Hyperthyroidism causes cardiac dysfunction by mitochondrial impairment and energy depletion

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Abstract
This study elucidates the role of metabolic remodeling in cardiac dysfunction induced by hyperthyroidism. Cardiac hypertrophy, structural remodeling, and expression of the genes associated with fatty acid metabolism were examined in rats treated with triiodothyronine (T3) alone (8 μg/100 g body weight (BW), i.p.) for 15 days or along with a peroxisome proliferator-activated receptor alpha agonist bezafibrate (Bzf; 30 μg/100 g BW, oral) and were found to improve in the Bzf co-treated condition. Ultrastructure of mitochondria was damaged in T3-treated rat heart, which was prevented by Bzf co-administration. Hyperthyroidism-induced oxidative stress, reduction in cytochrome c oxidase activity, and myocardial ATP concentration were also significantly checked by Bzf. Heart function studied at different time points during the course of T3 treatment shows an initial improvement and then a gradual but progressive decline with time, which is prevented by Bzf co-treatment. In summary, the results demonstrate that hyperthyroidism inflicts structural and functional damage to mitochondria, leading to energy depletion and cardiac dysfunction.

Key Words
- Thyroid hormone
- Cardiac dysfunction
- Mitochondria
- Fatty acid metabolism
- Energy depletion

Introduction
Thyroid hormone (TH) is required for normal functioning of nearly all tissues and affects growth, differentiation, metabolism, and oxygen consumption (Yen 2001). The heart is an important target organ of TH and is very sensitive to minimal changes in serum TH level (Kahaly & Dillmann 2005). Hyperthyroidism increases cardiac contractility, resting heart rate, lipolysis, blood volume, left ventricular (LV) muscle mass, cardiac output (CO), etc., and decreases vascular tone and afterload (Fadel et al. 2000, Klein & Ojamaa 2001, Degens et al. 2003, Dillmann 2010). TH supplementation initially improves heart function but chronic hyperthyroidism, if untreated, inflicts detrimental effects to the heart such as hypertrophy, dysfunction ultimately leading to heart failure. Cardiac hypertrophy ensuing hyperthyroidism is due to the hyper-dynamic circulatory system that results from an enhanced metabolic rate, increased blood volume, and decreased peripheral resistance, and all these factors lead to an increased energy demand in the hypertrophied myocardium (Degens et al. 2003).

Cardiac hypertrophy, though initially beneficial for maintaining the CO under a high workload, is an important predisposing risk factor for cardiac morbidity and mortality (Hunter & Chien 1999, Shohet et al. 2004). Hypertrophic growth of the myocardium occurs due to an increase in size of the individual cardiomyocytes triggered by many kinds of stimuli such as hypertension, valvular defects, ischemia, myocardial infarction, and neurohormonal factors (Chu et al. 2002, Drazner 2005).
Under normal conditions, fatty acids are the main source of energy, which undergo β-oxidation in mitochondria to provide about 70-90% of total ATP. However, during cardiac hypertrophy, the energy metabolism shifts from fatty acids to glucose, much like the fetal period when mitochondrial content is low (Barger & Kelly 2000, Garcia & Goldenthal 2002, Lehman & Kelly 2002, Goffart et al. 2004). It was demonstrated that metabolic remodeling of cardiomyocytes is one of the important adaptive changes that drive the shift in substrate preference from fatty acids in normal adult heart to glucose in hypertrophy that eventually becomes maladaptive leading to ATP depletion (Ingwall 2009).

A number of important genes involved in fatty acid metabolism (e.g. medium-chain fatty acyl co-A dehydrogenase (Mcad (Acadm)), carnitine palmitoyl transferase-1 beta (Cpt1β), fatty acid transport protein (Fatp (Sclk27a1)), fatty acid translocase (Fat), etc.) are downregulated and expression of peroxisome proliferator-activated receptor alpha (Ppara; Ppara) is also reduced in both rats and humans during cardiac hypertrophy and failure (Barger & Kelly 2000, Garcia & Goldenthal 2002, Goffart et al. 2004). Ppara, the transcription factor that belongs to the nuclear receptor superfamily, is the central regulator of mitochondrial fatty acid oxidation (FAO) and is involved in almost every step of FAO in myocardial mitochondria (Barger & Kelly 2000, Huss & Kelly 2005). It plays a key role in metabolic remodeling in cardiomyocytes by regulating expression of some of the important genes involved in FAO (Lehman & Kelly 2002, Goffart et al. 2004, Huss & Kelly 2004).

Mitochondria are important targets of TH and extensive changes in the mitochondrial compartment take place in response to increased TH levels. Mitochondria biochemically mediate the calorigenic effects of TH at the cellular level (Goglia et al. 1999). It is known that TH causes a hypermetabolic state resulting in metabolic remodeling in the heart. Although initially TH induces mitochondrial biogenesis, mitochondrial proliferation does not keep pace with the increased energy demand in the hypertrophied heart. Therefore, it is likely that there occurs a mismatch between energy demand and supply in the heart in the hyperthyroid condition (Garcia & Goldenthal 2002, Degens et al. 2003, Garcia 2010, Sarma et al. 2012). As PPARα is the central regulator of mitochondrial energy metabolism, it might have a direct role in myocardial bioenergetics imbalance in the hyperthyroid state. In this study, we demonstrate that the PPARα-mediated signaling pathway is crucial in development of LV hypertrophy and that mitochondrial impairment in hyperthyroid rat heart leads to cardiac dysfunction. Furthermore, we show that PPARα not only regulates FAO but is also critical for maintaining structural as well as functional integrity of myocardial mitochondria, which in turn is essential for the adequate energy supply needed to maintain the proper functioning of heart.

