Decreased cardiac SERCA2 expression, SR Ca uptake, and contractile function in hypothyroidism are attenuated in SERCA2 overexpressing transgenic rats

Roland Vetter,1 Uwe Rehfeld,1 Christoph Reissfelder,1 Henry Fechner,2,3 Enn Seppet,4 and Reinhold Kreutz1
1Institute of Clinical Pharmacology and Toxicology, and 2Department of Cardiology and Pneumology, Charité—Universitätsmedizin Berlin, Berlin; 3Institute of Biotechnology, University of Technology, Berlin, Germany; and 4Department of Pathophysiology, Institute of General and Molecular Pathology, Faculty of Medicine, University of Tartu, Tartu, Estonia

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Vetter R, Rehfeld U, Reissfelder C, Fechner H, Seppet E, Kreutz R. Decreased cardiac SERCA2 expression, SR Ca uptake, and contractile function in hypothyroidism are attenuated in SERCA2 overexpressing transgenic rats. Am J Physiol Heart Circ Physiol 300: H943–H950, 2011. First published January 7, 2011; doi:10.1152/ajpheart.00490.2010.—The sarcoplasmic reticulum (SR) Ca2+-ATPase SERCA2a has a key role in controlling cardiac contraction and relaxation. In hypothyroidism, decreased expression of the thyroid hormone (TH)-responsive SERCA2 gene contributes to slowed SR Ca2+ reuptake and relaxation. We investigated whether cardiac expression of a TH-insensitive SERCA2a cDNA minigene can rescue SR Ca2+ handling and contractile function in female SERCA2a-transgenic rats (TG) with experimental hypothyroidism. Wild-type rats (WT) and TG were rendered hypothyroid by 6-N-propyl-2-thiouracil treatment for 6 wk; control rats received no treatment. In vivo measured left ventricular (LV) hemodynamic parameters were compared with SERCA2a expression and function in LV tissue. Hypothyroidism decreased LV peak systolic pressure, dP/dtmax, and dP/dtmin in both WT and TG. However, loss of function was less in TG. Thus slowed relaxation in hypothyroidism was found to be 1.5-fold faster in TG compared with WT (P < 0.05). In parallel, a 1.4-fold higher Vmax value of homogenate SR Ca2+ uptake was observed in hypothyroid TG (P < 0.05 vs. hypothyroid WT), and the hypothyroidism-caused decline of LV SERCA2a mRNA expression in TG by −24% was markedly less than the decrease of −49% in WT (P < 0.05). A linear relationship was observed between the SERCA2a/PLB mRNA ratio values and the Vmax values of SR Ca2+ uptake when the respective data of all experimental groups were plotted together (r = 0.90). The data show that expression of the TH-insensitive SERCA2a minigene compensates for loss of expression activity of the TH-responsive native SERCA2a gene in the female hypothyroid rat heart. However, SR Ca2+ uptake and in vivo heart function were only partially rescued.

sarcoplasmic reticulum; Ca2+-ATPase; female; thyroid hormone; atrophy

THE SARCO-/ENDOPLASMIC RETICULUM Ca2+ pump SERCA2a has a key role in Ca2+ regulation during the contraction-relaxation cycle of the myocardium. The SERCA2a-mediated transport of Ca2+ from the cytosol of the cardiomyocyte to the lumen of the sarcoplasmic reticulum (SR) is the major mechanism for lowering the cytosolic Ca2+ concentration during muscle relaxation. Decreases in SERCA2a protein levels and/or its Ca2+-transporting activity contribute to impaired cardiac function in a variety of pathophysiological conditions, such as diabetes (13, 39), inflammation (12, 40), left ventricular (LV) hypertrophy/failure (1, 18, 32), and hypothyroidism (2, 7). In the latter, a characteristic feature of LV dysfunction is slowed contraction and relaxation. This particularly has been demonstrated using animal models with 6-N-propyl-2-thiouracil (PTU)-induced hypothyroidism (4, 5, 9). Altered cardiac relaxation in hypothyroidism is associated with abnormal cellular Ca2+ handling (3, 6, 22). In particular, the SERCA2a-catalyzed removal of cytosolic Ca2+ into the lumen of the SR is defective (4, 6, 7, 20, 34). A major underlying mechanism for this defect is reduced expression of the SERCA2a gene (4, 7, 20, 34), which is thyroid hormone (TH) controlled (16). In addition, altered abundance and phosphorylation of the negative SERCA2a-regulatory protein phospholamban (PLB) may also contribute to this effect (7, 20, 34).

