Regulation of Gene Expression in Cardiomyocytes by Thyroid Hormone and Thyroid Hormone Analogs 3,5-Diiodothyropropionic Acid and CGS 23425 [N-[3,5-Dimethyl-4-(4’-hydroxy-3’-isopropylphenoxy)-phenyl]-oxamic Acid]

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ABSTRACT

The heart is an important target of thyroid hormone actions. Only a limited number of cardiac target genes have been identified, and little is known about their regulation by T3 (3,3')-triiodothyronine) and thyroid hormone analogs. We used an oligonucleotide microarray to identify novel cardiac genes regulated by T3 and two thyroid hormone analogs, 3,5-diiodothyropropionic acid (DITPA) and CGS 23425 [N-[3,5-dimethyl-4-(4’-hydroxy-3’-isopropylphenoxy)-phenyl]-oxamic acid]. DITPA binds with lower affinity than T3 to thyroid hormone receptor α1 and β1 isoforms, whereas CGS 23425 binds selectively to β1. Fluorescent-labeled cDNA was prepared from cultured heart cells maintained in medium stripped of thyroid hormone (“hypothyroid” control) or treated with T3, DITPA, and CGS 23425 at concentrations 5 times their respective Kd values for 48 h. The arrays were scanned and analyzed using an analysis of variance program. Sixty-four genes were identified that were >1.5 times up- or down-regulated by one of the treatments with P < 0.05. The genes regulated by T3 and DITPA were nearly identical. Thirteen genes were differentially regulated by CGS 23425. Genes encoding contractile proteins, Ca2+ -ATPase of sarcoplasmic reticulum and several proteins of mitochondrial oxidative phosphorylation, were up-regulated by T3 and DITPA but not by CGS 23425. These results indicate that some, but not all, of the actions of thyroid hormone analogs can be explained by differences in gene activation.

Thyroid hormone plays an essential role in development, metabolism, and function of many organs. The physiological actions of 3,3',5'-triiodothyronine (T3), the intracellular form of the hormone, and its analogs are thought to be mediated through chromatin-associated nuclear thyroid hormone receptors (TRs), which are encoded by the α and β c-erbA proto-oncogene family (Lazar, 1993; Tsai and O'Malley, 1994). T3 has been reported to have similar binding affinity for the α1 and β1 receptor subtypes (Murray et al., 1988). The ability of thyroid hormone to improve cardiac performance (Morkin et al., 1983; Klein and Ojamaa, 2001) and to lower cholesterol when given to hypothyroid individuals (Peters and Man, 1950) have prompted efforts to design thyroid hormone analogs that utilize these properties in the treatment of heart failure and hypercholesterolemia. The purpose of this study is to compare the pattern of genes expressed in cardiomyocytes after treatment with T3 and two thyroid hormone analogs, 3,5-diiodothyropropionic acid (DITPA) and CGS 23425 [N-[3,5-dimethyl-4-(4’-hydroxy-3’-isopropylphenoxy)-phenyl]-oxamic acid], using microarray technology. Since small changes in the structure of the thyroxine molecule result in profound alterations in physiological activity, the question arises as to whether these effects are mediated through activation of different sets of genes.

DITPA was identified by screening compounds related to thyroid hormone for potential usefulness in treatment of...
congestive heart failure (Pennock et al., 1992). DITPA has a propionic acid side chain rather than an alanine side chain like T₃ and lacks iodides on the outer ring. DITPA binds with approximately equal affinities for the α₁ and β₁ subtypes of the c-erbA proto-oncogene family of nuclear TRs but with 100 times less affinity than T₃ (Pennock et al., 1992). In hypothyroid rats, DITPA increased cardiac performance with approximately half of the chronotropic effect and less metabolic stimulation than i-thyroxine. DITPA also improved left ventricular performance in rabbit and rat postinfarction models of heart failure when administered alone (Mahaffey et al., 1995) or in combination with an angiotensin I-converting enzyme inhibitor (Pennock et al., 1993). In a pilot clinical study, DITPA improved cardiac output and shortened diastolic relaxation in patients with moderately severe heart failure (Morkin et al., 2002).