Materials and methods

Treatment of animals

Female Sprague Dawley rats were received from the Institute’s animal facility. The animals were handled as per the guidelines of the Institutional Animal Ethics Committee (IAEC) of Indian Institute of Chemical Biology, in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice, Government of India. This study was approved by the IAEC. Rats with a body weight (BW) of 180–220 g were randomly assigned to the following treatment groups: control (n=12), triiodothyronine (T3) (n=12), T3+bezfibrate (Bzf) (n=12), and Bzf (n=8). Hyperthyroidism was induced by intraperitoneally injecting the rats daily with 8 μg/100 g BW T3 (Sigma Chemical Co.) for 15 days (T3) as described earlier (De et al. 2004). To study the progression of cardiac dysfunction induced by hyperthyroidism, rats were also treated with T3 for 2 days D2 (n=6), 5 days D5 (n=6), and 8 days D8 (n=6). The CON group was treated with the same volume of normal saline. To examine the effect of PPARα agonist in hyperthyroidism-induced cardiac hypertrophy, rats were given Bzf 30 μg/100 g BW (MP Biomedicals, Inc., Solan, OH, USA) (Davidoff et al. 2004) in 0.5% methyl cellulose (MP Biomedicals, Inc.) by oral gavage alone (Bzf) or along with T3 (T3+Bzf).

Determination of blood pressure

Noninvasive systolic blood pressure (SBP) of conscious rats was measured by tail cuff method using BIOPAC MP 36 (BIOPAC Systems, Inc., Goleta, CA, USA). The instrument has an animal heating controller (Tail heating B), small animal tail noninvasive blood pressure system (NIBP 200A), and MP 36 software for data integration.

Estimation of plasma T3 and thyroxine concentration

The concentration of T3 and thyroxine (T4) in rat plasma was estimated using Diagnova Eliscan T3 and Diagnova Eliscan T4 (RFCL Ltd., Faridabad, Haryana, India) kits respectively as per the manufacturer’s protocol.
Assessment of cardiac hypertrophy

The BW of rats was measured and then they were killed by administration of sodium pentobarbital (200 mg/kg BW) intraperitoneally. The hearts were surgically removed and immersed in ice-cold 0.9% NaCl. Excess blood, atrial tissues, visible fat, and connective tissues were removed. The weight of individual heart was recorded. The tissue samples used for isolation of RNA were immersed in TriPure reagent (Sigma Chemical Co.) and stored at −80 °C. Samples for biochemical assays were snap frozen in liquid nitrogen and stored at −80 °C. The degree of hypertrophy was calculated as the ratio of heart weight (HW) to BW (mg/g).

Real-time quantitative RT-PCR analysis

Expression levels of (Ppara), atrial natriuretic peptide (Anp (Nppa)), brain natriuretic peptide (Bnp (Nppb)), Cpt1β, and Mcad mRNAs were quantified by real-time quantitative RT-PCR using the Dynamo SYBR Green qPCR Kit (Finnzymes, Waltham, MA, USA) and the iCycler real-time detection system and software (Bio-Rad Laboratories). Equal amount (500 ng) of total RNA was used for RT-PCRs. GeneFisher software (http://bibiserv.techfak.uni-bielefeld.de/genefisher/) was used to design sequence-specific PCR primers (Table 1) according to the published gene sequences. The level of expression was calculated as fold change compared to control using the Ct value after normalizing with GAPDH. The experiment was repeated with RNA samples from LV tissue from six different rats.

Histological studies

After 15 days of treatment, hearts were collected and immediately fixed in 10% formalin and embedded in paraffin following a routine procedure (De et al. 2004). LV sections (5 μm thick) were prepared and stained with hematoxylin–eosin (HE; Sigma Chemical Co.). The stained tissue sections were examined under Olympus BX51 microscope (Olympus Corporation) at 400× magnification and images were captured with a digital camera attached to it. To measure the cross-sectional area (μm²) of the myocytes, the peripheral margin of 30 myocytes was manually traced and the area was measured using ImageJ software (NIH, Bethesda, MD, USA).

Western blot analysis

The whole homogenate from LV tissues was prepared as described earlier (Ghosh et al. 2007). Briefly, LV tissue was homogenized in a buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM each of phenylmethylsulphonyl fluoride and sodium orthovanadate, 1 μg/ml each of pepstatin A, leupeptin, and aprotinin. Protein from mitochondrial fraction was isolated using Mitochondria Isolation Kit for Tissue and Cultured Cells (BioChain Institute, Inc., Hayward, CA, USA) as per the manufacturer’s protocol. Equal amounts of protein (60 μg for PPARα and 40 μg for CPT1β, MCAD, prohibitin (PHB), VDAC, and SOD2) was subjected to SDS–PAGE in 10% polyacrylamide gels. Resolved proteins from gel were transferred to PVDF membranes (Millipore, Billerica, MA, USA) and kept immersed in blocking buffer (10% nonfat dry milk, 2 mM Tris–base, 13.7 mM NaCl, 0.1 M HCl, and 0.1% Tween 20, pH 7.6) for 1 h at room temperature. Then the membrane was incubated overnight at 4 °C with respective primary antibodies: PPARα (P0369, Sigma Chemical Co.); MCAD (101730, Cayman Chemical Company, Ann Arbor, MI, USA); CPT1β (sc-20670), PHB (sc-56467), SOD2 (sc-18503), and actin (sc-1616) (Santa Cruz Biotechnology, Inc.); and VDAC (PC548, Calbiochem, Darmstadt, Germany). Detection of PPARα and actin was conducted in whole homogenate and mitochondrial fraction was used to examine the expression of other proteins. Pixel density of the bands was quantified using ImageJ software (NIH) from a minimum of three different immunoblots from three different rats in each treatment group.

Electrophoretic mobility shift assay

An electrophoretic mobility shift assay (EMSA) was performed as described earlier (Cabrero et al. 2002).

