Changes in Adenylyl Cyclase Isoforms as a Mechanism for Thyroid Hormone Modulation of Cardiac β-Adrenergic Receptor Responsiveness

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Although thyroid hormones are known to modulate cardiac β-adrenergic receptor expression, the physiologic implications of these changes in the cardiac manifestations of altered thyroid hormone metabolism have been disputed. This study examined whether thyroid hormone modulates signaling via the cyclic adenosine monophosphate (cAMP) pathway by regulating cardiac adenylyl cyclase (AC) isoform expression. Northern blot analyses and AC enzyme assays were performed on preparations from hypothyroid, euthyroid, and hyperthyroid rat ventricles. Steady-state levels of cardiac AC mRNA types V and VI in hypothyroid ventricles were 173% ± 8% and 149% ± 12%, respectively, of the values in euthyroid ventricles ($P < .01$). This increase in AC mRNA isoforms was accompanied by a 1.5-fold increase ($P < .05$) in the activation of catalytic AC by forskolin and Mn. In contrast, the relative abundance of transcripts for types V and VI AC was similar in hyperthyroid and euthyroid ventricles, but catalytic AC activation by forskolin and Mn was significantly reduced by 35% in membranes obtained from hyperthyroid ventricles. AC activation through β-adrenergic receptor stimulation by isoproterenol was not altered by thyroid hormone status. Thus, the effect of thyroid hormone to repress AC catalytic activity would be anticipated to offset the increase in β-adrenergic receptor expression in hyperthyroidism. These studies identify cardiac AC enzymes as important targets for thyroid hormone–dependent regulation of signaling via the cAMP pathway, and support the finding that cardiac adrenergic responsiveness is unaltered in thyroid disease states.

CERTAIN CARDIOVASCULAR manifestations of hyperthyroidism resemble a state of increased β-adrenergic receptor stimulation, whereas the changes that accompany hypothyroidism suggest a diminished β-adrenergic receptor response.[1] These observations gave rise to the concept that the sympathoadrenal axis plays a role in the characteristic hemodynamic alterations of thyroid disease states. The failure to link thyroid hormone–dependent changes in sympathetic tone to the expected alterations in serum catecholamine levels[2] shifted the focus of investigations to the cell-surface β-adrenergic receptor complex as the likely locus for regulation by thyroid hormone in tissues that are the target for both thyroid hormone and catecholamines. Several laboratories have presented evidence that β-adrenergic receptor density varies directly with serum thyroid hormone levels (reviewed in Bilezikian and Loeb[3]). While this provides a logical and appealing explanation for many of the cardiovascular manifestations induced by thyroid hormone, the functional relevance of changes in β-receptor expression has been questioned.[4-6]

Recent studies have examined the effects of thyroid hormone on various components of the membrane adrenergic receptor complex. Studies examining the α and β subunits of the heterotrimeric G proteins in response to thyroid hormone have been inconsistent. The stimulatory Gαs protein that couples the β-adrenergic receptor to activation of adenylyl cyclase (AC) was shown to be unaltered by changes in thyroid hormone status in adult rat myocardium,[7] whereas it was induced by exposure to thyroid hormone in cultured neonatal ventricular cardiomyocytes.[8] The inhibitory Gαi that has recently been linked to signaling via the β_2-adrenergic receptor in cardiomyocytes[9] and the β subunit of the βγ dimer were found to be repressed by thyroid hormone in both whole animal and cell culture models.[7,8] In the cultured cardiomyocyte model, triiodothyronine was shown to enhance catecholamine-dependent stimulation of AC activity via Gαs, whereas endothelin- and carbachol-dependent inhibition of cyclase activity was attenuated, suggesting that thyroid hormone–dependent alterations in G protein subunit expression lead to physiologically relevant changes in the activation of AC.[8] However, in vivo, hypothyroidism did not alter the dose-response relationship for norepinephrine-dependent stimulation of AC activity,[10] and hyperthyroidism in subhuman primates did not alter isoproterenol sensitivity.[6]

The AC enzyme itself could be regulated by thyroid hormone in the heart. The few studies that examined this issue preceded the recognition that AC comprises a family of isoforms, with types V and VI being the predominant species detected in cardiac preparations.[11,12] While thyroid hormone failed to alter forskolin-activated enzyme activity which reflects direct activation of the catalytic subunit and potentiation of the activation of the AC catalytic moiety through Gs, changes in basal enzyme activity that could represent changes in enzyme expression were detected.[7] Since changes in AC catalytic activity per se could explain the apparent changes in sympathetic tone in thyroid disease, the current study directly tested the hypothesis that thyroid hormone regulates cardiac AC isoform expression and thereby influences the cell's capacity to receive and integrate signals via the cyclic adenosine monophosphate (cAMP) pathway.