CGS 23425 is a thyromimetic compound containing an outer ring isopropyl group, inner ring methyl groups in place of the iodides in T₃, and an oxamic acid side chain. The concentration of CGS 23425 required for half-maximal stimulation (EC₅₀) of the apoA1 promoter in transient transfection assays was reported to be about 80 times lower in the presence of TRβ1 than with TRα1 (Taylor et al., 1997). When tested in hypercholesterolemic rats, it produced significant lowering of cholesterol.

In the first application of microarray technology to thyroid hormone regulation of genes, Feng et al. (2000) prepared fluorescent-labeled cDNA from livers of T₃-treated and hypothyroid mice to a cDNA microarray representing 2225 different genes and analyzed relative changes in gene expression. Interestingly, only 55 genes were found to be T₃ regulated (2.5%), which was much lower than an earlier estimate of 8% (Oppenheimer et al., 1987). Among these genes, 14 were positively regulated and 41 were negatively regulated. Forty-five of the genes had not been previously known to be T₃ regulated. Weitzel et al. (2001) performed cDNA expression arrays using hepatic RNA of hypothyroid and hyperthyroid rats 6, 24, and 48 h after administration of T₃. Twenty-three of 588 genes were differentially regulated, 18 of which were previously not known to be regulated by T₃. Two different expression time courses were observed. In the early expressed genes, transcription levels rose within 6 h, dropped by 24 h, and increased again within 48 h. The late expression pattern suggests an additional mechanism of action, other than simple binding of ligand to nuclear receptors, may be involved in T₃ actions such as activation of transcription factors.

To test the hypothesis that thyroid hormone analogs differentially regulate gene expression, we have incubated primary cultures of fetal rat cardiomyocytes with T₃, DITPA, and CGS 23425 and examined the pattern of genes activated after 48 h using microarray analysis. An analysis of variance (ANOVA) design for the experiments and analysis was used as described by Kerr and Churchill (2001) and Kerr et al. (2000). This approach permits statistical evaluation of changes in gene expression and variability in the array data. The results indicate that the gene expression pattern of T₃ and DITPA are virtually the same, but there were a significant number of differences between these treatments and CGS 23425.

### Materials and Methods

#### Animals
Timed pregnant Sprague-Dawley rats at 17 to 18 days of gestation were obtained from Harland (St. Louis, MO) and maintained in the animal care facility. They were handled according to procedures approved by the Institutional Animal Care and Use Committee of the University of Arizona. To obtain fetal pup hearts, the mother was anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg). When an adequate level of anesthesia was obtained, the rat was decapitated with a guillotine. Fetal rat pups were decapitated with sharp scissors and the hearts removed.

#### Preparation of Cardiomyocytes
Primary cardiomyocytes were prepared using techniques described earlier (Nag and Cheng, 1984). Briefly, the hearts were collected in ice-cold Dulbecco’s modified essential medium (DMEM) with 10% fetal calf serum plus 100 U/ml penicillin and 100 μg/ml streptomycin. After removal of the atria, the ventricles were cut into small strips with scissors and transferred to a stopped, water-jacketed Erlenmeyer flask (37°C) with 10 ml of 0.125% pancreatin in calcium- and magnesium-free buffered salt solution. Cell disaggregated during 15 min of digestion were collected by centrifugation at 600g for 4 min. Fresh digestive solution was then added to the undigested tissue, and the cells from the second and subsequent digestions were collected and pooled. The pooled myocytes were centrifuged and resuspended in Ham’s F-12 media with 1% bovine serum albumin, 250 mg/ml fetuin, 20 mg/liter ascorbic acid, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cardiomyocytes were differentially plated for 1 h to remove fibroblasts and other contaminating cell types before being replated in DMEM with 4 mM l-glutamine adjusted to contain 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, 1.0 mM sodium pyruvate, 10% fetal bovine serum (HyClone Laboratories, Logan, UT), and phosphate-buffered saline, pH 7.4. Thyroid hormone was removed from serum by passage over AG-1 resin as described by Samuels et al. (1979). Greater than 90% of thyroid hormone was removed by this procedure. Heart cell preparations were checked for purity by staining for sarcomeric myosin (MF-20 antibody) and for endothelial cells, which are the principal contaminants, by staining for Von Willebrand factor (Factor VIII).

