Overexpression of sarcoplasmic reticulum \( \text{Ca}^{2+} \)-ATPase improves cardiac contractile function in hypothyroid mice

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Received 13 November 1998; accepted 12 February 1999

Abstract

Objective: Prolonged cardiac contraction and relaxation in hypothyroidism are in part related to diminished expression of the gene coding for the calcium pump of the sarcoplasmic reticulum (SERCA2a). Therefore, we examined whether or not transgenic SERCA2a gene expression in mice may compensate for the cardiac effects of hypothyroidism. Methods: SERCA2a mRNA and protein were analyzed from hearts of euthyroid and hypothyroid mice of wild-type or SERCA2a transgene status. Contractile function was studied in isolated left ventricular papillary muscles. Results: We found significant decreases of SERCA2a mRNA and protein levels in hearts of hypothyroid wild-type mice in comparison with euthyroid wild-type mice (controls). Papillary muscles from hypothyroid wild-type mice showed significant increases in time to peak contraction and relaxation times compared with controls. In contrast, SERCA2a mRNA and protein levels were significantly higher in hypothyroid SERCA2a transgenic mice than in hypothyroid wild-type mice. The transgene led to a functional improvement by compensating for the prolonged contraction and relaxation of papillary muscles. Conclusions: Our murine model of hypothyroidism revealed decreases in SERCA2a gene expression accompanied by prolonged contraction and relaxation of papillary muscles, and an improvement of the contractile phenotype due to compensated SERCA2a gene expression in SERCA2a transgenic mice. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Contractile function; Gene expression; SR; Cardiomyopathy; E-C coupling

See Editorial of this article by Ventura et al. (pages 282–284) in this issue.

1. Introduction

The calcium pump, or \( \text{Ca}^{2+} \)-ATPase, of the sarcoplasmic reticulum (SERCA2a) is a major regulator of cardiac contractile function. During cardiac relaxation, \( \text{Ca}^{2+} \) uptake into the sarcoplasmic reticulum (SR) accounts for the majority of \( \text{Ca}^{2+} \) removal from the cytoplasm. SERCA2a activity is therefore the most important determinant of the speed of cardiac relaxation. SERCA2a activity also affects the magnitude and rate of force development during cardiac contractions by altering the SR’s \( \text{Ca}^{2+} \) content. Decreases in SERCA2a protein levels or its activity contribute to impaired cardiac function in a variety of pathophysiologic conditions, such as hypothyroidism [1], diabetes [2], and cardiac hypertrophy and heart failure [3,4].

We recently reported increased SERCA2a mRNA and protein levels in a transgenic mouse line overexpressing SERCA2a [5]. Functionally, the mice showed enhanced relaxation of isolated cardiac myocytes, papillary muscles, and the in vivo heart, as well as accelerated calcium transients. Since these mice express a functionally active SERCA2a transgene that results in contractile alterations, we were interested in exploring its implications in pathophysiologic conditions with downregulated SERCA2a.

In this report, we examined a potential compensatory function of SERCA2a transgene expression in hypothyroidism [6]. Prolonged cardiac contraction and relaxation are prominent features of hypothyroidism in several...
species, including the mouse [7]. In addition to changes in myosin isoforms [8,9], decreases in SERCA2a mRNA [10] and protein levels [11] have been reported in hypothyroid rats. We postulated that decreased SERCA2a expression contributes to the impaired relaxation in hypothyroid mice, and that prolonged cardiac contraction and relaxation can be improved or compensated by SERCA2a transgene expression in transgenic mice.

To examine these hypotheses, wild-type and SERCA2a transgenic mice were made hypothyroid and compared with euthyroid wild-type and SERCA2a transgenic mice. Contractile function was studied in isolated left ventricular papillary muscles, as recently described [5]. In light of the prevalence of genetically engineered mice, this technique should be of major interest for characterizing murine cardiac contractile phenotypes. The papillary muscle preparation is particularly suitable for resolving changes in the time courses of contraction and relaxation, which are the predominant cardiac manifestations of hypothyroidism. Our results prove in principle the feasibility of a transgene-mediated compensation for a loss of endogenous gene expression and a resulting recovery of contractile function.

2. Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.1. Construction of SERCA2a transgenic mice

SERCA2a heterozygous transgenic mice were constructed as previously described [5], using standard techniques [11]. Transgene expression of a rat SERCA2a cDNA was driven by a human cytomegalovirus (CMV) immediate early enhancer and a chicken β-actin promoter. To identify transgene-positive animals, tail clips were taken from the mice at three weeks of age. DNA was then isolated and screened by polymerase chain reaction (PCR) using primers internal to the human CMV enhancer of the transgene. This reaction yields an amplified fragment of 300 bp in the transgenic animals.

2.2. Northern blot analysis

RNA was isolated from ventricular homogenates (n=6–7 in each group) as described by Chomczynski and Sacchi [12]. Standard protocols were used for gel electrophoresis, Northern transfer and hybridization [13]. Membranes were probed with a 1.8-kb EcoRI restriction fragment corresponding to the 5'-end of the rat SERCA2a cDNA. We further probed for myosin heavy chain β, and for 28S as a loading standard. Northern blots were scanned and normalized to the average of all control lanes (wild-type, euthyroid).

2.3. Western blot analysis

Proteins from ventricular homogenates (n=3 in each group) were resolved by gel electrophoresis and transferred onto nitrocellulose membranes with a Novex transfer apparatus (San Diego, CA, USA). A rabbit polyclonal antibody against rat SERCA2 was used as previously described [14]. Secondary antibody labeling and protein detection were performed with an ECL Western blotting kit by Amersham (Little Chalfont, UK). Western blots were scanned and normalized to the average of all control lanes (wild-type, euthyroid).

2.4. Hypothyroidism

Mice were made hypothyroid by the addition of 0.15% 5-propyl-2-thiouracil (PTU) to their food for four weeks or by injection of radioactive iodine (100 μCi of Na131I, i.p.) four weeks prior to sacrifice. Blood obtained by cardiac puncture or from the chest following removal of the heart was collected into Eppendorf tubes containing 10 USP units of heparin. Serum was obtained by centrifugation at 4°C and stored at −20°C for later analysis. Total serum levels of the thyroid hormone thyroxine (T4) were determined by a solid phase radioimmunoassay based on competitive binding of 125I-labeled T4 (Diagnostic Products Corporation, Los Angeles, CA, USA).

2.5. Isolated papillary muscle experiments

Left ventricular mouse papillary muscles were isolated as follows from 14 euthyroid wild-type, 14 euthyroid transgenic, six hypothyroid wild-type, and five hypothyroid transgenic mice. The mice were deeply anesthetized with ketamine (140 mg/kg i.p.) and xylazine (10 mg/kg i.p.). Hearts were removed and rinsed in oxygenated Tyrode solution (in mM: 136 NaCl, 5.4 KCl, 1 MgCl2, 0.33 NaH2PO4, 10 N-2-hydroxyethylpiperazine–N’-2-ethanesulfonic acid (HEPES), 10 glucose, 2.5 CaCl2, pH 7.40) containing 30 mM 2,3-butanedione monoxime (BDM). Left ventricular papillary muscles were excised, inserted into Ω-shaped clamps made from strips of platinum foil, and tied with 6.0 braided silk suture. The muscles were transferred to a 0.5-ml muscle chamber where they were mounted on hooks of platinum wire.

Muscles were perfused with Tyrode solution at 37°C and stimulated at 2 and 6 Hz stimulation frequencies through the platinum clamps (5 V, 0.1 ms duration). Force was measured with an isometric force transducer (model OPT1L, Scientific Instruments, Heidelberg, Germany) and recorded on a strip chart recorder. Muscles were stretched over a period of 30 to 60 min to the length at which active force development was maximal (Lmax). Forces (in mN)
were normalized by the muscle cross-sectional areas to yield stresses (in mN/mm²). The cross-sectional area was calculated for each muscle as the ratio of muscle volume (determined by weighing) to muscle length at \( L_{\text{max}} \).

Time to peak tension was determined as the time from 10% of maximum developed tension to the peak of contraction. The relaxation times, \( RT_{10} \) and \( RT_{90} \), were determined as the time from the peak of contraction to 50 or 90% of tension decline (50 or 10% of remaining tension) during relaxation.

2.6. Quantification of compensation

Hypothyroidism-induced delays in contraction and relaxation (in ms) were determined by comparing results from hypothyroid and euthyroid wild-type mice. Transgene-induced accelerations of contraction and relaxation were determined by comparing results from wild-type and transgenic hypothyroid mice. Compensation was calculated as the ratio of transgene-induced acceleration to hypothyroidism-induced delay (Table 1).

