Published in final edited form as:

Free Radic Biol Med. 2015 June ; 83: 252–261. doi:10.1016/j.freeradbiomed.2015.02.035.

Differential involvement of various sources of reactive oxygen species in thyroxine-induced hemodynamic changes and contractile dysfunction of the heart and diaphragm muscles

Mohammad T. Elnakish^{a,b,c}, Eric J. Schultz^{a,b}, Rachel L. Gearinger^{a,b}, Nancy S. Saad^{a,b,c}, Neha Rastogi^{a,b}, Amany A.E. Ahmed^c, Peter J. Mohler^{a,b}, and Paul M.L. Janssen^{a,b,*}

^aDepartment of Physiology and Cell Biology, College of Medicine, The Ohio State University, Columbus, OH 43210, USA

^bDorothy M. Davis Heart & Lung Research Institute, The Ohio State University, Columbus, OH 43210, USA

^cDepartment of Pharmacology and Toxicology, Faculty of Pharmacy, Helwan University, Cairo, Egypt

Abstract

Thyroid hormones are key regulators of basal metabolic state and oxidative metabolism. Hyperthyroidism has been reported to cause significant alterations in hemodynamics, and in cardiac and diaphragm muscle function, all of which have been linked to increased oxidative stress. However, the definite source of increased reactive oxygen species (ROS) in each of these phenotypes is still unknown. The goal of the current study was to test the hypothesis that thyroxine (T₄) may produce distinct hemodynamic, cardiac, and diaphragm muscle abnormalities by differentially affecting various sources of ROS. Wild-type and T₄ mice with and without 2-week treatments with allopurinol (xanthine oxidase inhibitor), apocynin (NADPH oxidase inhibitor), L-NIO (nitric oxide synthase inhibitor), or MitoTEMPO (mitochondria-targeted antioxidant) were studied. Blood pressure and echocardiography were noninvasively evaluated, followed by ex vivo assessments of isolated heart and diaphragm muscle functions. Treatment with L-NIO attenuated the T₄-induced hypertension in mice. However, apocynin improved the left-ventricular (LV) dysfunction without preventing the cardiac hypertrophy in these mice. Both allopurinol and MitoTEMPO reduced the T₄-induced fatigability of the diaphragm muscles. In conclusion, we show here for the first time that T₄ exerts differential effects on various sources of ROS to induce distinct cardiovascular and skeletal muscle phenotypes. Additionally, we find that T₄-induced LV dysfunction is independent of cardiac hypertrophy and NADPH oxidase is a key player in this process. Furthermore, we prove the significance of both xanthine oxidase and mitochondrial ROS pathways in T₄-induced fatigability of diaphragm muscles. Finally, we confirm the importance of the nitric oxide pathway in T₄-induced hypertension.

Keywords

Thyroxin; Allopurinol; Apocynin; L-NIO; MitoTEMPO; Cardiac muscle; Diaphragm; Free radicals

Thyroid hormones are key regulators of basal metabolic state and oxidative metabolism, with the potential to increase ROS¹ generation [1]. Hyperthyroidism has been reported to cause significant alterations in hemodynamics and in cardiac and skeletal muscle functions [2–8]. In the cardiovascular system thyroxin (T4) results in hypertension [2,9] and exerts striking effects on the heart, ranging from physiologic cardiac hypertrophy with enhanced function [3] to cardiac dilation and heart failure [4]. Antioxidants effectively decreased T4-induced hypertension, signifying a role for oxidative stress in this process [2]. Similarly, several reports revealed increased oxidative enzymes and decreased antioxidant enzymes in the hyperthyroid hearts, indicating a state of increased oxidative stress in these hearts [2,6,10–12]. On the other hand, thyroid hormone is known to reduce contractility in various skeletal muscles [5–8]. Diminished vital capacity and dyspnea are occasionally coupled with clinical hyperthyroidism, and a decline in peak respiratory muscle force has been presented [5,8]. Interestingly, T4-induced elevation in lipid peroxidation was detected in the mainly slow-twitch oxidative soleus but not in the fast-twitch glycolytic extensor digitorum longus [6,7,13]. Likewise, oxidative modifications of myofibrillar proteins have been shown to be involved in contractile dysfunction of the hyperthyroid diaphragms [8].

A significant link between cardiac and skeletal muscle dysfunction is based on the notion that some inflammatory diseases, including sepsis and heart failure, coupled with the progress of generalized muscle weakness, stir up higher ROS generation in skeletal muscle [14]. In particular, development of heart failure was reported to be coupled with marked changes in diaphragmatic function, resulting in a significant increase in fatigability and ROS generation [14,15]. Generally, improved cardiac pump function is the most documented upshot of hyperthyroidism [3]. Nevertheless, cardiac dysfunction has also been reported in animals after prolonged T4 treatment, as well as in human patients, indicating that excess T4 can be a potential risk factor for heart failure [4]. Recently, we [16] demonstrated that a T4 dose of 500 µg/kg/day results in hypertension, cardiac hypertrophy, and LV systolic dysfunction, in contrast to physiological cardiac hypertrophy and preserved cardiac function that we previously reported to be present at a lower T4 dose (200 µg/kg/day) [17,18]. In the latter study [18], we also showed increased ROS production in hyperthyroid hearts.

Taking these findings into account, we postulate that T4 may produce these distinct cardiovascular and skeletal muscle phenotypes by differentially affecting various sources of ROS. The goal of this study was to investigate this hypothesis by (1) examining the role of ROS in T4-induced hypertension, cardiac hypertrophy, and associated cardiac dysfunction in our model; (2) examining the effect of T4 on diaphragm muscle function as well as the possible contribution of ROS to this effect; and (3) identifying the source of ROS resulting in these distinct phenotypes. Sources of ROS include nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, uncoupled nitric oxide (NO) synthase (NOS), and mitochondria [19]. We inhibited each source sequentially as previously

described [20] and examined and quantified its effects on T4-induced cardiovascular and diaphragm muscle phenotypes.

Methods

Animals

Male FVB/N mice (7–9 months of age) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and kept at the Research Animal Facility of The Ohio State University. The experimental procedures and protocols used in this study were approved by the Animal Care and Use Committee of The Ohio State University, conforming to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85–23, revised 1996).

Antioxidant and T4 treatments

All drugs were freshly prepared and administered by intraperitoneal injection every day before T4 treatment for 2 weeks based on previous reports with slight modifications as follows: allopurinol from Cayman Chemical (Ann Arbor, MI, USA) was dissolved in phosphate-buffered saline (PBS) after heating at 75 °C for 1 h and administered at a dose of 20 mg/kg/day while it was warm [20–22], apocynin from Cayman Chemical was dissolved in dimethyl sulfoxide (DMSO) and diluted with PBS (final DMSO concentration 5%) and administered at a dose of 50 mg/kg/day [23,24], *N*⁵-(1-iminoethyl)-L-ornithine dihydrochloride (L-NIO) from Calbiochem, EMD Millipore (Taunton, MA, USA) was dissolved in PBS and administered at a dose of 25 mg/kg/day [20], and MitoTEMPO from Sigma–Aldrich (St. Louis, MO, USA) was dissolved in PBS and administered at a dose of 0.7 mg/kg/day [20]. Sodium-L-thyroxine, T4, from Sigma–Aldrich was prepared as previously described [18] and injected intraperitoneally at a dose of 500 µg/kg/day for 2 weeks as reported before [16].

Animals were divided into six groups based on treatment as follows: wild-type+vehicle (control, *n* = 15), wild-type+T4 (T4, *n* = 13), wild-type+allopurinol+T4 (allopurinol, *n* = 11), wild-type + apocynin+T4 (apocynin, *n* = 14), wild-type+L-NIO+T4 (L-NIO, *n* = 10), and wild-type+MitoTEMPO+T4 (MitoTEMPO, *n* = 12). At the end of the treatment period animals underwent blood pressure (BP) measurements and echocardiography. Thereafter, the animals were sacrificed; heart and diaphragm muscles were excised and processed for further ex vivo experiments.

Blood pressure measurements

BP was measured noninvasively in conscious mice by the tail cuff method using a six-channel CODA high-throughput acquisition system (Kent Scientific Corp., Torrington, CT, USA) as previously described [16,25]. BP recordings were obtained after the mice had been trained. Each training and experimental session consisted of 10 acclimatization cycles followed by 10 BP measurement cycles. Only accepted cycles as identified by the BP measurement software were included. The average of accepted cycles from one session was used for systolic, diastolic, and mean arterial BP in each mouse.

