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Overexpression Myocardial Inducible Nitric Oxide Synthase Exacerbates Cardiac Dysfunction and Beta-Adrenergic Desensitization in Experimental Hypothyroidism^{☆,☆☆}

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Abstract

Background—Altered nitric oxide synthase (NOS) has been implicated in the pathophysiology of heart failure (HF). Recent evidence links hypothyroidism to the pathology of HF. However, the precise mechanisms are incompletely understood. The alterations and functional effects of cardiac NOS in hypothyroidism are unknown. We tested the hypothesis that hypothyroidism increases cardiomyocyte inducible NOS (iNOS) expression, which plays an important role in hypothyroidism-induced depression of cardiomyocyte contractile properties, $[Ca^{2+}]_i$ transient ($[Ca^{2+}]_{iT}$), and β -adrenergic hyporesponsiveness.

Methods and Results—We simultaneously evaluated LV functional performance and compared myocyte three NOS, β -adrenergic receptors (AR) and SERCA2a expressions and assessed cardiomyocyte contractile and $[Ca^{2+}]_{iT}$ responses to β -AR stimulation with and without pretreatment of iNOS inhibitor (1400W, 10^{-5} mol/L) in 26 controls and 26 rats with

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hypothyroidism induced by methimazole (~30 mg/kg/day for 8 weeks in the drinking water). Compared with controls, in hypothyroidism, total serum T₃ and T₄ were significantly reduced followed by significantly decreased LV contractility (E_{ES}) with increased LV time constant of relaxation. These LV abnormalities were accompanied by concomitant significant decreases in myocyte contraction (dL/dt_{max}), relaxation (dR/dt_{max}), and [Ca²⁺]_{IT}. In hypothyroidism, isoproterenol (10⁻⁸ M) produced significantly smaller increases in dL/dt_{max}, dR/dt_{max} and [Ca²⁺]_{IT}. These changes were associated with decreased β₁-AR and SERCA2a, but significantly increased iNOS. Moreover, only in hypothyroidism, pretreatment with iNOS inhibitor significantly improved basal and isoproterenol-stimulated myocyte contraction, relaxation and [Ca²⁺]_{IT}.

Conclusions—Hypothyroidism produces intrinsic defects of LV myocyte force-generating capacity and relaxation with β-AR desensitization. Up-regulation of cardiomyocyte iNOS may promote progressive cardiac dysfunction in hypothyroidism.

1. Introduction

Hypothyroidism, or thyroid hormone deficiency, is a common condition that is more prevalent with advancing age.^{1–3} It can cause reduced cardiac performance, increased systemic vascular resistance, and reduced chronotropy.^{3,4} Recent studies link hypothyroidism to cardiomyopathy and heart failure (HF).⁵ Persistent subclinical thyroid dysfunction was recently associated with the development of HF in patients with and without underlying heart disease.^{5,6} Patients with systolic HF and hypothyroidism are at increased risk for death.^{1,2} Despite these data, the underlying mechanisms through which thyroid hormone deficiency results in myocardial damage are incompletely understood.

The direct cardiac effects of hypothyroidism, independent its effects on loading conditions have not been fully elucidated. Hypothyroidism results in complex changes within the heart. However, it is unclear whether the left ventricular (LV) systolic and diastolic dysfunction observed with hypothyroidism is an intrinsic property of cardiomyocytes and the concept of hypothyroid cardiomyopathy remains controversial. Recent evidence suggests that altered nitric oxide synthase (NOS) isoform activations and impaired cardiac β-adrenergic receptors (AR) subtype-mediated signal pathways may be important in hypothyroid-associated cardiomyopathy.^{4,7,8} However, the findings concerning the expression and function of three NOS isoforms and different cardiac β-AR subtypes, in particular β₁- and β₃-AR in hypothyroidism are limited and conflicting.

We designed the current study to address, for the first time, these limitations to characterize and assess in an integrated fashion hypothyroidism-induced alternations in global cardiac function, single cell mechanisms, dynamics of the cytosolic [Ca²⁺]_i, sarcoplasmic reticulum (SR) Ca²⁺-ATPase (SERCA2a), NOS and hormonal activation and related molecular and cellular signal transduction pathways. We tested the hypotheses that (1) defects in cardiomyocyte force-generating capacity and relaxation process with impaired [Ca²⁺]_i regulation is critical element in the development of hypothyroid cardiomyopathy and HF, and (2) up-regulation of cardiomyocyte inducible NOS (iNOS) with down-regulation of β₁-AR and SERCA2a importantly contribute to the intrinsic defects of cardiomyocytes. The increased cardiac iNOS activation may exacerbate already-existing dysfunctional [Ca²⁺]_i

regulation, enhance inhibition of cardiomyocyte contraction and relaxation, and promote progressive cardiac dysfunction in hypothyroidism.

2. Methods

2.1. Animal Model

This investigation was approved by the Wake Forest University Animal Care and Use Committee and conforms to the Guide for the Care and Use Laboratory Animals (Institute of Laboratory Animal Resources 1996). Forty male Sprague–Dawley rats weighing 280–300g (Charles River Laboratories International, Inc.) were used for this experiment. Rats were randomly and equally divided into control and hypothyroid groups (n=26 each). The hypothyroid group received methimazole (0.04%, 30mg/kg/day, Sigma-Aldrich Co.) in their drinking water for 8 weeks. Body weight was measured once per week, and average food and water intake were recorded twice per week. All animals were maintained in the same environment, including temperature and humidity, and free access to food and water.

2.2. Experimental Protocol

Studies were performed simultaneously examining intact animals, isolated cardiomyocytes, and intracellular molecular mechanisms. In the subgroup of animals without hemodynamic study, serum thyroid hormone levels and neurohormonal changes were determined from each animal group.

2.3. Intact Animal Study

2.3.1. Hemodynamic and Pressure-Volume Relation Measurements—Rats were anesthetized with intraperitoneal ketamine (Hospira, Inc.) (50 mg/kg) and xylazine (Phoenix Pharmaceutical, Inc.) (10 mg/kg). A heating pad was placed underneath each rat, and the core temperature, measured via a rectal probe, was maintained at 37°C. Animals were intubated and ventilated with a positive-pressure respirator (Model RSP1002, Kent Scientific Corp, Litchfield, CT). A polyethylene catheter was inserted into the left external jugular vein for drug administration. After adequate calibration, using the closed-chest approach, a 2-Fr microtip P-V catheter (SPR-838, Millar Instruments, Houston, TX) was inserted into the right carotid artery and advanced into the LV under pressure control. After stabilization for 10 min, signals were continuously recorded at a sampling rate of 500 samples/s using a P-V conductance system (MPCU-200, Millar Instruments) with BioBench software (National Instruments, Inc.) under steady-state conditions and during transient preload decline, achieved by manual compression of the inferior vena caval occlusion (VCO). Then isoproterenol (10^{-8} M, 0.5 ml i.v.) was infused; steady-state and VCO data were continuously acquired immediately and during 10-minute periods. As previously described, with the use of a special P-V analysis program (PVAN, Millar Instruments), standard steady-state hemodynamic parameters such as heart rate, LV pressures (P) and the time constant of LV relaxation (τ) and LV volume (V) were measured. Stroke volume (SV) and cardiac output (CO) were calculated and corrected according to in vitro and in vivo volume calibrations using PVAN software. LV P-V relations and its slopes were derived.^{9–12} Effective arterial elastance, E_a , was calculated as the ratio of end-systolic P (P_{ES}) and SV, and LV-arterial coupling was quantitated as the ratio of E_{ES} to E_a .^{13–16} After

hemodynamic measurements, animals were deeply anesthetized with sodium pentobarbital (50 mg/kg). The heart was rapidly removed and immediately placed in ice-cold, calcium-free HEPES buffer solution. At the end of experiment, heart and thyroid weights were measured. In the subsets of animals from both control and hypothyroidism groups without hemodynamic study, blood samples for the measurements of serum thyroid hormone levels and neurohormonal changes were drawn from the heart between 9:00 and 10:00 am as described previously.^{8, 17}

