Analysis of differentially expressed genes in hyperthyroid-induced hypertrophied heart by cDNA microarray

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Abstract

Experiments were carried out to identify the altered genes in hyperthyroid rat heart and their influence on the functions of cardiac myocytes. Chronic treatment of rats with 3,5,3' triiodo-L-thyronine (T3) resulted in a prominent increase in the size of the left ventricle with increased wall thickness and reduced chamber volume leading to concentric cardiac hypertrophy. The heart weight to body weight ratio (HW/BW) in hyperthyroid rats was increased by about 58% over that of normal rats. Using cDNA microarray comprising 588 genes, we compared the differences in mRNA expression of hyperthyroid and normal rat heart. Based on a threshold of greater than 10% change, about 37 genes were found to be regulated by T3. Further analyses by Western blotting, Northern blotting and real-time quantitative RT-PCR of some of the genes confirmed the microarray results. The T3-altered genes encode various types of proteins related to metabolism, matrix and cytoskeletal structures, growth factors, tran-

scription factors, Ca²⁺-channels etc. The physiological significance of one of these altered proteins in hyperthyroid heart, insulin-responsive glucose transporter (GLUT) type 4 (GLUT4), was studied in detail. The expression of GLUT4 was drastically reduced in the ventricular tissues of hyperthyroid heart. Insulin-induced glucose uptake in hyperthyroid cardiomyocytes was reduced significantly, indicating the impaired glucose transport in cardiac cells. Interestingly, a few genes such as GLUT4, cytochrome P450 isoforms, superoxide dismutase (SOD), collagens, matrix metalloproteinases (MMP), tissue inhibitors of matrix metalloproteinases etc. which had not been reported earlier were found to be altered in hyperthyroid heart. Our results show some new aspects of hyperthyroid heart which will be important in assessing the pathophysiology of hypertrophied cardiomyocytes.

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Introduction

Thyroid hormones have profound effects on metabolism, growth and development (Oppenheimer *et al.* 1987). Although the action of thyroid hormone involves virtually all organ systems, the heart responds to minimal changes in the serum thyroid hormone levels. It was reported that defects in thyroid hormone receptor expression and thyroid hormone signaling occur in the heart of failing/hypertrophied human patients (Kinugawa *et al.* 2001). Moreover hyperthyroid human patients experience cardiovascular complications with alteration of several cardiac parameters. These include increased resting heart rate, abnormal myocardial contractility and an increased left ventricular muscle mass (Fadel *et al.* 2000). Therefore, prolonged hyperthyroidism, if left untreated, results in abnormal myocardial function and cardiac hypertrophy that may lead to heart failure (Chien *et al.* 1993, Cohn 1996).

Cardiac hypertrophy is an adaptive enlargement of the myocardium characterized by increased size of individual myocytes in response to a wide variety of extrinsic as well as intrinsic stimuli. Extrinsic stimuli are pressure overload, volume overload and neurohumoral factors. Intrinsic stimuli include contractile abnormalities due to altered expression or mutations of the sarcomeric proteins. Although hypertrophic growth is initially compensatory in nature, the function in the hypertrophied heart eventually becomes abnormal and a transition from hypertrophy to heart failure occurs (Feldman *et al.* 1993). Due to abnormal heart function in the long run, the overall growth process initiating the cardiac disease has been termed as pathological hypertrophy (Scheuer & Batrick 1987).

Treatment of cardiac myocyte by 3,5,3' triiodo-L-thyronine (T3) causes beta adrenergic stimulation, increased protein kinase A (PKA) activity, cAMP production and phosphorylation of proteins related to Ca²⁺

cycling kinetics (Kiss *et al.* 1994, Ojamaa *et al.* 2000). Thus, beta-adrenoreceptor stimulation may be responsible for thyroid-mediated positive inotropic actions and enhanced atrial contractility (Dillman 1990). Because thyroid hormone also enhances cardiac functions characterized by faster myocardial relaxation and a decline in the cytosolic Ca^{2+} transient (MacKinnon *et al.* 1988, Bing *et al.* 1994, Kiss *et al.* 1994), thyroid hormone treatment has been suggested for improving cardiac performance in the postinfarction model of heart failure (Gay *et al.* 1988, Pennock *et al.* 1993, Mahaffey *et al.* 1995). However, the long-term effects of thyroid hormone on cardiac function and metabolism are incompletely known. Recently, it has been shown that thyroid hormone-induced cardiac hypertrophy is associated with an initial increase in cardiac performance followed by a decline in cardiac function indicating the detrimental effects of chronic hyperthyroidism (Degens *et al.* 2003).