Table 1 List of primers used in real-time quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>NCBI GenBank accession no.</th>
<th>Forward primer (5′→3′)</th>
<th>Reverse primer (5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anp</td>
<td>NM_012612</td>
<td>GTGTTCAACACACAGACTCTGATGG</td>
<td>GCGAGGCCAGAGACAGCCCTCA</td>
</tr>
<tr>
<td>Bnp</td>
<td>M25297</td>
<td>TGGAAGTCTCTACACAGCTCTC</td>
<td>GCGATCCGGTCTATTCTCTG</td>
</tr>
<tr>
<td>Gapdh</td>
<td>XM_216453</td>
<td>GCCATCAACAGACCCCTCC</td>
<td>AGCCGGGACCTTCCCA</td>
</tr>
<tr>
<td>Ppara</td>
<td>NM_013196</td>
<td>CCGTCCGGTGAGAGATGCTCACAC</td>
<td>GTGAGGGTGTCAGAGAGAGAGACAGTGA</td>
</tr>
<tr>
<td>Cpt1β</td>
<td>BC085761</td>
<td>GCTACAGGAGAGACCCAGAGCAGTACTG</td>
<td>TGGCTGTGTCAGCTGCTAG</td>
</tr>
<tr>
<td>Mcad</td>
<td>J02791</td>
<td>ATGGGTCAAGGTTGCTCTGA</td>
<td>TGAAGAICTCTTGGTCCTGCCACTAG</td>
</tr>
</tbody>
</table>

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Nuclear proteins from LV tissues of different treatment groups of rats were prepared using Nuclear Extraction Kit (Panomics, Fremont, CA, USA), and the protein concentration was determined using the Bio-Rad DC Protein Assay Reagents (Bio-Rad Laboratories). A double-stranded oligonucleotide containing the PPRE (Huss & Kelly 2004, Lefebvre et al. 2006) consensus sequence (underlined) sense: 5′-GGAACATAGGCATTTCCAGTCCAAGGA-3′; antisense: 5′-CCTTGATCCAGTTTCCAGTACAGG-3′ was end labeled with [γ-32P] ATP using T4 polynucleotide kinase (Fermentas, Inc., Waltham, MA, USA). Labeled probe was cleaned up using the QiAquick Nucleotide Removal Kit (Qiagen) according to the manufacturer’s protocol. Nuclear protein (5 μg) was incubated with labeled probe for 1 h at 37 °C. Where indicated, unlabeled oligonucleotide was added before the labeled probe as competitor and incubated for 10 min on ice. PPARα antibody (P0369, Sigma Chemical Co.) was added 15 min before incubation with the labeled probe. Protein–DNA complexes were resolved by electrophoresis at an 6% nondenaturing polyacrylamide gel. The protein–DNA complexes were visualized by autoradiography.

Transmission electron microscopy

For transmission electron microscopy, LV tissue was processed as described earlier (Lewis et al. 2001) with minor changes. Briefly, hearts were dissected out and immediately immersed in ice-cold 0.9% saline. Excess blood, atrial tissues, visible fat, and connective tissues were removed. A single 2 mm-thick slice was made at the ventricular apex, stored in 2% glutaraldehyde, and sectioned longitudinally and transversely to generate the ventricular apex, stored in 2% glutaraldehyde, and were removed. A single 2 mm-thick slice was made at blood, atrial tissues, visible fat, and connective tissues.

Mitochondria were isolated from LV tissue of rat using the Mitochondria Isolation Kit for Tissue and Cultured Cells (Bio Chain Institute, Inc., Hayward, CA, USA). A mitochondria activity assay was done by measuring mitochondria-specific enzyme cytochrome c oxidase activity in isolated mitochondria samples using Mitochondria Activity Assay (Cytochrome c Oxidase Activity Assay) kit (Bio Chain Institute, Inc.) as per the manufacturer’s protocol.

ATP assay

LV tissue homogenate from equal amounts of LV tissue (100 mg) from different treatment groups was prepared using the Deproteinizing Sample Preparation Kit (BioVision, Mountain View, CA, USA). ATP concentration was measured in the homogenates using ATP Colorimetric/Fluorometric Assay Kit (BioVision) as per the manufacturer’s protocol.

Determination of lipid peroxidation

Lipid peroxides in the cell-free homogenate obtained from LV tissue were determined as described earlier (Ghosh et al. 2007). Briefly, LV tissue (200 mg) was homogenized in ice-cold 0.9% saline (pH 7.0) with a Potter–Elvehjem glass homogenizer for 30 s. The homogenate was centrifuged at $800 \text{ g}$ for 3 min at 4 °C and the cell-free homogenate was mixed with thiobarbituric acid (TBA)–trichloro acetic acid reagent with thorough shaking. After boiling for 15 min, the samples were cooled to room temperature. The absorbance of the pink chromogen present in the clear supernatant obtained after centrifugation was measured at 532 nm using a u.v.–VIS spectrophotometer (Bio-Rad). The amount of lipid peroxide present in each sample was calculated as the amount of TBA reactive substance (TBARS)/mg protein.

Antioxidant assay

Blood was collected from rats from different treatment groups and allowed to clot for 30 min at 25 °C. Then, it was centrifuged at $2000 \text{ g}$ for 15 min at 4 °C and the top yellow layer (serum) was collected. Total antioxidant level including endogenous (enzymes such as SOD, catalase, and glutathione peroxidase; macromolecules such as albumin, ceruloplasmin, and ferritin; and an array of small molecules including α-tocopherol, β-carotene, reduced glutathione, uric acid, and bilirubin) and food-derived antioxidants in different serum samples were determined using the Antioxidant Assay Kit (Cayman Chemical Company) as per the manufacturer’s protocol.
Hemodynamic study

The rats were anesthetized with sodium pentobarbital (50 mg/kg BW) and heparin (500 units/kg BW). The right internal carotid artery was cranially ligated. A miniaturized conductance catheter (SPR-B38 Millar Instruments, Houston, TX, USA) was inserted into the LV through the carotid artery. Stable pressure-volume (PV) loops were obtained under steady-state conditions (Pacher et al. 2008). These PV loops were analyzed using the PVAN 3.5 software (Millar Instruments) to obtain the values of a range of hemodynamic parameters such as heart rate, stroke volume (SV), CO, and so on. The experiment was repeated with six animals in each treatment group.