2.3.2. Thyroid Hormone Measurement and Biochemical Assay

Measurement of Thyroid Hormone Levels: Serum levels of total triiodothyronine (TT3) and total thyroxine (TT4) were measured by radioimmunoassay by the Pathology laboratory at WFHS.⁸

Measurement of Plasma Catecholamine and 8-Isoprostane Levels: Blood was placed in chilled tubes containing EDTA and, after separation in a refrigerated centrifuge, stored at -20°C before analysis. Plasma levels of norepinephrine and 8-isoprostane (to reflect levels of systemic oxidative stress) were measured by the Hypertension Center Core Laboratory at WFHS.¹⁷ All assays were performed in duplicate.

2.4. Isolated Cardiomyocyte Study

2.4.1. Myocyte Isolation—Calcium-tolerant, high-yield myocytes were obtained from both normal and hypothyroid rats.^{14, 15, 18} Briefly, the hearts were retrograde perfused on a Langendorff apparatus with calcium-free HEPES solution (in mmol/L: NaCl 110.0, KCl 5.4, MgSO₄ 1.2, KH₂PO₄ 1.2, HEPES 10.0, D-mannitol 45.0 and glucose 15.0) for 5 minutes at 37°C. Then the perfusion solution was switched to a calcium-HEPES-collagenase solution containing CaCl₂ 35 µmol/L, type II collagenase, 10 mg (0.04%, w/v) (290 U/mg, Worthington, Freehold, NJ) and bovine serum albumin 60 mg (0.1%, w/v) (Sigma-Aldrich Co. USA) until cells were enzymatically digested. Then, the flaccid heart was removed from the cannula. Only LV tissues were transferred to sterilized centrifuge tubes and further digested with the HEPES–collagenase solution until myocytes were almost completely dissociated. The dispersed myocytes were checked under a microscope. The collected cells were centrifuged (500 rpm, 4 °C 1 min), and the supernatant with broken cells was removed. After each settling, the HEPES solution was changed stepwise to increase calcium concentrations (i.e. 250, 500 and 1000 µmol/L). Finally, the cells were suspended in the modified HEPES solution (“the study buffer”) with 1.2 mM CaCl₂ and stored at room temperature until ready for use. At the final concentration of calcium, LV myocytes were counted and viability and morphology evaluated.^{13, 14, 19, 20}

2.4.2. Myocyte Functional Performance: Myocyte Contractile Response and [Ca²⁺]_i Regulation Measurement of Contractile Response

Responses to β- and β₃-Agonists: After stabilization, isolated myocytes were placed in superfused culture dishes. Myocyte baseline contraction and relaxation and inotropic contractile responses were measured with the Fluorescence and Contractility System (IonOptix, Milton, MA). Contraction was elicited by field-stimulation.^{15, 20} First, steady-state baseline data were recorded. Myocytes were randomly exposed to a non-selective β-

AR agonist, isoproterenol (ISO, 10^{-8} M), or a selective β_3 -agonist, BRL-37344 (BRL, 10^{-8} M).¹⁵ Data were acquired after 3 to 8 minutes of drug exposure and 5 to 10 minutes after drug washout.

Responses to iNOS Inhibitor: To determine LV myocyte functional effects from iNOS activation, the above protocol was repeated after myocytes were pretreated with a selective iNOS inhibitor, 1400W (10^{-5} mol/L). Percent shortening (SA), the maximum rate of shortening ($dL/dt_{\max}$), and re-lengthening ($dR/dt_{\max}$) were obtained as previously reported.

Simultaneous Measurement of Contractile and Calcium Transient Responses

—In the second series of experiments, myocytes were incubated with 10 μ M indo-1-AM (Molecular Probes, Eugene, OR) and then placed in a flow-through T-culture dish. Contractile and peak systolic $[Ca^{2+}]_{i,T}$ responses in a single myocyte were measured simultaneously with a dual-excitation fluorescence photo-multiplier system (IonOptix).^{15, 19, 20} After stabilization, the isoproterenol protocol was repeated. When myocytes were loaded with indo-1-AM, compartmentalization of the indicator may have occurred in the mitochondria and, thus, the absolute value of $[Ca^{2+}]_i$ was not used. The ratio of the emitted fluorescence (410/490) was used to represent the relative changes in peak intracellular $[Ca^{2+}]_i$ before and after interventions.^{15, 21}

2.5. Molecular Study

To further delineate the molecular basis contributing to LV myocyte deficits caused by hypothyroidism, we took serial measurements of changes in expressions of myocyte NOS isoforms, Phospho-iNOS, SERCA2a, PLB and PLB Phosphorylation. To clarify the conflicting reports regarding cardiac β_1 - and β_3 -AR in hypothyroidism, we also measured myocyte β_1 - and β_3 -AR protein levels and activities in these animals.

2.5. 1. Determine LV Myocyte β_1 - and β_3 -AR mRNA and Protein Levels

Expression of β_1 - and β_3 -AR mRNA: Using our previously described methods,^{20, 22, 23} LV myocyte total RNA was extracted by RNAqueous™ Phenol-Free Total RNA Isolation Kit (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase I (Life Technologies, Grand Island, NY). The reverse transcription (RT) reaction was performed with RETROscript™ First-Strand Synthesis Kit (Invitrogen, Carlsbad, CA) using the antisense primers. The cDNA produced was amplified by polymerase chain reaction (PCR).

A 327 bp DNA fragment corresponding to rat β_1 -AR coding region (bases 307 to 634) was produced by PCR with published the primers for rat β_1 -AR: sense: 5'-GCCGATCTGGTCATGGGA-3' (bases 307 to 324) and antisense: 5'-GTTGTAGCAGCGGCGCG-3' (bases 617 to 634).²⁴

A 308 bp DNA fragment corresponding to rat β_3 -AR coding region (bases 12 to 319) was produced by PCR with the primers for rat β_3 -AR: sense: 5'-ATG GCT CCG TGGCCTCAC-3' (bases 12 to 29) and antisense: 5'-CCCAAGGGCCAGTGGCCAGTCAGCG-3' (bases 295 to 319); and the 308 bp DNA fragment corresponding to rat β_3 -AR was cloned into PCR 2.1 (Invitrogen, Carlsbad, CA).

DNA sequencing of the recombinant plasmid was performed with T7 and M13 specific primers. The sequence of the fragment was 100% homologous to the corresponding region of the published rat β_3 -AR cDNA (GenBank GI: 298306). GAPDH was co-amplified as an internal control.

