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Post-transcriptional downregulation of sarcolipin mRNA by triiodothyronine in the atrial myocardium

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Abstract **Thyroid hormone-mediated positive cardiotropic effects are differently regulated between the atria and ventricles. This regulation is, at least in part, dependent on sarcoplasmic reticulum (SR) proteins. Sarcolipin, a homologue of phospholamban, has been recently identified as an atrium-specific SR protein. The expression of sarcolipin mRNA was significantly decreased in the atria of mice with hyperthyroidism and in 3,5,3′-triiodo-L-thyronine-treated neonatal rat atrial myocytes. Promoter activity and mRNA stability analyses revealed that thyroid hormone post-transcriptionally downregulated the expression of sarcolipin mRNA. The atrium-specific effect of thyroid hormone may occur in part through the regulation of atrial sarcolipin gene expression.**

Keywords: Thyroid hormone; Calcium; Gene expression; Cardiomyocyte; Sarcoplasmic reticulum

1. Introduction

Thyroid hormone plays an important role in normal cardiovascular development and exerts positive inotropic, lusitropic, and chronotropic effects on the heart [1]. These effects are mediated in part by regulating the transcription of the genes encoding proteins involved in Ca^{2+} cycling, ion transport, β-adrenergic signaling, and myofibrillar organization. In addition to its physiological role, the excess of thyroid hormone, or the state of "hyperthyroidism", often induces pathological changes in the heart such as cardiac hypertrophy, sinus tachycardia and atrial arrhythmias including atrial fibrillation. The prevalence of atrial arrhythmias is much higher than that of ventricular arrhythmias in patients with hyperthyroidism, indicating that the atrial myocardium is more susceptible to rhythm disturbance by the excess of thyroid hormone than the ventricular myocardium. In this regard, it has been known that the responses to thyroid hormone differ between the atria and ventricles [2–4]. Kaasik et al. demonstrated that thyroid hormone activated sarcoplasmic reticulum (SR) Ca^{2+} ATPase (SERCA) more in the atria than in the ventricles [3]. Shenoy et al. showed that the Ca^{2+} transporter proteins were differently regulated in the atria and the ventricles [4]. Several recent studies have suggested that abnormal intracellular Ca^{2+} homeostasis and perturbations in Ca^{2+} cycling play an important role in atrial fibrillation-induced atrial remodeling [5,6]. Therefore, the differential effect of thyroid hormone on the proteins involved in Ca^{2+} cycling may account for the occurrence of atrial rather than ventricular arrhythmias.

We have found that sarcolipin (SLN), a homologue of phospholamban (PLN), is specifically expressed in the atrial myocardium and skeletal muscle, but not in the ventricular myocardium [7]. SLN interacts with SERCA and regulates excitation–contraction coupling in the atrial and skeletal muscles [8]. Accordingly, SLN may play an important role in characterizing the chamber-specific physiological properties of the atria. The atrial chamber-specific expression of SLN could be primarily regulated at the transcriptional level. Our previous studies have demonstrated that SLN mRNA expression is increased during development, and decreased by hypertrophic remodeling of the atria [7,9] as well as in patients with atrial fibrillation [10]. However, the effect of thyroid hormone on the transcriptional regulation of the SLN gene in the heart has not been investigated, although thyroid hormone is known to regulate the transcription of the SR genes such as PLN and cardiac SERCA2 isoform (SERCA2a) in the heart [11–13]. In the present study, we examined the effect of thyroid hormone on the transcription of SLN gene in the atrial tissue and myocytes.

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Abbreviations: ANF, atrial natriuretic factor; CSQ2, cardiac calsequestrin; EDD, end-diastolic left ventricular dimension; ESD, end-systolic left ventricular dimension; %FS, percent fractional shortening; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IVS, interventricular wall thickness; LVPW, left ventricular posterior wall thickness; MLC2a, atrial myosin light chain 2; NCX1, cardiac sodium calcium exchanger; PLN, phospholamban; RT-PCR, reverse transcription polymerase chain reaction; RyR2, cardiac ryanodine receptor; SERCA, SR Ca^{2+}-ATPase; SLN, sarcolipin; SR, sarcoplasmic reticulum; T3, 3,5,3′-triiodo-L-thyronine; TR, thyroid hormone receptor; TRE, thyroid hormone response element

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2. Materials and methods

2.1. Animals

All treatments were approved by the Animal Protocol Committee of Yokohama City University School of Medicine. Hyperthyroidism was induced in ddY mice (Nippon SLC Inc.) at 6.5 weeks of age ($n = 5$) by intraperitoneally injecting 3,5,3′-triiodo-L-thyronine (T3) at a dose of 2 μg/g body weight/day for 4 weeks. Hypothyroidism was induced in ddY mice at 3 weeks of age ($n = 5$) by feeding the animals with a 0.05% 5-propyl-2-thiouracil (PTU)-containing water for 8 weeks. Non-treated (euthyroid) mice ($n = 5$) were bred for the same period.