Statistical analysis

The data are presented as the mean ± S.E.M. All the experiments were repeated independently at least with three different animals in each group. The real-time PCR analysis for each gene was repeated with six different rats in each group. Data between two groups were evaluated by Student’s t-test (independent) and the complete data set among all treatment groups (three or more, as indicated) was evaluated by one-way ANOVA using Microcal Origin 6.0 (Microcal Software, Inc., MA, USA). A level of 0.05 was set as the threshold for statistical significance between the control and various experimental groups.

Results

Downregulation of PPARx in hyperthyroid-induced hypertrophied left ventricle

Development of overt hyperthyroidism was confirmed by determination of plasma T₃ level (Table 2). There was about a 3.5-fold increase in plasma T₃ level after 15 days of T₃ (8 μg/100 g BW) treatment compared with vehicle-treated control. In Bzf-co-treated rats, plasma T₃ level was found to be almost equal compared with T₃-treated rats.

Table 2 Plasma levels of thyroid hormones. n=4, for control, T₃, T₃+Bzf, and Bzf. T₄ was not detected (ND) in T₃ and T₃+Bzf samples. No significant difference was found between plasma T₄ levels of control vs Bzf alone

<table>
<thead>
<tr>
<th>Sample</th>
<th>T₃ (ng/ml)</th>
<th>T₄ (μg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.36 ± 0.05</td>
<td>3.48 ± 0.27</td>
</tr>
<tr>
<td>T₃</td>
<td>4.66 ± 0.22*</td>
<td>ND</td>
</tr>
<tr>
<td>T₃ + Bzf</td>
<td>4.59 ± 0.19*</td>
<td>ND</td>
</tr>
<tr>
<td>Bzf</td>
<td>1.27 ± 0.04</td>
<td>3.28 ± 0.11</td>
</tr>
</tbody>
</table>

*P<0.01 vs control.

Plasma T₄ was not detected in T₃-treated and T₃ and Bzf-co-treated rats. Bzf treatment alone did not significantly change plasma T₃ or T₄ levels compared with controls. To examine whether PPARx is involved in hyperthyroidism-induced cardiac hypertrophy, rats were treated with T₃ for 15 days in the absence or presence of the PPARx agonist, Bzf, and cardiac hypertrophy was assessed by measuring HW:BW ratio. After 15 days of T₃ treatment, HW (combined left and right ventricle) was increased by ~42% in T₃-treated rats compared with control rats (Table 3) leading to about a 60% increase in HW:BW ratio. Co-treatment of rats with Bzf along with T₃ prevented hypertrophy of the heart significantly indicating that PPARx is involved in cardiac hypertrophy induced by hyperthyroidism. Treatment with Bzf alone did not have a significant effect on either absolute HW or HW:BW ratio (Fig. 1A).

To confirm the induction of cardiac hypertrophy, the expression of molecular markers of hypertrophy, ANP, and BNP was also examined. The level of Anp mRNA was increased over fourfold (P<0.01) compared with control (Fig. 1B), whereas the level of Bnp mRNA was increased about fivefold (P<0.01) after 15 days of T₃ treatment (Fig. 1C). Co-treatment of rats with Bzf significantly prevented T₃-induced expression of Anp and Bnp in the LV (Fig. 1B and C). Histological examination of HE-stained LV cross-sections also showed the occurrence of myocyte hypertrophy under the hyperthyroid condition that was attenuated by Bzf co-treatment (Fig. 1D and E). Both mRNA and protein levels of PPARx were significantly reduced in T₃-treated rat heart compared with controls, while treatment with Bzf along with T₃ enhanced its expression. Bzf alone did not significantly change the expression of Ppara in the heart (Fig. 2A and B). Taken together, the results demonstrated that PPARx is involved in the development of pathological cardiac hypertrophy induced by hyperthyroidism.

Table 3 Induction of cardiac hypertrophy by T₃. n=12, for control, T₃, and T₃+Bzf; n=8 for Bzf. No significant difference was found between the parameters of control vs Bzf

<table>
<thead>
<tr>
<th>Sample</th>
<th>HW (mg)</th>
<th>BW (g)</th>
<th>HW/BW (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>619.9 ± 12.7</td>
<td>211.1 ± 3.39</td>
<td>2.94 ± 0.03</td>
</tr>
<tr>
<td>T₃</td>
<td>880.8 ± 12.6</td>
<td>187.4 ± 2.24</td>
<td>4.7 ± 0.06</td>
</tr>
<tr>
<td>T₃ + Bzf</td>
<td>680.1 ± 12.3</td>
<td>198.5 ± 2.00</td>
<td>3.46 ± 0.05</td>
</tr>
<tr>
<td>Bzf</td>
<td>616.9 ± 12.1</td>
<td>204.0 ± 2.63</td>
<td>3.02 ± 0.04</td>
</tr>
</tbody>
</table>

P<0.01 (Con vs T₃ and T₃ vs T₃+Bzf).
SBP was found to be elevated by 36% in T3-treated rats compared with controls (Table 4). Co-treatment with the PPARα agonist Bzf attenuated T3-induced hypertension by about 9%. Treatment with Bzf alone did not significantly alter blood pressure.

**Effect of hyperthyroidism on Pparα target genes associated with FAO in heart**

As PPARα was found to be downregulated in LV tissue of T3-treated rats, we examined the expression of two of its important target genes (Cpt1β and MCAD) involved in β-oxidation of fatty acid after 15 days of T3 treatment. The mRNA as well as protein levels of both CPT1β and MCAD were significantly reduced in T3-treated rat LV compared with control, which were enhanced when Bzf was co-administered (Fig. 3A, B, C and D). Treatment with Bzf alone did not significantly alter either CPT1β or MCAD expression.

**Mitochondrial structural damage in hyperthyroid rat heart**

Expression of the mitochondrial inner membrane protein, PHB, was decreased significantly in T3-treated hearts compared with controls (Fig. 4A). Expression of the outer membrane protein VDAC was also downregulated by T3 treatment (Fig. 4B). Bzf co-administration along with T3 significantly prevented the decrease in expression of both these proteins compared with T3 alone, whereas only Bzf treatment did not show any significant difference in the levels of these two membrane proteins of mitochondria (Fig. 4A and B).