Western Blot Analysis of β_1 - and β_3 -AR: LV myocytes were briefly washed with pre-chilled PBS before the addition of a membrane protein-extraction reagent (Pierce, Rockford, IL) with a proteinase inhibitor cocktail (Cell Signaling Technology, Danvers, MA)²⁰. Cell membrane lysate (30 μ g) was blotted to a PVDF membrane and then incubated with polyclonal IgG to β_1 - and β_3 -AR (1:1500 dilutions, Santa Cruz Biotechnology Inc. Santa Cruz, CA) at 4°C overnight. Following washes, the membrane was incubated with horseradish peroxidase–conjugated anti-rabbit IgG (1:3000 dilutions, Sigma, St. Louis, MO). For normalization, the same blot was stripped and reprobed with polyclonal IgG to actin at 1:2500 dilutions (Santa Cruz Biotechnology, Inc.).²⁰

2.5.2. Determine LV Myocyte Expression and Activity of SERCA2a, PLB and PLB Phosphorylation—Myocyte major calcium handling protein sarcoplasmic/endoplasmic-reticulum (SR/ER) was extracted using ER Isolation Kit following the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO). Protein content was determined with protein assay kit (Bio-Rad Laboratories, Hercules, CA). The levels of sarcoplasmic reticulum calcium ATPase 2a (SERCA2a) were analyzed with immunoblot. Sarcoplasmic reticulum (30 μ g) was blotted to a PVDF membrane and then incubated with specific primary IgGs for SERCA2a (1:1000 dilutions, Abcam, Cambridge, MA). For normalization, the same blot was stripped and reprobed with polyclonal IgG to calsequestrin at 1:1500 dilutions (Santa Cruz Biotechnology, Inc.).²⁰ SERCA2a activity assays were carried out on the basis of a pyruvate/NADH coupled reaction as previously described.²⁵ Ca^{2+} -ATPase activity was calculated as absorbance/(6.22 $\times$ protein $\times$ time) in nmol ATP/(mg protein $\times$ min). For detection total phospholamban (PLB) and phosphorylated PLB immunoblotting will be done with commercially available anti-PLB antibody, anti-PLB phosphorylated at Ser16 and Thr17 (Upstate Biotechnology, Greenland, NH),^{20, 23} all experiments were done in duplicate.

2.5.3. Determination of LV Myocyte Protein Levels of eNOS, nNOS, iNOS and phospho-iNOS expression—LV myocytes were treated as described above for Western blots; cell membrane lysate (50 μ g) was blotted to a PVDF membrane and then incubated with polyclonal IgGs to eNOS, nNOS and iNOS (1:1000 dilutions, Abcam) at 4°C overnight. Following washes, the membrane was incubated with horseradish peroxidase–conjugated anti-rabbit IgG (1:3000 dilutions, Sigma, St. Louis, MO). For normalization, the same blot was stripped and reprobed with polyclonal IgG to actin at 1:2500 dilutions (Santa Cruz Biotechnology, Inc.). In addition, anti-iNOS (phospho Y151) antibody (1:1000 dilutions, Abcam, Cambridge, MA) was used to detect endogenous levels of iNOS only when phosphorylated at tyrosine.

2.6. Statistical Analysis

All data are presented as mean $\pm$ SE or mean $\pm$ SD as indicated. Multiple comparisons were performed using analysis of variance. When a significant overall effect was present, intergroup comparisons were performed using a Bonferroni correction for multiple comparisons. Two-tailed unpaired student's t tests were used to evaluate mean differences in hemodynamic parameters and plasma hormone concentrations between groups. Measurements of myocyte contraction and $[Ca^{2+}]_{IT}$ were averaged from each animal and treated as a single data point. The mean differences in cell dynamics and indo-1-AM fluorescence ratios between groups were calculated. Significance was established as $p < 0.05$.

3. Results

3.1. Animal Follow-Up and General Features of Methimazole-Induced Hypothyroidism

Statistically significant lower serum levels of total thyroxine (TT4) (0.9 ± 0.2 vs 4.2 ± 0.4 $\mu\text{g/dl}$, 79% reduction) and total triiodothyronine (TT3) (22.3 ± 1.2 vs 88.8 ± 13 ng/dl , 75% reduction) in methimazole-treated rats were evidence that hypothyroidism was induced. Compared with controls, hypothyroid rats had markedly decreased food and fluid intakes and lower body temperatures. Body weight, heart weight, and heart weight/body weight ratio (329 ± 10 vs 357 ± 5 mg/100g) were all significantly decreased in treated rats. Hypothyroid caused a 46% increase in thyroid weight (95 ± 5 vs 65 ± 4 mg) with a 160% increase in the ratio of thyroid to body weight. The calculated ratio of wet lung to body weight was also increased 43%.

3.2. LV Systolic and Diastolic Dysfunction and Hormone Levels in Hypothyroidism

As summarized in Table 1 and displayed in Figure 1, heart rate, $dP/dt_{\max}$, $-dP/dt_{\min}$ and LV end systolic pressure (P_{ES}) all significantly decreased in treated rats versus controls. Arterial elastance (E_a) was increased in hypothyroid rats, but LV end diastolic volume and end diastolic pressure were not significantly altered. The time constant of LV relaxation (τ) was significantly increased (hypothyroid: 21.8 vs control: 10.9 ms) accompanied by 38% reduced LV peak filling rate of $dV/dt_{\max}$ (3443 vs 5531 $\mu\text{l/sec}$) and 28% decrease in stroke volume (SV). In the current study, anesthesia agent of ketamine was used to induce and maintain anesthesia; consistent with past reports^{10, 26}, we obtained relatively lower ejection fraction (EF) values in both groups^{10, 26}. However, compared with controls, in the hypothyroid rats, EF still significantly reduced 26% (hypothyroid: 40% vs control: 54%) due to the significantly decreased SV, and cardiac output decreased by 54%. In hypothyroid rats, LV contractility significantly decreased, as indicated by significant reductions in the slopes of P_{ES} - V_{ES} (E_{ES}) (39%, 0.65 vs 1.06 $\text{mmHg}/\mu\text{l}$) and SW - V_{ED} (M_{SW}) (31%, 56.3 vs 81.5 mmHg) (Figure 1). The E_{ES}/E_a ratio significantly decreased by about 47% (0.9 vs 1.7) (Table 1). Compared to controls, plasma norepinephrine levels significantly increased by about 3 folds (1028 vs 362 pg/ml), and 8-isoprostane concentrations increased by about 30% (238 vs 183 pg/ml) in hypothyroidism rats.

In controls, isoproterenol caused significant increases in heart rate, EF and $dV/dt_{\max}$ in controls, but significantly decreased V_{ES} and τ . There were no significant changes in V_{ED} with only slight increases in LV P_{ED} and P_{ED} ($p = \text{NS}$). We noted that after Isoproterenol,

although the $dP/dt_{\max}$, a convenient index for LV global contractility tended to increase, but failed to reach statistical significance. The large standard deviation (SD) of $dP/dt_{\max}$ (likely resulted by the signal noise on the pressure catheter tip recorded LVP^{27–30}, which may also attenuate LVP signal adequate frequency response to isoproterenol stimulation) or some other confounding factors (such as anesthetics we used; alterations in the heart rate and the loading conditions) may contribute to this effect^{27–30}. However, isoproterenol-produced increases in LV contractility were clearly demonstrated by using the load-insensitive measures of contractility, LV P-V analysis. As shown in Table 1 and Figure 1, after Isoproterenol, the P_{ES} - V_{ES} relationships were shifted to the left with about a 41% increase in slopes of E_{ES} and about 31% increase in M_{SW} , indicating significantly augmented LV contractility. By contrast, hypothyroid rats had attenuated positive inotropic and lusitropic responses to β -AR stimulation. Compared with baseline, isoproterenol failed to cause significant decreases in V_{ES} and τ . Isoproterenol resulted in significantly less increases in E_{ES} (15%) and in M_{SW} (12%) in hypothyroid rats, demonstrating impaired β -adrenergic regulation with decline of contractility reserve.

