FUNCTIONAL AND METABOLIC ADAPTATION OF THE HEART TO PROLONGED
THYROID HORMONE TREATMENT

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Running head: thyroid hormone and cardiac metabolism

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ABSTRACT

In heart failure thyroid hormone (TH) treatment improves cardiac performance. Long-term effects of TH on cardiac function and metabolism, however, are incompletely known. To investigate the effects of up to 28 days TH treatment, male Wistar rats daily received T3 (200 μg.kg⁻¹ sc) leading to a 2.5-fold rise in plasma fatty acids (FA) level and progressive cardiac hypertrophy (+47% after 28 days) (p < 0.001). Ejection fraction (echocardiography) was increased (+12%; p < 0.05) between 7 and 14 days and declined thereafter. Neither cardiac FA oxidation and glycolytic capacity (homogenates) per unit muscle mass, nor mRNA levels of proteins involved in FA and glucose uptake and metabolism (Northern blots and micro-array) were altered. After 28 days of treatment mRNA levels of uncoupling proteins 2/3 (UCP2/3) and atrial natriuretic factor (ANF) were increased (p < 0.05). This indicates that TH-induced hypertrophy is associated with an initial increase in cardiac performance, followed by a decline in cardiac function and increased expression of UCP’s and ANF, suggesting that detrimental effects eventually prevail.

Keywords: cardiac hypertrophy, fatty acid, oxidation, cardiac function, uncoupling protein
INTRODUCTION

Development of heart failure is accompanied by a variety of neuro-endocrine changes. Recently, cardiac failure was shown to be associated with both a decline in circulating thyroid hormone (TH) levels (18, 24) and altered cardiac TH signaling, as evidenced by changes in myocardial expression of TH receptor isoforms (21, 22). As a result, a state of relative hypothyroidism may ensue, which has been held responsible for the decline in expression of TH responsive genes during hypertrophy and failure. These include the genes encoding α-myosin heavy chain (MyHC) and sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2a), both of which are important determinants of cardiac function (11, 38). The observation that short-term TH administration improves cardiac performance, both in animal models of cardiac dysfunction and in patients suffering from cardiac failure (11, 18, 24, 29, 31) is in concert with this notion. This beneficial effect of TH was indeed associated with an elevation of α-MyHC mRNA and protein levels. The effect on cardiac SERCA2a mRNA content was less consistent (11, 31). One should realize, however, that hyperthyroidism itself is often associated with impaired cardiac function (24, 35, 45) and that in rats TH treatment of myocardial infarction only transiently improves cardiac performance (28). This discrepancy may result from the duration of exposure to elevated plasma concentrations of TH. So far, detailed information on the nature of the cardiac adaptive response to TH supplementation as a function of time is virtually lacking.

In hyperthyroidism cardiac hypertrophy is accompanied by an overall increase in metabolic rate and enhanced lipolysis (19). The absence of hypertrophy in heterotopic cardiac transplants in TH treated rats suggests that cardiac hypertrophy does not result from direct effects of TH on cardiac muscle (23). The hypertrophic response rather results from the hyperdynamic circulatory state as a consequence of the enhanced metabolic rate, increased blood volume and decreased peripheral resistance (23, 45). Each of these factors potentially increases myocardial energy demand. Accordingly, it has been reported that cardiac glucose
metabolism (34) and fatty acid oxidation (37, 39) are enhanced in hyperthyroid animals. On the other hand during pressure- and volume-overload induced cardiac hypertrophy, cardiac substrate metabolism shifts from fatty acids to glucose (1), possibly due to a marked reduction in the expression of β-oxidation enzymes (4, 32, 40). This raises the question as to whether changes in cardiac energy metabolism as a consequence of TH supplementation are also associated with changes in the expression of genes involved in substrate handling, and if so, whether such changes depend on the duration of TH supplementation and the extent of cardiac hypertrophy.

