Free Radical Biology and Medicine 83 (2015) 252–261

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

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

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ARTICLE INFO

Article history:
Received 24 November 2014
Received in revised form
12 February 2015
Accepted 27 February 2015
Available online 17 March 2015

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

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 functions, 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 thyroxin (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.

Thyroid hormones are key regulators of basal metabolic state and oxidative metabolism, with the potential to increase ROS 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

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; RT_{50}, time from peak force to 50% relaxation; RV, right ventricle; T4, thyroxin; TTP, time to peak force.

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http://dx.doi.org/10.1016/j.freeradbiomed.2015.02.035
0891-5849/

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 diaphragm [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 progression of generalized muscle weakness, 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 systolic 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-thyroxin, 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 $MgSO_4$, 1.2 NaH_2PO_4, 20 $NaHCO_3$, 0.25 Ca^{2+}, and 10 glucose (pH 7.4), equilibrated with 95% O_2–5% CO_2. 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 compared to the T4 group: 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