Echocardiography

In vivo LV dimension and contractile function in mice were evaluated using a high-frequency ultrasound imaging system (VEVO 2100, Visual Sonics, Toronto, ON, Canada) as previously described [16–18]. Experimental mice were anesthetized with isoflurane at a concentration of 2% and then maintained at 1.5% isoflurane using nasal prongs during the whole procedure. The measurements were taken from the parasternal short-axis view in M-mode to view the LV movement during systole and diastole corresponding to the electrocardiogram. All data and imaging were analyzed by the Visual Sonics Cardiac Measurements Package.

Cardiac muscle preparation and experimental setup

Five minutes after intraperitoneal heparin administration, mice were euthanized by cervical dislocation. After bilateral thoracotomy, hearts were rapidly excised and placed in Krebs–Henseleit buffer containing (in mmol/L) 120 NaCl, 5 KCl, 2 MgSO₄, 1.2 NaH₂PO₄, 20 NaHCO₃, 0.25 Ca²⁺, and 10 glucose (pH 7.4), equilibrated with 95% O₂–5% CO₂. Additionally, 20 mmol/L 2,3-butanedione monoxime (BDM) was added to the dissection buffer to prevent cutting injury [16,25–27]. Hearts were cannulated via the ascending aorta and retrogradely perfused with the same buffer for several minutes. Blood was thoroughly washed out, and from the right ventricle (RV), uniform linear papillary muscles were carefully dissected. The dimensions of the muscles were measured using a calibration reticule in the ocular of the dissection microscope (40 ×, resolution ~ 10 μm). The cross-sectional areas were calculated assuming ellipsoid cross-sectional shapes. Average dimensions were not significantly different among groups: control (0.46 × 0.30 × 1.05 mm), T4 (0.44 × 0.29 × 0.95 mm), allopurinol (0.46 × 0.31 × 0.96 mm), apocynin (0.52 × 0.35 × 1.05 mm), L-NIO (0.44 × 0.29 × 0.87 mm), and MitoTEMPO (0.47 × 0.31 × 1.06 mm).

With the use of the dissection microscope, muscles were mounted between the basket-shaped extension of a force transducer (KG7, Scientific Instruments, Heidelberg, Germany) and a hook (valve end) connected to a micromanipulator as previously described [16,25,27]. Muscles were superfused with the same buffer at 37.5 °C as above (with the exception that BDM was omitted) and stimulated at 4 Hz. Extracellular Ca²⁺ concentration was raised to 2 mmol/L and muscles were allowed to stabilize for at least 30 min before the experimental protocol was initiated. As in our previous reports [16,25,27], the 4 Hz baseline was selected rather than a more physiological 12 Hz. However, to study more physiological frequencies, 12-Hz contractions were also assessed, but only for brief periods. Generally, muscles were stretched to an optimal length at which a small increase in length resulted in nearly equal increases in resting tension and active developed tension. This length was selected to be comparable to the maximally attained length in vivo at the end of diastole [28].

To obtain a broad scope of quantitative data to dissect contractile function and dysfunction, the three main mechanisms utilized in vivo to physiologically modify the force of contraction, length-dependent activation, frequency-dependent activation, and β-adrenergic stimulation were assessed in mouse papillary muscles under near-physiological conditions as previously described [16,25,27]. To assess the effect of muscle length on developed force (F_{dev}), slack length (length of muscle without any preload) and optimal length were

determined. The difference was divided into three equal steps, and the muscle was stretched sequentially with each step, increasing the length of the muscle until the baseline length was achieved. Parameters were recorded when the muscle had stabilized at each length. We assessed the effect of increasing stimulation frequencies between 4 and 14 Hz, spanning the entire in vivo range of the mouse. At each frequency, forces were allowed to reach steady state before data were recorded. The effects of β -adrenergic stimulation were assessed by a concentration–response curve with isoproterenol (10^{-9} – 10^{-6} mol/L) at a baseline stimulation frequency of 4 Hz.

In all experiments performed peak isometric F_{dev} was determined and normalized to the cross-sectional area (CSA) of the muscle. Additionally, as a model-independent parameter of force decay kinetics, time to peak force (TTP), and time from peak force to 50% relaxation (RT_{50}) were determined.

Diaphragm force measurements and experimental setup

Diaphragm force and fatigue measurements were done as reported before [29]. After careful excision of mouse hearts, the diaphragm and ribcage were removed and placed in Krebs–Henseleit buffer equilibrated with 95% O_2 –5% CO_2 with the addition of BDM to prevent muscle damage during dissection. Two linear strips of muscle, approximately 2–3 mm in width, were carefully dissected from each diaphragm. This width was found to be optimal through earlier unpublished work in our lab. On one end of the muscle, the rib tissue was left intact, allowing the diaphragm strip to be held in place by inserting the muscle through a stainless steel basket connected to a force transducer (KG2, Scientific Instruments). The central tendon at the deep end of the diaphragm strip was then pierced over hooks that were connected to a linear micromanipulator. Each muscle bath contained the same oxygenated Krebs–Henseleit solution as described above (without BDM and with 2.0 mmol/L $CaCl_2$) at 37 °C. A single electrical stimulation pulse was delivered via two parallel platinum–iridium electrodes on either side of the muscle. The muscle was then stretched to its optimal length, defined as the length at which maximum twitch force is measured. The muscles were subsequently allowed to rest for 10 min. After equilibration and finding the optimal length using twitch contractions, the muscles were subjected to a protocol consisting of a series of six tetanic contractions within a frequency range of 20–180 Hz stimuli at a maximal voltage of 250 ms, with approximately 2 min between contractions. For comparative purposes, all force measurements are expressed per unit of CSA (normalized isometric force or tension: mN/mm^2). CSA was calculated using the following equation: muscle mass in mg/[$(\text{optimal length in mm}) \times (\text{muscle density in } mg/mm^3)$], where muscle density is assumed to be 1.056 mg/mm^3 .

With the same muscles, we assessed the response to a fatigue/ injury protocol. Muscles were stimulated once per second (frequency 100 Hz, duration 500 ms) for 66 s. Thereafter, the muscles were left to rest unstimulated for 15 min, and the 66-s on-off protocol was repeated.

Data analysis and statistics

Data are presented as the mean $\pm$ SEM and were analyzed by either one-way analysis of variance (ANOVA) followed by the Dunnett multiple comparisons post hoc test, comparing

all groups to T4, or by two-way ANOVA. A two-tailed value of $p = 0.05$ was considered statistically significant.

Results

First, we investigated BP after 2 weeks of treatment in all groups. T4-treated mice exhibited significant increases in systolic (108 ± 4 mm Hg; $p < 0.01$), diastolic (77 ± 3 mm Hg; $p < 0.05$), and mean arterial pressure (MAP) (87 ± 4 mm Hg; $p < 0.01$) compared to control (systolic, 90 ± 3 mm Hg; diastolic, 62 ± 3 mm Hg; and MAP, 70 ± 3 mm Hg; Figs. 1A–C), as we illustrated before [16]. Surprisingly, L-NIO resulted in a significant decrease in T4-induced increases in diastolic pressure (63 ± 4 mm Hg; $p < 0.05$) and MAP (73 ± 4 mm Hg; $p < 0.05$), but resulted in only a slight decrease in systolic BP (98 ± 5 mm Hg) compared to T4-treated mice (Figs. 1A–C). Conversely, none of the other antioxidant drugs were able to affect systolic (allopurinol, 107 ± 4 mm Hg; apocynin, 109 ± 4 mm Hg; and MitoTEMPO, 111 ± 4 mm Hg), diastolic (allopurinol, 76 ± 3 mm Hg; apocynin, 78 ± 3 mm Hg; and MitoTEMPO, 79 ± 4 mm Hg), or MAP (allopurinol, 86 ± 4 mm Hg; apocynin, 88 ± 4 mm Hg; and MitoTEMPO, 89 ± 4 mm Hg) compared to T4-treated mice (Figs. 1A–C). Consistent with our previous findings, there was no significant difference in HR between T4-treated mice (707 ± 18 bpm) and control (719 ± 12 bpm) during BP measurements (Fig. 1D). All antioxidant treatments exhibited comparable HR (allopurinol, 735 ± 11 bpm; apocynin, 685 ± 14 bpm; and Mito TEMPO, 692 ± 18 bpm) to that of T4-treated mice except for L-NIO, which showed significantly lower HR (631 ± 33 bpm; Fig. 1D).