The main aim of the present study was to investigate the time-related effects of elevated circulating TH levels on the development of cardiac hypertrophy, performance and energy metabolism. This study was performed in rats. As a measure of cardiac performance left ventricular ejection fraction was determined by means of echocardiography. MyHC isoform distribution, collagen content, and SERCA2a and atrial natriuretic factor (ANF) mRNA levels were assessed to delineate the hypertrophic phenotype. Furthermore, TH-induced changes in cardiac metabolism were assessed at the biochemical and molecular level by measuring fatty acid oxidation and glycolytic capacity in homogenates as well as mRNA levels of genes involved in fatty acid and glucose uptake and metabolism. In addition the mRNA levels of the uncoupling proteins UCP2 and UCP3 were determined because these proteins may be responsible for the decrease in mitochondrial efficiency during hyperthyroidism (9). Finally, the expression of peroxisome proliferator-activated receptor-α (PPARα) was determined as this transcription factor is considered to play a pivotal role in the regulation of the expression of genes involved in cardiac lipid metabolism (44). Moreover, its expression has been reported to be diminished in overload-induced cardiac hypertrophy (5).
Collectively, the present findings indicate that TH exposure, although beneficial on a short-term basis, may become detrimental for the heart when continued for longer time intervals.

MATERIALS AND METHODS

Animals
At the start of the experiments male Wistar rats (198 ± 13 g) were 7 weeks old. Rats, two per cage, were kept at a 12 hour light/dark cycle. Food (25% protein, 6% fat, 38% carbohydrates; Hope Farms BV, Woerden, the Netherlands) and water were provided ad libitum. The rats were randomly assigned to thyroid hormone treated (TH) and corresponding sham groups. Body weights (BW) were determined weekly. TH rats daily received subcutaneous injections of T3 (3,3',5-triiodo-L-thyronine, 200 µg·kg⁻¹ BW) for 3, 7 or 28 days. TH treatment started 28, 7 or 3 days, respectively, before the terminal experiment. Accordingly, all animals were of the same age (11 weeks) at the time of sacrifice. TH (40 µg·ml⁻¹) was dissolved in 1 mM NaOH, 0.9% NaCl. Rats of the sham group daily received subcutaneous injections of the solvent at the same volume. All procedures were approved by the local Committee on Animal Experimentation of the Maastricht University.

Echocardiography
In a subset of animals echocardiography was performed at weekly intervals following initiation of TH or vehicle administration. The first measurements were performed within 6 h after the first injection of TH or vehicle. For echocardiography the rats were anesthetized with pentobarbital (50 mg·kg⁻¹ i.p.). M-mode images obtained from the short axis of the heart at the level of the papillary muscles with a 10 MHz probe (LA14; Esaote Biomedica; Indiana; USA). From this
image heart rate (HR) was calculated. Left ventricular (LV) free wall thickness during diastole was determined and the LV ejection fraction (EF) measured from the internal ventricular diameter during diastole and systole. The ratio LV free wall thickness to internal LV diameter during diastole was used as a measure of eccentric or concentric hypertrophy.

**Terminal experiment**

At the end of TH treatment the rats were anesthetized with pentobarbital (60 mg·kg\(^{-1}\) i.p.). Following thoracotomy the heart was exposed and 1-ml blood samples were withdrawn from the LV cavity with a syringe containing 100 µl 3.8% sodium citrate. After 30 min the blood samples were spun for 5 min at 6000 rpm on a tabletop centrifuge and the supernatants were collected and stored at –80°C until use.

Immediately after blood sampling the heart was excised and adherent blood was removed in ice-cold saline. The heart was blotted dry and heart weight (HW) determined. After excision of the heart left tibia length (TL) was measured *ex situ* with a pair of calipers. The HW/TL ratio was used to assess the degree of cardiac hypertrophy, as this ratio takes into account differences in body weight, body composition, and/or growth that occur during TH treatment. After removal of the atria a 3 mm thick cross-sectional slice of the entire base of the heart was placed on cork and frozen in liquid nitrogen for histological analysis. Then the right ventricle was separated from the LV and septum and both parts were frozen in liquid nitrogen. All tissue samples were stored at –80°C until RNA and protein extraction.

**Metabolic capacity**

In another sub-set of animals, a 5% homogenate of the LV was made on ice in SET-buffer (0.25 M sucrose; 10 mM TRIS, 2mM EDTA; pH 7.4) for the measurement of palmitate oxidation and glycolytic rate. We choose homogenates as they allow one to assess whether the capacity for palmitate oxidation and glycolysis is affected, irrespective of changes in e.g. substrate
availability and transport, and alterations in the content of cofactors during hypertrophy of the in situ heart.