2.7. Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA). Twitch parameters were analyzed with repeated measures ANOVA with combined data from 2 and 6 Hz stimulation frequencies. Northern and Western blot data were analyzed by one-factor ANOVA. Post-hoc comparisons between multiple groups were performed in each case by a Student–Newman–Keuls test. Data are presented as mean ± standard error. Statistical significance was assumed for \( P<0.05 \).

3. Results

3.1. Gene expression

In order to examine the effects of hypothyroidism on the expression of SERCA2a and myosin heavy chain \( \beta \) in wild-type and SERCA2a transgenic animals, we used Northern blot analysis to determine the respective levels of mRNA from seven euthyroid wild-type, six euthyroid transgenic, seven hypothyroid wild-type and seven hypothyroid transgenic mice. Serum \( T_4 \) levels ranged from 0 to 0.6 ng/dL in hypothyroid mice and from 2.7 to 5.0 ng/dL in euthyroid mice. Representative Northern blots are shown in Fig. 1. Myosin heavy chain \( \beta \) expression was absent in euthyroid mice, but strongly induced in both wild-type and transgenic mice that were made hypothyroid. SERCA2a expression was decreased by hypothyroidism and increased by SERCA2a transgene expression.

Quantification of mRNA analysis for SERCA2a is shown in Fig. 2. SERCA2a mRNA was increased by 52% in transgenic euthyroid mice compared with wild-type euthyroid mice (\( P<0.05 \)). Hypothyroidism decreased SERCA2a mRNA by 36% in wild-type mice (\( P<0.05 \)). However, in SERCA2a transgenic animals, hypothyroidism decreased SERCA2a mRNA only by 25% (euthyroid transgenic vs. hypothyroid transgenic, \( P<0.05 \)), and there was no significant difference between SERCA2a mRNA levels of hypothyroid transgenic and euthyroid wild-type mice. Expression of the SERCA2a transgene compensated therefore for the loss of endogenous SERCA2a gene expression with hypothyroidism.

Western blot analysis was used to compare SERCA2a protein expression from wild-type mice and from hypothyroid transgenic mice (Fig. 3). We observed a 26% reduction in SERCA2a protein expression with hypothyroidism in wild-type mice (\( P<0.05 \)). However, this was partially compensated for in hypothyroid transgenic mice, where SERCA2a protein levels were reduced by only 18% (\( P<0.05 \)).

3.2. Papillary muscle experiments

Contractile function was measured in papillary muscles
Fig. 3. Western blot analysis of SERCA2a protein (top) and quantification of normalized protein levels ($n=3$ in each group, bottom). SERCA2a protein reduced with hypothyroidism in wild-type animals and was higher in hypothyroid transgenic mice than in hypothyroid wild-type mice.

from wild-type mice and from mice expressing a SERCA2a transgene. In our earlier study [5], we showed that overexpression of SERCA2a resulted in accelerated relaxation in vivo, in papillary muscles and isolated myocytes, and in an accelerated decline of intracellular calcium transients. Here, we analyzed the effects of hypothyroidism on contractile function in wild-type and SERCA2a transgenic mice.

Six wild-type mice and six SERCA2a transgenic mice were injected with radioactive iodine. At four weeks following injection, one transgenic mouse had a normal serum T$_4$ level (2.9 ng/dL) and was hence excluded from the analysis. The other eleven mice became markedly hypothyroid, with serum T$_4$ levels ranging from 0.1 to 0.7 ng/dL. Fourteen euthyroid wild-type mice and fourteen euthyroid SERCA2a transgenic mice were used for comparison, with serum T$_4$ levels ranging from 2.5 to 6.5 ng/dL. The papillary muscles isolated from the 39 mice had a length of 2.04±0.08 mm and a cross-sectional area of 0.40±0.03 mm$^2$, with no significant differences between groups.