Next, the weight of the mice was assessed. At the end of the treatment period there was a significant difference in the body weights of only the mice in the MitoTEMPO group compared to the T4-treated mice ($p < 0.05$; Table 1). Consistent with our previous findings [16], heart weight and heart weight/body weight as isolated parameters were significantly altered by T4 treatment compared to control ($p < 0.01$). However, none of the antioxidant drugs were able to attenuate these increases in the T4-treated mice (Table 1).

As we previously reported [16], echocardiograph analysis of the mouse hearts confirmed our morphological data and showed a significant increase ($p < 0.01$) in the LV mass of the hearts of T4-treated mice (139 ± 5 mg) compared to those of control (103 ± 4 mg). None of the antioxidant drugs were able to attenuate these increases in the hearts of T4-treated mice (allopurinol, 139 ± 9 mg; apocynin, 135 ± 7 mg; L-NIO, 137 ± 4 mg; and MitoTEMPO, 137 ± 5 mg; Fig. 2A). Echocardiography showed also that there was no significant difference in stroke volume between T4-treated mice (46 ± 1 μ l) and control (44 ± 1 μ l; Fig. 2B). All antioxidant treatments exhibited stroke volumes (allopurinol, 43 ± 2 μ l; apocynin, 48 ± 2 μ l; and L-NIO, 44 ± 1 μ l) comparable to that of T4-treated mice except for MitoTEMPO, which showed significantly lower stroke volume (40 ± 2 μ l; Fig. 2B). In line with our previous data, LV systolic functions were compromised in the hearts of T4-treated mice as evident by significantly ($p < 0.01$) decreased ejection fraction (EF) ($57 \pm 1\%$) and fractional shortening (FS) ($29 \pm 1\%$) relative to those of control (EF $68 \pm 1\%$ and FS $38 \pm 1\%$; Figs. 2C and D). Allopurinol and MitoTEMPO treatment did not affect the EF (57 ± 2 and $54 \pm 2\%$, respectively) or FS (30 ± 1 and $28 \pm 1\%$, respectively) compared to those of T4-treated mice.

L-NIO increased both EF ($63 \pm 1\%$) and FS ($34 \pm 1\%$), yet, this was not statistically significant compared to T4-treated mice. At variance with all drug treatments, apocynin significantly ($p < 0.01$) increased both EF ($65 \pm 2\%$) and FS ($35 \pm 2\%$) compared to those of T4-treated mice (Figs. 2C and D).

Physiological changes in cardiac contractile strength are mainly governed via three mechanisms: length-dependent activation (Frank-Starling mechanism) [30,31], frequency-dependent activation (Bow-ditch effect) [32], and adrenergic stimulation (fight/flight response). To characterize potential deficiencies in cardiac contractile strength, we tested the contractile performance on papillary muscles isolated from the RV of the mouse hearts while varying the above three mechanisms, thereby encompassing their entire physiological range.

Similar to our previous results [16], under near-physiological temperature and at a preload resulting in a sarcomere length around the in vivo end-diastolic value of $2.2 \mu\text{m}$ [28], peak isometric active F_{dev} of papillary muscles from the RV of the mouse hearts was maintained ($p=0.1926$) among the groups at a stimulation frequency of 4 Hz (control, $15 \pm 2 \text{ mN/mm}^2$; T4, $23 \pm 5 \text{ mN/mm}^2$; allopurinol, $22 \pm 4 \text{ mN/mm}^2$; apocynin, $16 \pm 4 \text{ mN/mm}^2$; L-NIO, $19 \pm 4 \text{ mN/mm}^2$; and MitoTEMPO, $27 \pm 4 \text{ mN/mm}^2$; Fig. 3A). In contrast, muscles from T4-treated mice contracted and relaxed more rapidly compared with those from control mice (TTP, T4 $39 \pm 1 \text{ ms}$ vs control $43 \pm 1 \text{ ms}$, $p < 0.05$, and RT_{50} , T4-treated $18 \pm 1 \text{ ms}$ vs control $22 \pm 1 \text{ ms}$, $p < 0.01$). None of the antioxidant treatments were able to change either TTP (allopurinol, $39 \pm 1 \text{ ms}$; apocynin, $38 \pm 1.00 \text{ ms}$; L-NIO, $38 \pm 1 \text{ ms}$; and MitoTEMPO, $38 \pm 1 \text{ ms}$) or RT_{50} (allopurinol, $18 \pm 1 \text{ ms}$; apocynin, $19 \pm 1 \text{ ms}$; L-NIO, $17 \pm 1 \text{ ms}$; and MitoTEMPO, $18 \pm 1 \text{ ms}$) compared to T4 group (Figs. 3B and C). F_{dev} was assessed for muscles from control and T4-treated mice with or without antioxidant administration at 85, 90, and 95% of the optimal muscle length. A nearly linear and highly positive relationship between length and F_{dev} was observed in all groups, indicating that the length-dependent activation mechanism was not significantly affected in the cardiac muscles after T4 treatment (Fig. 4A). Even though two-way ANOVA detected that at least one of the drugs/lengths differs from the others with respect to relative tension change ($p=0.001$), repeated one-way ANOVA at 85, 90, and 95% of the optimal muscle length showed no significant difference among groups and p values were 0.1516, 0.1898, and 0.1155, respectively (Fig. 4A).

F_{dev} was determined not only at the baseline frequency of stimulation of 4 Hz but also within the murine in vivo physiological range (8–12 Hz), thereby allowing for a less ambiguous extrapolation to in vivo outcome. Two-way ANOVA showed that at least one of the drugs/frequencies differs from the others with respect to relative tension change ($p = 0.000$).

Repeated one-way ANOVA at each frequency clearly showed that muscles from control mice positively responded to increasing frequencies signifying a positive force–frequency relationship (FFR); however, muscles from T4-treated mice showed significantly ($p < 0.01$) lower changes in the F_{dev} at all tested frequencies in regard to its value at the basal frequency of 4 Hz compared to control, as demonstrated before [16]. None of the

antioxidant treatments were able to reverse this negative FFR in the T4-treated mice (Fig. 4B).

The effects of β -adrenergic stimulation were assessed by a concentration–response curve with isoproterenol (10^{-9} – 10^{-6} mol/L) at a baseline stimulation frequency of 4 Hz. Two-way ANOVA revealed that at least one of the drugs/isoproterenol concentrations differs from the others with respect to relative tension change ($p=0.000$). One-way ANOVA showed that under full β -adrenergic stimulation (1 μ mol/L isoproterenol), muscles from T4-treated mice exhibited significantly lowered responses ($p<0.01$) versus those from control mice, as we showed before [16]. None of the antioxidant treatments were able to recover this blunted isoproterenol response in the T4-treated mice (Fig. 4C).

Last, we assessed the effect of T4 on tetanic contractions of diaphragm muscles in our model at a frequency range of 20–180 Hz. Our results show that tetanic forces were maintained among groups at all tested frequencies (Fig. 5A). Then, we tested a fatigue/injury protocol in which muscles were stimulated once per second (frequency 100 Hz, duration 500 ms) for 66 s (Fig. 5B). Two-way ANOVA showed that at least one of the drugs/time points differs from the others with respect to relative tension change ($p=0.000$). The relative force drop due to fatigue/injury was higher in the T4-treated mice compared to control at all time points; however, this reached significance only at 18 and 24 s ($p<0.05$), as indicated by repeated one-way ANOVA (Fig. 5B). Earlier in the fatigue/injury protocol, allopurinol reversed the T4-induced force drop, but repeated one-way ANOVA showed significance only at 24 s ($p<0.05$). Thereafter the effect of MitoTEMPO predominated and reversed the T4-induced force drop starting at 30 s ($p<0.05$) until the end of this protocol ($p<0.01$), as revealed by repeated one-way ANOVA (Fig. 5B). This may indicate a mutual role for both xanthine oxidase and mitochondrial-derived ROS at early and late stages, respectively, during the fatigue/injury process in diaphragm. Other antioxidant treatments did not show any improvements compared to T4 (Fig. 5B). After 15 min of rest, however, there was no significant difference in recovery (% change in relative tension at the end of the first 66 s of fatigue/injury protocol compared to that at the beginning of the following 66 s of the same protocol after 15 min rest) between T4 ($28 \pm 2\%$) and control ($32 \pm 2\%$) muscles (Fig. 5C). Muscles from all antioxidant treatments showed recovery (allopurinol, $22 \pm 2\%$; apocynin, $23 \pm 3\%$; L-NIO, $20 \pm 2\%$) comparable to that of T4 muscles, except for the MitoTEMPO group, which showed significantly lower recovery ($11 \pm 4\%$; $p < 0.01$) compared to T4 muscles (Fig. 5C). This is most probably because muscles from this group were initially set at significantly higher relative tensions at the end of the first part of the fatigue/injury protocol compared to T4 muscles (Fig. 5B). Again, during the second part of the fatigue/injury protocol two-way ANOVA showed that at least one of the drugs/time points differs from the others with respect to relative tension change ($p=0.000$). T4 muscles had a trend of showing higher force drop compared to control, and only MitoTEMPO was able to improve it at the late stage of this protocol, starting at 42 s until the end of the second part ($p < 0.01$), as shown by repeated one-way ANOVA (Fig. 5B). This again may confirm the vital role of mitochondrial-derived ROS at late stages of fatigue/injury in diaphragm.