*Palmitate oxidation* rates were determined in 100 µl of the LV homogenate in a total volume of 0.5 ml, essentially as described previously (17). The final composition of the incubation medium was in mM: 22.6 KCl; 114.5 TRIS; 15 KH₂PO₄; 7.5 MgCl₂; 1.9 EDTA; 87.5 sucrose; 5 ATP; 1 NAD⁺; 0.1 Co-enzyme A; 0.5 L-malate; 0.5 L-carnitine and 0.025 cytochrome c. After pre-incubation (5 min), the reaction was started by addition of unlabeled and [1-¹⁴C]-labeled palmitate bound to albumin in a 5:1 molar ratio (specific activity 40 Bq·nmol⁻¹; final concentration 0.12 mM). Incubations were carried out in an airtight vial at 37°C. Reactions were stopped by addition of 200 µl 3 M perchloric acid, either just before (t = 0) or 10 or 20 min after addition of palmitate. The CO₂ produced was trapped in 400 µl ethanolamine/ethyleneglycol (1:2 v/v). To trap all CO₂ produced, the vials were left overnight at 4°C. Acid soluble products (citric acid cycle intermediates) were separated from palmitate by centrifugation. The ¹⁴CO₂ trapped and ¹⁴C-labeled acid soluble products were determined by liquid scintillation counting. Palmitate oxidation rate was calculated as the sum of the ¹⁴CO₂ trapped and ¹⁴C-labeled acid soluble products and expressed as nmol·min⁻¹·g⁻¹ wet weight of tissue.

*Glycolytic* rates were determined using 100 µl of the LV homogenate in a total volume of 0.5 ml. The final composition of the incubation medium was, as described by Beatty et al. (6), in mM: 70 KCl; 100 TRIS; 8 KH₂PO₄; 5 MgSO₄; 0.5 EDTA; 60 sucrose; 1 ATP; 1 ADP; 0.5 NAD⁺; 0.2 NADP; 1 dichloro-acetate; 0.04 Co-enzyme A. Reactions were started by addition of unlabeled and [5-³H]-labeled glucose (3 Bq·nmol⁻¹; final concentration 11 mM) and stopped by addition of 200 µl 3 M perchloric acid immediately before addition of glucose (t = 0 min) or after 20 or 40 min. Subsequently the medium was neutralized with 70 µl 10 M KOH and 75 µl of the medium was filtered through an anion exchange resin (200-400 Mesh Dowex 1-X4; Sigma) pretreated with 0.4 M potassium borate (7). The amount of ³H₂O, reflecting the glycolytic rate, was
determined by liquid scintillation counting for $^3$H. [U-$^{14}$C]glucose was added to the incubation medium to correct for leakage of glucose through the column.

*Plasma glucose and fatty acid levels*

Plasma glucose and fatty acid levels were determined in *ad libitum* fed rats. Plasma (unesterified) fatty acids were determined by means of the NEFA C kit, following the instructions of the manufacturer (Wako Chemicals; Neuss; Germany). Plasma glucose was determined spectrophotometrically (8) on a COBAS BIO.

*Histological analysis*

Cross-sections (8 µm) of the LV of the heart were cut on a cryostat at −20°C and stored at −80°C until use. Sections were stained with Sirius red, which has a high affinity for collagen. Collagen-positive areas were quantified in 12 randomly selected fields by densitometrical analysis (Quantimet 570 Image analyser; Leica; Cambridge; UK). Data were expressed as percentage cross-sectional area of the LV occupied by collagen (12).

*Myosin Heavy Chain composition*

Frozen LV tissue was pulverized with a pestle and mortar pre-cooled in liquid nitrogen. Total RNA and protein were extracted from the pulverized left ventricles using TRIzol reagens (Gibco BRL Life Technologies; Gaithersburg; MD; USA). The protein precipitate was dissolved in 1% sodium dodecyl sulphate (SDS). After determination of the protein content (BCA kit; Pierce; Rockford; IL; USA) an aliquot was diluted in a SDS sample buffer to a concentration of 0.125 mg ml$^{-1}$ followed by ultrasonication for 5 min. The MyHC composition was determined with SDS-poly-acrylamide gel electrophoresis essentially as described previously (14). The running gel contained 5% acrylamide-bis (37.5:1) and 30% glycerol. Fifteen µl sample was loaded on the gel and run for 27 h at 15°C. The gels were stained by using a silverstain-plus kit (Biorad
Laboratories, Hercules, CA, USA), scanned on a Fluor-S Imager (Biorad) and the relative proportions of α- and β MyHC were determined using Quantity One (Biorad).