Although it is reported that thyroid hormone influences cardiac growth and metabolism via the signaling pathway distinct from other hypertrophic stimuli, the molecular mechanisms underlying hyperthyroid-induced versus pathological hypertrophy remain uncertain (Scheuer *et al.* 1982, Schaible *et al.* 1984, Izumo *et al.* 1987). Some of the unique features of thyroid hormone-induced gene expression in cardiac muscle are induction of alpha myosin heavy chain (MHC- α), sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) and repression of beta MHC (MHC- β) (Dillmann 1990, Kinugawa *et al.* 2001, Klein & Ojamaa 2001). It is also postulated that thyroid hormone-induced improvement in myocardial contractility is partly due to the differential MHC gene expression (Fadel *et al.* 2000). To understand the gene expression programme in thyroid hormone-induced hypertrophied heart, we have conducted cDNA microarray analysis of heart tissue obtained from rats treated/untreated with T3 for 15 days. In the present study, alterations of at least 37 genes involved in cardiovascular function are demonstrated and the physiological significance of one of these altered genes has been explored in detail. Microarray analysis revealed that a few genes involved in the pathological hypertrophy are also altered in hyperthyroid heart in addition to the genes that are not yet known to be thyroid hormone responsive in cardiac tissues. Accordingly, we demonstrated that prolonged treatment of rats with T3 results in abnormal glucose transport into cardiomyocytes, which could be one of the important detrimental effects of long-term thyroid hormone treatment.

Materials and Methods

Animals

Sprague–Dawley rats were obtained from the Institute's animal facility. The animals were handled as per the guidelines of the animal ethics committee of this Institute.

Treatment of rats with T3

Sprague–Dawley rats were intraperitoneally injected daily with 7.5 μg T3/100 g body weight (Hamasaki *et al.* 2000, Weitzel *et al.* 2001) for 15 days. The control group was treated with the same volume of saline. The animals were killed on the 16th day. In parallel groups, rats treated with T3 for 15 days were maintained untreated for another 15 days to study the effect of T3 withdrawal, i.e. these rats were killed 1 month after the start of the experiment. Animals were anesthetized and hearts were surgically removed and immersed in ice-cold 0.9% NaCl. The degree of hypertrophy was calculated in terms of heart weight:body weight ratio.

Estimation of serum T3 concentration

To ensure the development of the hyperthyroid condition the level of T3 in the serum was detected by radioimmunoassay using the T3 radioimmunoassay kit (Bhaba Atomic Research Center, India) as per the manufacturer's protocol.

Histology

Cardiac tissue from control, T3-treated or T3-withdrawn rats was fixed in 10% formalin and embedded in paraffin following routine procedures. Tissue sections of 5 μm thickness were prepared and stained with Mayer's hematoxylin and eosin. The sections were mounted and observed under microscope at $\times 400$ magnification.

Northern blot analysis

Total left ventricular RNA from control and T3-treated rats was isolated using TriReagent (Sigma-Aldrich). About 15 μg RNA was resolved on 1% agarose/formaldehyde gels and transferred to nylon membrane (Millipore). After transfer, RNA was fixed by u.v. cross linking, hybridized with [α - ^{32}P]dATP labeled (Hexalabel Plus DNA labeling kit, MBI Fermentas), atrial natriuretic factor (ANF) cDNA and GLUT4 cDNA (a gift from Dr Jeffrey E Pessin, University of Iowa, Iowa). The membranes were washed twice for 10 min each with $2 \times \text{SSC}$, 0.1% SDS at room temperature followed by a single wash with the same solution at 65 °C for 30 min. Membranes were exposed to Kodak autoradiographic film.

cDNA expression arrays

An Atlas cardiovascular array kit was purchased from Clontech (USA). These arrays consisted of 588 cDNAs (organized into broad functional groups), nine housekeeping control cDNAs and negative controls. Membrane hybridization was performed according to the manufacturer's protocol. Briefly, 5 μg total RNA from the left

ventricular tissues of control and T3-treated rat hearts were reverse transcribed in the presence of [α - 32 P]dATP (3000Ci/mmol; Amersham Pharmacia) using MMLV reverse transcriptase (100 units) and CDS primer mix at 50 °C for 25 min. Array membranes were hybridized overnight at 68 °C in Express Hybridization solution containing 100 µg/ml sheared salmon testes DNA. The array membranes were then washed twice with $2 \times$ SSC, 0.1% SDS at room temperature followed by a single wash with the same solution at 65 °C for 30 min. The array membranes were exposed to Kodak (X-OMAT, Sigma) autoradiographic film for 48 h. Spots that developed were normalized by equalizing the intensity of signals from a set of housekeeping genes provided on the array. The autoradiographs were scanned and analyzed using ImageJ software (National Institute of Health, Bethesda, MD, USA). The results presented are the means $\pm$ S.E.M. of three separate microarray experiments using RNA prepared from hearts from three different batches of treatments.