#### Drug Treatments
Fetal heart cells were plated at a density of 6 × 10⁶ cells/100-mm diameter culture dish and maintained for 3 days in DMEM with 10% fetal calf serum from which thyroid hormone had been removed by resin stripping. A stock solution of T₃ was prepared by dissolving in 0.1 N NaOH and diluting with methanol. Stock solutions of DITPA and CGS 23425 were dissolved in methanol. T₃ and DITPA were added to culture medium at 5 times their Kᵢₐ concentrations of 6 and 218 nM, respectively (Pennonk et al., 1994). CGS 23425 was added at 1 nM, which is approximately 5 times the EC₅₀ for transcriptional activity of the apoA1 promoter mediated through TRβ1 (Taylor et al., 1997). After addition of treatments, cardiomyocytes were cultured for an additional 48 h before scrapping plates to isolate RNA. The experiments were repeated three times with different batches of cardiomyocytes. T₃ and DITPA were supplied by Sigma-Aldrich (St. Louis, MO). CGS 23425 was a gift from Novartis (Summit, NJ).

#### RNA Isolation
Total RNA was isolated from cardiomyocytes using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction. The amount of RNA was determined by A₂₆₀ measurement, and its quality was checked by formaldehyde gel electrophoresis before using for probes.

#### Oligonucleotide Library
A library containing 4270 oligonucleotides corresponding to genes in the current rat UniGene database was purchased from QIAGEN (Valencia, CA). The UniGene database clusters all GenBank rat sequences into a nonredundant set of genes. From each cluster a sequence is selected based on the longest region of high-quality sequence. Each of the 70-mers was sequenced-optimized using BLAST to minimize cross-hybridization and to allow analysis of overlapping and homologous genes. The 70-mers also were normalized to a melting point of 78 ± 5°C, which allowed for
more stringent hybridization. Further information regarding the oligonucleotide library can be found at www.qiagen.com/oligos/omad.

**Preparation.** Glass arrays were prepared in the Diamond Microarray Facility in the Children’s Research Center of the University of Arizona. In brief, slides were coated with 2% silane solution (3-glycidoxypropyltrimethoxysilane) in hexane and rinsed with hexane three times.

**Printing of Oligonucleotide Library.** Each oligo was resuspended in 7.5 μl of DNase-free water (pH 7.5–8.0) in 384-well plates, which were rotated at 60 rpm overnight at 4°C on an orbital shaker. Plates were sealed with aluminum sealing tape to prevent evaporation and centrifuged at low speed after shaking. A final concentration of 50% dimethyl sulfoxide was added to each oligonucleotide before printing on coated slides the next day. The oligonucleotide library was printed in triplicate on each slide with VersArray Chipwriter Pro System (Bio-Rad, Hercules, CA). Before final printing, slides were tested with positive and negative control oligonucleotides obtained from QIAGEN. After printing, oligonucleotides on the slides were baked for 90 min at 90°C and stored in a desiccated cabinet. The array printing procedure was optimized for use of oligonucleotides by multiple trial hybridizations with positive and negative control oligonucleotides obtained from QIAGEN. Positive controls consisted of a mixture of all of the oligonucleotides in the library. Negative controls were oligonucleotides with random sequence.