**Micro-array analysis**

Total RNA was isolated from the LV of sham, and of 3, 7 and 28-days TH-treated rats. The micro-array was performed on Incyte Rat gene expression micro-arrays (GEM version 2.20 and 3.17, containing 8485 and 8958 sequences, respectively; Genome systems; St. Louis; MO; USA) as described previously (3). The raw data set was filtered using various algorithms and Incyte software to eliminate spots with poor signal to noise ratio, leaving more than 15,000 sequences in total (some of which are present on both GEMs or represented more than once on the same GEM). Sequences of which the expression changed by at least 1.7 fold in duplicate assays were considered to be up- or down-regulated.

**Northern blots**

Northern blots were performed as described previously (41-43). In short, 10 or 20 µg total RNA was size-fractionated on a denaturing gel (1% agarose, 1x MOPS, 2% formaldehyde) and blotted by capillary transfer to a nylon membrane (Hybond-NX; Amersham; Slough; UK). The blots were hybridized with cDNA probes labeled with $[\alpha^{32}\text{P}]dCTP$ (3000 Ci·mmol$^{-1}$; Amersham) by random priming (Radprime, Life Technologies). Then the blots were exposed to an imaging screen and scanned with a Personal FX Phosphor-Imager (Biorad). Signals were quantitated using the software package Quantity One (Biorad). In a blot the signals were normalized to the 18S signal to correct for possible loading and transfer differences. Possible interblot differences were accounted for by subsequent normalization to corresponding sham samples that were present on each blot. Probes not described elsewhere (41-43) are presented in more detail below.
As markers for cardiac hypertrophy we used a 0.7 kb *HindIII/BamHI* fragment of atrial natriuretic factor (ANF), a 0.32 kb *EcoRI/NsiI* fragment of cardiac sarcoplasmic reticulum calcium ATPase (SERCA2a) and a 1.3 kb *BamHI/PstI* fragment of Collagen Iα (kindly provided by J. Cleutjens, Maastricht University, Maastricht, the Netherlands). As markers for glucose metabolism GLUT4, hexokinase II (HKII), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and a 3 kb *EcoRI-PvuI* fragment of mouse pyruvate dehydrogenase (PDH) E-1α-subunit (generous gift from H.H. Dahl, Royal Children’s Hospital, Melbourne, Australia) were used. As markers of fatty acid metabolism fatty acid translocase (FAT/CD36), heart-type fatty acid-binding protein (FABP), acyl-CoA synthetase (ACS), muscle-type carnitine palmitoyl transferase I (mCPT-I) and long-chain acyl-CoA dehydrogenase (LCAD) were studied. In addition, a 0.7 kb *HindIII/Xbal* fragment of glycogen phosphorylase (GP) and a probe for citrate synthase (CS) were used as markers of glycogen metabolism and the citric acid cycle, respectively. In addition, a probe for PPARα was used. Furthermore, blots were probed for rat uncoupling protein-2 (UCP-2) and UCP-3.

**Statistics**

Data is presented as mean ± SD. Statistical analysis was performed using INSTAT software (V2.00; Graphpad Software Inc.; CA; USA). For multiple comparisons, ANOVA followed by the Tukey post-hoc test was applied to locate the differences. Differences were considered significant at p < 0.05.
RESULTS

Animal characteristics
At the time of sacrifice all rats were 11 weeks old. Tibia lengths (TL) were similar for all groups (Table 1), indicating that TH treatment for up to 28 days did not affect growth rate. Body weights (BW), however, were significantly lower in rats treated with TH for 28 days. The reduced BW/TL ratio in these rats most likely reflects the loss of adipose tissue due to enhanced lipolysis. Indeed, plasma fatty acid levels were more than doubled already after 3 days of TH treatment and remained elevated thereafter (Table 1). Plasma glucose levels were not significantly affected by TH treatment (Table 1).

Cardiac dimensions and function
TH induces a marked hypertrophy, both when expressed as absolute heart weight and when normalized to tibia length or body weight (Table 1). After 3 days of TH treatment the HW/TL ratio had increased by 21%. After 28 days this ratio was increased by 47%. The echocardiographic measurements corroborated these observations and showed that LV free wall thickness markedly increased over time in TH-treated rats, reaching statistical significance after 7 days of TH treatment (Figure 1a). The ventricular hypertrophy was concentric in nature as reflected by the increased ratio of end-diastolic LV wall thickness over LV inner diameter (data not shown).