Western blot analysis

For Western blot analysis, either whole homogenate (80 µg protein sample) or microsome preparations (50 µg proteins sample) were used. The whole homogenate from the left ventricular tissues was prepared as described earlier (Bandyopadhyay *et al.* 2000). Briefly, rat heart tissue was homogenized in a buffer containing 2 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 100 mg/ml pepstatin A. For the microsome preparation, the whole homogenate was centrifuged at 4000 *g* for 15 min at 4 °C. The post-nuclear supernatants were subjected to a centrifugation for 40 min at 20 000 *g* at 4 °C using a fixed-angle Beckman T50 rotor. The supernatants were then ultra-centrifuged at 105 000 *g* for 90 min (4 °C). The pellet was resuspended in the homogenizing buffer and the expression of GLUT1 and GLUT4 was examined in this preparation. Immunoblot analyses with the antibodies against SOD-1, GLUT1, GLUT4, COX1 (cyclo-oxygenase 1), collagen IV, MMP2, Na-Ca-exchanger and MHC- β were performed as described earlier (Bandyopadhyay *et al.* 2000). Anti-goat SOD-1, anti-rabbit GLUT1, anti-goat GLUT4, anti-rabbit COX1, anti-rabbit collagen IV antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). Anti-mouse Na-Ca-exchanger and anti-rabbit MMP2 antibodies were purchased from Sigma. Antibody against MHC- β was a gift from Dr Do Han Kim (Gwangju Institute of Science and Technology, Gwangju, South Korea). Immunoblotting in parallel sets was performed with anti-mouse β -actin antibody (Sigma) as loading controls both with whole homogenate and microsome preparations separately. For quantification, the pixel intensities of the respective bands of the immunoblots from three different individual rats were calculated using ImageJ

software (NIH). The intensities of the bands are expressed as relative pixel density (arbitrary units).

SOD activity

Cardiac tissues (200 mg) were homogenized in 0.5 ml of 0.05 M potassium phosphate buffer containing 0.1 mM EDTA (pH 7.8). The enzyme was extracted with 0.35 ml chloroform:ethanol (3:5). After centrifugation for 5 min at 12 000 *g*, the supernatant was carefully collected and used for the Cu,Zn-SOD assay (Martin *et al.* 1987). The SOD activity is expressed as units/mg protein.

Real-time quantitative RT-PCR analysis

One-step quantitative RT-PCR of each sample was performed in triplicate using the QuantiTect SYBR Green RT-PCR kit (Qiagen) and the iCycler realtime detection system and software (Bio-Rad) according to the manufacturers' instructions. Sequence-specific PCR primers were designed using the GeneFisher software (<http://bibiserv.techfak.uni-bielefeld.de/genefisher/>) based on published nucleotide sequences. A standard curve for each cDNA was obtained by performing amplifications from serial dilutions of myocardial total RNA. For all specific mRNA amplified, linear inverse correlations were observed between an amount of RNA and the C_T values (number of cycles needed to generate a fluorescent signal above a pre-defined threshold). A higher C_T value indicates a lower amount of mRNA in the sample and vice versa. Expression level of the 'housekeeping gene' GAPDH was used to normalize for variations in the amount of RNA, purity etc. The normalized C_T values of T3-treated samples for each cDNA were expressed as percentage change compared with the untreated control; they are depicted as histograms in Fig. 4.

Isolation of adult rat ventricular cardiomyocytes

Adult rat ventricular cardiomyocytes from control and T3-treated animals were isolated as described previously (Piper *et al.* 1982) with some modifications. The left ventricles were cut, minced thoroughly and the tissue was incubated at 37 °C in enzyme solution (0.1% collagenase and 0.1% hyaluronidase in calcium-free Krebs-Henseleit (KH) bicarbonate buffer (NaCl 119 mM, KCl 4.7 mM, CaCl₂ 1.25 mM, MgSO₄ 1.2 mM, NaHCO₃ 25 mM, KH₂PO₄ 1.2 mM, Hepes 12.5 mM, BSA 0.2%, pH 7.4) for 30 min in a CO₂ incubator with intermittent shaking. After digestion the cell suspension was centrifuged at 50 *g* for 1 min. The pellet obtained was suspended in wash medium (3 parts of KH buffer + 1 part of medium 199 + 10% FBS) and centrifuged at 50 *g* for 1 min. The pellet contained 90–97% rod-shaped viable ventricular myocytes which were resuspended in glucose-free KH buffer. The

cells were stored in the CO₂ incubator at 37 °C for 30 min before they were used for monitoring glucose uptake.