**Probe Preparation.** Twenty micrograms of total RNA was reverse transcribed with oligo(dT) in the presence of 0.2 mM aminomethyl dUTP (Molecular Probes, Eugene, OR) and a mixture of four dNTPs according to manufacturer's instruction (Ambion, Austin, TX). After base hydrolysis of RNA with 0.4 M NaOH at 65°C for 15 min, the cDNA was neutralized with the same concentration of HCl. cDNA was purified with spin columns supplied by QIAGEN according to the manufacturer's PCR purification protocol except that the columns were washed with 75% ethanol rather than the buffer supplied by the manufacturer and vacuum dried. cDNAs were dissolved in 3 μl of NaHCO₃ (25 mg/ml), and 5 μl of either Alexa Fluor 546 or 647 (Molecular Probes) in dimethyl sulfoxide were added in separate tubes. The labeling reaction was done for 1 h at room temperature in the dark. Labeled probes of the same treatment from two different tubes were mixed together and purified the same way as described above.

**Hybridization and Washing.** Hybridizations were performed using the Gene Tac Hybridization Station at 47°C in presence of 1× Gene Tac hybridization buffer, 20% formamide, and 1°C lysis of RNA with 0.4 M NaOH at 65°C using the Gene Tac Hybridization Station at 47°C for 15 min, the cDNA was

**Scanning and Analysis of Data.** Scanning was performed with Array Worx software (Applied Precision Inc., Issaquah, WA) in high-resolution setting (5 μm/pixel). Sensitivity setting was high signal-to-noise ratio. Average exposure time was 1 s for green dye and 4 s for red. Spot-finding was done with Soft Worx Tracker version 2.20 from Applied Precision. Signal intensities for each element (spot) on the array were calculated as the mean spot intensity minus the median local background intensity. These background-subtracted intensities were then transformed using a modified linlog transformation (Cui et al., 2003). Normalization between the two channels for each array was then performed using a robust locally weighted regression (lowess) (Cleveland, 1979) based on the log ratio of the two channels versus overall spot intensity (Yang et al., 2002) and included terms to remove both spatial and intensity-dependent biases (Cui et al., 2003). A gene-by-gene ANOVA (Kerr et al., 2000) was then performed to calculate the contribution and significance of the different treatments to changes in gene expression. ANOVA was only performed on genes that were consistently expressed (more than 75% of the time) at measurable levels (background-subtracted intensity >2 background standard deviations) for at least one of the treatments. To account for known sources of experimental variability, the linear model used for the ANOVA included terms to account for the contribution of each dye and hybridization (array) to the measured intensities, in addition to the term used to determine the contribution of each treatment (Kerr and Churchill, 2001). Genes were identified as differentially expressed based on a maximum value of 0.05 for the P value associated with the F-test for the treatment term in the ANOVA and a minimum 1.5-fold difference between any two treatments.

**Results**

**Experimental Design.** The design of the microarray experiments is shown in Fig. 1. This design, based upon the method described by Kerr and Churchill (2001) and Kerr et al. (2000), differs from the usual procedure in which RNA from cardiomyocytes treated with T₃, DITPA, and CGS would be individually compared with “hypothyroid” control by additional comparisons between treatments. Red-green and

<table>
<thead>
<tr>
<th>UniGene No.</th>
<th>Encoded Protein</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
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<td>ATPase Ca²⁺⁺ transporting cardiac muscle, slow twitch 2</td>
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<td>GACATCCTGGTTTGCTGGGAATTGTT</td>
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</tr>
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<td>AGACGTGTGTGGACAGATGAGG</td>
</tr>
<tr>
<td>2745</td>
<td>Creatine kinase, muscle form</td>
<td>GCCCATCGCTGAGGACTACCC</td>
<td>AGACGTGTGTGGACAGATGAGG</td>
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<tr>
<td>3184</td>
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<td>GCCCATCGCTGAGGACTACCC</td>
<td>AGACGTGTGTGGACAGATGAGG</td>
</tr>
<tr>
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<tr>
<td>4400</td>
<td>Apoptosis-regulating basic protein</td>
<td>GCCCATCGCTGAGGACTACCC</td>
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</table>
green-red comparisons (double arrows) were made among RNAs from hypothyroid control cells and cells treated with DITPA and T\textsubscript{3}. In the case of CGS, the RNA labeled with red dye was hybridized with green-labeled RNA from T\textsubscript{3}-treated cells, and with RNA from DITPA-treated cells the dyes were reversed (single arrows).