Echocardiographic assessment of cardiac function did not reveal any acute effects of TH. Within hours after the first bolus of TH or vehicle no significant differences in heart rate and LV ejection fraction (EF) were observed. However, at day 7 heart rate increased from 447 ± 9 to 536 ± 31 beats.min⁻¹ (p < 0.01) and EF was significantly elevated (Figure 1b), suggesting enhanced cardiac function following TH treatment. Heart rate remained consistently increased during the remainder of the protocol. EF was highest between day 7 and 14 and gradually declined thereafter.
Phenotypic markers of TH-induced cardiac hypertrophy

The TH-induced increase in cardiac mass was not accompanied by changes in LV volume fraction of collagen (Figure 2a) indicating that the hypertrophic response was not associated with fibrosis. In sham rats β-MyHC protein could be detected in the majority of hearts analyzed (5.5 ± 6.8 % of MyHC isoforms; n =16). β-MyHC expression was undetectable after 28 days of TH treatment (Figure 2b). mRNA levels of SERCA2a were not altered in hearts of TH-treated animals at any time point (Figure 2c), which was consistent with the micro-array analysis (data not shown). With the exception of M-band protein, the expression of none of the sarcomeric proteins represented on the gene chips changed significantly (data not shown). ANF mRNA levels of the LV of hyperthyroid animals were identical to those of the sham group prior to and during the first 7 days of TH treatment (Figure 2d). After 28 days of treatment, however, ANF expression was found to be substantially elevated.

TH and metabolic remodelling of the heart

Fatty acid oxidation capacity, measured in LV homogenates under optimal assay conditions and normalized to wet weight, was not significantly affected by TH treatment at any time point (Figure 3a). Likewise, the glycolytic capacity did not differ between the TH and sham groups (Figure 3b). Consistent with these biochemical observations, cardiac mRNA levels of proteins involved in fatty acid uptake and metabolism (FAT/CD36, FABP, ACS, mCPT-I, LCAD) (Figure 4a) and glucose uptake and metabolism (GLUT4, HKII, GAPDH, PDH) (Figure 4b) were not specifically affected by TH. Similarly, the mRNA level of citrate synthase (CS), a marker of mitochondrial citric acid cycle activity, did not change (Figure 4c). Correspondingly, micro-array analysis also did not reveal specific alterations in the expression of genes involved in FA metabolism (e.g. ACS, VLACS, CPT-I, SCAD, LCAD, VLCAD), or glucose handling (e.g.
Collectively, the biochemical and molecular data suggest that, despite the marked degree of ventricular hypertrophy in the TH-treated animals and contrary to what has been reported for other forms of cardiac hypertrophy (1, 32), fatty acid and glucose metabolism was not specifically affected in this type of hypertrophy, but both followed the increase in muscular mass.

The mRNA level of UCP2 was more than doubled already after 3 days of TH treatment and remained elevated thereafter (Figure 4c). Furthermore, after 28 days of TH treatment the LV mRNA content of UCP3 was also increased (Figure 4c). Finally, the mRNA level of PPARα was reduced to 60% after 3 days of TH treatment (p < 0.05), but was normal again after 7 and 28 days of treatment (Figure 4c).

Since hypertrophy and failure were found to be associated with changes in the expression of TH receptor isoforms (21, 22), attention was paid to nuclear receptors and signaling pathways that may be activated secondary to TH treatment. Interestingly, in the microarray analysis the expression of de-iodinase, an enzyme involved in the inactivation of TH, was the only gene in this category that was transiently (1.7 fold at 7 days) up-regulated in hearts of TH-treated rats (data not shown).
DISCUSSION

Clinical (18, 29) and experimental (11, 31) investigations have lent support to the contention that TH supplementation improves performance of the failing heart. Recent studies indicated that TH supplementation helps to overcome the otherwise impaired TH signaling that is associated with cardiac failure (21, 22). At the same time it is commonly acknowledged that hyperthyroidism ultimately may be detrimental to the heart. Indeed in the present time-course study in rats we show that TH supplementation leads to a massive hypertrophy, which is associated with an initial improvement in cardiac function. Prolonged TH treatment, however, may give rise to a pathological form of hypertrophy as evidenced by enhanced expression of ANF and uncoupling protein 2 and 3 in the left ventricular tissue, in association with a decline in LV ejection fraction.

