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 (T_3) alone (8 $\mu\text{g}/100$ g body weight (BW), i.p.) for 15 days or along with a peroxisome proliferator-activated receptor alpha agonist bezafibrate (Bzf; 30 $\mu\text{g}/100$ g BW, oral) and were found to improve in the Bzf co-treated condition. Ultrastructure of mitochondria was damaged in T_3 -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 T_3 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

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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 (*Cpt1b*), fatty acid transport protein (*Fatp* (*Slc27a1*)), 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 calorogenic 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 *PPAR α* 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 *PPAR α* -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

PPAR α 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 (T_3) ($n=12$), T_3 +bezafibrate (Bzf) ($n=12$), and Bzf ($n=8$). Hyperthyroidism was induced by intraperitoneally injecting the rats daily with 8 μ g/100 g BW T_3 (Sigma Chemical Co.) for 15 days (T_3) as described earlier (De *et al.* 2004). To study the progression of cardiac dysfunction induced by hyperthyroidism, rats were also treated with T_3 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 *PPAR α* agonist in hyperthyroidism-induced cardiac hypertrophy, rats were given Bzf 30 μ g/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 T_3 (T_3 +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 T_3 and thyroxine concentration

The concentration of T_3 and thyroxine (T_4) in rat plasma was estimated using Diagonova Eliscan T_3 and Diagonova Eliscan T_4 (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 (*Ppar α*), atrial natriuretic peptide (*Anp* (*Nppa*)), brain natriuretic peptide (*Bnp* (*Nppb*)), *Cpt1 β* , 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 $400\times$ magnification and images were captured with a digital camera

attached to it. To measure the cross-sectional area (μm^2) 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 phenylmethylsulfonyl fluoride and sodium orthovanadate, 1 $\mu\text{g}/\text{ml}$ 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 PPAR α and 40 μg for CPT1 β , 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: PPAR α (P0369, Sigma Chemical Co.); MCAD (101730, Cayman Chemical Company, Ann Arbor, MI, USA); CPT1 β (sc-20670), PHB (sc-56467), SOD2 (sc-18503), and actin (sc-1616) (Santa Cruz Biotechnology, Inc.); and VDAC (PC548, Calbiochem, Darmstadt, Germany). Detection of PPAR α 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

Gene	NCBI GenBank accession no.	Forward primer (5' $\rightarrow$ 3')	Reverse primer (5' $\rightarrow$ 3')
<i>Anp</i>	NM_012612	GTGTCCAACACAGATCTGATGG	GCCAGCGAGCAGAGCCCTCA
<i>Bnp</i>	M25297	TGGGAAGTCCTAGCCAGTCTC	GCCGATCCGGTCTATCTTCTG
<i>Gapdh</i>	XM_216453	GCCATCAACGACCCCTTC	AGCCCCAGCCTTCTCCA
<i>Pparα</i>	NM_013196	CCTGTCCGTCGGGATGTCACAC	GTGACGGTCTCCACGGACATGC
<i>Cpt1β</i>	BC085761	GCTACACGGAGACAGGACACTG	TGCCTTGTCCCGGAAATGAGC
<i>Mcad</i>	J02791	ATGGGTGTCAGCGGTGCTCTGA	TGAAACTCCTTGGTGCTCCACTAG

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 PPARE (Huss & Kelly 2004, Lefebvre *et al.* 2006) consensus sequence (underlined) sense: 5'-GGAAGTAGGTCAAAGGTCATCCCCCT-3'; anti-sense: 5'-CCTTGATCCAGTTTCCAGTAGGA-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 μ g) 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. PPAR α antibody (P0369, Sigma Chemical Co.) was added 15 min before incubation with the labeled probe. Protein-DNA complexes were resolved by electrophoresis at on a 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 1 mm cubes that were further stored in 2% glutaraldehyde for 24 h at 4 °C. Tissues were fixed in 1% OsO₄ in PBS for 4 h. After fixation, tissues were dehydrated with graded ethanols and embedded in Spurr. Samples were sectioned (100 nm), stained with uranyl acetate-lead citrate, and examined under a transmission electron microscope (Tecnai G² Spirit BioTWIN, Czech Republic). For quantification of damaged mitochondria, total number of mitochondria along with the damaged ones was counted in three different micrographs for each treatment group and the percentage of damaged mitochondria was calculated.