Glucose uptake

Glucose uptake was monitored following the protocol described earlier (Roy *et al.* 2003) with some modifications. Ventricular cardiomyocytes were incubated for 30 min either in the absence (basal) or presence of porcine insulin (100 nM). After 30 min of insulin treatment, 2-deoxy-D-[³H]glucose (0.4 nmol) was added to each incubation and, after 5 min, uptake was terminated by washing cells three times with ice-cold KH buffer in the presence of 0.3 mM Phloretin to correct the glucose uptake data for simple diffusion and non-specific trapping of radioactivity. Cells were pelleted down, washed twice with KH buffer and sonicated in presence of Nonidet P40 (NP40) and acetone. [³H]deoxy glucose was measured in a liquid scintillation counter (Packard, Tricarb 2100TR) and the results are expressed as c.p.m. per mg protein.

Statistical analysis

Data are presented as means $\pm$ S.E.M. The level of significance was calculated using ANOVA. $P \leq 0.05$ was considered to be significant.

Results

Development of left ventricular hypertrophy in hyperthyroid rats

Figure 1A shows a clear increase in size of the left ventricle in T3-treated rats (T3, 4.04 ± 0.04 ng/ml serum) compared with the vehicle-treated animals (T3, 1.4 ± 0.02 ng/ml serum) that was further evident from the longitudinal sections of hearts. The histological sections of the left ventricular tissues displayed a normal, healthy and compact arrangement of myofibrils in control hearts whereas a massive enlargement of individual myofibrils and myocyte disarray occurred in the hypertrophied heart; normal architecture was restored in the heart following T3 withdrawal (Fig. 1A).

To assess the development of cardiac hypertrophy, combined left/right ventricle:body weight (HW/BW) ratios were measured. Figure 1B shows that HW/BW ratio increased with the duration of thyroid hormone treatment; the ratio reached a maximum value, and exhibited a 58% increase ($P < 0.001$) over that of control rat group, after 15 days. When T3 injections were discontinued for 15 days following 15 days of daily treatment, there was a restoration of HW/BW ratio (3.0 ± 0.09) similar to that of control rats (2.88 ± 0.04) indicating the specificity of thyroid hormone action on heart in the present study (Fig. 1B).

Thyroid hormone induced ANF expression in left ventricular tissues

Induction of ANF synthesis in ventricular tissue is a common event in various forms of cardiac hypertrophy (Chien *et al.* 1993). As shown in Fig. 2, the ANF mRNA level was very high in the left ventricular tissue of T3-treated rat heart compared with saline-treated control. When the T3 treatment was discontinued after the 15th day, ANF level was reduced. These data indicated that a prolonged hyperthyroid condition also induces severe cardiomyopathy.

Gene profile in hyperthyroid rat heart

To examine the effect of thyroid hormone treatment on cardiac gene expression, RNA from normal and T3-treated rat hearts was prepared, reverse transcribed and radiolabeled with [α -³²P]dATP; the labeled probes thus prepared were used for hybridization of the array membranes separately as described in Materials and Methods. The autoradiographs were scanned and analyzed using ImageJ software (NIH). After normalization of the signals with the control, 37 genes were found to be altered in T3-treated heart (Table 1). Out of these, 11 genes were upregulated and 26 genes were downregulated in the heart due to T3 treatment. The altered genes can be grouped into five categories based on their functions. These are related to general metabolism, cytoskeletal/matrix protein, hormone/growth factor, Ca²⁺-channel proteins and the proteins related to receptor transcription. A difference of above 10% in the intensity of the spots in the microarray blots was validated by Western blot analysis (Fig. 3). Hence a difference of above 10% is considered to be the real signal and is included in the altered gene list. About 212% increase in the expression of ANF in the T3-treated heart was observed which is consistent with typical hypertrophic growth of cardiomyocytes (Chien *et al.* 1993). A plethora of metabolic enzyme genes of the cytochrome P450 system were upregulated in T3-treated heart. MHC- α was upregulated and the MHC- β was downregulated in hyperthyroid rat heart as reported earlier (Dillmann 1990, Fadel *et al.* 2000, Kinugawa *et al.* 2001, Klein & Ojamaa 2001). Some of the genes important for cardiovascular functions which are downregulated but not yet known to be influenced by thyroid hormone are Cu,Zn-SOD, GLUT4, tissue inhibitor of metalloproteinase 2 (TIMP2), collagen XVI α 1, collagen IV α 4, vascular endothelial growth factor receptor and estrogen-related receptor alpha (ERR α). Similarly, the fact that MMP2, retinoic acid receptor alpha (RAR α) and RAR γ are positively regulated by thyroid hormone in the heart is revealed for the first time.