TH-induced hypertrophy: physiological or pathological?

Supplementation of TH is associated with a rapid and profound increase in cardiac mass. Notwithstanding the overt hypertrophy (almost 50% increase after 28 days), the absence of changes in (pro-)collagen mRNA levels and collagen surface area indicates that fibrosis did not occur. The cardiac α-MyHC protein content progressively increases at the expense of β-MyHC during TH treatment. Along with the marked rise in ejection fraction, seen within 7 days after the onset of treatment, these findings all favor the idea that initially the hypertrophic response is physiological rather than pathological in nature.

The shift in myosin isoyme content in favor of α-MyHC is consistent with transcriptional regulation of the gene via its TH-response element (10, 27). In view of the presence of a TH response element in the SERCA2a promoter a rise in SERCA2a mRNA levels in hearts of TH-treated rats was anticipated (10). The present findings, however, indicate that the myocardial SERCA2a mRNA content did not change irrespective of the duration of hormone
supplementation. This is in striking contrast with the marked rise in SERCA2a mRNA observed in earlier studies on intact hearts (2, 30) and on neonatal cardiomyocytes (20; unpublished observations from our laboratory). At the present state of the art one can only speculate regarding the discrepancy between the lack of effect of TH on SERCA2a expression in intact hearts and the upregulation in isolated cardiac myocytes (20). We like to propose that in vivo the stimulatory effect of TH on SERCA2a expression is counteracted by its extra-cardiac effects, i.e., the hyperdynamic circulatory state (19, 45). Indeed, this suggestion is supported by experiments with heterotopically transplanted hearts, showing that SERCA2a expression in unloaded hearts is substantially more elevated than in the hemodynamically loaded host hearts in response to TH treatment (30). Moreover, Ojamaa and colleagues observed that following myocardial infarction, TH administration failed to restore the expression of SERCA2a in the viable region of the heart (31), stressing the notion that the relationship between TH and cardiac SERCA2a expression is highly complex.

In the clinical setting, the increased hemodynamic load may ultimately lead to high output failure (45). Given the fact that cardiac hypertrophy is a result of chronic hemodynamic overload, it is comprehensible that during prolonged TH treatment a pathological form of hypertrophy evolves. Consistent with this notion is the observation that TH supplementation is associated with activation of the intra-cardiac renin-angiotensin system (25, 26), which is commonly regarded as being involved in the development of pathological hypertrophy. Along with the present observations that TH supplementation only leads to a transient increase in cardiac performance and that ANF is expressed in ventricular tissue after prolonged TH treatment, the combined data suggest that TH-induced hypertrophy resembles physiological hypertrophy initially and gradually changes towards a more pathological form of hypertrophy.
**Cardiac metabolism**

Based on both extensive micro-array analysis and on Northern blot data of a large panel of candidate genes, we found no evidence for specific changes in the expression of genes involved in the uptake and metabolism of either glucose or fatty acids in TH-induced hypertrophied cardiac tissue. Consistent with this, the flux of both fatty acids and glucose through their corresponding metabolic pathways, as measured under optimal conditions in cardiac homogenates, was not affected by TH at any time point. It should be emphasized, however, that the rate of glycolysis and fatty acid oxidation was normalized to wet weight of LV tissue. This finding implies that the metabolic capacity as measured in homogenates follows the increase in tissue mass. The corollary of this notion is that in TH-induced hypertrophy the intracellular capacity of substrate utilization most likely remains in balance with the increase in cardiac energy demand. Earlier findings of Seymour and colleagues (37), indicating no change in Vmax of glycolytic enzymes in TH-treated hearts, when normalized to wet weight of tissue, are in line with the present observation.

The absence of changes in mCPT-I expression is consistent with previous findings indicating that, unlike liver, heart mCPT-I mRNA levels do not change in response to TH (13). However, in intact isolated cardiac myocytes from hyperthyroid rats, treated with a 5-fold higher dose of TH as compared to the present study, CPT-I enzyme activity was enhanced in association with an increased rate of fatty acid oxidation. The discrepancy between these and our findings may be related to differences in the experimental models used (cardiac homogenates, isolated myocytes) and dose of TH applied. The use of cardiac homogenates in the present study precludes any confounding secondary effects due to differences in cardiac work between euthyroid and hyperthyroid animals, and changes in substrate supply and concentrations of intracellular cofactors. However, possible differences in transsarcolemmal transport rate of substrates can not be appreciated in this preparation. In addition, it should be
stressed that protein (enzyme) levels may be changed even in the absence of changes in tissue content of their corresponding mRNA.