MATERIALS AND METHODS

Animals

Thirty-three male Sprague-Dawley rats weighing about 175 g each were divided into 3 groups of 11. The animals were rendered hypothyroid or hyperthyroid by 6-*n*-propyl-2-thiouracil (PTU) at a concentra-

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tion of 750 mg/L in the drinking water or daily injections of 20 μg L-thyroxine in 0.5 mL phosphate-buffered saline, pH 7.4, respectively. They had access to rat chow and water ad libitum and were weighed every 4 to 5 days. After 12 days of treatment, they were killed, and their left ventricles were frozen in liquid nitrogen and stored at −80°C for subsequent RNA and enzyme analyses. The treatment protocol efficacy was validated by the observations that the mean ratio for left ventricular weight to total body weight (milligrams/grams) varied directly with the thyroid hormone status (2.084 ± 0.036 for hypothyroid, 2.256 ± 0.026 for euthyroid controls, and 2.741 ± 0.054 for hyperthyroid, $P < .05$ by ANOVA) and that β-myosin heavy chain (β-MHC) mRNA expression was confined to the samples derived from PTU-treated animals (Fig 1).

Northern Blot Analysis

Total cellular RNA was extracted from left ventricular tissue and its integrity verified by agarose gel electrophoresis as previously described.[13] Following oligo d(T)-cellulose affinity chromatography to enrich for poly(A)$^+$ RNA, 5 μg of sample was size-fractionated by denaturing agarose gel electrophoresis, transferred to nylon membrane (Duralon UV; Stratagene, San Diego, CA) by capillary blotting, and cross-linked by UV light. AC type V and VI cDNA probes (provided by Dr R. Iyengar, Mount Sinai Medical School, New York, NY) were radiolabeled (~10^8 dpm/pmol) and the hybridization reaction was performed as described previously.[13] β-MHC and α-tubulin mRNAs were measured using radiolabeled oligonucleotide probes[13] (Oncogene Science, Uniondale, NY). Quantitation of the mRNA was performed by phosphorimage analysis (Molecular Imager System; Biorad, Hercules, CA).

AC Activity

Ventricular myocardial membranes were prepared for measurement of AC enzyme activity according to standard methods published previously.[14] In these studies, the average membrane protein yield was 0.15% to 0.30% of the initial tissue wet weight and did not differ between hypothyroid, euthyroid, and hyperthyroid hearts. Basal AC activity was measured in the absence of activators and compared with activation by the nonhydrolyzable guanosine triphosphate analog, Gpp(NH)p (0.1 and 10 μmol/L), Gpp(NH)p (0.1 μmol/L) plus isoproterenol (5×10^{-9} to 2×10^{-6} mol/L), manganese (10 mmol/L), forskolin (10^{-8} to 10^{-5} mol/L), and forskolin (10 μmol/L) plus Mn (10 mmol/L). All assays were performed in triplicate for 30 minutes at 37°C; preliminary studies established that the assay is linear with time and protein concentration under these conditions.

Data Analysis

All results are expressed as the mean ± SEM. ANOVA was used for statistical comparison of multiple groups, and significance was assumed at a P level less than .05.

RESULTS

Cardiac AC mRNA Expression

Consistent with reports by other groups,[15] 2 major splice variants for type V AC (5.5 and 7.5 kilobase [Kb]) were detected in rat ventricle. The relative abundance of each splice variant did not change as a function of thyroid hormone status; therefore, both variants were combined in the quantitation of total type V AC mRNA. Type VI AC was detected as a single 6.5-kb transcript (Fig 1A). Steady-state levels of type V and VI AC mRNA in hypothyroid ventricles were 173% ± 8% and 149% ± 12%, respectively, of euthyroid values ($P < .01$). The relative abundance of transcripts for type V and VI AC was not significantly different in samples obtained from hyperthyroid ventricles versus euthyroid ventricles (Fig 1B).