Discussion

The goal of the current study was to test the hypothesis that T4 may produce distinct hemodynamic, cardiac, and diaphragm muscle abnormalities by differentially affecting various sources of ROS. Consistent with published data [2,9,16,33–35], T4 increased the BP of mice in the current study. Among all antioxidant treatments, only L-NIO, a nonspecific NOS inhibitor, attenuated T4-induced hypertension. NO is a well-known regulator of BP. Three isoforms of NOS have been recognized: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) [36]. Whereas eNOS-derived NO consistently shows a protective role in hypertension [36,37], there are, however, some inconsistencies in the literature regarding the role of nNOS- and iNOS-derived NO in BP regulation. For instance, several reports show decreased BP after nNOS inhibition [36,38,39], whereas others show the opposite [40–42]. Likewise, some studies describe a role for iNOS in the pathogenesis of hypertension [37,43,44] even as other studies show protective effects [45–47].

Thyroid hormone has been shown to stimulate the expression of all three NOS isoforms, with greater generation of NO in both aorta and vascular smooth muscle cells mainly by iNOS and nNOS without any involvement of eNOS [48]. Similarly, another group confirmed increased NOS activity in hyperthyroidism in tissues primarily related to BP regulation including blood vessels, heart, and kidney [49]. This group also demonstrated that T4-induced hypertension was associated with elevated urinary isoprostane F2 α excretion and that antioxidant treatment with tempol decreased isoprostane F2 α excretion as well as the hypertension in hyperthyroid rats [2]. In further studies, the same group showed the activation of NOS in hyperdynamic circulation characteristic of hyperthyroidism. Whereas eNOS and iNOS may have a homeostatic role, nNOS was proposed to be involved in the development of hyperdynamic circulation and hypertension in their model [33–35].

Generally, T4-induced hypertension is considered a model of cardiogenic hypertension. Nevertheless, we [16] and others [9] showed that increased cardiac output may not be the key factor responsible. At variance with those studies [2,33–35], hyperdynamic circulation does not seem to have a significant role in T4-induced hypertension in our model. Our previous findings [16], as well as the current results, showed that neither HR nor stroke volume was significantly changed by T4 treatment in mice. The exact mechanisms for the development/attenuation of hypertension in our model remain to be determined; however, this may be through one or more of the following: (1) vascular effects: disruption of the initial vasodilating outcomes of T4 as well as increased vessel rigidity secondary to increased oxidative stress. (2) Renal effects: T4-induced hypertension has been linked to increased sodium loading as well increased renal oxidative stress [2,50]. nNOS, which has been shown to be an important source of ROS in the kidney under conditions of oxidative stress [51] and to increase tubular sodium reabsorption [52], may play a part in these renal effects. (3) Central effects: despite the facts that BP in our model was increased independent of HR and NOS inhibitors have been previously shown to decrease HR without affecting BP [53], the contribution of the depressor effect of L-NIO on HR in attenuating hypertension in this study should not be ignored. Inhibition of nNOS in the rostral ventrolateral medulla of rat, where sympathetic vasomotor tone begins, resulted in a decline in systemic BP and HR [54].

It is worth mentioning that currently available NOS inhibitors add a further level of complexity by exhibiting a marked heterogeneity in their selectivity for different NOS isoforms. For example, the selectivity for nNOS of 7-nitroindizaole, which attenuated hypertension in hyperthyroid rats [35], is questionable, and its ability to inhibit eNOS in vivo has been reported [40,41,55]. Likewise, the nonspecific NOS inhibitor used in the current study, L-NIO [20,56,57], has been repeatedly used as a specific eNOS inhibitor [39,42,58]. The ability of L-NIO to decrease both HR and BP in the current study confirms the nonspecific inhibition of NOS by L-NIO. It also shows that its inhibitory effects on both nNOS and iNOS predominate. Whether L-NIO affected HR and BP in this study by inhibiting nNOS, iNOS, or both needs further investigation.

Overall, our current data confirm previous reports [35] showing that the NOS pathway is a major determinant of T4-induced hypertension. However, we cannot exclude the role of other pathways, at least the NADPH oxidase pathway, owing to concerns raised about the inability of the specific NADPH oxidase inhibitor used in this study, apocynin, to inhibit the vascular NADPH oxidase [59,60].

The development of cardiac hypertrophy after T4 treatment is well documented [2–4,10–12,16,18,33–35,61–63]. Likewise, signs of increased oxidative stress in hyperthyroid hearts have been consistently reported [2,6,10–12,18,52,61–63]. Different reports from the same group showed the efficiency of vitamin E, an antioxidant, at decreasing the T4-induced increase in heart weight [11,61–63]. On the contrary, several reports show the ineffectiveness of multiple antioxidants with divergent mechanisms of action, such as tempol [2], carvedilol [8], and vitamin E [12], as well as specific and nonspecific NOS inhibitors [33–35], at reversing the increase in heart weight after T4 treatment. Consistent with these reports, our data confirm the development of cardiac hypertrophy in the hearts of hyperthyroid mice and show the inability of any of the studied antioxidants to recover this hypertrophic response. Nevertheless, this does not completely rule out the contribution of ROS to the development of T4-induced cardiac hypertrophy. Our previous findings demonstrate that pravastatin, by inhibiting myocardial Rac1, a major component of the NADPH oxidase complex, did not decrease the gross heart weight, but significantly decreased the cardiomyocyte size to a level that was still higher than control, indicating a partial role for ROS in this response [16].

Considering these results, one reasonable question arises: what is the main role and source of this elevated oxidative stress in hyperthyroid hearts? Initially, T4 was reported to increase mitochondrial ROS [6,64], expression and activity of NOS, and NO production [11,52,63], as well as the expression of NADPH oxidase [63] in the heart. In addition, the ROS (mainly through NADPH oxidase)/NO balance has been proposed to play a role in the control of T4-induced cardiac hypertrophy [63]. Thus, to answer the above question, we further investigated the effects of the selected antioxidants on the associated cardiac dysfunction in hyperthyroid hearts. Treatment with L-NIO showed a strong trend toward improving the LV functions as evident by increased LV EF and LV FS; however, these increases did not reach significance ($p=0.062$ and 0.069 , respectively) compared to T4. In fact, NO is essential for normal cardiac physiology and has a protective role in cardiac diseases. However, it can also exert cytotoxic effects mostly through the toxic actions of the highly reactive oxidant