2.2. Transthoracic echocardiography

Transthoracic echocardiography was performed as previously described [14].

2.3. Cell culture and stimulation with T3

Neonatal rat atrial myocytes were prepared as the same method described previously for neonatal rat ventricular myocytes [15]. Cardioblast H9C2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 1% fetal calf serum (FCS) for 7 days after confluence. Neonatal rat atrial myocytes and H9C2 cells were treated with 1 nM of T3 for 24 h, and were then collected.

2.4. Northern blot and quantitative reverse transcription polymerase chain reaction (RT-PCR) analyses

Isolation of total RNA and Northern blot analysis were performed as previously described with modification [7,9,16]. Mouse cDNA probes including the SLN gene were used as reported previously [9]. The quantitative RT-PCR was performed by using SYBR Green PCR reagents (Applied Biosystems) as previously described [10]. The forward and reverse primer sequences of SLN were 5′-CTGAGGTCCTTGGTAGCCTG-3′ and 5′-GGTGTGTCAGGCATTGTGAG-3′, respectively.

2.5. Cloning of mouse sarcolipin promoter

During the present study was prepared, the genomic sequence of mouse chromosome 9 including 5′ flanking sequence of the SLN gene has been published (NCBI Accession No.: NT_039474). The primers for PCR amplification of 5′ flanking sequence of the SLN gene were designed as based on the nucleotide sequence (forward: 5′-CAGCTAACCAGGCACAACAA-3′, reverse: 5′-CACTCAGGCTACCAAGGACC-3′). A 2299-bp fragment between −2237 of the 5′ flanking sequence and +62 of the non-coding exon 1 of the SLN gene was amplified and was subcloned into a pDrive vector (Qiagen). The nucleotide sequence was confirmed by direct DNA sequencing.

2.6. Promoter activity assay

The 2299-bp and fragments of the SLN promoter region was cloned into the promoter-less firefly luciferase expression vector, pGL3-Basic (Promega). This construct was named −2237/SLNluc. A shorter construct, −563/SLNluc, containing 563 upstream nucleotides from the putative transcriptional start site was made by self-ligation after digestion of *Mlu*I restriction enzyme. Neonatal rat atrial myocytes were incubated with serum-free medium for 24 h and then they were transiently co-transfected with 300 ng of each SLN promoter luciferase test plasmid and 75 ng of phRL-TK control plasmid (Promega), using FuGene 6 (Roche). Three hours after the transfection, the myocytes were incubated with or without T3 (1 nM) for 24 h, and the luciferase activity was measured with Dual-Luciferase Reagents (Promega).

2.7. mRNA stability assay

To determine whether T3 alters SLN mRNA stability, H9C2 cells were treated with 5 μg/ml actinomycin D, a blocker for transcription, in the presence or absence of 1 nM T3 for up to 8 h. At the indicated times, total cellular RNA was prepared and analyzed by quantitative RT-PCR analysis.

2.8. SERCA activity

SERCA activity was measured as previously described [7].

2.9. Statistics

Data are expressed as means ± S.E.M. Differences were considered significant at $P < 0.05$ (one- or two-way ANOVA with Student–Newman–Kuel's post hoc test).

3. Results

3.1. The expression of SLN mRNA was decreased in the atria of hyperthyroid mice

Serum concentrations of free triiodothyronine were >32.6, 2.75 ± 0.17, and 1.71 ± 0.12 pg/ml in hyperthyroid, euthyroid and hypothyroid mice, respectively. Hyperthyroidism was associated with significant increases in the left ventricle/body weight ratio and the atria/body weight ratio when compared with euthyroid mice (Fig. 1A and B). Transthoracic echocardiography revealed that heart rate, LV chamber size and LV ventricular wall thickness were significantly increased in hyperthyroid mice when compared with those in euthyroid mice (Fig. 1C–E). However, these parameters except heart rate were not different between hypothyroid and euthyroid mice (Fig. 1). Percent fractional shortening (%FS) was not different among three groups (Fig. 1F).