The effect of T3 on the ultrastructure of mitochondria was examined by transmission electron microscopy. As shown in Fig. 4C, the normal architecture of the mitochondria was lost and cristae are damaged in T3-treated rat heart. Also, the ordered distribution pattern of mitochondria in-between the myofibrils was disrupted.
marker for oxidative stress in LV tissue. As shown in Fig. 6A, a more than twofold increase in lipid peroxidation was observed in LV of T3-treated rat. Bzf prevented T3-induced elevation in lipid peroxidation. The total antioxidant level in LV was significantly reduced by T3, which was found to be enhanced compared with controls in Bzf-co-treated tissue (Fig. 6B). Bzf alone did not significantly affect lipid peroxidation and total antioxidant level.

Prolonged T3 treatment causes cardiac dysfunction via downregulation of PPARα

Initially, all the hemodynamic parameters were improved significantly by T3 (Table 5). However, the SV and diastolic index (−dP/dt) declined after 5 days. This trend further continued through 8 days and reduced to a minimum level after 15 days, indicating diastolic dysfunction. The marked decrease in SV leads to strikingly reduced CO despite an increased heart rate. After the initial increase, the systolic index (+dP/dt) also started decreasing, which was prominent after 8 days, indicating a systolic dysfunction. Co-treatment with Bzf prevented the deterioration in hemodynamic parameters resulting in improved cardiac function.

Discussion

This study demonstrates a possible mechanism of cardiac dysfunction in the hyperthyroid condition involving PPARα-mediated mitochondrial impairment. The molecular basis of hyperthyroidism-induced cardiac malfunction could be attributed to metabolic remodeling and bioenergetic inefficiency of the myocardium due to structural and functional abnormalities of the mitochondria leading to an energy crisis in myocytes. Prevention of hyperthyroidism-induced mitochondrial damage as well as amelioration of hypertrophy by Bzf signifies a pivotal role of the PPARα pathway in the functioning of the cardiac power house.

A shift from fatty acids to glucose as the main source of energy is known to occur in many models of cardiac dysfunction. As considerable ultrastructural damage and functional impairment of myocardial mitochondria was caused due to T3 treatment, we measured lipid peroxidation as a marker for oxidative stress in LV tissue. As shown in Fig. 6A, a more than twofold increase in lipid peroxidation was observed in LV of T3-treated rat. Bzf prevented T3-induced elevation in lipid peroxidation. The total antioxidant level in LV was significantly reduced by T3, which was found to be enhanced compared with controls in Bzf-co-treated tissue (Fig. 6B). Bzf alone did not significantly affect lipid peroxidation and total antioxidant level.

Table 4  Mean ± S.E.M. of SBP of six animals in each group (n = 6 for control, T3, T3 + Bzf, and Bzf)

<table>
<thead>
<tr>
<th>Sample</th>
<th>SBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>112.9 ± 3.0</td>
</tr>
<tr>
<td>T3</td>
<td>153.7 ± 1.4</td>
</tr>
<tr>
<td>T3 + Bzf</td>
<td>129 ± 1.0</td>
</tr>
<tr>
<td>Bzf</td>
<td>110.4 ± 2.0</td>
</tr>
</tbody>
</table>

P < 0.01 (Con vs T3 and T3 vs T3 + Bzf).
hypertrophy and heart failure (Barger & Kelly 2000, Garcia & Goldenthal 2002, Lehman & Kelly 2002, Goffart et al. 2004, Kieck-Wilk et al. 2005). PPARα is the central regulator of cardiac fatty acid metabolism (Huss & Kelly 2004, 2005) and is found to be downregulated in various models of cardiac hypertrophy (Barger & Kelly 2000, Garcia & Goldenthal 2002, Ventura-Clapier et al. 2003, Kieck-Wilk et al. 2005). Consistently, here, we show that downregulation of PPARα is crucial for myocardial remodeling (Figs 1 and 2) as its agonist Bzf prevented cardiac hypertrophy in the hyperthyroid condition.

Anti-thyroidal effect of fibrates has been reported by previous studies (Rodriguez-Gomez et al. 2008). One of the major mechanisms of fibrates in lowering plasma T3 level is by inhibition of deiodinases apart from the induction in uridine diphosphate glucuronosyltransferases (Luci et al. 2006). However, in this study, Bzf co-treatment did not reduce the plasma T3 level (Table 2). This apparent discrepancy might be explained by two facts. First, hyperthyroidism is induced in this study by T3 instead of T4 (Rodriguez-Gomez et al. 2008), and secondly, Bzf is used instead of clofibrate (Rodriguez-Gomez et al. 2008). Despite increased plasma T3 levels, Bzf co-treatment minimally but significantly lowered SBP in our study (Table 4).

Subsequently, Bzf co-administration along with T3 also enhances the expression of the target genes of PPARα involved in fatty acid transport (CPT1β) and oxidation (MCAD) compared with the expression levels in control rats (Fig. 3, B, C and D). This is further supported by EMSA, in which the diminished binding of PPARα protein with its response element in the target gene is seen in the hyperthyroid heart and is enhanced compared with control (Luci et al. 2006).