Previous studies have shown that in pressure- and volume-overload cardiac hypertrophy, substrate preference is shifted from fatty acids utilization to glucose (1, 16, 46), which is generally considered a hallmark of the return to the fetal gene program of the hypertrophied heart (4, 15). This shift was reported to go along with diminished expression of a set of β-oxidation genes (32, 33, 46). Prolonged TH treatment, resulting in a substantial increase in cardiac mass, obviously did not evoke the shift in fuel selection from fatty acids to glucose. On the contrary, the chronically elevated plasma fatty acid levels may even favor the utilization of fatty acids in the intact heart due to increased availability of these substrates.

It is of note that similar to what has been observed by others (9), TH was found to increase cardiac mRNA levels of the uncoupling proteins UCP2 and UCP3, which are believed to reduce the mitochondrial efficiency by dissipating the electrochemical proton gradient. In this way, the elevated expression of UCP might hamper energy conversion in the hypertrophied heart, and consequently cardiac function. However, the significance of this TH-mediated effect in muscle has been questioned, as ATP production by skeletal muscle mitochondria of hypertrophied rats was found to increase, rather than decrease under these conditions (36). Previously, others and we (42, 47) showed that the expression of UCPs is stimulated by fatty acids, most likely in a PPAR-dependent manner. Similarly the rise in plasma fatty acid concentration, induced by fasting, was accompanied by an enhanced expression of other PPAR-responsive genes in the heart (43). At first sight, the enhanced expression of UCP2 and UCP3 in the hearts of TH-treated animals seems consistent with the marked rise in circulating fatty acid levels. However, the time course of changes in UCP expression, in particular of UCP3, does not parallel the rapid rise in plasma fatty acid levels. Moreover, since we observed a transient decrease in PPARα mRNA, a direct relationship between the expression if this
transcription factor and that of the UCPs is neither apparent. Finally, the expression of other PPAR-regulated genes, among which FAT, ACS, CPT-1 and LCAD is not changed at all. This seems to exclude a specific role of PPARα in the regulation of the expression of cardiac genes by elevated TH.

Summary
The present biochemical and molecular data indicate that irrespective of the duration of TH supplementation and the severity of the ensuing hypertrophy, the increase in cardiac mass is met by a parallel increase in glycolytic and fatty acid oxidative capacity. In this respect the TH-induced hypertrophy compares favorably to hypertension-induced cardiac hypertrophy. However, a number of typical markers of physiological hypertrophy is not present, and cardiac performance is only transiently enhanced. Furthermore, with prolonged TH supplementation ANF and UCP2/3 expression becomes elevated. This suggests that with time beneficial effects of TH are getting overruled by detrimental, most likely extra-cardiac effects of the hormone.
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LEGENDS

Figure 1. Effect of thyroid hormone (TH)-treatment on A) left ventricular (LV) free wall thickness measured during diastole and B) ejection fraction (LV EF). Values are mean ± SD; *: significantly different from Sham; †: significantly different from day 0; ‡: significantly different from 7 days; ¶: significantly different from 14 days (p < 0.05).

Figure 2. Effect of thyroid hormone (TH)-treatment for 3, 7 or 28 days on left ventricular A) collagen content as determined in histological cross-sections stained by Sirius Red, B) protein β-myosin heavy chain (MyHC) isoform content, C) sarcoplasmic reticulum calcium ATPase 2a (SERCA2a) mRNA and D) Atrial Natriuretic Factor (ANF) mRNA content. S: Sham. Values are mean ± SD *: significantly different from Sham, 3 days and 7 days, respectively (p< 0.001).

Figure 3. Metabolic capacity in left ventricular homogenates of Sham (S) rats and rats treated with thyroid hormone for 3, 7 or 28 days. A) Palmitate oxidation and B) glycolytic rate in nmol·min⁻¹·g⁻¹ wet weight. Values are mean ± SD. Statistical differences were not observed.