The expression of SLN mRNA was significantly decreased in the atria of the hyperthyroid mice when compared with both euthyroid and hypothyroid mice (Fig. 2A and B). The expression of SLN mRNA was slightly higher in the hypothyroid atria than the euthyroid atria, although the difference did not reach statistical significance. No SLN transcript was detected in the ventricles of both hyperthyroid and hypothyroid mice (data not shown). The expression levels of PLN, SERCA2a, cardiac sodium calcium exchanger (NCX1), RyR2, CSQ2, ANF and atrial myosin light chain 2 (MLC2a) mRNAs were also examined in these atria. We found that the expression level of PLN (Fig. 2C) and MLC2a (data not shown) RNAs were significantly decreased in the hyperthyroid mice.

SERCA activity in the hyperthyroid atria ($n = 4$) was significantly higher than that in the euthyroid atria ($n = 4$) (590 ± 66 nmol/mg protein/min and 270 ± 66 nmol/mg protein/min, respectively). SR Ca^{2+} ATPase activity was much higher in the atria than that in the ventricles (hyperthyroid: 142 ± 25 nmol/mg protein/min; euthyroid: 56 ± 20 nmol/mg protein/min).

3.2. Thyroid hormone downregulated the expression of SLN in neonatal rat atrial myocytes

To avoid a secondary effect of thyroid state-mediated hemodynamics on the expression of SLN mRNA, we examined the effect of T3 in cultured neonatal rat atrial myocytes. T3 decreased the expression of SLN mRNA by 20% ($P < 0.05$) in neonatal rat atrial myocytes that did not exhibit a significant enlargement in size, whereas T3 increased the expression levels of SERCA2a and NCX1 mRNAs by 80% ($P < 0.001$) and 37% ($P < 0.01$), respectively (Fig. 3A and B). PLN mRNA was undetectable in neonatal rat atrial myocytes in our experiments.

3.3. Thyroid hormone treatment did not suppress the SLN transcriptional activity, but accelerated SLN mRNA degradation

Transfection with −2237/SLNluc or −563/SLNluc resulted in a 40-fold increase in transcriptional activity in neonatal