3.3. LV Myocyte Dysfunction and Impaired $[Ca^{2+}]_i$ Regulation with Hypothyroidism

3.3.1. Basal Myocyte Functional Performance—Compared with controls, LV myocytes isolated from Hypothyroidism rats showed significantly decreased basal contractile functional performance and impaired $[Ca^{2+}]_i$ regulation (Table 2A, Figure 2). In hypothyroid rats, LV myocyte lengths tend to shortened (107.8 ± 4.4 vs 120.0 ± 3.3 μm), cell systolic amplitude decreased by 43%, about 60% reductions in the maximum rate of myocyte shortening ($dL/dt_{\max}$, 57.8 ± 2.5 vs 141.7 ± 5.6 $\mu m/s$) and relengthening ($dR/dt_{\max}$, 46.2 ± 2.3 vs 112.9 ± 5.8 $\mu m/s$). The peak systolic $[Ca^{2+}]_{iT}$ (0.18 ± 0.01 vs 0.25 ± 0.01) was markedly reduced and the decline of $[Ca^{2+}]_i$ was also slower.

3.3.2. Myocyte Functional Responses to β -AR Stimulation and iNOS activation

Effects of Isoproterenol: Compared with controls, in hypothyroid rats, not only the basal myocyte contraction and relaxation were depressed, but the ability of the β -adrenergic agonist isoproterenol (ISO) to increase myocyte contractility was also significantly reduced (Figures 2–3, Table 2A). Compared with controls, cardiomyocytes from hypothyroid rats showed significantly decreased isoproterenol-induced increases in SA (24% vs 42%), $dL/dt_{\max}$ (36% vs 66%) and $dR/dt_{\max}$ (33% vs 48%) and the peak systolic $[Ca^{2+}]_{iT}$ (16% vs 36%).

Effect of BRL-37344: Compared with control myocytes, in cardiomyocytes from treated rats, a selective β_3 -AR agonist, BRL-37344 (BRL) produced similarly significant decreases in SA (hypothyroid: -15% vs control: -14%), $dL/dt_{\max}$ (-16% vs -14%), $dR/dt_{\max}$ (-13% vs -12%) and $[Ca^{2+}]_{iT}$ (-15% vs -17%), indicating unaltered myocyte functional response to β_3 -AR stimulation in hypothyroid rats (Figure 3, Table 2B).

Effect of iNOS Activation: Figure 4 and Table 3 show the contributions of iNOS activation to hypothyroidism-caused myocyte dysfunction. Pretreatment of control myocytes with the iNOS inhibitor of 1400W, has no effect on myocyte functional performance. In contrast, compared with untreated myocytes from hypothyroid rats, 1400W pretreatment improved

myocyte basal $dL/dt_{\max}$ by 36%, $dR/dt_{\max}$ by 43%, and $[Ca^{2+}]_{IT}$ by 22% (Table 3A). Moreover, after 1400W pretreatment, the ISO-induced percent increases in SA (42% vs 24%), $dL/dt_{\max}$ (57% vs 36%), $dR/dt_{\max}$ (61% vs 33%) and $[Ca^{2+}]_{IT}$ (30% vs 16%) were also significantly augmented compared to myocytes from hypothyroid rats without 1400W pretreatment (Figure 4 and Table 3B).

3.4. Molecular Alterations Contributing to LV Myocyte Dysfunction in Hypothyroidism

Hypothyroidism-induced alterations (both examples and group means) of cardiomyocyte β -ARs and NOS expressions are displayed in Figures 5 and 6.

3.4.1. Myocyte β_1 - and β_3 -AR Expression—Using RT-PCR, β_1 - and β_3 -AR mRNA expression was detected in myocytes of both groups of rats (Figure 5a). Both β_3 - and β_1 -AR protein levels were quantified by using Western blot analyses. Compared with control myocytes, β_1 -AR protein levels were significantly decreased in myocytes from hypothyroid rats. The signal ratio of β_1 -AR protein to actin was reduced by about 29% (0.56 vs 0.79). In contrast, the signal ratios of β_3 -AR protein to actin were similar for myocytes from rats in both groups (Figure 5). The complete examples of western blot experiments on myocyte β_1 - and β_3 -AR expression were presented as supplemental data of online Figure 1.

3.4.2. Myocyte SERCA2a, PLB and p-PLB Protein Levels—As shown in Figure 6 (also exhibited in supplemental data of online Figure 2), compared with control myocytes, the signal ratio of SERCA2a protein to calsequestrin was significantly reduced by about 54% in myocytes from hypothyroid rats (0.25 vs 0.54), and Ca^{2+} -dependent SERCA2a activity significantly decreased by about 30% (1448 ± 203 vs 2187 ± 489 nmol/mg/min), demonstrating that SERCA2a is down-regulated in hypothyroidism. In addition, compared with normal control myocytes, there were no significant changes in the total phospholamban (PLB), but the signal ratio of SERCA2a/PLB (0.82 vs 1.69) was reduced with significant decreases in the phosphorylated PLB (PLB^{ser16}) levels and the ratio of PLB^{ser16}/PLB in myocytes from hypothyroid rats.

3.4.3. Myocyte NOS Protein Levels and iNOS phosphorylation—As displayed in Figure 7A, compared with control myocytes, the signal ratio of iNOS protein to actin significantly increased in myocytes from hypothyroid rats by about 93% (0.29 vs 0.15). The eNOS protein levels decreased much less (25%) but still significantly (0.39 vs 0.52); and nNOS protein levels (0.55 vs 0.52) were relatively unchanged between groups. The complete examples of western blot experiments on myocyte NOS protein expression were presented as supplemental data of online Figure 3. Importantly, as shown in Figure 7B, in myocytes from hypothyroid rats, the elevated iNOS protein levels were accompanied with significantly increased iNOS phosphorylation at Tyrosine position 151. Compared with control myocytes, the signal ratio of phospho-protein to actin was increased by about 123% (0.29 vs 0.13), demonstrating that iNOS is up-regulated in hypothyroidism.

4. Discussion

We examined the direct cardiac effects and underlying cellular and molecular mechanisms in a rat model with methimazole-induced hypothyroidism.^{2, 5, 7, 8} The major new findings

are: 1) Chronic hypothyroidism produces increased activation of the sympathetic nervous system (SNS), followed by direct depression of LV contractile function and impaired LV filling. This was associated with defects in cardiomyocyte contraction, relaxation and $[Ca^{2+}]_i$ regulation with diminished β -AR inotropic reserve. 2) Hypothyroidism causes up-regulation of cardiomyocyte iNOS, but down-regulation of β_1 -ARs and SERCA2a with unchanged β_3 -ARs. 3) The increased cardiac iNOS activation exacerbates already-existing dysfunctional $[Ca^{2+}]_i$ regulation and β -adrenergic desensitization, enhances inhibition of cardiomyocyte contraction and relaxation, and contributes to progressive cardiac dysfunction. Our observations support the existence of a specific hypothyroid cardiomyopathy. The defect in cardiomyocyte force-generating capacity and relaxation process with impaired $[Ca^{2+}]_i$ regulation is a critical element and up-regulation of cardiomyocyte iNOS promoting cardiac dysfunction in hypothyroidism.

