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Original article

Hypothyroidism leads to increased collagen-based stiffness and re-expression of large cardiac titin isoforms with high compliance

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Abstract

Because long-term hypothyroidism results in diastolic dysfunction, we investigated myocardial passive stiffness in hypothyroidism and focused on the possible role of titin, an important determinant of diastolic stiffness. A rat model of hypothyroidism was used, obtained by administering propylthiouracil (PTU) for times that varied from 1 month (short-term) to 4 months (long-term). Titin expression was determined by transcript analysis, gel electrophoresis and immunoelectron microscopy. Diastolic function was measured at the isolated heart, skinned muscle, and cardiac myocyte levels. We found that hypothyroidism resulted in expression of a large titin isoform, the abundance of which gradually increased with time to become the most dominant isoform in long-term hypothyroid rats. This isoform co-migrates on high-resolution gels with fetal cardiac titin. Transcript analysis on myocardium of long-term PTU rats, provided evidence for expression of additional PEVK and Ig domain exons, similar to what has been described in fetal myocardium. Consistent with the expression of a large titin isoform, titin-based restoring and passive forces were significantly reduced in single cardiac myocytes and muscle strips of long-term hypothyroid rats. Overall muscle stiffness and LV diastolic wall stiffness were increased, however, due to increased collagen-based stiffness. We conclude that long term hypothyroidism triggers expression of a large cardiac titin isoform and that the ensuing reduction in titin-based passive stiffness functions as a compensatory mechanism to reduce LV wall stiffness.

Keywords: Passive stiffness; Diastolic stiffness; Titin; Connectin; Collagen; Extra cellular matrix; Fetal cardiac isoforms

1. Introduction

Thyroid hormones have significant effects on cardiac contractility with hyperthyroidism associated with a general hyperdynamic state and hypothyroidism leading to hypodynamic hearts. The molecular basis for changes in contractility and relaxation include effects of thyroid hormone on transcription of myosin, SERCA, and the SERCA inhibitor phospholamban (for reviews and original citations, see [1,2]). Furthermore, thyroid hormone is a negative regulator of expression of collagen [3–5], but the functional consequence of changes in collagen expression in hyper(hypo)thyroidism have not been investigated. Except for a study in the rat that revealed that manipulating thyroid hormone levels results in significant changes in the left ventricular end-diastolic stress-strain relation [6], little is know about effects of thyroid hormonal status on passive myocardial stiffness. Here we studied LV chamber and myocardial stiffness in rats with short- and long-term hypothyroidism, and focused on titin, a well-recognized major contributor to passive stiffness of the heart.

Titin is an endosarcomeric protein that spans from the Z-disk to the M-line of the sarcomere [7]. Passive stiffness of sarcomeres is largely due to the extensible I-band region of titin that functions as a molecular spring, developing passive force in sarcomeres stretched above the slack length (length at which passive force is zero) and restoring force when sarcomeres

Abbreviations: SERCA, Sarcoplasmic reticulum $Ca^{(2+)}$-ATPase; CAD, coronary artery disease (CAD); DCM, dilated cardiomyopathy; PTU, propylthiouracil; SL, sarcomere length.

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shorten to below this length [7]. The sequence composition of titin's molecular spring segment varies greatly in different titin isoforms (due to differential splicing) and titin's stiffness varies accordingly [8]. Stiffest is the adult N2B isoform, less stiff is the adult N2BA isoform, and most compliant is fetal cardiac titin (fetal cardiac titin belongs to the N2BA class of cardiac isoforms), which is expressed during fetal and neonatal development [8–10]. Sarcomeres of adult myocardium co-express the N2BA and N2B isoforms and their expression ratio determines the level of titin-based passive stiffness [11,12]. This expression ratio is not fixed but instead responds to altered cardiovascular hemodynamics. For example in the canine rapid pacing model, 4 weeks of pacing results in elevated N2B titin expression and down regulation of N2BA titin [13]. A similar change (i.e., increased expression of stiff N2B titin) has been reported in hypertensive rats [14]. Interestingly, opposite changes are present in patients with CAD or DCM: compliant N2BA isoforms are upregulated at the expense of N2B titin [15–17]. We have shown that passive myocardial stiffness is lowest in DCM patients with the highest degree of N2BA upregulation and, furthermore, echocardiography-derived diastolic filling parameters revealed that these patients have the lowest grade of diastolic dysfunction [15]. In summary, titin is an important contributor to myocardial passive stiffness and differential splicing of titin controls passive stiffness during heart development and disease.