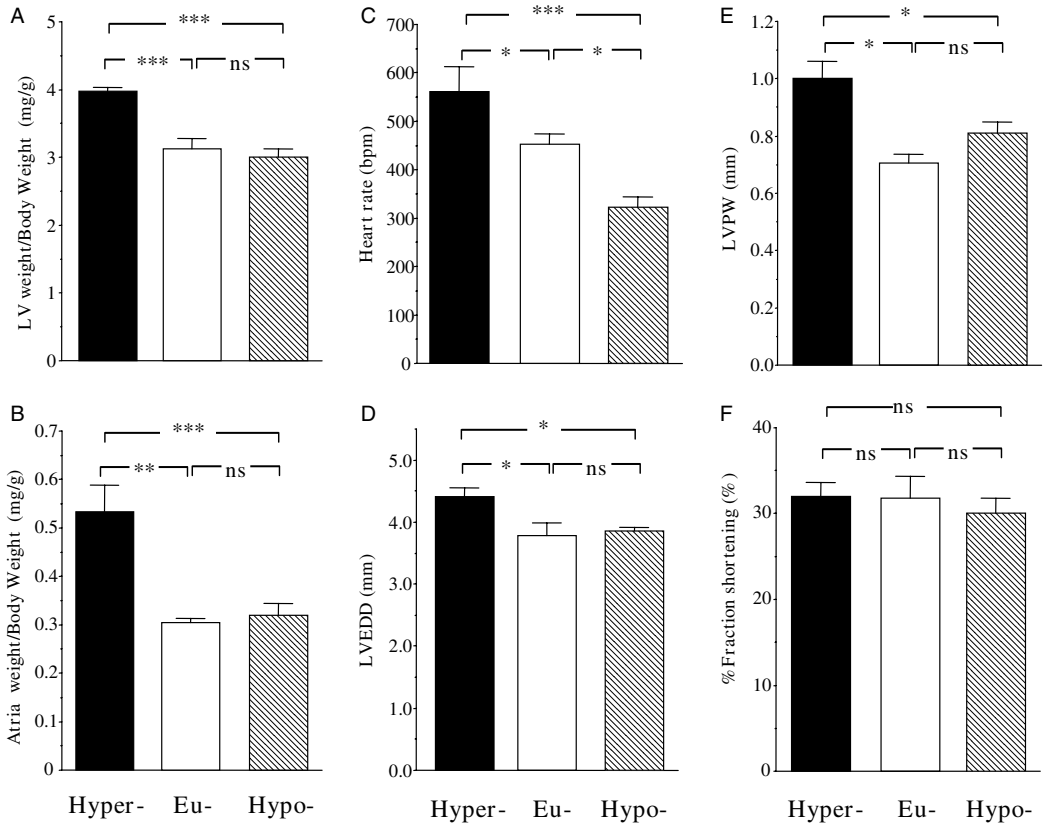

Fig. 1. Cardiac morphology and function in hyperthyroid and hypothyroid mice. (A) Left ventricle/body weight ratio; (B) atria/body weight ratio; (C) heart rate; (D) left ventricular end-diastolic diameter (EDD); (E) left ventricular posterior wall thickness (LVPW); (F) percent fractional shortening (%FS). The left ventricle/body weight ratio and the atria/body weight ratio were significantly increased in hyperthyroid mice. Transthoracic echocardiography revealed that heart rate, EDD and LVPW were significantly increased in hyperthyroid mice, whereas %FS was not different among three groups.

rat atrial myocytes when compared with an empty luciferase control plasmid. The transcriptional activity, however, was not changed in the presence or absence of T3 (Fig. 4A). We then examined the effect of T3 on SLN mRNA stability in H9C2 cells in the presence or absence of T3. The decay of SLN mRNA levels was greater in H9C2 cells in the presence of T3 for 4 and 8 h after actinomycin D treatment (Fig. 4B).

4. Discussion

The present in vivo and in vitro studies demonstrated that thyroid hormone decreased the expression of SLN mRNA in the atrial tissue and myocytes. To our knowledge, this is the first report showing that the expression of the SLN gene are regulated by thyroid hormone. Thyroid hormone binds to high-affinity nuclear receptors that consist of thyroid hormone response elements (TREs), resulting in activating or repressing gene transcription. The thyroid hormone repressed-genes generally contain one or more negative TREs. In the present study, we found four putative TREs in the SLN promoter region between −2237 of the 5′ flanking sequence and +62 of the partial non-coding exon 1 of the SLN gene. However, our data indicated that these TREs were not responsible for the suppressive SLN transcription by thyroid hormone. Instead, we found that thyroid hormone accelerated the decay of the expression of SLN mRNA, suggesting that thyroid hormone altered SLN mRNA stability and thus increased its degradation. In this regard, a number of studies have demonstrated that thyroid hormone post-transcriptionally downregulates the expression of several genes [17,18]. The in vitro study described here indicated that T3 (1 nM) was sufficient to decrease the SLN transcripts in cultured neonatal rat atrial myocytes, where the stresses such as tachycardia and increased afterload due to ventricular hypertrophy were eliminated. Therefore, we think that the excess of thyroid hormone directly downregulates the expression of SLN mRNA. However, the depressing effect of T3 on SLN mRNA levels on in vitro neonatal atrial cardiomyocytes was much less pronounced than the in vivo atrial tissues. Accordingly, we have demonstrated that local pressure overload or atrial fibrillation decreases the expression of SLN mRNA in the atria [9,10]. Since the hyperthyroid mice investigated here displayed cardiac hypertrophy and sinus tachycardia, we postulated that these stresses augmented the decrease in the expression of SLN mRNA in the in vivo atria.

On the other hand, the present study demonstrated that the hypothyroid mice exhibited no change in cardiac morphology and function except slower heart rates. Accordingly, we found that the expression of SLN mRNA was not significantly increased in the atria of hypothyroid mice. We assume that the expression of SLN mRNA would reach the maximal level in the adult atria even in the euthyroid state and that a decrease in thyroid hormone does not enhance SLN mRNA stability. There is an alternative possibility that the intensity and dura-