Validation of microarray results

Some of the genes altered in hyperthyroid rat heart, as revealed by the cDNA microarray analysis (Table 1), were

Figure 1 Assessment of left ventricular hypertrophy in hyperthyroid rat. (A) Morphology, a; longitudinal section, b; and histological examination, c ($\times 400$ magnification) of hearts from rats injected with vehicle (C) or T3 ($7.5 \mu\text{g}/100 \text{ g}$ body weight) for 15 days (T) or 15 days withdrawal after T3 administration (W) showing the increased wall thickness (thick line) and reduced chamber volume in hyperthyroid rat heart. (B) Heart weight:body weight ratio (mg/g) of rats treated without T3 (Con) or with T3 for 4 days (D4), 8 days (D8), 12 days (D12) or 15 days (D15) or 15 days withdrawal following T3 treatment (W). The data represent means $\pm$ S.E.M. of 12 individual animals. * $P < 0.01$ vs control; ** $P < 0.001$ vs control.

Figure 2 Thyroid hormone induced ANF expression in rat heart. Ventricular tissues from vehicle (C), T3-treated for 15 days (T) or T3-treated for 15 days followed by 15 days without any treatment (W) were used to extract total RNA which was transferred to nylon membrane and subjected to Northern blot analysis with rat ANF cDNA as a probe. The lower panel shows the loading control of total RNA in three lanes.

further examined by Western blot analysis using homogenate prepared from the left ventricular tissues of control and hyperthyroid rat heart. Figure 3A shows significantly increased ($P < 0.05$) level of expression of MMP2 and COX1, and a decreased ($P < 0.05$) level of expression of GLUT4, SOD, MHC- β , collagen IV α 4, Na-Ca $^{2+}$ exchanger consistent with the cDNA microarray analysis. Since SOD expression was reduced, we additionally examined the activity of this antioxidant enzyme in the homogenate prepared from control and T3-treated rat hearts. It is interesting that SOD activity was also reduced significantly ($P < 0.01$) in hypertrophied heart tissues (Fig. 3B).

Alteration of some of the nuclear hormone receptors was validated by real-time quantitative PCR. The real-time quantitative PCR analysis revealed that the expression of ERR α and mineralocorticoid receptor (MR) was reduced while that of RAR α was increased in hyperthyroid rat heart compared with the control, consistent with microarray analysis (Fig. 4).

Thyroid hormone inhibits GLUT4 expression in heart

The inhibition of GLUT4 expression as evident from microarray analysis prompted us to investigate the expression of GLUT4 with the progression of cardiac hypertrophy following T3 treatment. Microsomes were prepared from left ventricular tissues after treatment of rats without or with T3 for 4 and 15 days and were subjected to Western blot analysis. As shown in Fig. 5, the expression of GLUT4 was reduced after 4 days and was further reduced after 15 days of T3 treatment. The level of GLUT4 was restored to normal when T3 treatment was

discontinued for 15 days after 15 days of injection. These data indicate that hyperthyroidism repressed GLUT4 transcription in the heart.

Thyroid hormone inhibits glucose uptake in cardiac myocytes

To examine the effect of low levels of GLUT4 expression on the heart, glucose uptake in cardiomyocytes prepared from normal and T3-treated animals was examined. Cardiomyocytes from the adult rats were prepared and 2-deoxy-D-[3 H]glucose uptake was monitored in the absence or presence of insulin. Figure 6A shows that insulin-stimulated 2-deoxy-D-[3 H]glucose uptake was reduced in the cardiomyocytes prepared from T3-treated rat, indicating the impairment of glucose transport in the hyperthyroid heart. Since the basal level of 2-deoxy-D-[3 H]glucose uptake was also reduced in cardiomyocytes of hyperthyroid rat, the level of GLUT1 in ventricular microsome preparations was examined by Western blot analysis with anti-GLUT1 antibody. As shown in Fig. 6B, the level of GLUT1 was also significantly reduced ($P < 0.05$) in T3-treated rat heart compared with the control.

Discussion

The key points of this study are: (1) thyroid hormone induces changes in gene expression in the heart, some of which were previously not known to be thyroid hormone responsive and associated with cardiac hypertrophy *in vivo*; (2) one of the consequences of long-term thyroid hormone treatment is a reduction of GLUT1 and GLUT4 expression and inhibition of glucose entry into the cardiac myocytes. Analysis of gene expression reveals that thyroid hormone signals the hypertrophic growth of the cardiac myocytes through a gene expression program distinct from other types of hypertrophic stimuli and altered genes are associated with the pathological hypertrophy. Whether the altered genes are directly regulated by T3 needs further study. Considering the characteristics of altered genes and specifically abnormal glucose handling of the cardiomyocytes, it is reasonable to suggest that in the event of increased excitation-contraction coupling, the hyperthyroid heart could fail due to defects in the energy metabolism.