4.1. LV and Myocyte Dysfunction and Impaired $[Ca^{2+}]_i$ Regulation with Hypothyroidism

In a rat model with methimazole-induced thyroid-hormone deficiency, we made important observations that mimic some cardiovascular injuries and functional and neurohormonal changes of clinical hypothyroidism.^{2, 5, 7, 8} Consistent with past experimental and clinical reports, these animals showed increased SNS activation, with significantly decreased heart rate, stroke volume, cardiac output, ejection fraction, and increased Ea without significant change in V_{ED} (Table 1).^{3, 4, 31} The relative unchanged V_{ED} may be related hypothyroidism produced LV atrophy.

To avoid the potentially confounding effects of hypothyroidism-induced changes in loading conditions on conventional measures of LV performance, for the first time, we evaluated LV contractile performance in the P-V plane. Hypothyroidism produced direct inhibition of basal LV contractility and caused progressive LV systolic and diastolic functional impairment, with significantly decreased E_{ES} (−39%) and M_{SW} , slowed LV relaxation (τ was doubled), and reduced LV filling (dV/dt_{max} reduced 38%). In addition, hypothyroid rats had attenuated positive and lusitropic responses to no-selective β -AR agonist stimulation. Isoproterenol failed to cause significant decreases in V_{ES} and τ , and caused significantly smaller increases in E_{ES} and M_{SW} , demonstrating impaired β -adrenergic regulation with decline of contractility reserve (Table 1). These changes were similar to those observed in our canine model with pacing-induced HF¹² and rat model with isoproterenol-induced HF.¹⁴

We further studied cardiomyocyte functional performance in freshly-isolated single myocyte from the LV in normal and hypothyroid rats, thereby removing potentially confounding effects of extracardiac factors that may influence contractility. Hypothyroidism produced direct inhibition of LV myocyte contractility and relaxation associated with depressed peak systolic $[Ca^{2+}]_{iT}$ and elevated diastolic $[Ca^{2+}]_i$. Moreover, compared with control myocytes, in myocytes from hypothyroid rats, not only did basal myocyte contraction, relaxation and peak systolic $[Ca^{2+}]_{iT}$ decrease, but also β -AR agonist-stimulated myocyte functional performance and $[Ca^{2+}]_{iT}$ response were significantly attenuated. These findings indicate that LV contractile dysfunction observed with hypothyroidism is directly attributable to changes of LV myocytes, and demonstrate the existence of hypothyroid cardiomyopathy.

The defects in $[Ca^{2+}]_i$ regulation may be the primary driver of the altered cardiomyocyte force-generating capacity and relaxation of hypothyroid cardiomyopathy.

4.2. Molecular Basis Contributing the Intrinsic Defects of Cardiomyocytes with Hypothyroidism

Thyroid hormone signaling is extremely complex with several clearly defined cellular mechanisms and many molecular players contributing to ventricular dysfunction. These proteins function in intracellular calcium cycling and thereby regulate systolic and diastolic function characteristic of hypothyroidism.^{4, 32} The defects in $[Ca^{2+}]_i$ regulation we observed in the current study resulted from hypothyroidism produced changes in cardiac gene expressions and activities, specifically SR Ca^{2+} -ATPase and PLB. We found that in myocytes from hypothyroid rats, SERCA2a is down-regulated (the protein levels of SERCA2a decreased 54% and SERCA2a activity reduced by 30%). Also the proteins that “turns on” SERCA2a, phosphorylated-PLB (p-PLB) was significantly lower in the myocytes from hypothyroid rats. Thus, compared with controls, in hypothyroidism, the SERCA2a and PLB interaction was altered, resulting in decreased SERCA2a/PLB ratio with reduced the p-PLB. These alterations contributed to the LV and myocyte dysfunction. This finding is consistent with past studies demonstrating the capacity of sarcoplasmic reticulum to accumulate and retain Ca^{2+} and to inhibit excitation-contraction coupling in cardiomyocytes is depressed in hypothyroidism.^{9, 12, 13}

Importantly, in the current study, we found that not only did basal, but also β -AR stimulated myocyte $[Ca^{2+}]_i$ regulation is also impaired. It is not the up-regulation of cardiac β_3 -ARs, but increased iNOS activation with decreased β_1 -ARs is important contributing cause for this defect in hypothyroidism. It is well known that the SNS controls $[Ca^{2+}]_i$ regulatory systems via β -AR-activation. We found that hypothyroidism is associated with increased sympathetic nervous system activation,^{33, 34} but a diminished positive inotropic response to β -AR stimulation. Previously, we and others have shown that both down-regulation of β_1 -AR and up-regulation of a newly-described β_3 -AR-mediated inhibitory pathway play an important role in β -adrenergic desensitization in HF and cardiomyopathies.^{17, 20, 24, 35, 35} Whether hypothyroidism produces similar changes in the subtypes of β -ARs is uncertain; published results are inconsistent. Herein, we found that plasma norepinephrine levels were significantly elevated in hypothyroid rats, but the ability of β -adrenergic agonist, isoproterenol, to increase LV contractility and augment LV myocyte contraction and relaxation and peak $[Ca^{2+}]_i$ all were significantly blunted. This was accompanied by significantly reduced myocyte β_1 -AR mRNA and β_1 -AR protein levels, with relatively unaltered β_3 -AR mRNA and β_3 -AR protein levels.

We further examined myocyte response to stimulation with BRL-37344, a selective β_3 -AR agonist; we observed similar depressions of myocyte contraction and relaxation with significant reductions in SA, dL/dt_{max} and dR/dt_{max} compared to controls. These data indicate that desensitization of β -AR is mainly due to the down-regulation of β_1 -ARs. These results are consistent with past reports,^{4,36} but contrary to the findings of Arioglu⁷ et al, who reported that decreased positive inotropic responses to both isoprenaline and noradrenaline in isolated papillary muscle but unchanged β_1 -AR mRNA expression in hypothyroidism. Of

note, Arioglu's group was the first to measure hypothyroidism-induced changes in β_3 -AR. They reported that, contrary to our results, the negative inotropic effect of BRL-37344 was decreased in hypothyroid rats versus controls, but β_3 -AR mRNA expressions were increased. We observed no significant changes in either β_3 -AR mRNA expression or β_3 -AR protein levels in hypothyroid rats. Furthermore, myocyte functional responses to BRL-37344 were also similar in normal and hypothyroid rats. This discrepancy might result from several confounding factors, such as different tissue (or cell) preparations. Arioglu et al.⁷ studied isolated papillary muscles, and we examined single LV myocytes. There are different distributions of β_3 -AR in different cardiac chambers. Also, fat contamination may complicate the accurate assessment of β_3 -AR mRNA. Most importantly, in the earlier study, β_3 -AR protein levels were not measured. Thus, we showed, for the first time, that cardiac β_3 -AR protein levels and function were not modified by hypothyroidism. This was different from the increased β_3 -AR expression and function reported in patients with HF and diabetes.^{17, 20, 24, 35, 35}

Compelling data have shown important differences in the regulatory behavior components of the β -AR-G-protein-adenylate cyclase complex that relate to different types of heart muscle disease.³⁷ Thus, the down-regulation of β_1 -AR with unaltered β_3 -AR may be consistent with the view that chronic hypothyroidism-induced heart muscle disease has a distinct pathophysiology.^{2, 3, 5, 37} However, the different severity of cardiac injury and failure in hypothyroid rats may also contribute to this difference. Further studies to address this issue are currently ongoing.