Figure 3
Bzf improves the expression of FAO genes. Expression level of mRNA and protein for CPT1β and MCAD (A, B, C and D) was evaluated by real-time quantitative RT-PCR analysis and western blot analysis, respectively, in LV tissue samples from vehicle (CON) or T3 (T3) or Bzf along with T3 or only Bzf-treated rats. *P<0.01 control vs T3, #P<0.01 T3 vs T3+Bzf (n=6) (A and C). Representative immunoblots (B and D) show the level of CPT1β and MCAD protein in the mitochondrial fraction of LV tissues. Lower panel shows the average pixel density of three immunoblots from different rats (n=3) after normalizing with SOD2 protein expression. *P<0.01 control vs T3 and #P<0.01 T3 vs T3+Bzf (n=3) for both CPT1β and MCAD proteins. The complete set of data (for A, B, C and D) are significant at <0.01 level (one-way ANOVA). No significant difference was seen in control vs Bzf (A, B, C and D).
control by Bzf co-treatment (Supplementary Figure 1, see section on supplementary data given at the end of this article). To explain this, we measured time-dependent expression of PPARα in LV tissue of T3-treated rats and also after Bzf administration in the presence or absence of T3. The expression of PPARα is initially increased and then gradually decreased in T3-treated rats. In Bzf-co-treated rats, PPARα expression is improved as seen initially in T3-treated rats, whereas treatment with Bzf alone did not significantly alter its expression (Supplementary Figure 2). This indicates that after the initial increase, Bzf prevented the downregulation of PPARα expression. Bzf treatment alone did not significantly affect either PPARα expression or its target genes. It is possible that Bzf only enhances the binding of PPARα to PPRE. As 70–90% of energy in the heart is derived from FAO, it implicates a high involvement of PPARα in FAO regulation under normal conditions. Therefore, treatment with agonist alone might not significantly increase the binding of PPARα to PPRE and produce any additional detectable effect in the expression of target genes of PPARα or FAO in the heart.

TH receptors (TRs) and PPARs interact in various tissues including the heart, and these interactions play an important role in regulating PPAR target gene expression. TRs and PPARs compete for binding with retinoid X receptor, the common heterodimerization partner and also to binding sites in the target genes. Several previous studies have shown diminished or enhanced expression of PPAR target genes by TRs (Hyyti & Portman 2006). In this study, in addition to the decrease in PPARα expression, TR–PPARα interaction in the myocardium might be another possible mechanism responsible for the decrease in Ppara target gene expression.

CPT1β activity is crucial in FAO as it catalyzes the transesterification of long-chain fatty acids, the rate-limiting step in mitochondrial fatty acid uptake.

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CPT1β activity is crucial in FAO as it catalyzes the transesterification of long-chain fatty acids, the rate-limiting step in mitochondrial fatty acid uptake.
MCAD catalyzes the first step in the mitochondrial fatty acid β-oxidation spiral. Defective expression, function, or mutation in this gene is one of the important causes of in-born errors of metabolism that often lead to childhood cardiomyopathy and sudden death, underscoring the importance of FAO in proper cardiac functioning (Barger & Kelly 2000, Garcia & Goldenhall 2002).

Downregulation of these two important target genes of PPARα in hyperthyroid rat heart might lead to reduced FAO and bioenergetic disorders. Cardiomyocytes have the highest volume density of mitochondria (occupying about 30% space) in the body and are well organized under the sarcolemma and between the myofilaments so as to maintain efficient diffusion distance. Nearly 90% of total energy is produced by mitochondrial respiration in the heart and mitochondria are one of the major targets of T₃ (Goglia et al. 1999, Ventura-Clapier et al. 2003, Goffart et al. 2004, Huss & Kelly 2004). In this study, it is interesting to observe that in addition to genes involved with FAO, excess T₃ in the heart downregulates the mitochondrial membrane proteins PHB and VDAC (Fig. 4A and B). PHB1 along with its heteromeric partner PHB2 is present in the inner mitochondrial membrane as a macromolecular complex and is known to play a critical role in maintaining normal mitochondrial function and morphology. It acts as a chaperone that stabilizes unassembled membrane proteins and protects newly imported proteins from degradation by m-AAA protease (Coates et al. 2001). Hence, decreased PHB expression in mitochondria might lead to enhanced degradation of membrane proteins and diminished activity of the mitochondrial respiratory chain resulting into increased oxidative stress and loss of mitochondrial membrane potential (Artal-Sanz & Tavernarakis 2009). VDACs are

Figure 5
Bzf prevents mitochondrial dysfunction. (A) The histogram represents the mitochondrial activity evaluated by measuring the cytochrome c oxidase activity (U/mg) in mitochondria isolated from the LV tissues of vehicle (CON) or T₃ (T₃) or Bzf in combination with T₃-treated rats (T₃+Bzf) or alone (Bzf) for 15 days. Each bar represents mean ± S.E.M. of five rats (n = 5), *P < 0.01 control vs T₃ and #P < 0.01 T₃ vs T₃+Bzf. P value for complete data set is < 0.01, analyzed by one-way ANOVA. Cytochrome c oxidase activity was not significantly different from control in only Bzf-treated LV tissue. (B) The histogram represents the total ATP concentration in homogenate of LV tissue from control (CON), T₃-treated rats at different time points (viz. 2 days (D2), 5 days (D5), 8 days (D8), and 15 days (D15)) as well as T₃ along with Bzf for 15 days (D15+Bzf) or only Bzf for 15 days treated rats. Each bar represents ATP concentration as mean ± S.E.M. from three independent experiments (n = 3). *P < 0.01 control vs D2, #P < 0.01 control vs D5, *P < 0.01 control vs D8, and *P < 0.01 D15 vs D15+Bzf. No significant difference was found between ATP concentrations of control and only Bzf-treated rats.