Cytochrome c oxidase activity assay

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 *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 *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-838 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 $\pm$ 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 $P < 0.05$ was set as the threshold for statistical significance between the control and various experimental groups.

Results

Downregulation of PPAR α in hyperthyroid-induced hypertrophied left ventricle

Development of overt hyperthyroidism was confirmed by determination of plasma T_3 level (Table 2). There was about a 3.5-fold increase in plasma T_3 level after 15 days of T_3 (8 μ g/100 g BW) treatment compared with vehicle-treated control. In Bzf-co-treated rats, plasma T_3 level was

Table 2 Plasma levels of thyroid hormones. $n=4$, for control, T_3 , T_3 +Bzf, and Bzf. T_4 was not detected (ND) in T_3 and T_3 +Bzf samples. No significant difference was found between plasma T_4 levels of control vs Bzf alone

Sample	T_3 (ng/ml)	T_4 (μ g/dl)
Control	1.36 ± 0.05	3.48 ± 0.27
T_3	$4.66 \pm 0.22^*$	ND
T_3 +Bzf	$4.59 \pm 0.19^*$	ND
Bzf	1.27 ± 0.04	3.28 ± 0.11

* $P < 0.01$ vs control.

Table 3 Induction of cardiac hypertrophy by T_3 . $n=12$, for control, T_3 , and T_3 +Bzf; $n=8$ for Bzf. No significant difference was found between the parameters of control vs Bzf

Sample	HW (mg)	BW (g)	HW/BW (mg/g)
Control	619.9 ± 12.7	211.1 ± 3.39	2.94 ± 0.03
T_3	880.8 ± 12.6	187.4 ± 2.24	4.7 ± 0.06
T_3 +Bzf	680.1 ± 12.3	198.5 ± 2.0	3.46 ± 0.05
Bzf	616.9 ± 12.1	204.0 ± 2.63	3.02 ± 0.04

$P < 0.01$ (Con vs T_3 and T_3 vs T_3 +Bzf).

found to be almost equal compared with T_3 -treated rats. Plasma T_4 was not detected in T_3 -treated and T_3 - and Bzf-co-treated rats. Bzf treatment alone did not significantly change plasma T_3 or T_4 levels compared with controls. To examine whether PPAR α is involved in hyperthyroidism-induced cardiac hypertrophy, rats were treated with T_3 for 15 days in the absence or presence of the PPAR α agonist, Bzf, and cardiac hypertrophy was assessed by measuring HW:BW ratio. After 15 days of T_3 treatment, HW (combined left and right ventricle) was increased by $\sim 42\%$ in T_3 -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 T_3 prevented hypertrophy of the heart significantly indicating that PPAR α 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 T_3 treatment (Fig. 1C). Co-treatment of rats with Bzf significantly prevented T_3 -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 PPAR α were significantly reduced in T_3 -treated rat heart compared with controls, while treatment with Bzf along with T_3 enhanced its expression. Bzf alone did not significantly change the expression of *Ppar α* in the heart (Fig. 2A and B). Taken together, the results demonstrated that PPAR α is involved in the development of pathological cardiac hypertrophy induced by hyperthyroidism.

Figure 1

Inhibition of cardiac hypertrophy by Bzf. Separate groups of rats were administered either with vehicle (CON, $n=12$) or T₃ (T₃, $n=12$) or T₃ in combination with Bzf (T₃+Bzf, $n=12$) or Bzf (Bzf, $n=8$) alone for 15 days and the degree of hypertrophy was expressed as HW to BW ratio in mg/g (HW:BW). These data represent mean $\pm$ s.e.m. of HW:BW ratio (A). * $P<0.01$ control vs T₃, # $P<0.01$ T₃ vs T₃+Bzf. The mRNA expressions of *Anp* (B) and *Bnp* (C) in the LV were examined by real-time quantitative RT-PCR. * $P<0.01$ control vs T₃, # $P<0.01$ T₃ vs T₃+Bzf, ($n=6$) for both (B) and (C) and the complete data set (for A, B and C) is also significant at <0.01 level,