Evidence has been provided that the diminished expression of the cardiac SR Ca2+-ATPase due to hypothyroidism can be restored by correcting the hormonal disorder by using TH therapy (7, 34). In addition, interventions directed specifically at increasing SERCA2a expression without influencing the hormonal status of hypothyroid animals appear to be beneficial. Thus Bluhm et al. (4) have reported that cardiac expression of a TH-insensitive SERCA2a minigene in a transgenic (TG) mouse line with PTU-induced hypothyroidism compensated for loss of SERCA2a molecules encoded by the TH-sensitive endogenous SERCA2 gene, and this prevented the typical slowdown of contraction and relaxation. The latter finding relies mainly on a comparative analysis of load-independent time parameters, which were determined in experiments with isometrically contracting isolated papillary muscle from mice (4). So far, hemodynamic effects of TG SERCA2a expression in the hypothyroid heart have not been studied in vivo. Furthermore, caution is merited in extrapolating results obtained in mice directly to other species with experimental hypothyroidism due to specific features of Ca2+ cycling and contractile activation in mouse cardiac muscle (14).

We, therefore, set out to investigate whether LV function could be rescued in SERCA2a TG rats (39) with experimental PTU-induced hypothyroidism. Furthermore, to examine whether TG SERCA2a expression preserves the SERCA2a-catalyzed Ca2+ transport function of the cardiac SR in experimental hypothyroidism, the Ca2+-transporting activities of the SR together with the SERCA2a steady-state mRNA and protein

Address for reprint requests and other correspondence: R. Vetter, Inst. of Clinical Pharmacology and Toxicology, CCM, CharitéCenter 04 for Therapeutic Research, Charité—Universitätsmedizin Berlin, Charitéplatz 1, D-10117 Berlin, Germany (e-mail: roland.vetter@charite.de).

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levels were compared in euthyroid and hypothyroid SERCA2a TG and wild-type (WT) female rats.

MATERIALS AND METHODS

Animal model. The investigation conforms to institutional guidelines for care and use of laboratory animals and the German Animal Protection Law (approval no. G 0054/00, Landesamt für Gesundheit und Soziales, Berlin). We used a previously generated and described SERCA2a overexpressing TG rat line carrying in addition to the endogenous SERCA2 gene a rat SERCA2a minigene driven by human cytomegalovirus enhancer chicken β-actin promoter (39). The SERCA2a minigene included the first exon, first intron, second exon, second intron, and part of the third exon to which the rat SERCA2a cDNA was ligated. This construct has also been used for generation of the SERCA2a TG mice line that was used in the work of Bluhm et al.(4).

Female WT and heterozygous SERCA2a TG rats weighing 250 g were made hypothyroid with administration of 0.05% PTU (Sigma-Aldrich, Deisenhofen, Germany) in drinking water for 6 wk. Euthyroid control rats received drinking water without the thyrostatic drug. Hypothyroid states of WT and TG rats were confirmed by serum T4 levels were 6.6 ± 0.4 μg/dl in euthyroid WT, and <0.4 μg/dl in both hypothyroid WT and TG, respectively. Six weeks after the treatment began, hemodynamic measurements were performed followed by the removal of the heart and other tissue samples.

Hemodynamic measurements. To measure LV functional parameters, the animals were anesthetized with a mixture of ketamine 40 mg/kg body wt (Ketanest, Parke Davis, Berlin, Germany) and xylazine 12 mg/kg body wt (Rompun Bayer, Leverkusen, Germany) administered intraperitoneally. Anesthesia at the given dosage lasted ~45 min. After reaching the tolerance stage, the animals were endotracheally intubated and ventilated with room air using a rodent respirator (Hugo-Sachs-Elektronik, March-Hugstetten, Germany). Ventilation volume was 100–120 ml/min, ventilation rate 60 breaths/min. To measure pulse rate and aortic blood pressure, a 3-Fr Millar tip catheter was advanced via the right carotid artery into the ascending aorta. Then, left-sided thoracotomy was performed at the level of the apex of the heart. The Millar tip catheter removed from the carotid artery was introduced into the left ventricle via the apex to measure LV peak systolic pressure (LVPSP), LV end-diastolic pressure, and maximum rates of pressure rise (dP/dtmax) and decay (dP/dtmin). This protocol was used due to difficulties to advance the catheter into the left ventricle of hypertrophic animals without damaging the aortic valve. The pressure recording system consisted of a computer-attached TSE amplifier with special TSE interface (TSE, Bad Homburg, Germany). The system was equipped with data acquisition and analysis software BioSys (TSE). A sampling rate of 0.4 kHz was used for data recording. After completion of the hemodynamic measurements, a blood sample was collected via apex puncture. Thereafter, the heart of the anesthetized animal was quickly excised and immediately immersed in an ice-cold solution containing 130 mM NaCl, 30 mM KCl, and 10 mM histidine (pH 7.4). Tissue samples were blotted and shock-frozen in liquid nitrogen. Frozen tissue samples were stored at −80°C until used for preparation of RNA or homogenates.