peroxynitrite [65]. Increased expression and activity of iNOS and nNOS along with NO overproduction have been reported in the failing myocardium as well as in heart failure models including experimental hyperthyroidism [11,52,63,65]. On the other hand, inhibition of NADPH oxidase by apocynin significantly improved LV systolic dysfunction. To confirm that this effect is solely due to apocynin without any interference from its vehicle (5% DMSO), a separate group of mice ($n=5$) was injected with 5% DMSO, and T4 was followed by echocardiography evaluation. Indeed, we did not find any significant change in LV function compared to the T4 group ($EF 57 \pm 5\%$ and $FS 30 \pm 3\%$; data not shown). NADPH oxidase, through redox-sensitive signal transduction, has been presented as a key player in the pathogenesis of several aspects of cardiac remodeling and its antecedent conditions both in human patients and in heart failure models, including experimental hyperthyroidism [63,66]. Additionally, apocynin has been shown to reduce NADPH oxidase activity and oxidative stress and ameliorate LV dysfunction after myocardial infarction [67]. Interestingly, this happened in the absence of any effects of apocynin on the cardiac mass, which means that NADPH oxidase plays a major role in T4-induced LV dysfunction regardless of the cardiac hypertrophy. In line with this finding, NADPH oxidase has been reported to participate in the progress of cardiac contractile dysfunction in pressure overload, even though it is not necessary for the growth of morphologic hypertrophy per se [68]. This also indicates that T4-induced LV dysfunction is independent of the development of cardiac hypertrophy. Unlike apocynin, in our previous study [16], pravastatin significantly decreased myocardial Rac-GTPase activity; however, it did not show any improvement in LV systolic function. The reasons for this discrepancy are not clear. Still, there are apparent differences in the nature and the doses of both drugs that were used in the two studies. In general, oxidative and nitrosative stresses can result in cardiac dysfunction through: (1) desensitization of contractile protein, (2) changes in cellular energetics, (3) alterations in excitation–contraction coupling, (4) variations in myofilament calcium responsiveness, and/or (5) endothelial dysfunction [65,68]. However, the precise mechanism(s) by which apocynin and L-NIO attenuated LV dysfunction in this study remain to be elucidated.

T4-induced cardiac hypertrophy has been described as relative hypertrophy that encompasses the whole heart (RV and LV). T4-induced RV hypertrophy is linked to marked contractile abnormalities as described in detail in our previous report [16]. Similarly, isolated RV papillary muscles in this study exhibit the hallmarks of T4 treatment, including decreased contraction/relaxation times, negative FFR, and blunted β -adrenergic response. However, none of the antioxidant treatments in this study was able to reverse T4-induced contractile abnormalities of the RV. Actually, LV and RV have differences in structure, function, and response to stress and disease [69]; hence, their differential responses to treatment could be expected. Improved LV but not RV function has been reported in human patients after treatment with carvedilol, a mixed α - and β -blocker with antioxidant properties [70].

The role of ROS in muscle contractility has been extensively inspected and there is growing evidence proposing that oxidative modification of contractile and regulatory proteins is a key player in the depression of force development in skeletal muscles [8]. In this regard, T4

has been reported to reduce various skeletal muscles' contractility, including the diaphragm muscle via a mechanism involving increased oxidative stress [5–8,13]. In contrast to previous studies that show decreased diaphragm contractility after T4 treatment [5,8], our data revealed preserved contractility at all frequencies, but increased fatigability in hyperthyroid diaphragm muscles compared to control. Consistent with these data, maintained tetanic force and increased fatigability of diaphragm muscles have been reported in normal hamsters after T4 treatment compared to placebo [71].

Generally, the force drop during fatigue may be accredited to: (1) alteration in the maximum Ca^{2+} -activated force, (2) alteration in the intracellular tetanic Ca^{2+} , and/or (3) alteration in the myofibrillar Ca^{2+} sensitivity. Among these, ROS have been shown to accelerate muscle fatigue mainly by reducing the myofibrillar Ca^{2+} sensitivity [72]. Despite mitochondria being the principal source of ROS generation in skeletal muscles, ROS can also be generated from xanthine oxidase and NADPH oxidase [72]. Here, we showed that inhibition of xanthine oxidase by allopurinol at early stages during the injury/fatigue process decreased the T4-induced force drop in diaphragm muscles. In agreement with these results, treatment with allopurinol or its active metabolite oxypurinol has been coupled with a decline in the levels of oxidative damage indicators as well as muscle damage markers subsequent to exhaustive exercise protocols in humans and rats [73,74]. However, at later stages of the injury/fatigue process the effect of targeted inhibition of mitochondrial ROS by MitoTEMPO predominated, with clear improvement in the force drop. This may be due to the fact that the diaphragm contains quite large amounts of mitochondria. Indeed, myofibrillar protein oxidation and contractile dysfunction in diaphragm muscle compared to the extensor digitorum longus, formed of a similar fiber type, after T4 treatment has been proposed to be attributed to the abundance of mitochondria in diaphragm muscle [8]. It is also clear that the improvement in force drop by MitoTEMPO in this study is higher than that of control muscles, which coincides with the fact that mitochondria are the principal source of ROS in skeletal muscles and in the diaphragm per se [8,72], and this pathway could be activated during the injury/fatigue process even in the absence of T4. In agreement with these results, Powers et al. [75] showed that mitochondria-targeted antioxidants protect against mechanical ventilation-induced diaphragm weakness.

Conclusion

We have shown for the first time that T4 exerts differential effects on various sources of ROS to induce distinct cardiovascular and skeletal muscle phenotypes. Another novel finding is that T4-induced LV dysfunction seems to be independent of cardiac hypertrophy and that NADPH oxidase is a key player in this process. Furthermore, we proved the significance of both xanthine oxidase and mitochondrial ROS pathways in T4-induced fatigability of diaphragm muscles. Finally, we confirmed previous reports showing the importance of the NO pathway in T4-induced hypertension.

The biochemical and cellular responses to antioxidant treatments used in this study, however, are yet to be entirely investigated. Exposing the effects of pharmacological interventions on the development of T4-induced cardiovascular and diaphragm muscle

abnormalities can help us comprehend the primary mechanisms and endorse further studies to explore the fundamental biochemical and cellular mechanisms beyond these effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding was provided in part through start-up funds to P.M.L.J. from The Ohio State University and by P30 Core Grant NINDS P30 NS045758-06 (PI: C. Beattie).

References

1. Giavarotti S, Rodrigues L, Rodrigues T, Junqueira VB, Videla LA. Liver microsomal parameters related to oxidative stress and antioxidant systems in hyperthyroid rats subjected to acute lindane treatment. *Free Radic Res.* 1998; 29:35–42. [PubMed: 9733020]
2. Moreno JM, Rodríguez Gómez I, Wangenstein R, Osuna A, Bueno P, Vargas F. Cardiac and renal antioxidant enzymes and effects of tempol in hyperthyroid rats. *Am J Physiol Endocrinol Metab.* 2005; 289:E776–783. [PubMed: 15941780]
3. Kuzman JA, Vogelsang KA, Thomas TA, Gerdes AM. L-Thyroxine activates Akt signaling in the heart. *J Mol Cell Cardiol.* 2005; 39:251–258. [PubMed: 15890358]
4. Kuzman JA, Thomas TA, Vogelsang KA, Said S, Anderson BE, Gerdes AM. Effects of induced hyperthyroidism in normal and cardiomyopathic hamsters. *J Appl Physiol.* 2005; 99:1428–1433. [PubMed: 15976357]
5. Miyashita A, Suzuki S, Suzuki M, Numata H, Suzuki J, Akahori T, Okubo T. Effect of thyroid hormone on in vivo contractility of the canine diaphragm. *Am Rev Respir Dis.* 1992; 145:1452–1462. [PubMed: 1596018]
6. Asayama K, Dobashi K, Hayashibe H, Megata Y, Kato K. Lipid peroxidation and free radical scavengers in thyroid dysfunction in the rat: a possible mechanism of injury to heart and skeletal muscle in hyperthyroidism. *Endocrinology.* 1987; 121:2112–2118. [PubMed: 2824181]
7. Yamada T, Mishima T, Sakamoto M, Sugiyama M, Matsunaga S, Wada M. Oxidation of myosin heavy chain and reduction in force production in hyperthyroid rat soleus. *J Appl Physiol.* 2006; 100:1520–1526. [PubMed: 16397059]
8. Yamada T, Mishima T, Sakamoto M, Sugiyama M, Matsunaga S, Wada M. Myofibrillar protein oxidation and contractile dysfunction in hyperthyroid rat diaphragm. *J Appl Physiol.* 2007; 102(1985):1850–1855. [PubMed: 17303704]
9. García del Río C, Moreno MR, Osuna A, et al. Role of the rennin–angiotensin system in the development of thyroxine-induced hypertension. *Eur J Endocrinol.* 1997; 136:656–660. [PubMed: 9225731]
10. Gredilla R, Barja G, Lopez-Torres M. Thyroid hormone-induced oxidative damage on lipids, glutathione and DNA in the mouse heart. *Free Radic Res.* 2001; 35:417–425. [PubMed: 11697138]
11. Araujo AS, Schenkel P, Enzweiler AT, Fernandes TR, Partata WA, Llesuy S, Ribeiro MF, Khaper N, Singal PK, Belló-Klein A. The role of redox signaling in cardiac hypertrophy induced by experimental hyperthyroidism. *J Mol Endocrinol.* 2008; 41:423–430. [PubMed: 18787053]
12. Asayama K, Dobashi K, Hayashibe H, Kato K. Vitamin E protects against thyroxine-induced acceleration of lipid peroxidation in cardiac and skeletal muscles in rats. *J Nutr Sci Vitaminol (Tokyo).* 1989; 35:407–418. [PubMed: 2632676]
13. Zaiton Z, Merican Z, Khalid BA, Mohamed JB, Baharom S. The effects of propranolol on skeletal muscle contraction, lipid peroxidation products and antioxidant activity in experimental hyperthyroidism. *Gen Pharmacol.* 1993; 24:195–199. [PubMed: 8482496]