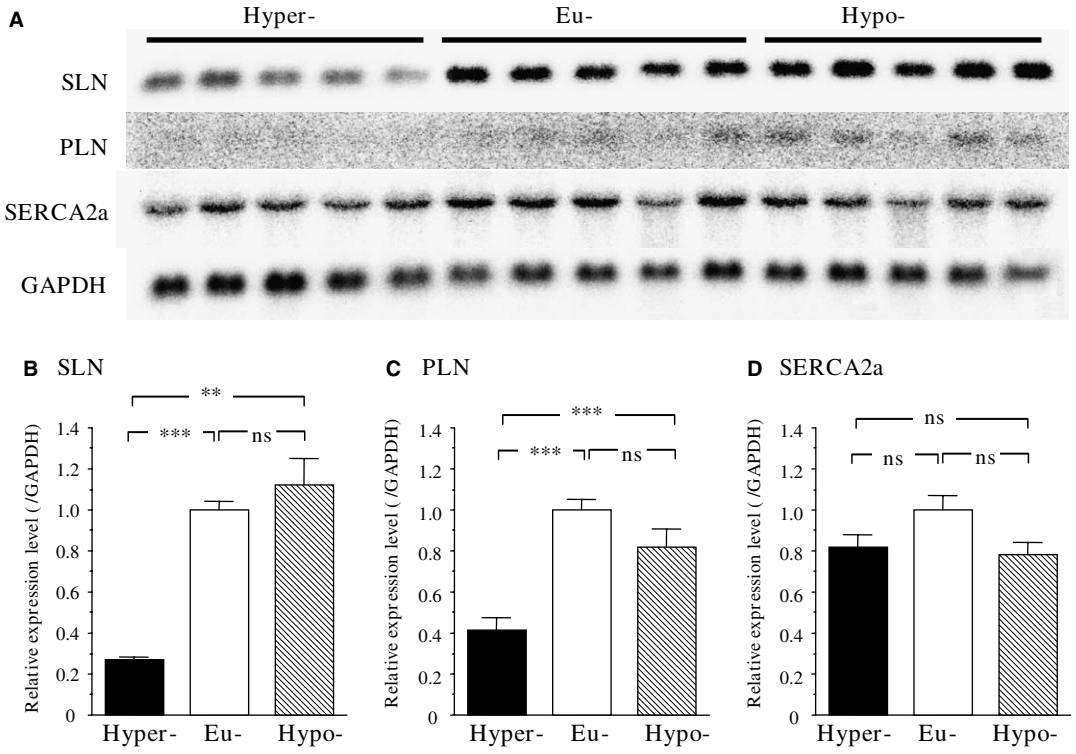

Fig. 2. The expression of SLN mRNA was decreased in the atrial myocardium of the hyperthyroid mice. (A) The raw data of the expression of SLN mRNA by Northern blot analysis. Ten μg of total RNA was loaded per lane. (B–D) The summary of Northern blot analyses. The expression levels of SLN and PLN mRNAs were significantly decreased in the atria of the hyperthyroid mice (Hyper-) when compared with euthyroid and hypothyroid mice (Eu- and Hypo-, respectively). The expression levels of SERCA2a mRNA were not different between three groups. Each signal intensity was standardized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as an internal control. The data are presented as means ± S.E.M. $^{**}P < 0.01$; $^{***}P < 0.001$; ns, not significant.