Isolation of RNA and Northern blot analysis. Total cellular RNA was isolated from LV myocardium using TRIzol Reagent (GIBCO-BRL, Life Technology, Grand Island, NY). The integrity of the RNA was checked by agarose gel electrophoresis using ethidium bromide staining. Steady-state expression levels of rat SERCA2a, PLB, β-actin, and glycolaldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were detected by Northern dot blot analysis of total mRNA from rat heart. Dot blot analysis was performed after confirmation that each of the respective probes hybridized specifically to its mRNA and showed a single band (SERCA2a, GAPDH, β-actin) or three specific bands (PLB) on Northern blots. Total RNA was denatured in 50% formaldehyde, and 1× standard saline citrate solution by heating at 65°C for 15 min and cooling on ice. RNA samples (1.5 μg and 3 μg per dot) were loaded onto the nitrocellulose filter of a dot blot microfiltration apparatus (Bio-Rad Laboratories, München, Germany). The same amounts of tRNA were also loaded to detect nonspecific binding of probes. The nylon membrane (Amersham Buchler, Braunschweig, Germany) was hybridized with 32P-labeled cDNA probes as described earlier for Northern blotting (7, 39). To characterize the SERCA2a transcript expression, a 1.6-kb EcoRI-EcoRI I restriction fragment corresponding to the 5′-end of the rat SERCA2a cDNA was used. Blots were also hybridized with a 1.2-kb Pst I-Pst I chicken GAPDH fragment, a β-actin PCR probe, and a 0.7-kb EcoRI-I-BamHI I PLB fragment as described earlier for Northern blotting (7). The fragments were labeled with [32P]dCTP by using a multiprim type DNA labeling kit (Stratagene, La Jolla, CA). The radioactivity associated with the individual mRNA dots was quantified using a bio-imaging analyzer (model BAS 2000, Fuji). For normalization of the obtained signal, the optical density measured using the SERCA2a probe was divided by the optical density obtained by using either GAPDH, β-actin, or PLB.

Preparation of cardiac homogenates and membranes. LV homogenates were prepared from tissue samples that were initially shock-frozen in liquid nitrogen and stored at −80°C. Tissue was first powdered in liquid nitrogen, and homogenates were prepared by Polytron homogenization using an ice-cold phosphoprotein protection buffer as described earlier (7). Crude membrane preparations were isolated at 4°C from powdered ventricular tissue of single hearts essentially as described previously (7). Final membrane samples were shock-frozen in liquid nitrogen and stored at −80°C until use. Crude cardiac membranes isolated according to this protocol comprised ~30% of total ventricular protein and showed a similar enrichment of membranes in all experimental groups. Protein was determined according to Lowry’s method by using bovine serum albumin as standard after incubating samples in 1 M NaOH for 30 min as in Ref. 7.