Figure 4. Left ventricular mRNA levels, as determined by Northern blotting, of Sham (S) rats and rats, treated with TH for 3, 7 or 28 days of proteins involved in A) fatty acid metabolism, B) glucose metabolism and of C) citrate synthase (CS), uncoupling protein (UCP) 2 and 3, and peroxisome-proliferator-activated receptor-α (PPARα). FAT: fatty acid translocase; FABP: heart type fatty acid-binding protein; ACS: acyl-CoA synthetase; mCPT-I: muscle type carnitine palmitoyl transferase-I; LCAD: long-chain acyl-CoA
dehydrogenase; GLUT4: glucose transporter 4; HKII: hexokinase II; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; PDH: pyruvate dehydrogenase; GP: glycogen phosphorylase; ‘: significantly different from Sham at p < 0.05.
FIGURE 1

A

Duration of TH-treatment (days)

LV wall thickness (mm)

- Sham
- TH

B

Duration of TH-treatment (days)

LV EF (%)

- Sham
- TH
FIGURE 2

A. Collagen

<table>
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B. β-MyHC

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<th>3d</th>
<th>7d</th>
<th>28d</th>
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C. SERCA2a

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D. ANF

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</table>

* Indicates statistical significance.
FIGURE 3

Panel A: Palmitate oxidation (nmol·g⁻¹·min⁻¹) over time (S, 3d, 7d, 28d).

Panel B: Glycolysis (nmol·g⁻¹·min⁻¹) over time (S, 3d, 7d, 28d).
FIGURE 4

A

![Bar chart showing gene expression levels for different conditions](chart-a)

B

![Bar chart showing gene expression levels for different conditions](chart-b)

C

![Bar chart showing gene expression levels for different conditions](chart-c)
**Table 1: Animal characteristics**

<table>
<thead>
<tr>
<th></th>
<th>BW (g)</th>
<th>TL (cm)</th>
<th>HW (mg)</th>
<th>BW/TL (g cm⁻¹)</th>
<th>HW/TL (mg cm⁻¹)</th>
<th>HW/BW (mg g⁻¹)</th>
<th>Fatty acids (mmol l⁻¹)</th>
<th>Glucose (mmol l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>305 ± 21</td>
<td>3.65 ± 0.09</td>
<td>918 ± 86</td>
<td>86 ± 5</td>
<td>261 ± 20</td>
<td>3.01 ± 0.17</td>
<td>0.15 ± 0.07</td>
<td>6.9 ± 1.5</td>
</tr>
<tr>
<td>TH, 3d</td>
<td>310 ± 30</td>
<td>3.63 ± 0.09</td>
<td>1144 ± 114</td>
<td>85 ± 7</td>
<td>315 ± 28</td>
<td>3.69 ± 0.24</td>
<td>0.43 ± 0.14 *</td>
<td>7.3 ± 0.9</td>
</tr>
<tr>
<td>TH, 7d</td>
<td>285 ± 13</td>
<td>3.68 ± 0.07</td>
<td>1208 ± 45</td>
<td>78 ± 4 *</td>
<td>328 ± 13 *</td>
<td>4.24 ± 0.24 †</td>
<td>0.46 ± 0.15 *</td>
<td>6.8 ± 0.7</td>
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<tr>
<td></td>
<td>(9)</td>
<td>(9)</td>
<td>(9)</td>
<td>(9)</td>
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<td>(6)</td>
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<tr>
<td>TH, 28d</td>
<td>256 ± 21 †¶</td>
<td>3.61 ± 0.04</td>
<td>1356 ± 117 †¶</td>
<td>73 ± 6 *</td>
<td>384 ± 34 †¶</td>
<td>5.32 ± 0.48 †¶</td>
<td>0.39 ± 0.12 *</td>
<td>6.0 ± 1.5</td>
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<td>(17)</td>
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<td>(10)</td>
</tr>
</tbody>
</table>

All animals were the same age at the end of the experiment; S: sham; TH: treated with thyroid hormone (200 µg·kg⁻¹ daily) for 3, 7 or 28 days; BW: body weight; TL: tibia length; HW: heart weight; fatty acids and glucose were analyzed in plasma as indicated in Material and Methods. Values are mean ± SD; number (n) of experiments are indicated in brackets; *: significantly different from Sham; †: significantly different from 3 days; ‡: significantly different from 7 days at p < 0.05.