Figure 6
Bzf prevents oxidative damage in hyperthyroid myocardium. Lipid peroxidation (A) in LV tissue homogenates from rats treated with either vehicle (CON) or T₃ (T₃) or Bzf in combination with T₃ (T₃+Bzf) or alone (Bzf) for 15 days. Each bar in the histogram represents mean ± S.E.M. of TBARS in nM/mg protein estimated from LV tissue of five different animals; (n = 5). *P < 0.01 control vs T₃ and #P < 0.01 T₃ vs T₃+Bzf. Total antioxidants (B) present in serum samples of similar groups of rats are shown in the histogram. Each bar represents mean ± S.E.M. of total antioxidants in three rats; (n = 3). *P < 0.01 control vs T₃ and #P < 0.01 T₃ vs T₃+Bzf. The P value for the complete data set is < 0.01, analyzed by one-way ANOVA for both (A and B). Lipid peroxidation or total antioxidant level was not significantly different in control vs only Bzf-treated rats.
Table 5 Effect of T3 on heart function. No significant difference was found between the parameters of control vs Bzf (15 days)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Heart rate (bpm)</th>
<th>SV (µl)</th>
<th>+ dP/dt (mmHg/s)</th>
<th>− dP/dt (mmHg/s)</th>
<th>CO (µl/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>363 ± 6</td>
<td>128 ± 2</td>
<td>7642 ± 346</td>
<td>−6266 ± 210</td>
<td>46 352 ± 326</td>
</tr>
<tr>
<td>T3 (2 days)</td>
<td>519 ± 2</td>
<td>149 ± 2</td>
<td>10 245 ± 185</td>
<td>−8289 ± 152</td>
<td>77 408 ± 904</td>
</tr>
<tr>
<td>T3 (5 days)</td>
<td>522 ± 2</td>
<td>111 ± 4</td>
<td>9234 ± 461</td>
<td>−5283 ± 249</td>
<td>58 197 ± 2758</td>
</tr>
<tr>
<td>T3 (8 days)</td>
<td>558 ± 2</td>
<td>58 ± 1</td>
<td>6763 ± 125</td>
<td>−3176 ± 83</td>
<td>32 179 ± 589</td>
</tr>
<tr>
<td>T3 (15 days)</td>
<td>570 ± 1</td>
<td>43 ± 1</td>
<td>4338 ± 84</td>
<td>−2525 ± 47</td>
<td>24 606 ± 1005</td>
</tr>
<tr>
<td>T3 + Bzf (15 days)</td>
<td>467 ± 15</td>
<td>131 ± 3</td>
<td>8798 ± 398</td>
<td>−6211 ± 250</td>
<td>61 982 ± 2890</td>
</tr>
<tr>
<td>Bzf (15 days)</td>
<td>365 ± 5</td>
<td>126 ± 2</td>
<td>7432 ± 343</td>
<td>−6220 ± 208</td>
<td>46 099 ± 1074</td>
</tr>
</tbody>
</table>

n = 6, P < 0.05 (for all the parameters of Con. vs T3 (2 days), Con. vs T3 (5 days), Con. vs T3 (8 days), Con. vs T3 (15 days), and T3 (15 days) vs T3 + Bzf (15 days). SV, stroke volume; CO, cardiac output.

ion channel proteins most abundant in the outer mitochondrial membrane. The physiological function ofVDACs is to control the movement of adenine nucleotides, NADH, and other metabolites across the outer membrane. ATP produced in mitochondria is available to cytosolic enzymes such as hexokinase, glucokinase, and glycerol kinase via VDAC (Blachy-Dyson & Forte 2001, Colombini 2004, Shoshan-Barmatz et al. 2010). Therefore, reduced expression of VDAC might adversely affect various metabolic pathways, resulting into insufficient energy production in cardiomyocytes in the hyperthyroid condition. The loss of mitochondrial cristae density and cristae damage (Fig. 4C) leads to the disruption of the respiratory chain complex (Abel & Doenst 2011). This could be the cause of reduced cytochrome c oxidase enzyme activity (Fig. 5A) and diminished ATP concentrations (Fig. 5B) rendering the hypertrophied heart energy deficient (van Bilsen et al. 2009, Tsutsui et al. 2009). Phosphocreatine (PCr) serves as the primary energy reserve in the heart and the phosphoryl group from it is transferred to ADP for resynthesis of ATP that is used for muscle contraction. This reaction is catalyzed by creatine kinase (CK). The CK system plays an important role under stressed conditions such as hypoxia and ischemia by transferring the phosphoryl group to ADP from PCr and slowing the rate of ATP depletion. It also helps myocytes to sustain increased workload under stressed conditions (Liao et al. 1996). A decrease in PCr along with ATP content in the heart has been reported in LV hypertrophy and failing myocardium in earlier studies (Liao et al. 1996, Pinz et al. 2011). Mitochondrial enzyme activity and ATP levels have also been reported to be decreased in pacing induced heart failure (Marín-García et al. 2001). Consistently, our study demonstrates ATP depletion in the heart under the hyperthyroid condition and is probably accompanied by depletion in PCr level and/or CK activity. Also, the initial increase in ATP concentration during T3 treatment might partly be attributed to PCr along with oxidative phosphorylation in the myocardium to sustain enhanced workload.

Lower myocardial energy production indicates reduced oxygen consumption and limits cardiac work under high workload conditions. The disorganized distribution and diminished ratio of mitochondria to myofibrils would further diminish the effective diffusion of ATP from mitochondria to myofibrils and would adversely affect cardiac function (Ventura-Clapier et al. 2003).

Mitochondria produces a major part of reactive oxygen species (ROS) in cell as a byproduct of the electron transport chain, which is detoxified by the cellular antioxidant system under physiological conditions (Gustafsson & Gottlieb 2008, Zhang et al. 2008). Excess T3 induces a hypermetabolic state in the heart and also increases cellular respiration leading to increased ROS production, which is not completely neutralized by the cellular antioxidants, and there is gradual and progressive accumulation of ROS in cardiomyocytes (Ghosh et al. 2007, Abel & Doenst 2011). This is evident from increased lipid peroxidation and depletion of cellular antioxidants seen after 15 days of T3 treatment (Fig. 6A and B). The level of antioxidants is increased compared with control when Bzf is co-administered along with T3 (Fig. 6B). This indicates that the increased amounts of antioxidants are required to neutralize the increased ROS generated due to higher metabolic rates and hence we see no net increase in lipid peroxidation. Mitochondrial ROS also leads to opening of the mitochondrial permeability transition pore (Garcia & Goldenthal 2002, Gustafsson & Gottlieb 2008, Abel & Doenst 2011), resulting in loss of proton gradient and electrical potential across the inner mitochondrial membrane. This may lead to uncoupling of
oxidative phosphorylation, influx of water to cause mitochondrial swelling, cristae remodeling, and rupture of outer membrane (Gustafsson & Gottlieb 2008). Excessive ROS generation has been linked to transition from compensatory to decompensatory hypertrophy and eventually to heart failure (Gustafsson & Gottlieb 2008, Tsutsui et al. 2009, Abel & Doenst 2011).