Western blot analysis. For Western blotting, 100 μg of homogenate or membrane protein was solubilized either for 15 min at 37°C or for 5 min at 95°C in either 2% SDS, 50 mM H2PO4, 5 mM EDTA, 10% glycerol, 2 mM diethiothreitol, 0.04% bromphenol blue or 2% SDS, 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2 mM diethiothreitol, 0.04% bromphenol blue. Proteins were separated by SDS-PAGE according to Swank and Munkres (35) (total monomer concentration 13.8%, cross-linking monomer concentration 3.2% for SERCA2a and PLB) or Laemmli (total monomer concentration 7.0%, cross-linking monomer concentration 2.67% for Na-Ca exchanger, NCX; and β-myosin heavy chain, β-MHC) using 1.5-mm-thick mini gels. Proteins of four gels were transferred onto PVDF membranes (Roche, Mannheim, Germany) simultaneously in a wet transfer apparatus (PerfectBlue Tank-Elektroblotter WebS, PEQLAB Biotechnology, Erlangen, Germany) for 90–105 min at 20°C and constant voltage of 100 V. Membrane blocking and washing was done as described earlier (7). For detecting the SR Ca2+-ATPase, PLB, NCX mouse monoclonal anti-SERCA2 (clone 2A7-A1, 1:1,000 dilution; ABR), anti-PLB (clone A1, 1: 2,000 dilution; Millipore), and anti-NCX (R3F1, 1:3,000 dilution; Swant) were used as reported previously (7). The monoclonal anti-myosin (skeletal, slow) antibody (clone NOQ7.5.AD, 1:10,000 dilution, Sigma) was used to detect β-MHC. Anti-GAPDH (clone 6C2, 1:10,000 dilution; ABR) primary antibody was used to detect GAPDH as a homogenate protein loading control. The signal from horseradish peroxidase-conjugated affinity purified goat anti-mouse (H+L)IgG (Dianova) used as secondary antibody was developed using either Super Signal West Pico or Super Signal West Duro Extended Duration substrates (Thermo Scientific). The signals were collected in a Fusion FX7 detection system (PEQLAB Biotechnology, Erlangen, Germany) and quantified by densitometry analysis using Bio-ID software (version 12.12; PEQLAB Biotechnology). Determined optical densities were considered to reflect the relative amounts of the detected proteins.
**RESULTS**

**Experimental animals.** As shown in Table 1, treatment with PTU resulted in diminished body and heart weight in both hypothroid WT and hypothroid SERCA2a TG female rats. The observed decrease in the LV to body weight indicates LV atrophy in these animals. However, the degree of atrophi was similar in WT and TG rats as indicated by a similar reduction of LV wet weight by 30% in WT and by 29% in TG. Tibia length did not differ significantly between the groups. The measured heart rate values were found to be low in all experimental groups due to anaesthesia. Irrespective of the latter condition, the heart rate of hypothroid WT and hypothroid TG was ~20% lower than in the respective euthyroid controls. This was associated with lowered systolic and diastolic aortic blood pressure values in hypothroid animals. Although aortic blood pressure decline was smaller in hypothroid SERCA2a TG (Table 1), the relative differences in decline of systolic and diastolic were similar.

**Northern blot and dot blot analysis of LV total RNA revealed that the steady-state**

![Table 1. General characteristics of wild-type and SERCA2-transgenic rats without and with hypothyroidism](http://ajpheart.physiology.org/DownloadedFrom)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Euthyroid</th>
<th>Hypothyroid</th>
</tr>
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<tbody>
<tr>
<td>BW, g</td>
<td>293 ± 7</td>
<td>283 ± 5</td>
</tr>
<tr>
<td>Tibia length, mm</td>
<td>39.6 ± 0.3</td>
<td>39.6 ± 0.4</td>
</tr>
<tr>
<td>HW, mg</td>
<td>954 ± 21</td>
<td>1012 ± 33</td>
</tr>
<tr>
<td>LV + S, mg</td>
<td>663 ± 14</td>
<td>685 ± 22</td>
</tr>
<tr>
<td>HW/BW, mg/g</td>
<td>3.26 ± 0.04</td>
<td>3.58 ± 0.11</td>
</tr>
<tr>
<td>LV+/S/BW, mg/g</td>
<td>2.27 ± 0.03</td>
<td>2.42 ± 0.08</td>
</tr>
<tr>
<td>RV/BW, mg/g</td>
<td>0.71 ± 0.02</td>
<td>0.82 ± 0.04</td>
</tr>
<tr>
<td>A/BW, mg/g</td>
<td>0.30 ± 0.02</td>
<td>0.34 ± 0.02</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>122.1 ± 3.6</td>
<td>116.7 ± 4.4</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>85.5 ± 4.0</td>
<td>86.4 ± 4.6</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>4.1 ± 0.1</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>253.8 ± 4.2</td>
<td>249.9 ± 8.1</td>
</tr>
</tbody>
</table>

Values are means ± SE of 12 euthyroid and 15 hypothyroid female rats per group. WT, wild-type rats; TG, SERCA2-transgenic rats; BW, body wt; HW, whole heart wet wt; LV + S, left ventricular + septum wet wt; RV, right ventricular wet wt; A, weight of left plus right atrium; SBP and DBP, systolic and diastolic blood pressure, respectively, measured in the ascending aorta of anesthesized animals; LVEDP, left ventricular end-diastolic pressure. *Significant difference vs. euthyroid WT ($P < 0.05$). †Significant difference vs. euthyroid TG ($P < 0.05$).