14. Supinski GS, Callahan LA. Diaphragmatic free radical generation increases in an animal model of heart failure. *J Appl Physiol.* 2005; 99:1078–1084. [PubMed: 16103520]
15. Supinski G, DiMarco A, Dibner-Dunlap M. Alterations in diaphragm strength and fatiguability in congestive heart failure. *J Appl Physiol.* 1994; 76:2707–2713. [PubMed: 7928904]
16. Elnakish MT, Moldovan L, Khan M, Hassanain HH, Janssen PM. Myocardial Rac1 exhibits partial involvement in thyroxine-induced cardiomyocyte hypertrophy and its inhibition is not sufficient to improve cardiac dysfunction or contractile abnormalities in mouse papillary muscles. *J Cardiovasc Pharmacol.* 2013; 61:536–544. [PubMed: 23429587]
17. Elnakish MT, Awad MM, Hassona MD, Alhaj MA, Kulkarni A, Citro LA, Sayyid M, Abouelnaga ZA, El-Sayed O, Kuppusamy P, Moldovan L, Khan M, Hassanain HH. Cardiac remodeling caused by transgenic over-expression of a corn Rac gene. *Am J Physiol Heart Circ Physiol.* 2011; 301:H868–H880. [PubMed: 21622832]
18. Elnakish MT, Hassona MD, Alhaj MA, Moldovan L, Janssen PM, Khan M, Hassanain HH. Rac-induced left ventricular dilation in thyroxine-treated ZmRacD transgenic mice: role of cardiomyocyte apoptosis and myocardial fibrosis. *PLoS One.* 2012; 7:e42500. [PubMed: 22936985]
19. Santos CX, Anilkumar N, Zhang M, Brewer AC, Shah AM. Redox signaling in cardiac myocytes. *Free Radic Biol Med.* 2011; 50:777–793. [PubMed: 21236334]
20. Sovari AA, Rutledge CA, Jeong EM, Dolmatova E, Arasu D, Liu H, Vahdani N, Gu L, Zandieh S, Xiao L, Bonini MG, Duffy HS, Dudley SC Jr. Mitochondria oxidative stress, connexin43 remodeling and sudden arrhythmic death. *Circ Arrhythm Electrophysiol.* 2013; 6:623–631. [PubMed: 23559673]
21. Engberding N, Spiekermann S, Schaefer A, Heineke A, Wiencke A, Müller M, Fuchs M, Hilfiker-Kleiner D, Hornig B, Drexler H, Landmesser U. Allopurinol attenuates left ventricular remodeling and dysfunction after experimental myocardial infarction: a new action for an old drug? *Circulation.* 2004; 110:2175–2179. [PubMed: 15466649]
22. Burch TG, Prewitt RL. The effects of allopurinol on skeletal muscle microcirculation in normal and dystrophic mice. *Res Commun Chem Pathol Pharmacol.* 1979; 26:329–335. [PubMed: 523775]
23. Haruta M, Bush RA, Kjellstrom S, Vijayasarathy C, Zeng Y, Le YZ, Sieving PA. Depleting Rac1 in mouse rod photoreceptors protects them from photo-oxidative stress without affecting their structure or function. *Proc Natl Acad Sci USA.* 2009; 106:9397–9402. [PubMed: 19470639]
24. Paterniti I, Galuppo M, Mazzon E, Impellizzeri D, Esposito E, Bramanti P, Cuzzocrea S. Protective effects of apocynin, an inhibitor of NADPH oxidase activity, in splanchnic artery occlusion and reperfusion. *J Leukocyte Biol.* 2010; 88:993–1003. [PubMed: 20807703]
25. Elnakish MT, Hassanain HH, Janssen PM. Vascular remodeling-associated hypertension leads to left ventricular hypertrophy and contractile dysfunction in profilin-1 transgenic mice. *J Cardiovasc Pharmacol.* 2012; 60:544–552. [PubMed: 22967989]
26. Janssen PML, Hunter WC. Force, not sarcomere length, correlates with prolongation of isosarcometric contraction. *Am J Physiol Heart Circ Physiol.* 1995; 269:H676–H685.
27. Janssen PML, Hiranandani N, Mays TA, Rafael-Fortney JA. Utrophin deficiency worsens cardiac contractile dysfunction present in dystrophin-deficient mdx mice. *Am J Physiol Heart Circ Physiol.* 2005; 289:H2373–H2378. [PubMed: 16024571]
28. Rodriguez EK, Hunter WC, Royce MJ, Leppo MK, Douglas AS, Weisman HF. A method to reconstruct myocardial sarcomere lengths and orientations at transmural sites in beating canine hearts. *Am J Physiol Heart Circ Physiol.* 1992; 263:H293–H306.
29. Murray JD, Canan BD, Martin CD, Stangland JE, Rastogi N, Rafael-Fortney JA, Janssen PM. The force-temperature relationship in healthy and dystrophic mouse diaphragm; implications for translational study design. *Front Physiol.* 2012; 3:422. [PubMed: 23162469]
30. Frank O. Zur dynamik des Herzmuskels. *Z Biol.* 1895; 32:370–447.
31. Starling, EH. The Lineacre Lecture on the Law of the Heart. London: Longman Greens; 1918.
32. Bowditch HP. Ueber die Eigenthümlichkeiten der Reizbarkeit, welche die Muskelfasern des Herzens zeigen. *Ber Sachs Ges (Akad) Wiss.* 1871; 23:652–689.