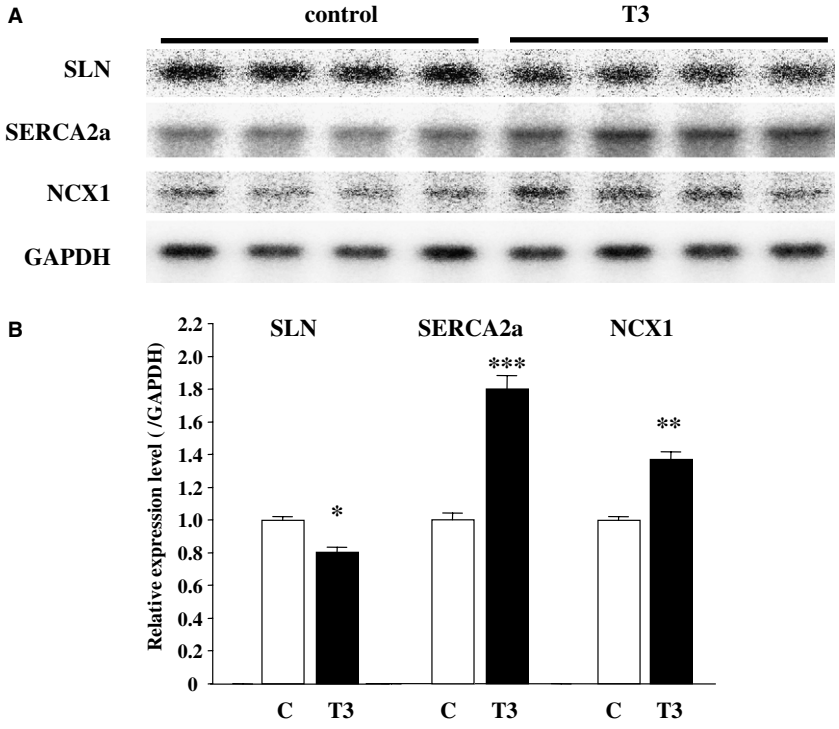

Fig. 3. Thyroid hormone treatment decreased the expression of SLN mRNA in neonatal rat atrial myocytes. (A) The raw data of the expression of SLN mRNA by Northern blot analysis. Ten μg of total RNA was loaded per lane. (B) The summary of Northern blot analyses. The expression level of SLN mRNA was significantly decreased in neonatal rat atrial myocytes in the presence of T3 (T3), whereas the expression levels of SERCA2a and NCX1 mRNAs were significantly increased by T3. Each signal intensity was standardized by GAPDH used as an internal control. The data are presented as means ± S.E.M. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (C) Control myocytes in the absence of T3.

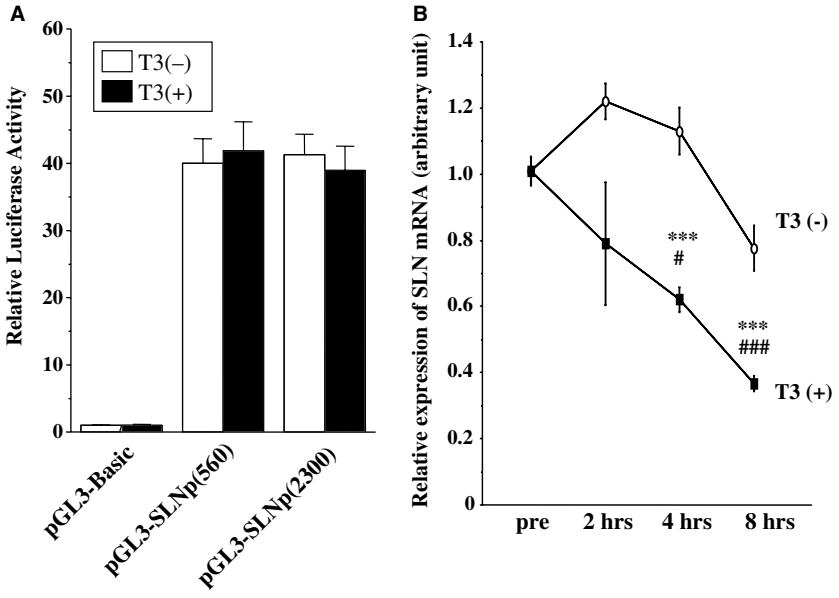

Fig. 4. Thyroid hormone treatment accelerated the decay of SLN mRNA. (A) The relative luciferase activity of the mouse SLN promoter in neonatal rat atrial myocytes. (B) The relative expression of SLN mRNA in H9C2 cells treated by actinomycin D. The luciferase activity was not changed in the presence or absence of T3. The decay of SLN mRNA levels was greater in H9C2 cells in the presence of T3 for 4 and 8 h after actinomycin D treatment. The data are presented as means ± S.E.M. $^{***}P < 0.001$ versus T3(−). # and ###, $P < 0.05$ and $P < 0.001$ versus pre, respectively.

tion of the hypothyroid state were not enough to increase the expression of SLN mRNA in the present study.

A large body of evidence has indicated that thyroid hormone plays an important role in the expression of the SR Ca^{2+} genes [19–21]. It has been known well that PLN is downregulated by thyroid hormone, which was confirmed in the atria by the present study. The downregulation of PLN mRNA contributes to the positive inotoropic effects mediated by thyroid hormone treatment. Like PLN, SLN also inhibits SERCA2a function and reduces SR Ca^{2+} stores [22]. Accordingly, the atrial tissues may be more sensitive to the stimulation of thyroid hormone than the ventricular tissues because of a decrease in SLN expression in addition to a decrease in PLN. It is of great interest that the downregulation of SLN mRNA by thyroid hormone would result in a decrease in SLN protein in the atria. In the present study, we could not show it, because it is difficult to obtain a SLN antibody probably due to the very short cytoplasmic region of the structure. Further investigation will be required to clarify whether thyroid hormone-mediated downregulation of SLN mRNA increases Ca^{2+} uptake in the atria and thus function.

In conclusion, the SLN transcripts in the atrial tissue and myocytes were post-transcriptionally downregulated by thyroid hormone. The atrium-specific effect of thyroid hormone may occur, at least in part, through the regulation of atrial SLN gene expression.

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