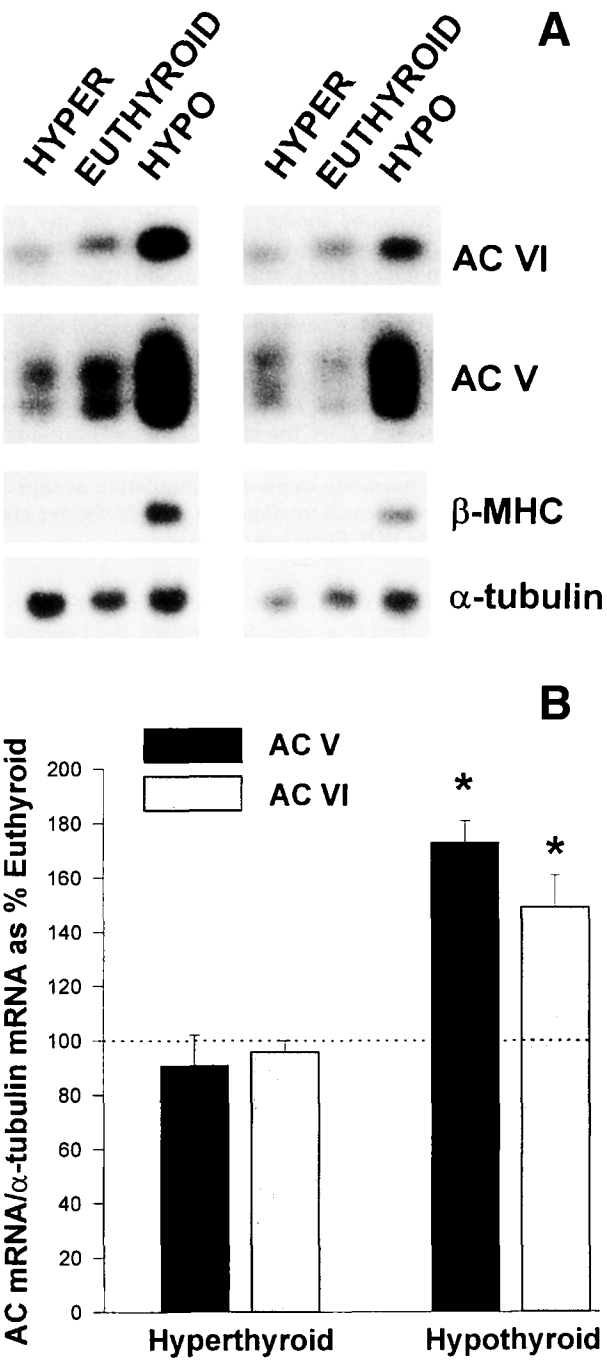

Fig 1. AC mRNA levels in hypothyroid, euthyroid, and hyperthyroid ventricles. (A) Representative Northern blot of RNA samples that were size-fractionated and sequentially probed for type V and type VI AC, β-MHC, and α-tubulin. (B) Changes in AC mRNA expression. Results in hyperthyroid and hypothyroid samples are expressed as a percent of euthyroid values after normalization to α-tubulin mRNA (n = 4-6 per group). *$P < .01$ *v* euthyroid and hyperthyroid.

Due to the low abundance of the AC mRNA isoforms in rat ventricles, poly(A)$^+$ RNA was isolated from each sample for analysis by Northern blotting in which rat α-tubulin mRNA was

used as the nonvariant housekeeping gene for normalization. The validity of using α-tubulin mRNA for normalization was verified by determining that its expression was not altered by thyroid status. The amount of α-tubulin mRNA per 1 μg poly(A)$^+$ RNA was 7.2 ± 0.6, 7.0 ± 0.5, and 6.3 ± 0.6 arbitrary units for euthyroid, hypothyroid, and hyperthyroid ventricles, respectively (n = 7 per group).

Cardiac AC Enzyme Activity

Figure 2 shows that basal AC activity tended to vary inversely with thyroid hormone status, but these differences were not significant. Activation of AC through G proteins, by either a submaximal (0.1 μmol/L) or maximal (10 μmol/L) concentration of the nonhydrolyzable guanosine triphosphate analog Gpp(NH)p, was similar in preparations from hypothyroid, euthyroid, and hyperthyroid ventricles. To probe for β-adrenergic receptor function, isoproterenol-dependent AC activity was measured in the presence of a low concentration of Gpp(NH)p (0.1 μmol/L) as a cofactor for receptor-dependent stimulation. Under these conditions, thyroid hormone status had no effect on either the incremental stimulation of AC by a maximal concentration of isoproterenol (2 μmol/L) (Fig 2) or the 50% effective concentration for isoproterenol-dependent activation of AC activity (Fig 3A). The failure of thyroid hormone to influence β-receptor–dependent activation of AC is noteworthy in the context of the significant thyroid hormone–dependent changes in β-adrenergic receptor expression observed in this preparation.[3]