We sampled 4270 genes with the oligonucleotide microarray. This represents approximately 10 to 20% of the expressed genes in heart, assuming the number of genes expressed is similar to other organs, which express 10,000 to 20,000 genes (Zhang et al., 1997). Among the genes spotted on the array, an average of 597 genes were detected using RNA from hypothyroid control, 654 with RNA from T\textsubscript{3}-treated cells, 645 with DITPA treatment, and 723 with CGS treatment.

Genes were selected for comparison based upon the criteria that expression was increased/decreased >1.5 times over hypothyroid control cells by at least one of the three treatments with \( P < 0.05 \). Using these criteria, 64 genes were selected. The genes have been organized into functional categories, and the results are presented as -fold change and \( \log_{10} \) ratio of treatment versus hypothyroid control in Table 2.

**Effects of T\textsubscript{3} on Cultured Heart Cells.** Addition of T\textsubscript{3} to culture media up-regulated three transcription factors or cofactors including hairless, cardiac ankyrin repeat protein (CARP), and four-and-a-half LIM domains 2. T\textsubscript{3} down-regulated two transcripts encoding glycolytic enzymes, fructose-1,6-biphosphatase and aldolase C, and up-regulated several components of mitochondrial respiration. Cytochrome c and subunit VIII-H (heart/muscle) of cytochrome c oxidase were among those transcripts that were up-regulated.

Acyl CoA synthetase, which catalyzes the first step in the entry of fatty acids into mitochondria, was down-regulated. Possibly, this may reflect tissue culture conditions in which heart cells preferentially utilize glucose and lactate rather than fatty acids to meet energy requirements. The glucose transporter 4, the principal regulator of glucose uptake in response to insulin, was also up-regulated. The \( \alpha \) subunit of Na\(^{+}/K\(^{-}\))/ATPase, a known target for T\textsubscript{3} actions, was up-regulated. However, the T\textsubscript{3}-induced increase in Na\(^{+}/K\(^{-}\))/ATPase mRNA apparently is not associated with increased transcription, but is mediated at the post-transcriptional level (Awais et al., 2000).

The largest number of genes strongly expressed after treatment with T\textsubscript{3} and DITPA were those involved with signal transduction. The Ca\(^{2+}\)-ATPase of sarcoplasmic reticulum (SERCA2a) was strongly up-regulated by T\textsubscript{3}, as was brain natriuretic factor and natriuretic peptide precursor. Among the contractile protein genes, treatment with T\textsubscript{3} caused the expected induction of adult cardiac myosin heavy chain isofrom (\( \alpha \)-MHC) and repression of the fetal cardiac myosin heavy chain type (\( \beta \)-MHC). A number of proteins found in the extracellular matrix were up-regulated by T\textsubscript{3}, including \( \alpha \) subunit of prolyl 4-hydroxlyase, matrix Gla protein, and \( \alpha \)7A integrin.

**Effects of DITPA and CGS on Cultured Heart Cells.** Among the genes spotted on the microarray, those regulated by T\textsubscript{3} and DITPA were the nearly identical. One exception was the gene encoding the nuclear matrix protein matrin 3, which was up-regulated by DITPA and CGS 23425, but not by T\textsubscript{3} (Table 2). Another exception was presenilin 2, which was down-regulated more by DITPA than by T\textsubscript{3}. Of particular note, the Ca\(^{2+}\)-ATPase of the cardiac sarcoplasmic reticulum was significantly up-regulated by T\textsubscript{3} and DITPA, but not by CGS 23425. This is consistent with physiological studies indicating that T\textsubscript{3} and DITPA increase cardiac inotropic activity, which is dependent upon stimulation of Ca\(^{2+}\)-ATPase activity (see Discussion).