analyzed by one-way ANOVA. (D) Representative images (400 $\times$ magnification) of HE-stained cross-sections through LV of rats belonging to different treatment groups as indicated in figure. Scale bar: 25 μm . The histogram (E) shows the cardiomyocyte cross-sectional area in (μm^2) measured from 30 randomly selected cardiomyocytes from three different HE-stained sections from three individual rats in each treatment group. * $P<0.01$ control vs T₃, # $P<0.01$ T₃ vs T₃+Bzf ($n=3$). P value for the complete data set is <0.01 analyzed by one-way ANOVA. Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-12-0304>.

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

Effect of hyperthyroidism on Ppar α target genes associated with FAO in heart

As PPAR α was found to be downregulated in LV tissue of T₃-treated rats, we examined the expression of two of its important target genes (*Cpt1 β* and *Mcad*) involved in β -oxidation of fatty acid after 15 days of T₃ treatment. The mRNA as well as protein levels of both CPT1 β 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 CPT1 β 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.

Figure 2

Downregulation of *Pparα* expression in LV tissue. (A) Change in *Pparα* mRNA expression in LV of vehicle (CON) or T_3 treated (T_3) or T_3 in combination with bezafibrate (Bzf; T_3 + Bzf) or Bzf alone for 15 days is shown in the histogram. Each bar represents mean $\pm$ S.E.M. of six rats ($n=6$ in each group). (B) The representative immunoblot showing the level of PPAR α protein in the LV tissue. Each bar in the lower panel shows the mean $\pm$ S.E.M. of pixel intensity of three immunoblots each from different individual rats after normalizing with actin. * $P<0.01$ control vs T_3 , # $P<0.01$ T_3 vs T_3 + Bzf; $n=6$ for (A) and * $P<0.01$ control vs T_3 , # $P<0.01$ T_3 vs T_3 + Bzf; $n=3$. No significant difference was seen in control vs Bzf (A and B).

The overall normal architecture and distribution of mitochondria were preserved when Bzf was co-administered along with T_3 . Treatment with Bzf alone did not detectably affect mitochondrial architecture. The percentage of damaged mitochondria was quantified in the transmission electron micrographs of the above-mentioned treatment groups (Fig. 4D). The histogram shows a sharp increase in the number of damaged mitochondria in the hyperthyroid condition compared with control that was prevented by Bzf.

Effect of hyperthyroidism on mitochondrial activity and myocardial ATP level

Mitochondrial activity, measured as cytochrome *c* oxidase activity, was found to be reduced in T_3 -treated LV tissue. ATP level too after an initial increase after 2 days of treatment steadily declined. These two parameters were significantly enhanced upon Bzf co-treatment as shown in Fig. 5A and B. Bzf alone did not have any significant effect on either cytochrome *c* activity or ATP level.

Oxidative stress in hyperthyroid rat heart by PPAR α downregulation

As considerable ultrastructural damage and functional impairment of myocardial mitochondria was caused due to T_3 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 T_3 -treated rat. Bzf prevented T_3 -induced elevation in lipid peroxidation. The total antioxidant level in LV was significantly reduced by T_3 , 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 T_3 treatment causes cardiac dysfunction via downregulation of PPAR α

Initially, all the hemodynamic parameters were improved significantly by T_3 (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 PPAR α -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 PPAR α 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

Table 4 Mean $\pm$ S.E.M. of SBP of six animals in each group ($n=6$ for control, T_3 , T_3 + Bzf, and Bzf)

Sample	SBP
Control	112.9 $\pm$ 3.0
T_3	153.7 $\pm$ 1.4
T_3 + Bzf	139 $\pm$ 1.0
Bzf	110.4 $\pm$ 2.0

$P<0.01$ (Con vs T_3 and T_3 vs T_3 + Bzf).