We measured the time to peak tension of left ventricular papillary muscles to determine the effects of SERCA2a transgene expression and of hypothyroidism on the time course of active force development (Fig. 4). In papillary muscles from euthyroid mice, time to peak tension was unaffected by SERCA2a transgene expression. Hypothyroidism produced a significant prolongation of the time to peak tension, but this prolongation was more pronounced in wild-type mice than in SERCA2a transgenic mice, i.e., SERCA2a transgene expression significantly restored the speed of contraction of hypothyroid mice. However, this compensation was only partial, since time to peak tension of muscles from hypothyroid transgenic mice was still significantly increased compared with that of euthyroid controls.

We measured the relaxation time, RT$_{50}$, of isolated papillary muscles to determine the effects of altered SERCA2a expression on cardiac relaxation in hypothyroidism of wild-type and SERCA2a transgenic mice (Fig. 5).

Fig. 4. Time to peak tension of isolated left ventricular papillary muscles. Twitch parameters were determined at 2 and 6 Hz stimulation frequencies, as described in Section 2. Statistical comparisons between all groups were made by repeated measures analysis of variance with combined data at 2 and 6 Hz. Relevant comparisons are indicated in the Figure (n.s., not significant).
In euthyroid mice, SERCA2a transgene expression slightly but significantly reduced RT_{50}. Muscles from hypothyroid wild-type mice had significantly increased relaxation times, RT_{50}, compared with those of euthyroid wild-type mice. However, this delay in relaxation was compensated for in hypothyroid transgenic mice, and RT_{50} in this group was not significantly different from the RT_{50} of muscles from euthyroid wild-type mice.

We further measured the relaxation time, RT_{90}, (to 90% of tension decline), which includes also the late phase of relaxation (Fig. 6). The changes in RT_{90} were qualitatively similar to the changes in RT_{50}: a significant acceleration of relaxation with transgene expression in both euthyroid and hypothyroid mice, and a significant delay in hypothyroid mice compared with euthyroid mice. The relaxation times, RT_{90}, of muscles from hypothyroid transgenic mice were not significantly different from those of euthyroid wild-type mice.

The functional compensation of SERCA2a transgene expression in hypothyroidism is quantified and summarized in Table 1. While the impaired relaxation was mostly (RT_{50}) or fully (RT_{90}) compensated for, the prolonged contraction (time to peak tension) was only partially compensated for.

### 4. Discussion

Prolonged cardiac contraction and relaxation are prominent manifestations of hypothyroidism. These prolongations are mediated in part through decreased sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2a) expression. The murine model of hypothyroidism presented in this study reveals decreased SERCA2a mRNA levels as well as prolonged cardiac contraction and relaxation of isolated papillary muscles from wild-type mouse. However, when transgenic mice overexpressing SERCA2a were made hypothyroid, SERCA2a mRNA was not different from that of wild-type euthyroid mice. Contraction and relaxation

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\(^{a}\) RT_{50}, relaxation time to 50% of tension decline. ‘Prolongation with hypothyroidism’ compares euthyroid wild-type mice with hypothyroid wild-type mice. ‘Acceleration with transgene’ compares hypothyroid wild-type mice with hypothyroid transgenic mice. ‘Compensation’ was determined by the ratio of the acceleration to the prolongation. RT_{90}, relaxation time to 90% of tension decline. TPT, time to peak tension.
were faster in hypothyroid transgenic mice than in hypothyroid wild-type mice, i.e., SERCA2a overexpression accelerated the prolonged time courses normally observed in hypothyroidism. Therefore, our results provide a promising example of a transgene-mediated functional improvement of a pathophysiologic cardiac condition.

Prolonged cardiac contraction and relaxation with hypothyroidism have been shown in several species, including the mouse [7]. Induction of myosin heavy chain β has been identified as a contributing factor in both the rat [9] and the mouse [8]. In addition, decreases in SERCA2a mRNA [10] and protein [1] have been shown in the rat. We have shown in this study that a decrease in SERCA2a mRNA concurs with prolonged contraction and relaxation in papillary muscles from hypothyroid mice.

Induction of myosin heavy chain β and reduction of endogenous SERCA2a are not expected to contribute equally to the delays in contraction and relaxation observed in hypothyroidism. SERCA2a overexpression, for example, did not alter the time to peak tension in euthyroid mice but affected primarily the speed of relaxation. On the other hand, changes in myosin isoforms have marked effects on the speed of contraction. Nevertheless, contraction and relaxation and their underlying molecular mechanisms are not entirely independent of each other. In contrast to the euthyroid animals, overexpression of SERCA2a did accelerate the prolonged tension development of hypothyroid mice, possibly through increased SR Ca²⁺ loading and accelerated release of Ca²⁺ from the SR. The fact that this compensation of time to peak tension was incomplete is consistent with the assumption that myosin isoforms are an important determinant of the speed of contraction. On the other hand, SERCA2a is the most important regulator of the speed of relaxation. Therefore, the more complete compensation of relaxation times is consistent with the full compensation of SERCA2a gene expression, as revealed by Northern blot analysis.