**Measurements of Ca$^{2+}$ transport.** Initial rates of SR oxalate-supported Ca uptake were estimated in LV homogenates by a standard procedure (7). The reaction medium contained 40 mM imidazole (pH 7.0), 100 mM KCl, 5 mM MgCl$_2$, 5 mM Tris-ATP, 6 mM phosphocreatine, 10 mM K-oxalate, 0.2 mM EGTA, 10 mM NaN$_3$, 0.025 to 0.25 mM $^{45}$CaCl$_2$ (1.9 x 10$^{11}$ Bq/mol), 2 μM synthetic protein kinase A inhibitor peptide [PKI(6-22)amide] (GIBCO, Life Technologies, Eggenstein, Germany), and 30 μg of homogenate protein per 0.2 ml. The free Ca$^{2+}$ concentration was calculated using Fabiato's computer program as described elsewhere (7). After 2 min of preincubation at 37°C, the duplicate measurement was started by addition of protein. After 2 min, a 0.15-ml sample was filtered through a 0.45-μm membrane filter ME25 (Schleicher & Schuell) using a vacuum pump. Filters were then washed twice with 3 ml of ice-cold solution containing 100 mM KCl, 2 mM EGTA, and 40 mM imidazole (pH 7.0). Radioactivity associated with the dry filters was determined by liquid scintillation counting. Solutions for Ca$^{2+}$ transport measurements were made with deionized water, pa (Merck, Darmstadt, Germany); contaminant Ca$^{2+}$ did not exceed 0.005 mg/l. The $^{45}$CaCl$_2$ was obtained from Amersham Buchler (Braunschweig, Germany).

**Statistical analysis.** Data are presented as means ± SE. Statistical analysis was performed by Kruskal-Wallis one-way ANOVA. When analysis was performed by Kruskal-Wallis one-way ANOVA. When applicable, pairwise comparisons following ANOVA were made using the Student-Newman-Keuls test. In case of unequal group size, pairwise comparisons following ANOVA were made using the Dunn’s test. Statistical significance was set at $P < 0.05$.
level of SERCA2a mRNA (Fig. 1) was elevated in female euthyroid SERCA2a TG rats by 51% \( (P < 0.001 \text{ vs. euthyroid WT}) \). In hypothyroid WT, the expression of SERCA2a mRNA was reduced by 49%, whereas this decline amounted to only 24% in hypothyroid TG \( (P < 0.001 \text{ vs. respective euthyroid controls}) \). The hypothyroid TG animals showed a 2.3-fold higher SERCA2a mRNA level than the hypothyroid WT \( (P < 0.001) \), and the SERCA2a mRNA abundance of the latter did not differ from that in euthyroid WT (Fig. 1, A and B). Similar differences between the experimental groups were also obtained when the respective SERCA2a/PLB mRNA ratios were compared (Fig. 1C). Different dot blots hybridized with either β-actin or GAPDH probes did not show significant alterations in the mRNA levels of hypothyroid WT and SERCA2a TG compared with their euthyroid equivalents. This holds also partially true for PLB mRNA levels, which were found to be only slightly but not significantly elevated in hypothyroid WT only (data not shown).

**Cardiac protein expression.** Figure 2 shows that, in non-TG WT, hypothyroidism caused a decrease of cardiac SERCA2a protein by 19% \( (P < 0.05 \text{ vs. euthyroid WT}) \). There was also a marked significant decrease of SERCA2a protein expression in hypothyroid SERCA2-TG. However, the resulting SERCA2a protein abundance of the latter was 16% higher than in hypothyroid WT \( (P < 0.05) \). By contrast, PLB levels were moderately increased in hypothyroid hearts of both WT and TG (Fig. 2). As a consequence, the SERCA2a-to-PLB ratio of hypothyroid TG exceeded that of hypothyroid WT rats by 21%.

Experimental hypothyroidism also caused typical increases in the abundance of non-reticular proteins such as the sarcoplasmal NCX and the myofibrillar β-MHC, but these changes did not differ between WT and TG (Fig. 2).