Upon binding with thyroid hormone receptors localized in the DNA, thyroid hormone regulates expression of a wide variety of genes. Using cDNA microarray analysis, it has been shown recently that thyroid hormone regulates the expression of about 55 genes in its main target organ, the liver (Feng *et al.* 2000). Microarray analysis also demonstrated a thyroid hormone-induced gene profile in hypothyroid animals (Weitzel *et al.* 2003). Consistent with hyperthyroid-induced cardiovascular complications such as increased resting heart rate, myocardial contractility and

Table 1 Gene profile in hyperthyroid heart

Group	Gene/protein name	GenBank accession no.	Induction/repression
Metabolism	Cytochrome P450 IIB6, Cytochrome P450 2B3	M29874; J02864	+92.3
	Cytochrome P450 VIIIA1	D38145	+33.49
	Cytochrome P450 III A3	M13785	+194.6
	HDL-binding protein	M83789	+120
	Adipocyte fatty acid binding protein	J02874	-35.5
	Phospholipid transporter protein	L26232	-56
	Cyclo-oxygenase 1 (COX1)	M59979	+16.44
Cytoskeletal/matrix proteins	Insulin responsive glucose transporter (GLUT) type 4 (GLUT4)	M20747	-24
	Cu,Zn-SOD	J02947	-40.4
	Cardiac muscle myosin heavy chain α isoform (MHC- α)	D00943	+24
	Cardiac muscle myosin heavy chain β isoform (MHC- β)	M58018	-30.41
	Matrix metalloproteinase 2 (MMP2)	J03210	+15
	Tissue inhibitor of metalloproteinase 2 (TIMP2)	J05593	-60.86
	Collagen IV α 1	M11315	-12
	Collagen XVI α 1	M92642	-56.28
	L-selectin	M25280	+18
	Junction placoglobulin	M23410	-42.55
	Desmoplakin III	Z68228	
	Erythrocyte adducin alpha subunit	X58141	-21.4
	Atrial natriuretic factor (ANF)	HS75640	+211
Hormone/growth factor	Placental growth factor 1	X54936	-30
Ca ²⁺ channel protein	Na ⁺ -Ca ²⁺ exchanger	M91368	-15
	Cardiac phospholamban	M63603	-77
Receptor/transcription factors	Estrogen-related receptor alpha (ERR α)	X51416	-45.58
	Retinoic acid receptor alpha (RAR α)	X06614	+26
	Retinoic acid receptor gamma (RAR γ)	M24857	+13.74
	V-erb-related protein 3, transcription factor COUP2;	X12795; X16155;	-41.63
	nuclear receptor subfamily 2 group F member 2 (NR2F2)	X58241	
	Progesterone receptor	M15716	-26
	Mineralocorticoid receptor	M16801	-45.6
	Purinoreceptor	U41070	-41.5
	Vascular endothelial growth factor receptor	AF016050	-43.71
	Vasopressin V-3 receptor	D31833	-23
	Sterol regulatory element-binding transcription factor 1	U00968	-18.3
	Protein tyrosine phosphatase receptor type-F	Y00815	+29.16
Functionally unclassified protein		X69398	
	Tafazzin	X92762	-26.1

left ventricular muscle mass (Fadel *et al.* 2000), we show here that T3 resulted in up- and downregulation of a diverse range of genes in the heart tissue. These gene products influence metabolism, growth and transcriptions, cytoskeletal structures and Ca²⁺-signaling (Table 1). Interestingly, out of the 37 differentially expressed genes in the left ventricular tissue of hyperthyroid rat, the majority (26) are negatively regulated, as reported in mouse liver (Feng *et al.* 2000).

It is well known that ANF is released as an important molecular marker of cardiac hypertrophy, which is also differentially expressed in left ventricular tissue as revealed by our microarray and Northern blot analysis. Similar to other hypertrophic stimuli, the dramatic increase in the level of ANF in left ventricular tissue is associated with hyperthyroid-induced cardiac hypertrophy, which was

reduced following withdrawal of T3 treatment after 2 weeks (Table 1, Fig. 2). However, the reason for the greater reduction in ANF level in the ventricular tissues after T3 withdrawal, compared to the control, is unclear. It is likely that some of the molecular phenotypes in the hearts of the withdrawal group may not be identical to the control hearts although the hypertrophy is completely regressed. This could be an interesting mechanism, which needs further detailed study.