TH is known to enhance force and speed of systolic contraction and speed of diastolic relaxation through upregulation of sarcoplasmic reticulum Ca\(^{2+}\) ATPase-2 (Serca2 (Atp2a2)) transcription and downregulation of phospholamban in cardiomyocytes (Klein & Ojamaa 2001, Kahaly & Dillmann 2005). Heart rate (chronotropy) and CO also increases due to hyperthyroidism. Increased Serca2 expression increases the speed of diastole by increasing the rate of Ca\(^{2+}\) reuptake by the sarcoplasmic reticulum, consuming ATP in the process (Klein & Ojamaa 2001, Yen 2001, Kahaly & Dillmann 2005). Heart rate (chronotropy) and CO also increases due to hyperthyroidism. Increased Serca2 expression increases the speed of diastole by increasing the rate of Ca\(^{2+}\) reuptake by the sarcoplasmic reticulum, consuming ATP in the process (Klein & Ojamaa 2001, Yen 2001, Kahaly & Dillmann 2005). Consistently, our findings show an initial increase in heart rate, SV, and systolic and diastolic indexes, leading to greater CO (Table 5). The increase in SERCA2 expression increases cardiac energy demand. The concomitant ATP depletion in the hypertrophied heart would adversely affect the efficacy of the SERCA pump, compromising the rate of Ca\(^{2+}\) reuptake and consequently prolonging Ca\(^{2+}\) transient. This would result into impaired diastolic function of the heart, diminishing the beneficial effect of increased SERCA2 expression and might trigger ventricular fibrillation (Braunwald & Bistrow 2000, Yen 2001, Ashrafian et al. 2003, Ingwall & Weiss 2004, Kenzel et al. 2008, Pinz et al. 2011). Consistently, our findings from the hemodynamic analysis show a gradual decrease in diastolic index (−dp/dt) (Table 5). With progression of pathological hypertrophy, impaired diastole would lead to progressive decreases in sarcomeric length/stretch resulting in lesser force generation during systole (Ashrafian et al. 2003). This would progressively diminish the efficiency of the cardiac pump reflected in the significant decrease in SV that is evident after 5 days of T\(_3\) treatment. The decline in SV is so pronounced after 8 days of T\(_3\) treatment that even an increased heart rate is unable to prevent the decrease in CO, which reaches a remarkably minimum level after 15 days of treatment. Decline in systolic index (+dp/dt) after 8 days of treatment is also evident and significantly contributes to the decrease in SV and CO. Our data reveal that co-treatment with Bzf improves the cardiac function and the parameters lie within the range of 2 days and 5 days of T\(_3\) treatment. This indicates that probably this is the transition period of adaptive to maladaptive hypertrophy and Bzf intervenes during this period to prevent the progressive decline in functional parameters.

Another probable contributor responsible for reduced force generation could be the increase in myosin heavy-chain alpha (MHC\(_\alpha\)) in hyperthyroid heart, which has a higher ATPase activity and increased velocity of contraction than MHC\(_\beta\) (Klein & Ojamaa 2001, Yen 2001, Ashrafian et al. 2003). The resultant hyper-contractility coupled with increased heart rate and decreased sarcomeric length due to impaired diastolic function could lead to increased cross-bridge detachment and premature ventricular beats even before the contraction is complete without contributing to force generation, consuming ATP in the process. Thus, enhanced contractility seen during the initial stage of treatment T\(_3\) would result in reduced mechanical efficiency and increased energy cost for force generation (Ashrafian et al. 2003, Kenzel et al. 2008). Consistently, our data show that though enhanced systolic index (+dp/dt) is seen even after 5 days of T\(_3\) treatment, the trend of decline in CO is apparent at this stage with a significant decrease in diastolic index (−dp/dt), which might be the reason for the decrease in CO and SV. This continuum of hemodynamic events suggests that probably diastolic dysfunction paves the way for systolic abnormalities in hyperthyroidism-induced cardiac hypertrophy.

Bzf has been reported to reduce heart rate and blood pressure in hypertriglyceridemic patients, and these changes are likely to be caused by Bzf-mediated improvement of endothelial function (Jonkers et al. 2001). It is possible that NO-mediated induction of the parasympathetic nervous system might be involved in Bzf-mediated heart rate reduction in this study. However, further studies are warranted to unveil this mechanism.

In summary, TH has distinct effects on nuclei and mitochondria. Prolonged TH administration down-regulates nuclear encoded genes involved in \(\beta\)-oxidation of fatty acid, the major energy-generating pathway in cardiomyocytes, by reducing the expression of the central transcription factor for these enzymes, PPAR\(_\alpha\). Hyperthyroidism also reduces expression of mitochondrial membrane proteins causing structural damage and functional impairment of mitochondria, leading to reduced ATP generation, rendering the hypertrophied heart energy starved. ROS generation is also enhanced in the hyperthyroid condition that leads to cardiac dysfunction. Downregulation of PPAR\(_\alpha\) might be contributing to enhanced ROS generation as its agonist could successfully attenuate ROS levels. Therefore, PPAR\(_\alpha\) agonists might act as cardio-protective agents by preventing mitochondrial dysfunction in addition to their lipid-lowering actions.
Bzf, used in this study as PPARα agonist, may also activate PPARβ/δ and PPARγ. As PPARβ/δ is expressed in comparable amounts along with PPARα in the adult heart and is said to influence FAO, the effects of Bzf might partly be attributed to PPARβ/δ.

**Supplementary data**

This is linked to the online version of the paper at [http://dx.doi.org/10.1530/JOE-12-0304](http://dx.doi.org/10.1530/JOE-12-0304).

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**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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