We further investigated NOS participation in the cardiac manifestations of hypothyroidism. We found that iNOS is activated in rats with hypothyroidism. Blocking iNOS activation improved myocyte basal and β -AR-stimulated LV myocyte contractility, relaxation and increased the peak systolic $[Ca^{2+}]_{IT}$. importantly, the enhanced iNOS-mediated negative modulation on LV myocyte function performance is due to the up-regulation iNOS in hypothyroidism. We found that compared with control myocytes, both the iNOS and phosphorylated iNOS protein levels were all significantly increased. It is noted that although recent evidence suggest that altered activations of cardiac NOS isoforms are of pathophysiological importance in hypothyroid cardiomyopathy,^{4, 7, 8, 38} the changes in, and functional consequence of, the three NOS isoforms in hypothyroidism have not been systematically evaluated previously.^{7, 8, 39} Thus, in the current study, for the first time, we reported upregulation and adverse functional effects of cardiac iNOS-coupled pathway in hypothyroidism. We found that hypothyroidism up-regulates myocyte iNOS, thereby exacerbating extant dysfunctional $[Ca^{2+}]_i$ regulation, reduced β -adrenergic reserve, and enhanced inhibition of cardiomyocyte contraction and relaxation-all of which promote cardiac dysfunction in the setting of thyroid-hormone deficiency.

The potential mechanisms linking iNOS to hypothyroidism are uncertain, but may be involved in the oxidative stress and/or inflammation. It has been reported that unlike eNOS and nNOS, iNOS has not been found to be constitutively present in the normal, younger adult heart, but can be induced by pro-inflammatory substances such as inflammatory cytokines, lipopolysaccharide,^{40, 41} and mechanical stress.^{42, 43} In the current study, we found chronic hypothyroidism produced significantly increased plasma norepinephrine

levels with elevated a biomarker of oxidative stress of 8-isoprostane concentrations. Whether increased cardiac iNOS expression triggers iNOS uncoupling, leading to oxidative stress and thus promoting intrinsic myocyte dysfunction in hypothyroidism remains to be investigated.

Also as yet, the relation between iNOS expression and reduced β_1 -AR expression is unclear. There is no study that has assessed a potential cause/effect relationship between these two alterations in hypothyroidism. It is likely; there is an important interaction between these two changes. The upregulation of iNOS may aggravate β_1 -ARs down-regulation. Perhaps the down-regulation of cardiac β_1 -ARs is as a consequence of hypothyroidism-induced sympathetic overdrive with high levels of catecholamines. β_1 -adrenergic signaling abnormalities may be an adaptive response to prevent pathway over-stimulation. However, hypothyroidism-induced up-regulation of iNOS signaling pathway may be a maladaptive change, directly depressing LV and cardiomyocyte contraction and relaxation, exacerbating SNS activation which may further down-regulate cardiac β_1 -ARs and driving hypothyroid cardiomyopathy progression. More insight may be gained from further work designed to test whether cardiac iNOS-deficient animals can be protected from methimazole-induced hypothyroidism.

5. Study limitations

Several methodological issues should be considered in the interpretation of our data. First, we studied a rat model with rather severe hypothyroidism induced by methimazole that reproduces many of the functional and neurohormonal features similar with clinical hypothyroidism; we cannot ascertain that these results are applicable to clinical hypothyroidism or thyroid-hormone deficiency from other causes (such as experimental models with surgical removal or with 5-propyl-2-thiouracil (PTU)-induction). It remains to be determined if similar cardiac changes may occur in humans with low thyroid function and what level of thyroid dysfunction is necessary to produce these changes. Second, general anesthesia is an essential element of experimental medical procedures. Administration of ketamine was reported to be associated with significantly decreased LV EF^{10, 26}. In the current study, ketamine was used to induce and maintain anesthesia. Thus, we obtained relatively lower EF (54%) values in the control and hypothyroid (40%) rats. However, compared with controls, in the hypothyroid rats, EF still significantly reduced 26%. Third, in the intact control animals, the isoproterenol-produced increase in LV contractility was clearly demonstrated by using the load-insensitive measures of contractility, LV P-V analysis, but failed to show significantly marked changes in the load-sensitive measures of LV contractility, specifically EF and dP/dt_{max} . We noted that compared with the baselines, isoproterenol caused increases in EF (although significant), but the changes were very small. In addition, isoproterenol failed to cause significant increase in the dP/dt_{max} . This may be due to several factors: 1) ketamine use could blunting sympathetic tone^{10, 26} which may attenuate normal isoproterenol-produced increases in EF and dP/dt_{max} . 2) We used a very small amount of isoproterenol, only 0.5 ml of 10^{-8} M. We are trying to mimic the physiologic stress to determine the β -AR reserve; 3) The maximum rate of LV pressure, dP/dt_{max} is a convenient index for LV contractile state, it is determined by myocardial contractility, heart rate, and the loading conditions on the ventricle. Although the

V_{ED} was relatively unchanged, but heart rate, P_{ED} and E_a changes may play some role^{27–30}. The large standard deviation (SD) of dP/dt_{max} also contributes to the no significant increase in the dP/dt_{max} in response to isoproterenol stimulation. The large SD of dP/dt_{max} may resulted by the signal noise on the pressure catheter tip recorded LVP^{27–30}, which may also attenuate LVP signal adequate frequency response to isoproterenol stimulation. Forth, we observed hypothyroidism caused increased LV myocyte iNOS expression and reduced β_1 -AR expression, but our study did not assess the relation between these two contrast changes. Further studies are warranted.

6. Conclusions

Hypothyroidism produces defects in the cardiomyocyte force-generating capacity and relaxation process with impaired $[Ca^{2+}]_i$ regulation, which may be critical element in the development of hypothyroid cardiomyopathy and HF. Up-regulation of cardiomyocyte iNOS with down-regulation of β_1 -AR and SERCA2a also importantly contribute to LV and myocyte dysfunction. Our data provide new evidence that low thyroid function is not simply a risk factor, but can actually cause cardiomyopathy and HF. These findings advance our understanding of the pathophysiology of hypothyroid cardiomyopathy, have implications for future therapeutic directions and reinforce the importance of early detection and effective treatment of cardiac abnormalities in patients who have hypothyroidism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Examples on hypothyroidism-induced contractile dysfunction of the left ventricle (LV) at baseline, and impaired response to β -adrenergic receptor stimulation. Shown are LV pressure-volume loops produced by inferior vena cava occlusions obtained from one control and one hypothyroid rat at baseline (dotted line) and after isoproterenol (ISO) infusion (solid line). P_{ES} - V_{ES} relation is indicated by the line. The slope and position of this line provide a load-insensitive measure of LV contractility.