Cytochrome P450 enzymes catalyze oxidation of a wide range of drugs and other xenobiotics, and are responsible for the metabolism of endogenous compounds. Expression of cytochrome P450 mono-oxygenases is reported for extrahepatic tissues including lung, kidney, gastrointestinal tract, endothelium and heart (Thum & Borlak 2000a). Heart tissue is metabolically competent and disease may

Figure 3 Validation of microarray results. (A) Representative immunoblots showing alteration of proteins in ventricular tissues obtained from rat treated with vehicle (C) or T3 (T) for 15 days. The approximate molecular masses of the proteins are given in parentheses. The levels of the proteins were quantitated using the software ImageJ (NIH, USA) and the pixel intensities are shown as histograms. Immunoblots with β -actin antibody confirmed the equal amount of protein loading of the whole homogenate and microsome preparations (Fig. 6B). Each immunoblot was repeated with the samples from three different hearts ($n=3$). * $P<0.05$ vs control. (B) Activity of Cu,Zn-SOD in the heart tissues obtained from control (C) and T3-treated (T) rats. The results are means $\pm$ S.E.M. of three individual rats. * $P<0.01$ vs control.

Figure 4 Validation of microarray results by real-time quantitative RT-PCR analysis. Expression patterns of ERR α , RAR α and MR were examined by real-time quantitative RT-PCR analysis using RNA samples prepared from left ventricular tissues of rat treated with vehicle (control) or T₃ for 15 days. Each bar represents the percentage change in C_T value over the control after normalization with the C_T value of GAPDH mRNA as described in Materials and Methods. Each experiment was repeated with three different batches of samples. **P* < 0.05 vs control.

Figure 5 Effect of T₃ on GLUT4 expression in ventricular tissues. Representative immunoblots showing GLUT4 expression in the ventricular microsome preparations obtained from rats treated without (Con) or with T₃ for 4 days (D4) and 15 days (D15) or treated for 15 days and then left for 15 days without treatment (W).

influence the expression of cytochrome P450 mono-oxygenases (Thum & Borlak 2000b). In the hypertrophied heart of spontaneously hypertensive rat, the expression and

activities of cytochrome P450 mono-oxygenases were shown to be induced resulting in increased lipid peroxidation and oxidative stress (Thum & Borlak 2002). In agreement with these results, we also show a dramatic increase in the expression of cytochrome P450 isoforms in the left ventricular tissues of hyperthyroid rat. This is likely to be due to a direct effect of T₃ on cardiac metabolism. Furthermore an increased amount of lipid peroxidation (data not shown) and a decreased level and activity of SOD1 (Table 1, Figs 3A and B), in thyroid hormone-induced rat heart indicates the occurrence of severe oxidative stress. Such a decrease in the level and activity of SOD due to increased oxidative stress has also been reported by others in a number of different experimental situations (Indik *et al.* 2001, Simon-Giavarotti *et al.* 2002).

Thyroid hormone-induced hypermetabolism, involving all major fuel sources, is a hallmark of hyperthyroidism (Moller *et al.* 1996). As a direct consequence of hypermetabolism, T₃ also alters a series of enzymes of the metabolic pathway in the heart such as high-density lipoprotein-binding proteins, adipocyte fatty acid binding protein, phospholipid transporter, COX1 and glucose transporter (Table 1). The lowering of plasma lipid levels by T₃ (Trost *et al.* 2000) could be linked to the reduced expression of fatty acid and lipid targeting proteins such as adipocyte fatty acid binding protein and phospholipid transporters respectively. Excess of thyroid hormone affects intermediary metabolism (Moller *et al.* 1996) and as a result, COX1 might have increased in the heart of hyperthyroid rat.

Consistent with earlier reports, microarray analysis reveals the differential expression of MHC genes, i.e. upregulation of MHC- α and repression of MHC- β in the heart of hyperthyroid rat (Table 1, Fig. 3A). Cardiac tissues that contain relatively more MHC- α than MHC- β contract faster than those in which the ratio of MHC- α to MHC- β is low. This could explain the increased heart rate in the hyperthyroid condition.

It is interesting to observe that MMP2 is increased as revealed by microarray and Western blot analyses whereas TIMP2 expression is decreased (Table 1). We examined the expression of TIMP2, the endogenous inhibitor of MMP2, by semi-quantitative RT-PCR. Concomitant with the increased expression of MMP2 by microarray and immunoblotting, the expression of TIMP2 was decreased. Consistent with these effects, expressions of collagen IV α 4 and collagen XVI α 1 are decreased in the heart of hyperthyroid rat. A possible mechanism involves reduction of collagen synthesis coupled with increased MMP2 activity leading to the low collagen deposition in the heart of hyperthyroid rat.