In the present study we investigated titin expression and passive stiffness in a rat model of hypothyroidism, obtained by administering PTU, for times that varied from 1 month (short-term) to 4 months (long-term). Titin was studied by gel electrophoresis, transcript analysis using a titin exon microarray, and by immunoelectron microscopy. Passive stiffness was studied at the cellular, tissue and isolated heart levels. It was found that long-term hypothyroidism results in expression of a large titin isoform that significantly reduces titin-based passive stiffness.

2. Materials and methods

2.1. Animal model

Two month old male Sprague–Dawley (SD) rats were randomly divided into control and PTU (a commonly used anti-thyroid drug) treatment groups. The control group received regular feed and drinking water. PTU rats received 0.15% PTU feed (Harlan Tekland Co., Madison, WI) and drinking water to which 0.05% PTU had been added. Both control and PTU treated rats were sacrificed 0, 1, 2, 3, and 4 month after PTU treatment was initiated. Body weight (BW) and weight of ventricles (left ventricle + right ventricle) were collected. Left ventricular myocardium was quick-frozen in liquid nitrogen for subsequent analysis.

2.2. Gel electrophoresis

Myocardial samples were analyzed by SDS-agarose gels [18]. Gels were Coomassie-blue stained, scanned and analyzed with one-D scan software (Scanalytics Inc, Fairfax, VA). The N2BA/N2B ratios were calculated as well as total titin, and MHC (for details, see [9]). MHC isoform expression was determined by SDS-PAGE as explained in Warren et al. [19].

2.3. Transcript studies

The method that was followed has been described in detail in Lahmers et al. [9] Briefly, total RNA was isolated from left ventricular myocardium from control and PTU treated rats and was converted to biotinylated cDNA. A 50-mer oligonucleotide array containing 385 probes was used representing all of titin's human gene exons, plus various controls. Biotinylated target was mixed, heat denatured and hybridized to the oligonucleotide array. Detection chemistries and signal amplification were achieved using the TSA™ biotin System (PerkinElmer, Boston, MA). A minimum of three independent experiments from each group was conducted. Results from different groups were compared with a two-tailed *t*-test for samples of unequal variance and $P<0.05$ was used as criterion for statistical significance. (For additional details, see [9]).

2.4. Immunoelectron microscopy (IEM)

Myocardial samples from control and PTU treated rats were skinned, fixed in 3% para-formaldehyde/PBS solution for 20 min, then washed and blocked in 1% BSA/PBS solution for 1 h. The samples were labeled with anti-N2B-Uc antibody [20] and then secondary antibodies for 48 h each. The samples were fixed in 3% glutaraldehyde/tannic acid and osmium–tetroxide solutions, and embedded in araldite. Sections were cut using a Leica microtome, and stained with 2% potassium permanganate and lead citrate. For additional details, see [20].

2.5. Collagen

For collagen analysis, the left ventricle was quick frozen in liquid nitrogen. Sections of 12 μm were cut that were fixed with 100% acetone at 4 °C. Sections were double labeled using primary antibodies against collagen type I (goat polyclonal antibody raised against collagen type I of mouse, Cat. Sc-8788, Santa Cruz Biotechnology) and collagen type III (rabbit polyclonal antibody raised against C-terminus of collagen Type III of human, Cat. Sc-28888, Santa Cruz Biotechnology). Secondary antibodies were goat anti-rabbit Alexafluor 594 conjugated IgG and chicken anti-goat Alexafluor 488-conjugated IgG (Molecular Probes). Sections were analyzed on a Bio-Rad MRC 1024 Confocal Laser Scanning Imaging System.

2.6. Measurement of passive force in skinned myocardium

The methods that were used have been published [12]. Briefly, small wall muscle strips (diameter ~0.2 mm) were carefully dissected from control and 4 month PTU treated rat LV and skinned overnight at 4 °C in relaxing solution [12] containing 1% w/v Triton X-100. The strips were then washed thoroughly with relaxing solution. To prevent degradation, all