A large group of genes that were more stimulated by T\textsubscript{3} and DITPA than by CGS 23425 were the contractile protein genes. The \( \alpha \)-MHC gene was up-regulated and the \( \beta \)-MHC was down-regulated. The reciprocal change in MHC isoforms was greatest with T\textsubscript{3} and DITPA and least with CGS 23425. In addition, there was an interesting group of transcriptional regulators including hairless, CARP, and four-and-a-half LIM domains 2 that were up-regulated by T\textsubscript{3} and DITPA but not by CGS 23425. The fourth transcription factor, transition protein 2, was down-regulated by all three treatments. In all, 13 genes were significantly more up- or down-regulated by CGS 23425 than by T\textsubscript{3} with \( P < 0.05 \) (Table 2). DITPA and T\textsubscript{3} up-regulated several transcripts that were down-regulated by CGS 23425. Among these were several mRNAs encoding mitochondrial enzymes or enzyme subunits. These transcripts included cytochrome c oxidase VII-H subunit, mitochondrial H\(^{+}\)-ATP synthase, F\(_{0}\) complex \( \alpha \) and \( \beta \) subunits, ATP-synthase H\(^{+}\) transporting mitochondrial F\(_{0}\) complex, and the muscle form of creatine kinase.

**Real-Time PCR.** To test the validity of the array experiments and ANOVA procedure, we performed real-time quantitative PCR on 10 genes using cDNA from hypothyroid cells and cells treated with T\textsubscript{3}, DITPA, and CGS 23425 (Table 3). Fold change for treatments represents cDNA produced from treated cells relative to hypothyroid control. These results should be compared with -fold changes by microarray in Table 2. Five of the genes were more strongly up-regulated by T\textsubscript{3} and DITPA than CGS 23425. One gene was more strongly down-regulated by T\textsubscript{3} and DITPA. Two genes were regulated more by CGS 23425 than T\textsubscript{3} and DITPA. One gene (presenilin-2) was down-regulated more strongly by DITPA than T\textsubscript{3}. In each case RT-PCR confirmed the relative differences between treatments observed in microarrays.

**Discussion**

Cell culture was selected as a means for obtaining information about the effects of T\textsubscript{3} and its analogs on gene ex-
presumption because tissue accessibility, volume of distribution, and other factors that could influence drug availability in vivo have not been established for the analogs used in this study or for most other thyroid hormone analogs. Furthermore, chemically induced hypothyroidism causes a significant reduction in body and organ weight at all stages of...
development. By using fetal cells harvested before exposure to T₃ and maintained in serum devoid of the hormone, it was possible to create hypothyroid control cells without chemical treatment of animals prior to harvest.

The results indicate that pattern of genes expressed in cardiomyocytes after treatment with T₃ and DITPA were nearly identical, whereas there were a number of differences with CGS 23425. Among the 64 genes that were increased more than 1.5 times, there were 13 differences in gene expression between T₃ and its analogs CGS 23425 (20.3%). The genes activated by CGS 23425 correspond to those activated via the TRβ1 receptor at ligand concentrations less than 5 times the Kᵦ. Presumably, the remaining T₃-activated genes are regulated via TRα under these conditions. To our knowledge, this is the first large-scale profiling of the effects of T₃ on cardiomyocytes and the first microarray analysis of the effects of T₃ on gene expression versus its analogs.

Surprisingly, there were few common targets between heart and earlier reports of the effects of T₃ on gene expression in liver. Using the study by Feng et al. (2000) for comparison with liver genes activated by T₃, common targets included cardiac α-actin, smooth muscle γ-actin, and matrix metalloproteinase. The message for the putative Src domain 3 (SH3)-containing protein identified as a T₃-regulated message in our arrays (Table 2) is in the same family as ponsin, which was found to be a T₃-regulated message in liver, but the product of a separate gene. Cardiac-specific targets were numerous and included α- and β-myosin heavy chains, brain natriuretic peptide, Ca²⁺-ATPase, Na⁺/K⁺-ATPase, etc. These results attest to the unique targeting of thyroid hormone actions in each organ and tissue.

