactivity, which indicate sterile inflammation, were higher in the hypothyroid group when compared to the control group. These results suggest that hypothyroidism increases the amount of proteins involved in sterile inflammation (Fig. 2).

Figure 4. The TUNEL assay results. Control (Figure 4A) cardiomyocytes were stained negatively with TUNEL assay. However, in the hypothyroid group (Figure 4B) heart sections showed abundant TUNEL positive cardiomyocytes (→). All bars are 50 μ M.

Lipid peroxidation results

Heart lipid peroxidation was measured by a TBA test. The lipid peroxide levels, expressed in terms of MDA in tissue homogenates, were significantly higher in the hypothyroid group than in the control group ($p < 0.05$) (Fig. 3).

The TUNEL assay results

Control group cardiomyocytes were stained negatively with TUNEL assay. However, in the hypothyroid group, abundant TUNEL positive cardiomyocytes were present (Fig. 4).

Blood sample results

Administration of PTU for 6 weeks resulted in significant increase in TSH levels, decreases in fT3 and fT4 compared with the levels in the untreated rats ($p < 0.05$). These results indicate the establishment of hypothyroidism (Fig. 5).

Western blot results

To determine the effect of hypothyroidism

Figure 5. T3, T4 and TSH levels in blood serum. The ft3 and ft4 levels were significantly decreased and TSH levels increased in the hypothyroid group. *: (p<0.05).

on sterile inflammation-associated proteins, we measured the NLRP3 and caspase-1 levels in the heart tissue by western blotting. The results showed that hypothyroidism increases the NLRP3 (33.15 fold) and caspase-1 (37.48 fold) (Fig. 6) levels compared with the control group.

DISCUSSION

In this study, we showed that hypothyroidism causes lipid peroxidation, pathological changes and formation of NLRP3 inflammasome complex. These pathways can cause apoptosis and necrosis in cardiomyocytes (Fig. 7).

Thyroid hormones play crucial roles in cell differentiation, growth and metabolism regulation as well as in the function and development of many organs. The main targets for thyroid hormone in the heart tissue are cardiomyocytes. Thyroid hormone receptors are widespread in the heart. Thyroid hormone influences the myocardial contraction and relaxation, heart rate, peripheral vascular resistance, blood pressure and synthesis of myocardial fibers, myosin ATPase activity, glycogenolysis and mitochondrial metabolism (20, 21). Due to thyroid hormone deficiency, many cardiovascular diseases are seen in hypothyroid patients (22). Thyroid hormone deficiency also causes inflammation in the heart (12). In this study, we investigated the effects of hypothyroidism on the cardiac tissue. Our histopathological results showed inflammatory mononuclear cell infiltration, myocardial atrophy, pyknotic nucleated cells and cytoplasmic vacuolation in parallel to Hajje *et al.* Our results suggested that hypothyroidism caused pathologic changes in cardiomyocytes, and that cardiac

Figure 6. Western blot results. Relative density of NLRP3 increases 33.15 and caspase 1 increases 37.48 fold in the hypothyroid group compared to the control group.

Figure 7. The graph summarizing the result of our study.

dysfunctions in hypothyroidism may result from these pathologic differences. The thyroid hormone deficiency increases lipid peroxidation (21). Lipid peroxidation is determined by MDA level, which is a final product of lipid peroxidation. Our results showed that MDA level was higher in the hypothyroid group. Lipid peroxidation is known to cause apoptosis in the cell through the caspase pathway and DNA fragmentation (23). Our TUNEL results suggest that increased lipid peroxidation increases the number of apoptotic cells in the hypothyroid group. The main cause of lipid peroxidation is oxidative stress. The antioxidant and oxidant balance is affected by many hormones, and these balances go through in the direction of oxidative stress especially in excess or lack of thyroid hormone. Oxidative stress and inflammation are two closely related processes in cardiovascular diseases. Increased oxidative stress promotes inflammation (24, 25). Sterile inflammation is a common outcome of a

number of different clinical organ disorders. Sterile inflammation occurs in solid organs, such as the heart, when an organized inflammatory response occurs in the absence of any infection. Sterile inflammation can be triggered by many receptors such as toll-like receptors (TLRs) and nucleotide binding domain leucine-rich repeat collecting receptors (NLR). These receptors can recognize parts associated with either cellular damage (DAMP; danger-associated molecular patterns) or invading microorganisms (PAMP: pathogen associated molecular patterns). Activation of these receptors ultimately leads to the formation of NLRP3 complex and production of cytokines that drive the inflammatory response (14, 15, 26-29). This is the first study to investigate the effect of hypothyroidism on the heart tissue in terms of sterile inflammation markers (NLRP3, Caspase 1).

In conclusion, hypothyroidism increased oxidative stress, determined pathologic findings related to inflammation in cardiac tissue. In addition, these findings suggest that thyroid hormone deficiency may increase the amount of NLRP3 and caspase 1 protein in the rat heart, which are markers of sterile inflammation. This increase suggests that hypothyroidism may exert its destructive effects on the heart tissue through sterile inflammation. Investigation of different inflammatory pathways associated with sterile inflammation may give promising results in the treatment of hypothyroidism.

Conflict of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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