**SR Ca\(^{2+}\) transport activity.** In hypothyroid TG and WT, the reticular Ca\(^{2+}\) transport rates measured at different free Ca\(^{2+}\) concentrations were markedly depressed compared with the respective values of euthyroid animals (Fig. 3). In particular, the maximum rates \( (V_{max}) \) of Ca\(^{2+}\) uptake were significantly decreased by 54% and 52%, respectively, in hypothyroid TG and WT rats (Fig. 3; Table 2). Although the TG hypothyroid hearts displayed close to normal WT SERCA2a mRNA and protein levels, the rates of SR Ca\(^{2+}\) uptake of the former did not reach the normal rate values of euthyroid WT. However, the \( V_{max} \) value of SR Ca\(^{2+}\) uptake of SERCA2a overexpressing hypothyroid TG was 1.4-fold higher than that in hypothyroid WT.

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Fig. 2. Relative levels of SERCA2a, phospholamban (PLB), Na-Ca exchanger (NCX), and β-myosin heavy chain (βMHC) protein in left ventricular myocardium of euthyroid and hypothyroid WT and SERCA2a-TG rats. A: Western blots for SERCA2a (100-kDa signals) plus PLB (6.5- and 27-kDa signals), NCX (120-kDa signals), and β-MHC (200-kDa signals) are shown for one representative animal of each experimental group. Membrane protein per lane: 20 μg for SERCA2a and PLB; 30 μg for NCX; 3 μg for β-MHC. B: bar graphs show the relative immunoreactive protein levels of SERCA2a, PLB, NCX, and β-MHC in left ventricular myocardium of euthyroid and hypothyroid WT and TG of 6–7 rats in each group. Values are means ± SE. *Significant difference vs. respective euthyroid controls \( (P < 0.05) \). †Significant difference vs. hypothyroid WT \( (P < 0.05) \).
Hypothyroidism caused a marked decrease in the developed LV peak systolic pressure (LVPSP), the pressure-rate product, as well as the maximum rates of pressure rise (dP/dt\text{max}) and decay (dP/dt\text{min}) in both open-chest, anaesthetized WT and TG harboring the SERCA2a minigene. The hypothyroidism-caused depression of the LV systolic and diastolic function was markedly attenuated in hypothyroid animals expressing the SERCA2a minigene. The maximum rate of pressure decay of hypothyroid TG, although markedly depressed compared with euthyroid controls, was 52% higher than that of hypothyroid WT (P < 0.05).

Table 2. Kinetic characteristics of oxalate-facilitated sarcoplasmic reticulum Ca\textsuperscript{2+} uptake in left ventricular homogenates of WT and TG with and without hypothyroidism

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>(V_{\text{max}}), nmol Ca\textsuperscript{2+}/mg protein (\cdot) min(^{-1})</th>
<th>EC(_{50}) for Ca\textsuperscript{2+}, (\mu)M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euthyroid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>16.6 ± 0.4</td>
<td>0.49 ± 0.03</td>
</tr>
<tr>
<td>TG</td>
<td>24.8 ± 0.7*</td>
<td>0.51 ± 0.03</td>
</tr>
<tr>
<td>Hypothyroid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>8.0 ± 0.2*</td>
<td>0.50 ± 0.03</td>
</tr>
<tr>
<td>TG</td>
<td>11.3 ± 0.3*</td>
<td>0.51 ± 0.03</td>
</tr>
</tbody>
</table>

Values are means ± SE for 6 hearts in each group. Initial rates of Ca\textsuperscript{2+} uptake were determined at varying free Ca\textsuperscript{2+} concentrations between 0.083 and 3.68 \(\mu\)M. \(V_{\text{max}}\), maximum rate of Ca\textsuperscript{2+} uptake; EC\(_{50}\), free Ca\textsuperscript{2+} concentration required for half-maximal Ca\textsuperscript{2+} uptake. \*Significant difference vs. respective WT value (P < 0.05). †Significant difference vs. euthyroid WT and TG (P < 0.05).