Fig. 2.

Examples of hypothyroidism (Hypo)-induced LV myocyte dysfunction and impaired $[\text{Ca}^{2+}]_i$ regulation at baseline, and response to β -adrenergic receptor stimulation. Analog recordings in the electrically-stimulated myocytes obtained from one control and one hypothyroid rat. Freshly-isolated myocytes were tested after being superfused with a modified HEPES solution and field stimulated at 0.5 Hz, and then after superfusion of isoproterenol (ISO). Percent of shortening (SA), peak velocity of shortening (dL/dt_{max}) and relengthening (dR/dt_{max}), and peak $[\text{Ca}^{2+}]_{iT}$ were assessed in myocytes.

Fig. 3.

LV myocyte functional responses to β -AR or β_3 -agonists in hypothyroidism (Hypo). Group means of the changes on LV myocyte contraction (velocity of shortening, dL/dt_{max}) and peak velocity ($[Ca^{2+}]_{ir}$) in control ($n=11$) and hypothyroid ($n=9$) rats, in response to isoproterenol (ISO) stimulation, a non-selective β -AR agonist (10^{-8} M), or BRL-37344 (BRL), a selective β_3 -agonist (10^{-8} M). Values shown mean ($\pm$ SE). [†] $p < 0.05$, ISO and BRL-induced changes vs baselines; [‡] $p < 0.05$, ISO-induced changes between groups.

Fig. 4A

Fig. 4B

Fig. 4.

Fig. 4A. Examples on LV myocyte functional responses to iNOS inhibition in hypothyroidism. The superimposed tracings of analog recordings of contractile and $[Ca^{2+}]_{iT}$ responses at baseline and after isoproterenol (ISO, $10^{-8}M$) in myocytes from hypothyroid rats without (a) and with (b) pretreatment with the iNOS inhibitor, 1400W (10^{-5} mol/L). Inhibition of iNOS improved both myocyte basal and β -adrenergic-stimulated functional

performance, supporting adverse effects of cardiac iNOS activation in hypothyroidism-induced LV myocyte dysfunction.

Fig. 4B. Group means (n=8 per group) of LV myocyte functional responses to iNOS inhibition in hypothyroidism (Hypo). Data shown the effects of iNOS inhibition with 1400W (10^{-5} mol/L) on cardiomyocyte basal (a) and β -adrenergic-stimulated (b) functional performance and $[Ca^{2+}]_i$ regulation in myocytes from normal and hypothyroid (Hypo) rats. Results (mean $\pm$ SE) of experiments comparing myocyte contraction (velocity of shortening, dL/dt_{max}), myocyte relaxation (dR/dt), and peak velocity ($[Ca^{2+}]_{iT}$) are shown. White bars = baseline values in myocytes from control and hypothyroid rats; Black bars = myocytes responses after 1400W. * $p<0.05$, hypothyroid baseline vs. normal control baseline; † $p<0.05$, hypothyroid with 1400W vs hypothyroid baseline.

Fig. 5.

β_1 - and β_3 -AR expression in LV myocytes from control and hypothyroid rats. Examples of β_1 - and β_3 -AR mRNA (A) and protein levels (B) of LV myocytes obtained from one control and one hypothyroid rat. (a) β_1 - and β_3 -AR mRNA were detected with the predicted size of 327bp and 308bp, respectively. For normalization, GAPDH was co-amplified with the predicted size of 500bp. (b) Western blot analysis. Single bands of β_1 - (about 55 kDa) and β_3 -AR (about 75 kDa) were detected. Actin was reprobated as a loading control. B Group means ($\pm$ SD) (n=8 per group) of β_1 - and β_3 -AR protein levels in myocytes from control and hypothyroid. * $p < 0.05$.

Fig. 6.

Expressions and activities of SERCA2a, phospholamban (PLB) and phosphorylated PLB (p-PLB) in LV myocytes from control and hypothyroid (Hypo) rats. (a) Examples of SERCA2a, PLB and p-PLB expressions. (b) Group means ($\pm$ SD) ($n=6$ per group) of SERCA2a protein levels, expressed as a ratio of calsequestrin signal, and Ca^{2+} -dependent SERCA2a activity. (c) Group means ($\pm$ SD) of p-PLB. $*p<0.05$.

Fig. 7A

Fig. 7B

Fig. 7.

Fig. 7A. Expression of three forms of nitric oxide synthase (iNOS, eNOS, and nNOS) expression in LV myocytes of control and hypothyroid (Hypo) rats. (a) Examples of western blots of each NOS form, with actin as a control. (b) Group means ($\pm$ SD) ($n=8$ per group) of LV myocyte iNOS, eNOS and nNOS protein levels. $*p<0.05$.

Fig. 7B. Expression of phospho-iNOS in LV myocytes from control and hypothyroid (Hypo) rats. (a) Examples of Western blots of phospho-iNOS, with actin as a control. (b) Group means ($\pm$ SD) ($n=6$ per group) of phospho-iNOS protein levels. $*p<0.05$.

Table 1
LV Function and Hemodynamics at Baseline and Response to Isoproterenol Stimulation

	Control (n=9)		Hypothyroidism (n=8)	
	Baseline	Isoproterenol	Baseline	Isoproterenol
Heart rate (beats/min)	261 ± 12	299 ± 11 [†]	164 ± 7 [*]	171 ± 7 [†] [‡]
Maximum dP/dt (mmHg/sec)	7402 ± 410	7555 ± 583	3006 ± 178 [*]	3085 ± 186
Minimum dP/dt (mmHg/sec)	-5933 ± 374	-5864 ± 476	-2563 ± 166 [*]	-2577 ± 161
Stroke volume (μl)	169.5 ± 15.1	183.3 ± 15.9 [†]	122.3 ± 5.9 [*]	124.4 ± 5.7 [‡]
LV end-diastolic pressure (mmHg)	5.9 ± 0.6	6.5 ± 0.8	4.6 ± 0.5	5.1 ± 0.6
LV end-systolic pressure (mmHg)	110 ± 3	112 ± 7	90 ± 2 [*]	91 ± 3
LV end-diastolic volume (μl)	310.7 ± 15.2	308.3 ± 15.7	304.2 ± 6.6	305.6 ± 7.1
LV end-systolic volume (μl)	142.2 ± 8.3	125.0 ± 8.5 [†]	181.9 ± 6.8 [*]	181.2 ± 7.3 [‡]
Ejection Fraction (%)	54 ± 3	58 ± 3 [†]	40 ± 2 [*]	41 ± 2 [‡]
Maximum dV/dt (μl/sec)	5531 ± 386	7181 ± 568 [†]	3443 ± 307 [*]	3908 ± 321 [†] [‡]
Time constant of relaxation (ms)	10.9 ± 0.6	8.9 ± 0.6 [†]	21.8 ± 1.4 [*]	21.1 ± 1.6 [‡]
Arterial Elastance (mmHg/μl)	0.64 ± 0.05	0.61 ± 0.07	0.76 ± 0.04	0.75 ± 0.03
E _{ES} (mmHg/μl)	1.06 ± 0.04	1.49 ± 0.07 [†]	0.65 ± 0.02 [*]	0.75 ± 0.01 [†] [‡]
M _{SW} (mmHg)	81.5 ± 2.4	106.8 ± 2.5 [†]	56.3 ± 1.9 [*]	63.2 ± 2.1 [†] [‡]

Values are mean ± SE; n = number of rats.