In contrast, an effect of thyroid hormone to influence AC activity was evident when the enzyme was stimulated by Mn, which directly activates catalytic activity.[14] In this case, the AC catalytic activity derived from hypothyroid plasma membranes was significantly ($P < .05$) greater versus either euthyroid or hyperthyroid conditions (Fig 2). Similar changes in catalytic activity were evident with diterpene forskolin, which activates catalytic AC both directly and via Gs,[14] or forskolin plus Mn in combination, which leads to stimulation of catalytic activity that is no longer influenced by G proteins. In both reaction conditions (forskolin and forskolin + Mn), AC activity varied inversely with thyroid hormone status. AC activity was 1.5-fold higher ($P < .05$) in hypothyroid membrane preparations compared with euthyroid ventricles, whereas AC activity in hyperthyroid ventricles was decreased by 35% compared with the euthyroid condition (Fig 2). Figure 3B shows the dose-response relationship between the forskolin concentration and AC activity, indicating a similar threshold for AC activation of approximately 10^{-7} mol/L forskolin in all 3 thyroid conditions.

DISCUSSION

Thyroid hormone has profound effects on the heart and vascular system. These include increased heart rate, cardiac output, and ventricular contractility. The extent to which increased cardiac β-adrenergic sensitivity contributes to the heightened contractile function of the hyperthyroid heart remains controversial. While clinical findings have suggested increased adrenergic activity, studies have consistently shown that serum levels of catecholamines are decreased in hyperthyroidism and increased in hypothyroidism.[1,2,4,16] The numerous carefully controlled studies concluding that the cardiac effects of hyperthyroidism are mediated partly by the sympathetic nervous system[8,17-19] are balanced by an equally compelling literature that refutes this concept.[4,5,20,21] The failure to demonstrate that thyroid hormone–induced changes in β-adrenergic receptor expression result in coordinate changes in catecholamine-dependent cAMP accumulation or contractile performance[6] has been attributed to various confounding experimental factors.[22] Alternatively, changes in β-adrenergic receptor

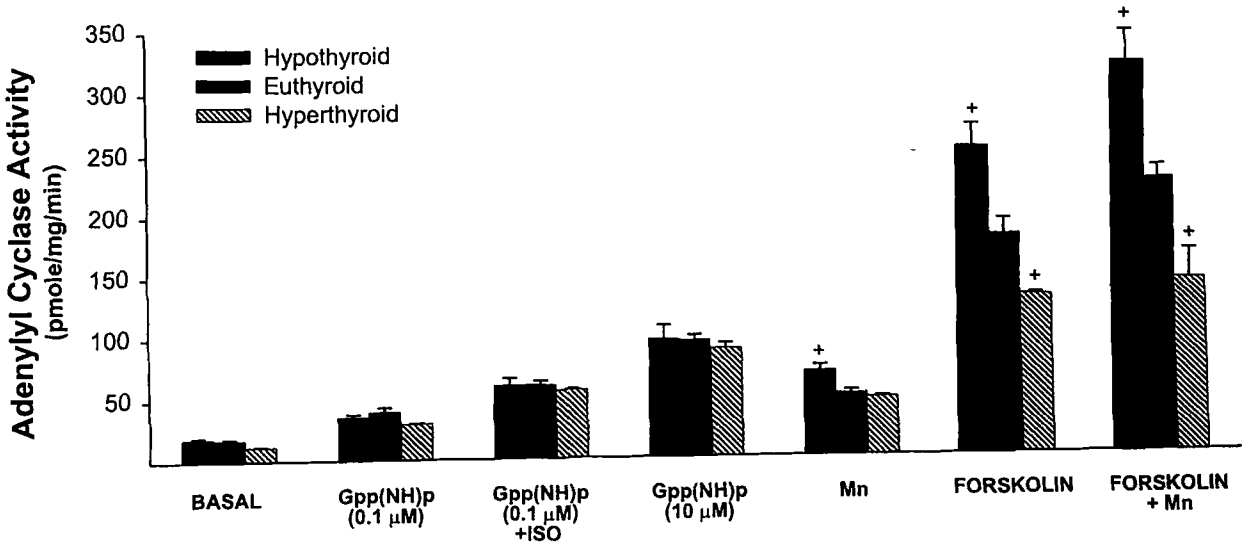

Fig 2. AC activity in membranes from hypothyroid, euthyroid, and hyperthyroid ventricles. AC activity was measured in the absence or presence of Gpp(NH)p (0.1 or 10 μmol/L), isoproterenol (ISO, 2 μmol/L), forskolin (10 μmol/L), or Mn (10 mmol/L) as indicated. In each set of samples (hypothyroid, euthyroid, and hyperthyroid), all agonists induced a statistically significant increase in AC activity over basal. There was a significant inverse relationship between AC activity in the presence of forskolin and forskolin + Mn and thyroid hormone status (+$P < .05$ *v* euthyroid).