**DISCUSSION**

Prolonged contraction and relaxation are prominent features of impaired cardiac performance in hypothyroidism (4, 15, 21, 22, 24, 28, 30, 31). In experimental hypothyroidism, this is mediated in part through depressed uptake of Ca\textsuperscript{2+} by the SR (7, 21, 22, 30). A decreased expression of the SR Ca\textsuperscript{2+} ATPase SERCA2a leading to reduced abundance of Ca\textsuperscript{2+} pumps in the SR membranes is one of the contributing mechanisms (4, 7, 20, 28, 34). In this work, we corroborate these previous findings by using PTU-treated female rats as an experimental model of hypothyroidism. In fact, the PTU-induced hypothyroid rat model used in this study reveals significantly decreased cardiac SERCA2a mRNA and protein levels, and this concurs with marked decreases in the rates of SERCA2a-catalyzed uptake of Ca\textsuperscript{2+} by the SR and slowed contraction and relaxation.

Our results suggest that expression of the TH-insensitive SERCA2a transgene can functionally substitute, at least partially, for loss of SERCA2a encoded by the endogenous TH-
sensitive SERCA2a gene in the hypothyroid heart. Indeed, in SERCA2a TG hypothyroid rats, the SERCA2a mRNA and protein levels did not differ significantly from those of euthyroid WT. However, although this resulted in a higher SR Ca\(^{2+}\)/H\(^{+}\)-ATPase-mediated Ca\(^{2+}\)/H\(^{+}\) uptake activity in hypothyroid TG compared with non-TG WT with hypothyroidism, complete normalization of the SR Ca\(^{2+}\) transport deficit was not achieved. At present, the reason for that type of limitation is unclear. It is important to consider that the effects of TH are pleiotropic, and, therefore, the TH deficiency may affect several cardiac genes involved in cardiac SR Ca\(^{2+}\) handling and excitation-contraction coupling (for review, see Refs. 6, 10). Thus the interplay between altered expression and/or function of SR proteins other than SERCA2a such as its negative modulator protein PLB and the reticular ryanodine receptor should not be dismissed while interpreting the SR Ca\(^{2+}\) pump- ing capacities in hypothyroid WT and TG myocardium. As shown in Table 2, we observed similar EC\(_{50}\) values for Ca\(^{2+}\) of the SERCA2a-mediated SR Ca\(^{2+}\) uptake for WT and TG hearts. This may suggest that under the experimental settings of the present study neither an altered phosphorylation of PLB (not studied in this work) nor the subtle changes in the PLB protein levels may have contributed to the observed partial improvement of the SR Ca\(^{2+}\) transport in hypothyroid TG hearts.

Our study revealed several interesting aspects on the relationships between TH-dependent changes in Ca\(^{2+}\) handling and contractile functions in rat heart. Along with the confirmation of earlier data that the reduced contractile function of the hypothyroid heart is associated with reduced expression and function of the SR Ca\(^{2+}\)-ATPase (4, 7, 20, 34), the present results provide the first direct experimental evidence for a SERCA2a minigene-mediated improvement of the contraction and relaxation in the heart of hypothyroid rat in vivo. These results indicate that the previously published results of a beneficial effect of TG SERCA2a expression in hyperthyroid murine hearts can be extrapolated to the rat heart irrespective of the well known distinct features of Ca\(^{2+}\) cycling and contractile activation in mouse and rat cardiac muscle (14). Of note, the beneficial changes of SR Ca\(^{2+}\) transport and LV hemodynamics in hypothyroid TG were observed despite the persistence of the hypothyroidism. This indicates that an intervention directed specifically at SERCA2a expression without influencing the overall thyroid status may be beneficial for improving the SR Ca\(^{2+}\) handling and ameliorating the LV contractile dysfunction of the hypothyroid heart. This conclusion is in line with earlier reports in constitutively SERCA2a overexpressing rat (8, 27, 39) and mouse models (19, 38) as well as with results obtained by using other approaches for specifically targeting the SR Ca\(^{2+}\) ATPase (26, 33) to rescue SR Ca\(^{2+}\) handling and myocardial contractile function in diseased states other than hypothyroidism.