The largest number of genes affected by T₃ and DITPA were those involved with signal transduction. Of particular note, the Ca²⁺-ATPase of the cardiac sarcoplasmic reticulum was significantly up-regulated by T₃ and DITPA but not by CGS 23425. This is consistent with physiological studies indicating that T₃ and DITPA increase inotropic activity of the heart, which is dependent upon stimulation of the Ca²⁺-ATPase activity (Arai et al., 1991; Pennock et al., 1992). Except at the highest doses tested, CGS 23425 and other TRβ1-selective drugs do exhibit inotropic effects (Taylor et al., 1997; Trost et al., 2000).

The next largest group of genes that were more stimulated by T₃ and DITPA than by CGS 23425 were the contractile protein genes. In adult rats with fast contracting ventricles, the V₁ form predominates, whereas during embryonic and fetal development the V₃ form is the predominate isoform (Morkin, 1993). As anticipated, the α-MHC gene was up-regulated by T₃ treatment and the β-MHC was down-regulated. The reciprocal change in MHC isoforms was greatest with T₃ and DITPA and least with CGS 23425.

There is evidence for myosin isoform-specific regulation by TRs from transgenic knockout experiments (Mansen et al., 2001). β-MHC is overexpressed in mice deficient in TRα1, suggesting that TRα1 plays a specific role in the negative regulation of β-MHC. In the experiments reported here, β-MHC is not as strongly down-regulated by the TRβ1-selective TH analog CGS 23425 as by T₃ and DITPA, which is consistent with the need for TRα1 activation to suppress β-MHC.

In addition to genes involved in signal transduction and contractile protein genes, there was an interesting group of transcriptional regulators that were up-regulated by T₃ and DITPA but not by CGS 23425. These genes included hairless, CARP, and four-and-a-half LIM domains 2. The fourth transcription factor, transition protein 2, was down-regulated by all three treatments. Hairless has recently been identified as a corepressor of TRs and vitamin D receptors (Hsieh et al., 2003). The lack of sequence similarity with previously identified TR corepressors suggests it may serve a more specialized role than ubiquitous corepressors. CARP is a nuclear coregulator for cardiac gene expression during development. Four-and-a-half LIM domains 2 is a member of a family of LIM proteins defined by the presence of one or more double zinc-finger domains. Four-and-a-half LIM domains 2 is primarily expressed in heart (Morgan and Madgwick, 1999). The LIM family members that have been characterized act as transcriptional regulators and are involved in muscle development and differentiation.

The ATP-synthesizing enzyme system of the inner mitochondrial membrane has two major components or factors, F₉ and Fₑ. Two subunits of these components were up-regulated by T₃. These were ATP synthase, H⁺-transporting, mitochondrial F₉ complex, and mitochondrial H⁺-ATP synthase, α subunit. The α and β subunits of F₁-ATPase have been reported to be less responsive to T₃ than other components of the oxidative phosphorylation system (Li et al., 1997) and in these experiments, were slightly down-regulated. Several mitochondrial proteins known to be stimulated by T₃, such as cytochrome c oxidase, mitochondrial H⁺-ATP synthase, and mitochondrial ATPase, were also stimulated by DITPA but not by CGS 23425. Generally, the TRβ1-specific analog, CGS

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**TABLE 3**

<table>
<thead>
<tr>
<th>UniGene No.</th>
<th>Encoded Protein</th>
<th>T₃</th>
<th>DITPA</th>
<th>CGS 23425</th>
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<tr>
<td>1051</td>
<td>ATPase, Ca²⁺-transporting cardiac muscle, slow twitch 2</td>
<td>1.11 ± 0.05</td>
<td>1.27 ± 0.06</td>
<td>0.54 ± 0.06</td>
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<td>1.32 ± 0.25</td>
<td>6.85 ± 0.09</td>
<td>3.32 ± 0.19</td>
</tr>
<tr>
<td>4400</td>
<td>Apoptosis-regulating basic protein</td>
<td>1.32 ± 0.05</td>
<td>1.84 ± 0.03</td>
<td>0.70 ± 0.05</td>
</tr>
</tbody>
</table>

* P < 0.01 vs. hypothyroid control.
* P < 0.05 vs. hypothyroid control.