The present study shows that phospholamban is down-regulated in the hyperthyroid heart as reported earlier (Kiss *et al.* 1994). Downregulation of phospholamban expression has further strengthened our microarray analysis since the regulation of phospholamban in relation to pathophysiology of the hyperthyroid heart is well established (Kiss *et al.*

Figure 6 Effect of T3 on glucose transport into adult rat ventricular cardiomyocytes. (A) Cardiomyocytes were prepared from the adult rat heart injected with vehicle (C) or T3 (T) for 15 days. The cells prepared from vehicle or T3-treated rats were stimulated without (C) or with 100 nM insulin (Ins) for 30 min followed by incubation for 5 min in the presence of 2-deoxy-D- $[^3\text{H}]$ -glucose (0.4 nmol) as described in Materials and Methods. $[^3\text{H}]$ deoxy glucose was measured in a liquid scintillation counter and results are expressed as c.p.m. per mg protein. Each bar represents the mean $\pm$ S.E.M. of three individual animals, each in duplicate. * $P < 0.01$ vs control; ** $P < 0.05$ vs control. (B) The expression level of GLUT1 in the ventricular tissues of hyperthyroid rat heart is significantly ($P < 0.05$, $n = 3$) reduced as detected by Western blot analysis. Equal amounts of protein (50 μg) of microsome preparations were used for immunoblotting with the β -actin antibody as loading control.

1998, Holt *et al.* 1999). However, SERCA is not depicted in the present study since the microarray blot does not include this cDNA. In the cardiac myocytes, Ca^{2+} release and reuptake by the sarcoplasmic reticulum regulate the rate of myocardial fiber contraction and relaxation. Phospholamban phosphorylates SERCA and inhibits its ability to reuptake Ca^{2+} into the lumen of sarcoplasmic reticulum. It is known that thyroid hormone allows an accelerated reuptake of calcium by the sarcoplasmic reticulum by upregulation of SERCA and downregulation of phospholamban protein expression (Fadel *et al.* 2000). This is a mechanism by which thyroid hormone improves the diastolic relaxation properties of the hyperthyroid heart.

Hyperthyroid rat heart shows an altered level of expression of some of the nuclear hormone receptors such as $\text{ERR}\alpha$, $\text{RAR}\alpha$, $\text{RAR}\gamma$, mineralocorticoid receptors etc. Retinoid receptors such as specific RARs and retinoic acid X receptors (RXRs) hetero- or homodimerize with specific regulatory sequences in gene promoters (Leng *et al.* 1994). Retinoids have long been recognized as important for cardiac morphogenesis and differentiation (Colbert *et al.* 1997). The present study shows for the first time that thyroid hormone mediated cardiac hypertrophy is signalled through the retinoid signaling pathway specifically via upregulation of $\text{RAR}\alpha$ (Table 1, Fig. 4). It is likely that hyperthyroid rat heart requires upregulation of its transactivators RARs and thus $\text{RAR}\alpha$ expression is

increased by more than 26% of its normal level. Microarray analysis show an altered expression of other nuclear hormone receptors as mentioned in Table 1, however the physiological significance of such alterations requires further study.

To meet energy demands for contractile function or increased workload, the heart utilizes a variety of substances including glucose, fatty acids, lactate, ketone bodies and amino acids. The rate of glucose utilization in the heart is greater than in many other tissues such as skeletal muscle, adipose tissues or lung (James *et al.* 1985a,b, Zorzano *et al.* 1997). Thus, glucose transport/utilization is critical for the maintenance of normal function. Glucose uptake in the heart is mediated by the facilitative transporter GLUT4 and to a lesser extent by GLUT1 (Young *et al.* 1997). Insulin stimulates GLUT4 translocation in the heart from the intracellular storage membrane to the cell surface where they facilitate glucose entry into the cell (Sun *et al.* 1994, Young *et al.* 1997). Surprisingly, microarray, and Northern and Western blot analyses show a reduction of GLUT4 expression in the T3-treated left ventricular tissues in a situation when cardiomyocytes require more energy under increased workload. Initially, the expression of GLUT4 is reduced a little which was considerably declined after 15 days of T3 treatment suggesting that GLUT4 expression is affected due to the prolonged hyperthyroid condition (Fig. 5). A

recent report shows that reduction of GLUT4 results in the development of cardiac hypertrophy in mice (Kaczmarczyk *et al.* 2003). The cardiac myocytes prepared from adult rat treated with T3 show a decreased glucose uptake in response to insulin compared with the untreated cardiomyocytes (Fig. 6). Depolarization across the plasma membrane results in Ca^{2+} -induced Ca^{2+} release, which is directly linked to excitation–contraction coupling of myofibrils (Hoffmann *et al.* 1995). It is known that KCl induces depolarization of cardiac myocytes *in vitro* and this process stimulates glucose entry into the myocytes. It is interesting that KCl-induced glucose uptake was also inhibited in cardiomyocytes of T3-treated rat heart compared with the untreated control (data not shown).

Taken together, we demonstrate the gene expression profile in left ventricular tissues of hyperthyroid rat in which some of the altered genes are common to pathological hypertrophy. Alteration of GLUT4 expression is one of the distinct features of hyperthyroid-induced altered gene expression leading to impaired glucose transport into the myocytes which could be detrimental to cardiac physiology if prolonged hyperthyroidism is unchecked.

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