The effects of hypothyroidism and of SERCA2a transgene expression were not related to developmental changes or changes in other genes related to calcium handling (except for myosin heavy chain β). Animals were at least two to three months old at the onset of hypothyroidism. SERCA2a transgene expression is sustained throughout the life span of the animals. In earlier studies, the contractile phenotype of SERCA2a transgenic mice was unchanged with age for mice between two and eleven months of age (data not shown). In our previous study that characterized the SERCA2a transgenic mice [5], we found no changes in mRNA levels of ryanodine and calsequestrin and no significant changes in protein levels of phospholamban and the Na⁺/Ca²⁺-exchanger. Furthermore, two recent studies that investigated protein levels of calsequestrin, phospholamban, actin and tropomyosin in mice overexpressing SERCA1a [15] as well as phospholamban and actin in mice overexpressing SERCA2a [16] found no differences.

The compensation of impaired cardiac function in hypothyroidism with SERCA2a transgene expression points to potential therapeutic benefits of SERCA2a transgene expression. However, due to the transgenic animal approach used in this study, transgene expression was already present prior to the onset of hypothyroidism. As a result, there were already small differences in relaxation time in the euthyroid animals. However, these differences were larger in the hypothyroid animals. Furthermore, there was no difference in time to peak tension in the euthyroid animals, but the transgene corrected for about half of the considerable prolongation in contraction in the hypothyroid animals. These considerations underline that our results indicate indeed a compensatory function of the transgene under hypothyroid conditions rather than merely a similar, and perhaps expected, phenotype in both euthyroid and hypothyroid animals.

A definitive ‘rescue’ of a hypothyroid phenotype could only be documented by an actual gene therapy approach consisting of a transgene-induced correction of impaired function following the onset of hypothyroidism. Currently, these techniques are not readily available in the mouse, but advances in gene delivery to the heart or improved methods of conditional transgene expression may soon offer additional avenues.

Genetically engineered animals offer exciting opportunities for studying the molecular mechanisms of pathophysiologic conditions and for exploring possible gene therapy approaches to cardiovascular diseases [17,18]. The prevalence of genetically altered mice has motivated the development of experimental techniques for characterizing the murine cardiac phenotype. Despite the undisputed importance of in vivo measurements [19–21] for characterizing integrated physiological responses, much insight into fundamental mechanisms may be obtained from in vitro methods ranging from the isolated cardiac myocyte [22,23] to the isolated working heart [7].

Isolated cardiac muscle preparations such as papillary muscles or trabeculae have greatly contributed to our understanding of cardiac physiology in many species. In the mouse, however, there are only a very limited number of reports from isolated left atrial [24], right ventricular [25] or left ventricular [26] preparations. In a recently published study [5], we examined for the first time the contractile function of isolated left ventricular papillary muscles from a transgenic mouse and found enhanced relaxation in muscles overexpressing the sarcoplasmic reticulum Ca²⁺-ATPase (SERCA2a). In this report, we have applied this model to the study of the cardiac manifestations of hypothyroidism [6]. The technique should be of further interest to other investigators for characterizing cardiac contractile phenotypes in the mouse, especially when time courses of contraction and relaxation or frequency-dependent behaviors are of primary interest.

In summary, we have shown decreased SERCA2a gene expression and prolonged cardiac contraction and relaxation in a murine model of hypothyroidism, and have
demonstrated the feasibility of a transgene-mediated functional improvement.

Acknowledgements

This study was supported by US Public Health Service grants DK-07494 (Dr. Bluhm), R01-HL-25022 (Dr. Dillmann) and R01-HL-52946 (Dr. Dillmann) from the National Institutes of Health, and by grant Me 1477/2-1 from the Deutsche Forschungsgemeinschaft (Dr. Meyer). We would like to thank Michele Bluhm for her assistance with the manuscript.

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