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*Downloaded from jpet.aspetjournals.org on October 18, 2017.*
23425, had less effect on nuclear encoded mitochondrial enzymes than T₃ and DITPA.

In addition to modulating mRNA levels of key nuclear-encoded genes of the mitochondrial oxidative phosphorylation system, T₃ modulates the steady-state concentration of all mitochondrial DNA encoded mRNAs (Endrīquez et al., 1999). Surprisingly, among over 100 nuclear encoded mitochondrial respiratory components only nine have been shown to directly respond by binding of T₃ to TRs in their promoter regions (Fillar and Seitz, 1997). However, there is evidence for indirect thyroid hormone regulation of additional mitochondrial components through activation of transcriptional factors (Weitzel et al., 2003). There have been no previous studies on the effects of thyroid hormone analogs on nuclear or mitochondrial encoded genes of mitochondrial respiration.

One of the notable physiological differences between DITPA and T₃ is that DITPA produces increases in left ventricular dP/dt comparable with those obtained with L-thyroxine but with significantly less tachycardia (Pennonck et al., 1992). A possible explanation for the lesser effect of DITPA on heart rate might be that DITPA down-regulated presenilin 2, whereas expression of this gene was less affected by T₃ and CGS 23425. Mutations in presenilin genes have been linked to early-onset familial Alzheimer’s disease. These genes encode membrane proteins presenilin 1 and 2, which are thought to constitute the catalytic subunits of γ-secretase that promote the cleavage of the amyloid precursor protein, creating the amyloid-β peptide that accumulates in the brain of individuals with this disorder. In addition to their catalytic role, presenilins form high molecular weight complexes with other proteins. One of these proteins, CALPK/KChIP4 (calcinilin-like protein), is a member of a family of KChIPs (Kv channel-interacting proteins) that has been shown to alter the voltage-gating and inactivation properties of voltage-gated potassium channel subunit Kv4 (Morohashi et al., 2002). Expression of wild-type presenilins increases outward K⁺ current densities in HIK-293 cells relative to untransfected cells (Malin et al., 1998). Potassium channel genes that code for K⁺ channels involved in action potential repolarization, like Kv 4.2 and minK, are TRα1 targets and strongly regulated by changes in thyroid status (Gloss et al., 2001). Additional experiments will be necessary to determine whether the differences in expression of presenilin 2, when stimulated with T₃ and DITPA, can account for differences in heart rate response.

There are other possible explanations for differences between DITPA and T₃ in their physiological effects. First, the microarrays used in these experiments contained a little over 4000 genes, which probably represents only 20 to 40% of the genes expressed in rat heart. Differences in expression of genes not represented in these arrays might be responsible for physiological differences between T₃ and DITPA. Second, several actions of thyroid hormone do not require intranuclear binding of T₃ by TR and would not necessarily be detected in microarray experiments. Some of these actions of thyroid hormone occur at the plasma membrane, involving ion channel and ion pump activities at the ribosome and Golgi apparatus and upon the cytoskeleton (Davis and Davis, 2002). Other actions that do not require T₃ binding to nuclear receptors include thyroid hormone activation of the mitogen-activated protein kinase cascade (Lin et al., 1999). T₃-induced cardiac hypertrophy recently has been shown to involve activation of the cytosolic protein, calcineurin, perhaps by promoting calcium entry into the cell (Vanamala et al., 2003). Activation of both the mitogen-activated protein kinase cascade and calcineurin ultimately result in induction of transcription factors, which should be detectable in microarray experiments. Possibly one or more of the mechanisms given above may explain differences between the physiological effects of T₃ and DITPA.

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References


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