33. Rodríguez-Gómez I, Sainz J, Wangenstein R, Moreno JM, Duarte J, Osuna A, Vargas F. Increased pressor sensitivity to chronic nitric oxide deficiency in hyperthyroid rats. *Hypertension*. 2003; 42:220–225. [PubMed: 12821601]
34. Rodríguez-Gómez I, Wangenstein R, Moreno JM, Chamorro V, Osuna A, Vargas F. Effects of chronic inhibition of inducible nitric oxide synthase in hyperthyroid rats. *Am J Physiol Endocrinol Metab*. 2005; 288:E1252–1257. [PubMed: 15644461]
35. Wangenstein R, Rodríguez-Gómez I, Moreno JM, Alvarez-Guerra M, Osuna A, Vargas F. Effects of chronic treatment with 7-nitroindazole in hyperthyroid rats. *Am J Physiol Regul Integr Comp Physiol*. 2006; 291:R1376–1382. [PubMed: 16778061]
36. Kurihara N, Alfie ME, Sigmon DH, Rhaleb NE, Shesely EG, Carretero OA. Role of nNOS in blood pressure regulation in eNOS null mutant mice. *Hypertension*. 1998; 32:856–861. [PubMed: 9822444]
37. Cau SB, Tostes RC. Inducible nitric oxide synthase inhibition as a target for the treatment of vascular dysfunction in hypertension. *Hypertension*. 2012; 59:e21. [PubMed: 22252393]
38. Walkowska A, Sadowski J, Kompanowska-Jezierska E. Oxidative stress and neuronal NOS activity: putative determinants of rapid blood pressure increase after renal denervation in anesthetized rats. *Physiol Res*. 2013; 62:257–266. [PubMed: 23489187]
39. Guo ZL, Tjen-A-Looi SC, Fu LW, Longhurst JC. Nitric oxide in rostral ventrolateral medulla regulates cardiac-sympathetic reflexes: role of synthase isoforms. *Am J Physiol Heart Circ Physiol*. 2009; 297:H1478–1486. [PubMed: 19684188]
40. Ollerstam A, Pittner J, Persson AEG, Thorup C. Increased blood pressure in rats after long-term inhibition of the neuronal isoform of nitric oxide synthase. *J Clin Invest*. 1997; 99:2212–2218. [PubMed: 9151793]
41. Zagvazdin Y, Sancesario G, Wang YX, Share L, Fitzgerald ME, Reiner A. Evidence from its cardiovascular effects that 7-nitroindazole may inhibit endothelial nitric oxide synthase in vivo. *Eur J Pharmacol*. 1996; 303:61–69. [PubMed: 8804912]
42. Ishikawa M, Quock RM. Role of nitric-oxide synthase isoforms in nitrous oxide antinociception in mice. *J Pharmacol Exp Ther*. 2003; 306:484–489. [PubMed: 12721331]
43. Hong HJ, Loh SH, Yen MH. Suppression of the development of hypertension by the inhibitor of inducible nitric oxide synthase. *Br J Pharmacol*. 2000; 131:631–637. [PubMed: 11015317]
44. Smith CJ, Santhanam L, Bruning RS, Stanhewicz A, Berkowitz DE, Holowatz LA. Upregulation of inducible nitric oxide synthase contributes to attenuated cutaneous vasodilation in essential hypertensive humans. *Hypertension*. 2011; 58:935–942. [PubMed: 21931069]
45. Förstermann U, Closs EI, Pollock JS, Nakane M, Schwarz P, Gath I, Kleinert H. Nitric oxide synthase isozymes: characterization, purification, molecular cloning and functions. *Hypertension*. 1994; 23:1121–1131. [PubMed: 7515853]
46. Ni Z, Oveisi F, Vaziri ND. Nitric oxide synthase isotype expression in salt-sensitive and salt-resistant Dahl rats. *Hypertension*. 1999; 34:552–557. [PubMed: 10523325]
47. Ishimitsu T, Uehara Y, Numabe A, Tsukada H, Ogawa Y, Yagi S. Antihypertensive effect of interleukin-2 in salt-sensitive Dahl rats. *Hypertension*. 1994; 23:68–73. [PubMed: 8282332]
48. Carrillo-Sepúlveda MA, Ceravolo GS, Fortes ZB, Carvalho MH, Tostes RC, Laurindo FR, Webb RC, Barreto-Chaves ML. Thyroid hormone stimulates NO production via activation of the PI3K/Akt pathway in vascular myocytes. *Cardiovasc Res*. 2010; 85:560–570. [PubMed: 19734167]
49. Quesada A, Sainz J, Wangenstein R, Rodriguez-Gomez I, Vargas F, Osuna A. Nitric oxide synthase activity in hyperthyroid and hypothyroid rats. *Eur J Endocrinol*. 2002; 147:117–122. [PubMed: 12088928]
50. Vargas F, Atucha N, Sabio JM, et al. Pressure-diuresis-natriuresis response in hyper- and hypothyroid rats. *Clin Sci (London)*. 1994; 87:323–328. [PubMed: 7955909]
51. Edlund J, Ffashing A, Liss P, Hansell P, Palm F. The roles of NADPH-oxidase and nNOS for the increased oxidative stress and oxygen consumption in the diabetic kidney. *Diabetes Metab Res Rev*. 2010; 26:349–356. [PubMed: 20583310]

52. Cervenka L, Kramer HJ, Malý J, Vanecková I, Bäcker A, Bokemeyer D, Bader M, Ganten D, Mitchell KD. Role of nNOS in regulation of renal function in hypertensive Ren-2 transgenic rats. *Physiol Res*. 2002; 51:571–580. [PubMed: 12511180]
53. Kelly PA, Ritchie IM, Arbuthnott GW. Inhibition of neuronal nitric oxide synthase by 7-nitroindazole: effects upon local cerebral blood flow and glucose use in the rat. *J Cereb Blood Flow Metab*. 1995; 15:766–773. [PubMed: 7545690]
54. Chan SH, Wang LL, Wang SH, Chan JY. Differential cardiovascular responses to blockade of nNOS or iNOS in rostral ventrolateral medulla of the rat. *Br J Pharmacol*. 2001; 133:606–614. [PubMed: 11399678]
55. Prickaerts J, Steinbusch HW, Smits JF, de Vente J. Possible role of nitric oxide-cyclic GMP pathway in object recognition memory: effects of 7-nitroindazole and zaprinast. *Eur J Pharmacol*. 1997; 337:125–136. [PubMed: 9430406]
56. Babu BR, Griffith OW. N5-(1-imino-3-butenyl)-l-ornithine: a neuronal isoform selective mechanism-based inactivator of nitric oxide synthase. *J Biol Chem*. 1998; 273:8882–8889. [PubMed: 9535869]
57. Kane AJ, Barker JE, Mitchell GM, Theile DR, Romero R, Messina A, Wagh M, Fraulin FO, Morrison WA, Stewart AG. Inducible nitric oxide synthase (iNOS) activity promotes ischaemic skin flap survival. *Br J Pharmacol*. 2001; 132:1631–1638. [PubMed: 11309233]
58. Xu Z, Park SS, Mueller RA, Bagnell RC, Patterson C, Boysen PG. Adenosine produces nitric oxide and prevents mitochondrial oxidant damage in rat cardiomyocytes. *Cardiovasc Res*. 2005; 65:803–812. [PubMed: 15721860]
59. Heumüller S, Wind S, Barbosa-Sicard E, Schmidt HH, Busse R, Schröder K, Brandes RP. Apocynin is not an inhibitor of vascular NADPH oxidases but an antioxidant. *Hypertension*. 2008; 51:211–217. [PubMed: 18086956]
60. Schlüter T, Steinbach AC, Steffen A, Rettig R, Grisk O. Apocynin-induced vasodilation involves Rho kinase inhibition but not NADPH oxidase inhibition. *Cardiovasc Res*. 2008; 80:271–279. [PubMed: 18596059]
61. Araujo AS, Enzweiler AT, Schenkel P, Fernandes TR, Ribeiro MF, Partata WA, Llesuy S, Belló-Klein A. Oxidative stress activates insulin like growth factor I receptor protein expression, mediating cardiac hypertrophy induced by thyroxine. *Mol Cell Biochem*. 2007; 303:89–95. [PubMed: 17447016]
62. Araujo AS, Ribeiro MF, Enzweiler A, Schenkel P, Fernandes TR, Partata WA, Irigoyen MC, Llesuy S, Belló-Klein A. Myocardial antioxidant enzyme activities and concentration and glutathione metabolism in experimental hyperthyroidism. *Mol Cell Endocrinol*. 2006; 249:133–139. [PubMed: 16574313]
63. Araujo AS, Diniz GP, Seibel FE, Branchini G, Ribeiro MF, Brum IS, Khaper N, Barreto-Chaves ML, Belló-Klein A. Reactive oxygen and nitrogen species balance in the determination of thyroid hormones-induced cardiac hypertrophy mediated by renin–angiotensin system. *Mol Cell Endocrinol*. 2011; 333:78–84. [PubMed: 21168472]
64. Mano T, Sinohara R, Sawai Y. Effects of thyroid hormone on coenzyme Q and other free radical scavengers in rat heart muscle. *J Endocrinol*. 1995; 145:131–136. [PubMed: 7798018]
65. Pacher P, Schulz R, Liaudet L, Szabó C. Nitrosative stress and pharmacological modulation of heart failure. *Trends Pharmacol Sci*. 2005; 26:302–310. [PubMed: 15925705]
66. Elnakish MT, Hassanain HH, Janssen PM, Angelos MG, Khan M. Emerging role of oxidative stress in metabolic syndrome and cardiovascular diseases: important role of Rac/NADPH oxidase. *J Pathol*. 2013; 231:290–300. [PubMed: 24037780]
67. Qin F, Simeone M, Patel R. Inhibition of NADPH oxidase reduces myocardial oxidative stress and apoptosis and improves cardiac function in heart failure after myocardial infarction. *Free Radic Biol Med*. 2007; 43:271–281. [PubMed: 17603936]
68. Grieve DJ, Byrne JA, Siva A, Layland J, Johar S, Cave AC, Shah AM. Involvement of the nicotinamide adenosine dinucleotide phosphate oxidase isoform Nox2 in cardiac contractile dysfunction occurring in response to pressure overload. *J Am Coll Cardiol*. 2006; 47:817–826. [PubMed: 16487851]