LV, left ventricular; dP/dt, rate of rise of LV pressure; maximum dV/dt, the peak rate of mitral flow; E_{ES}, the slope of linear PES-VES relation; M_{SW}, the slope of stroke work-VES relation.

^{*} $p < 0.05$, hypothyroidism baseline vs. control baseline;

[†] $p < 0.05$, isoproterenol vs. corresponding baseline value;

[‡] $p < 0.05$, isoproterenol-induced percent changes between hypothyroidism vs. normal control.

Table 2AMyocyte Function and $[Ca^{2+}]_i$ Transient at Baseline and Responses to Isoproterenol Stimulation

	Normal Control (n=11)		Hypothyroidism (n=9)	
	Baseline	Isoproterenol	Baseline	Isoproterenol
Resting length (μm)	120.0 $\pm$ 3.3	119.5 $\pm$ 3.2	107.8 $\pm$ 4.4	107.6 $\pm$ 4.4
Percent shortening (SA; %)	10.0 $\pm$ 0.4	14.0 $\pm$ 0.5 [†]	5.7 $\pm$ 0.3 [*]	7.0 $\pm$ 0.3 ^{† ‡}
Velocity of shortening ($\mu\text{m}/\text{sec}$)	141.7 $\pm$ 5.6	232.3 $\pm$ 11.1 [†]	57.8 $\pm$ 2.5 [*]	78.7 $\pm$ 3.5 ^{† ‡}
Velocity of relengthening ($\mu\text{m}/\text{sec}$)	112.9 $\pm$ 5.8	163.3 $\pm$ 8.5 [†]	46.2 $\pm$ 2.3 [*]	60.7 $\pm$ 3.5 ^{† ‡}
Peak systolic $[Ca^{2+}]_i$ transient	0.25 $\pm$ 0.01	0.34 $\pm$ 0.02 [†]	0.18 $\pm$ 0.01 [*]	0.21 $\pm$ 0.01 ^{† ‡}

Values are mean $\pm$ SE; n=number of rats.* $p < 0.05$, hypothyroidism baseline vs. control baseline;† $p < 0.05$, isoproterenol vs. corresponding baseline value;‡ $p < 0.05$, isoproterenol-induced percent changes between hypothyroidism vs. normal control.

Table 2BMyocyte Functional and $[Ca^{2+}]_i$ Transient at Baseline and Responses to BRL-37344 Stimulation

	Normal Control (n=11)		Hypothyroidism (n=9)	
	Baseline	BRL-37344	Baseline	BRL-37344
Resting length (μm)	116.9 $\pm$ 2.8	117.7 $\pm$ 2.6	108.9 $\pm$ 2.6	109.0 $\pm$ 2.6
Percent shortening (SA; %)	10.6 $\pm$ 0.4	9.1 $\pm$ 0.5 [†]	5.9 $\pm$ 0.4 [*]	5.0 $\pm$ 0.4 [†]
Velocity of shortening ($\mu\text{m}/\text{sec}$)	146.6 $\pm$ 5.8	126.1 $\pm$ 5.7 [†]	62.4 $\pm$ 4.2 [*]	52.7 $\pm$ 4.1 [†]
Velocity of relengthening ($\mu\text{m}/\text{sec}$)	114.1 $\pm$ 7.5	100.1 $\pm$ 5.8 [†]	47.6 $\pm$ 4.1 [*]	41.8 $\pm$ 4.1 [†]
Peak systolic $[Ca^{2+}]_i$ transient	0.25 $\pm$ 0.01	0.21 $\pm$ 0.02 [†]	0.18 $\pm$ 0.01 [*]	0.15 $\pm$ 0.01 [†]

Values are mean $\pm$ SE; n=number of rats.* $p < 0.05$, hypothyroidism baseline vs. control baseline;† $p < 0.05$, BRL-37344 vs. corresponding baseline value.

Table 3AEffect of 1400w on Myocyte Basal Contractile Performance and $[Ca^{2+}]_i$ Transient

	Normal Control (n=8)		Hypothyroidism (n=8)	
	Baseline	1400W	Baseline	1400W
Resting length (μm)	116.9 $\pm$ 2.6	116.3 $\pm$ 2.6	110.4 $\pm$ 2.9	110.3 $\pm$ 3.0
Percent shortening (SA; %)	10.7 $\pm$ 0.3	10.8 $\pm$ 0.4	5.7 $\pm$ 0.3 [*]	7.2 $\pm$ 0.3 ^{† ‡}
Velocity of shortening ($\mu\text{m}/\text{sec}$)	149.1 $\pm$ 3.8	152.1 $\pm$ 4.9	60.9 $\pm$ 3.4 [*]	82.9 $\pm$ 4.2 ^{† ‡}
Velocity of relengthening ($\mu\text{m}/\text{sec}$)	119.7 $\pm$ 2.8	121.7 $\pm$ 2.6	46.0 $\pm$ 3.8 [*]	66.0 $\pm$ 3.9 ^{† ‡}
Peak systolic $[Ca^{2+}]_i$ transient	0.25 $\pm$ 0.01	0.25 $\pm$ 0.01	0.18 $\pm$ 0.01 [*]	0.22 $\pm$ 0.01 ^{† ‡}

Values are mean $\pm$ SE; n=number of rats.^{*}
^{*} $p < 0.05$, hypothyroidism baseline vs. control baseline;[†]
[†] $p < 0.05$, 1400w vs. corresponding baseline value;[‡]
[‡] $p < 0.05$, 1400w-induced percent changes between hypothyroidism vs. normal control.

Table 3BEffect of 1400w on Myocyte Contractile and $[Ca^{2+}]_i$ Transient Responses to Isoproterenol Stimulation

	Normal Control (n=6)		Hypothyroidism (n=6)	
	1400W	Isoproterenol	1400W	Isoproterenol
Resting length (μm)	114.8 $\pm$ 0.8	113.8 $\pm$ 1.0	115.2 $\pm$ 6.1	114.4 $\pm$ 5.9
Percent shortening (SA; %)	9.5 $\pm$ 0.7	13.6 $\pm$ 0.9 [†]	7.1 $\pm$ 0.2 [*]	10.1 $\pm$ 0.3 [†]
Velocity of shortening (dL/dt _{max} ; $\mu\text{m}/\text{sec}$)	142.2 $\pm$ 10.9	226.7 $\pm$ 12.6 [†]	86.7 $\pm$ 4.6 [*]	135.2 $\pm$ 8.6 [†]
Velocity of relengthening (dR/dt _{max} ; $\mu\text{m}/\text{sec}$)	123.8 $\pm$ 9.6	186.4 $\pm$ 17.2 [†]	66.6 $\pm$ 3.9 [*]	106.0 $\pm$ 5.6 [†]
Peak systolic $[Ca^{2+}]_i$ transient	0.25 $\pm$ 0.01	0.33 $\pm$ 0.02 [†]	0.21 $\pm$ 0.01 [*]	0.27 $\pm$ 0.01 [†]

Values are mean $\pm$ SE; n=number of rats.* $p < 0.05$, hypothyroidism 1400w baseline vs. control 1400w baseline;† $p < 0.05$, isoproterenol vs. corresponding 1400w baseline value.