An interesting novel finding of this work is that the partial rescue of LV relaxation (dP/dt\(_{\min}\)) in hypothyroid TG, which is linked to an enhanced rate of SERCA2a-catalyzed SR Ca\(^{2+}\) uptake, was considerably stronger than the rescue of contraction (dP/dt\(_{\max}\)). This phenomenon is most likely due to the fact that the SR Ca\(^{2+}\) transport is the major direct player controlling the relaxation process. By contrast, the contraction depends on the SR Ca\(^{2+}\) transport activity indirectly only, because the latter process contributes to the Ca\(^{2+}\) load of the SR and,
hence, influences the amount of Ca$^{2+}$ available for release to activate the contractile machinery. However, other factors that may have contributed to the differential rescue of cardiac relaxation and contraction of hypothyroid SERCA2a TG animals should not be dismissed. In particular, alterations of the contractile proteins at the level of myosin ATPase are of interest in this regard. As shown in Fig. 2B, we found an approximately fourfold increase in β-MHC protein in hypothyroid hearts of both WT and TG compared with respective euthyroid controls. This completely corresponds to earlier observations that the myosin isoenzyme profile is profoundly affected in hypothyroidism due to downregulation of the α-MHC gene that is linked to an increased expression β-MHC in both rat and mouse heart (25, 29). This molecular switch is known to slow the cardiac contraction (11, 17). Note that the found marked increase in β-MHC protein levels in hypothyroid hearts did not differ between WT and TG. This may suggest that an increased SR Ca$^{2+}$ handling in the latter is without any effect on the TH deficiency caused increase in β-MHC expression. On the other hand, the marked expression shift to β-MHC with a very low ATPase activity may have contributed, at least in part, to the less pronounced improvement of the rate of pressure rise in hypothyroid TG compared with the stronger effect of the SERCA2a minigene expression on relaxation (Fig. 5). Most interestingly, despite such a possible limitation, the hypothyroid ventricles of TG exhibited LVSP values similar to those in euthyroid animals and were able to do almost the same amount of work (rate × LVSP) as the euthyroid ventricles of WT and TG (Fig. 5, A and B). These observations allow us to think that a possible increase in the consumption of ATP due to expression of additional SERCA2a molecules coded by the SERCA2 minigene was obviously not associated with a significant energy deficit in hypothyroid TG hearts. Most probably, this can result from an increased economy of ATP utilization due to both the improvement of cellular Ca$^{2+}$ handling and slowed cross-bridge cycling of β-MHC-rich isomyosins with low ATPase activity (11, 17) in the latter animals. Thus it appears that the improvement of SR Ca$^{2+}$ handling by introducing SERCA2a minigene is an energetically favorable treatment for preventing contractile dysfunction in diseased states.

The data in Table 1 show a similar reduction of the LV mass of ~30% in hypothyroid WT and TG, whereas the tibia length did not differ significantly between the experimental groups. This indicates a hypothyroidism-induced cardiac atrophy that is in line with earlier reports for female hypothyroid rat hearts (24, 36). Although this LV atrophy and its underlying mechanism in hypothyroidism was not the goal of our work, the presented data for hypothyroid WT and TG with clearly distinct SR Ca$^{2+}$ handling and LV performance may suggest that neither impaired SR Ca$^{2+}$ handling nor altered cardiac hemodynamics appears to play a role for the observed hypothyroidism-caused cardiac atrophy. By contrast, these animals exhibit a similarly reduced heart rate, which is known to be a major determinant for hemodynamic unloading of the heart in clinical and experimental hypothyroidism. It thus appears that the reduced heart rate is most probably the principal determinant of ventricular mass loss in both hypothyroid WT and TG animals.

It should be noted that the performance of the hemodynamic measurements in open-chest animals with deep anaesthesia may be a limitation of the present work. In fact, the peak LV systolic pressure values of all experimental groups were ~20 mmHg lower than the values of aortic systolic pressure measured just before thoracotomy. The use of the above-described hemodynamic protocol together with the application of cardiodpressive anaesthetics appears to be the most likely reason that the measured maximum rates of pressure rise and fall were only approximately one-third of the values usually observed using with the Millar tip catheter technique in vivo. Irrespective of this limitation, the used protocol allowed a reasonable detection of hemodynamic differences between the experimental groups reported in this work.

In summary, our data show that constitutive expression of the TH-insensitive SERCA2a minigene in the female rat heart compensates for loss of expressionional activity of the TH-responsive native SERCA2 gene. This resulted in partial rescue of SR Ca$^{2+}$ uptake and in vivo heart function. Because of the pleiotropic effects of TH, a complete restoration of normal heart function in TH-deficient states appears to be achievable only by therapeutic interventions that are capable of restoring normal TH status of the heart by either TH substitution therapy of the whole organism (7, 23, 34) or a cardiac-specific elevation in TH as recently reported by Trivieri et al. (37).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