69. Haddad F, Hunt SA, Rosenthal DN, Murphy DJ. Right ventricular function in cardiovascular disease, part I: anatomy, physiology, aging, and functional assessment of the right ventricle. *Circulation*. 2008; 117:1436–1448. [PubMed: 18347220]
70. Hiroi Y, Fujii K, Komatsu S, Sonoda M, Sakomura Y, Imai Y, Oishi Y, Nakamura F, Ajiki K, Hayami N, Murakawa Y, Ohno M, Hirata Y, Ohtomo K, Nagai R. Carvedilol therapy improved left ventricular function in a patient with arrhythmogenic right ventricular cardiomyopathy. *Jpn Heart J*. 2004; 45:169–177. [PubMed: 14973363]
71. Singh YN, Schlenker EH, Singh BN, Burbach JA. Consequences of thyroxine treatment on diaphragm and EDL of normal and dystrophic hamsters. *Can J Physiol Pharmacol*. 2004; 82:345–352. [PubMed: 15213735]
72. Moopanar TR, Allen DG. Reactive oxygen species reduce myofibrillar Ca^{2+} sensitivity in fatiguing mouse skeletal muscle at 37 degrees C. *J Physiol*. 2005; 564:189–199. [PubMed: 15718257]
73. Gomez-Cabrera MC, Pallardo FV, Sastre J, Vina J, Garcia-del-Moral L. Allopurinol and markers of muscle damage among participants in the Tour de France. *JAMA*. 2003; 289:2503–2504. [PubMed: 12759321]
74. Gomez-Cabrera MC, Borrás C, Pallardo FV, Sastre J, Ji LL, Vina J. Decreasing xanthine oxidase-mediated oxidative stress prevents useful cellular adaptations to exercise in rats. *J Physiol*. 2005; 567:113–120. [PubMed: 15932896]
75. Powers SK, Hudson MB, Nelson WB, Talbert EE, Min K, Szeto HH, Kavazis AN, Smuder AJ. Mitochondria-targeted antioxidants protect against mechanical ventilation-induced diaphragm weakness. *Crit Care Med*. 39:1749–1759. [PubMed: 21460706]

Abbreviations

BDM	2,3-butanedione monoxime
BP	blood pressure
CSA	cross-sectional area
DMSO	dimethyl sulfoxide
EF	ejection fraction
eNOS	endothelial nitric oxide synthase
F_{dev}	peak isometric developed force
FFR	force–frequency relationship
FS	fractional shortening
HR	heart rate
iNOS	inducible nitric oxide synthase
L-NIO	N^5 -(1-iminoethyl)-l-ornithine dihydrochloride
LV	left ventricular
MAP	mean arterial pressure
NOS	nitric oxide synthase
nNOS	neuronal nitric oxide synthase
PBS	phosphate-buffered saline

ROS	reactive oxygen species
RT₅₀	time from peak force to 50% relaxation
RV	right ventricle
T4	thyroxin
TTP	time to peak force

Fig 1.

Blood pressure measurement showed that the NOS pathway is a major determinant of T4-induced hemodynamic changes in mice. Representative bar graphs show (A) systolic, (B) diastolic, and (C) mean arterial blood pressure and (D) heart rate in mice. T4, thyroxine; L-NIO, *N*⁵-(1-iminoethyl)-L-ornithine dihydrochloride; BPM, beats per minute. Control, *n*=13; T4, *n*=12; allopurinol, *n*=11; apocynin, *n*=12; L-NIO, *n*=10; MitoTEMPO, *n*=12.

*Significant change compared with T4 group (one-way ANOVA followed by Dunnett multiple comparisons post hoc test, comparing all groups to T4).

Fig 2.

Echocardiography analysis of the mouse hearts showed that the NADPH oxidase pathway is a key player in the T4-induced LV dysfunction regardless of the increased LV mass. Representative bar graphs show (A) left-ventricular (LV) mass, (B) stroke volume, (C) ejection fraction (EF), and (D) fractional shortening (FS) in mice. T4, thyroxine; L-NIO, *N*⁵-(1-iminoethyl)-L-ornithine dihydrochloride. Control, *n*=12; T4, *n*=13; allopurinol, *n*=11; apocynin, *n*=13; L-NIO, *n*=10; MitoTEMPO, *n*=12. *Significant change compared with T4 group (one-way ANOVA followed by Dunnett multiple comparisons post hoc test, comparing all groups to T4).

Fig 3.

Contractile profile of isolated right-ventricular papillary muscles indicates rapid contraction and relaxation in T4-treated mice. Representative bar graphs show (A) mean isometric developed force (F_{dev}), (B) their corresponding time to peak (TTP), and (C) 50% relaxation time (RT₅₀) at 4 Hz stimulation frequency and 2 mmol/L Ca^{2+} . Control, $n=12$; T4, $n=11$; allopurinol, $n=10$; apocynin, $n=10$; L-NIO, $n=9$; MitoTEMPO, $n=9$. *Significant change compared with T4 group (one-way ANOVA followed by Dunnett multiple comparisons post hoc test, comparing all groups to T4).

Fig 4.

Isolated right-ventricular papillary muscles from T4-treated mice preserved their length-dependent activation, but lost their ability to respond to increased frequency or β -adrenergic stimulation. (A) Length-dependent activation. Peak isometric developed force (F_{dev}) value is expressed as a fraction of its corresponding F_{dev} at optimal length (L_{opt}) and presented as the mean $\pm$ SEM. (B) Force–frequency relationship. F_{dev} value is expressed as a fraction of its corresponding F_{dev} at the basal frequency of 4 Hz and presented as the mean $\pm$ SEM. (C) β -Adrenergic stimulation. F_{dev} value is expressed as a fraction of its corresponding F_{dev} at the basal frequency of 4 Hz before isoproterenol addition and presented as the mean $\pm$ SEM. Control, $n=12$; T4, $n=11$; allopurinol, $n=10$; apocynin, $n=10$; L-NIO, $n=9$; MitoTEMPO, $n=9$. ⁺Significant change as revealed by two-way ANOVA. *Significant change as revealed by one-way ANOVA followed by Dunnett multiple comparisons post hoc test, comparing all groups to T4.

Fig 5.

T4-induced fatigability in diaphragm can be repressed by xanthine oxidase inhibitors and mitochondrial-targeted antioxidants at early and late stages, respectively. (A) Representative bar graphs show mean tetanic forces at different frequencies in the range of 20–180 Hz. (B) Fatigue/injury induced by repetitive contractions, once per second (frequency 100 Hz, duration 500 ms) for 66 s, followed by 15 min of rest and a repeat in diaphragm muscles. (C) Representative bar graphs show mean % change in relative tension before and after rest (rest recovery). Control, $n=12$; T4, $n=11$; allopurinol, $n=13$; apocynin, $n=11$; L-NIO, $n=12$; MitoTEMPO, $n=13$; n represents the number of muscles isolated from 8 mice/group. ⁺ Significant change as revealed by two-way ANOVA; *only control exhibited a significant change compared to T4, as revealed by one-way ANOVA; #both control and allopurinol exhibited significant changes compared to T4, as revealed by one-way ANOVA; ^xonly MitoTEMPO exhibited a significant change compared to T4, as revealed by one-way ANOVA. One-way ANOVA was followed by Dunnett multiple comparisons post hoc test, comparing all groups to T4.

Table 1

Morphological data confirm the development of cardiac hypertrophy in T4-treated mice.

	Control (n=15/14)	T4 (n=13/13)	Allopurinol (n=11/11)	Apocynin (n=14/12)	L-NIO (n=10/10)	MitoTEMPO (n=12/10)
BW (g)	28.1±0.4	28.1±0.6	28.3±0.8	28.1±1.1	27.1±1.0	25±0.8*
HW (mg)	131±4*	170±4	172±8	180±6	166±5	160±5
HW/BW (mg/g)	4.68±0.13*	6.05±0.11	6.06±0.14	6.47±0.20	6.18±0.20	6.49±0.10

T4, thyroxine; L-NIO, N⁵-(1-iminoethyl)-L-ornithine dihydrochloride; BW, body weight; HW, heart weight; n, number of mice for body weight data/number of hearts for heart weight data.

* Significant change compared with T4 group (one-way ANOVA followed by Dunnett multiple comparisons post hoc test, comparing all groups to T4).