Figure 3

Bzf improves the expression of FAO genes. Expression level of mRNA and protein for CPT1 β 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 CPT1 β 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 CPT1 β 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).

hypertrophy and heart failure (Barger & Kelly 2000, Garcia & Goldenthal 2002, Lehman & Kelly 2002, Goffart *et al.* 2004, Kieck-Wilk *et al.* 2005). PPAR α 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 PPAR α 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 PPAR α involved in fatty acid transport (CPT1 β) 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 PPAR α protein with its response element in the target gene is seen in the hyperthyroid heart and is enhanced compared with

Figure 4

Bzf inhibits structural damage of mitochondria in thyroid hormone-treated rat heart. Representative immunoblots (A and B) show the levels of prohibitin and VDAC protein in the mitochondrial fraction of LV tissues of vehicle (CON) or T₃ (T₃) or Bzf along with T₃ (T₃+Bzf) or only Bzf-treated rats for 15 days. The histograms show 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 $^{\#}P<0.05$ T₃ vs T₃+Bzf ($n=3$) and complete data set is significant at <0.01 level (one-way ANOVA) for both (A and B). No significant difference was seen in control vs Bzf

(A and B). Also shown are the representative transmission electron micrographs (C) of LV tissues from similar treatment groups of rats. Arrows point mitochondria in different micrographs, scale bar: 2 μ m, ($n=3$). The histogram (D) shows the percentage of damaged mitochondria in the micrographs of the above-mentioned treatment groups. * $P<0.01$ control vs T₃ and $^{\#}P<0.01$ T₃ vs T₃+Bzf ($n=3$). No significant difference from control was found in the number of damaged mitochondria in only Bzf-treated hearts.

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-dependant expression of PPAR α in LV tissue of T₃-treated rats and also after Bzf administration in the presence or absence of T₃. The expression of PPAR α is initially increased and then gradually decreased in T₃-treated rats. In Bzf-co-treated rats, PPAR α 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 PPAR α expression. Bzf treatment alone did not significantly affect either PPAR α expression or its target genes. It is possible that Bzf only enhances the binding of PPAR α to PPRE. As 70–90% of energy in the heart is derived from FAO, it implicates a high involvement of PPAR α in FAO regulation under normal conditions. Therefore, treatment with agonist alone

might not significantly increase the binding of PPAR α to PPRE and produce any additional detectable effect in the expression of target genes of PPAR α 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 PPAR α expression, TR–PPAR α interaction in the myocardium might be another possible mechanism responsible for the decrease in PPAR α target gene expression.

CPT1 β activity is crucial in FAO as it catalyzes the transesterification of long-chain fatty acids, the rate-limiting step in mitochondrial fatty acid uptake.

Figure 5

Bzf prevents mitochondrial dysfunction. (A) The histogram represents the mitochondrial activity evaluated by measuring the cytochrome c oxidase activity (U/ml) 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 $\pm$ 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 $\pm$ 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 * $P<0.01$ control vs D15 and # $P<0.01$ D15 vs D15+Bzf. No significant difference was found between ATP concentrations of control and only Bzf-treated rats.

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 & Goldenthal 2002).

Downregulation of these two important target genes of PPAR α 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 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 Bzf alone (Bzf) for 15 days. Each bar in the histogram represents mean $\pm$ 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 $\pm$ 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)

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

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 of VDACs 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, Dillmann 2010). 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 ($-\text{dP/dt}$) (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 ($+\text{dP/dt}$) 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 ($\text{MHC}\alpha$) in hyperthyroid heart, which has a higher ATPase activity and increased velocity of contraction than $\text{MHC}\beta$ (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 ($+\text{dP/dt}$) 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 ($-\text{dP/dt}$), 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 β -oxidation of fatty acid, the major energy-generating pathway in cardiomyocytes, by reducing the expression of the central transcription factor for these enzymes, $\text{PPAR}\alpha$. 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 $\text{PPAR}\alpha$ might be contributing to enhanced ROS generation as its agonist could successfully attenuate ROS levels. Therefore, $\text{PPAR}\alpha$ agonists might act as cardio-protective agents by preventing mitochondrial dysfunction in addition to their lipid-lowering actions.

Bzf, used in this study as PPAR α agonist, may also activate PPAR β/δ and PPAR γ . As PPAR β/δ is expressed in comparable amounts along with PPAR α in the adult heart and is said to influence FAO, the effects of Bzf might partly be attributed to PPAR β/δ .

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-12-0304>.

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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