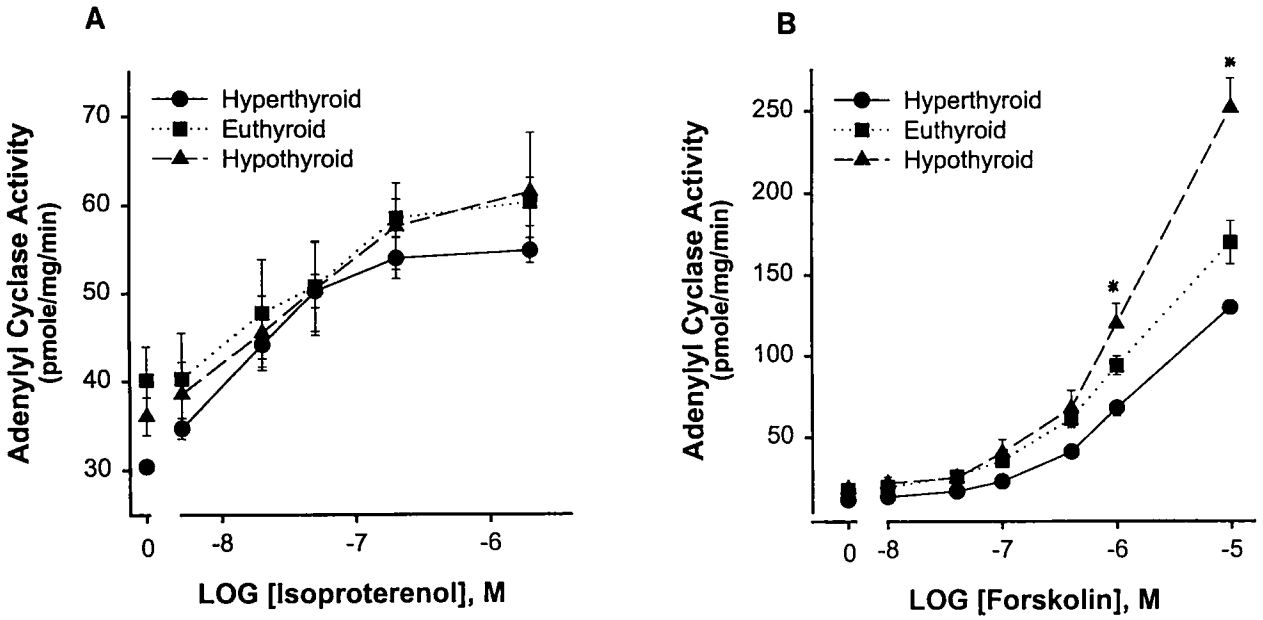

Fig 3. Concentration-response relationships for (A) isoproterenol-dependent and (B) forskolin-dependent AC activity. Results are the mean ± SEM of 5 separate experiments performed in triplicate.

expression without altered catecholamine sensitivity suggest that thyroid hormone may modulate the expression and function of other components of the AC signaling pathway.

The present results establish that thyroid hormone acts as a negative modulator of types V and VI AC in rat heart and suggest a plausible explanation for previous apparently contradictory findings. Recent studies in transgenic mice suggest that increased β-adrenergic receptor expression does not lead to a proportional increase in cAMP accumulation.[23] Rather, the low abundance of the AC enzyme membrane proteins may set the limit on transmembrane β-adrenergic receptor signaling.[24] According to this formulation, it is the ratio of the individual components of the AC signaling pathway that calibrates catecholamine responsiveness. Thus, the increase in β-adrenergic receptor density in the hyperthyroid heart is offset by a reciprocal decrease in AC enzyme abundance which limits cAMP accumulation. Similarly, increased AC expression in the hypothyroid heart amplifies the signal from the reduced number of cell-surface β-adrenergic receptors and preserves cAMP accumulation. This paradigm emphasizes that changes in AC expression are a critically important mechanism for the regulation of transmembrane receptor signaling. A similar dissociation of thyroid hormone–mediated changes in β-adrenergic receptor expression and cAMP accumulation was reported in brown adipose tissue (BAT).[25] Plasma membranes from ventricular tissue in the present study and from BAT[26] showed similar increases in AC activity in hypothyroid compared with euthyroid animals in response to forskolin, whereas in dispersed brown adipocytes AC activity appeared higher in euthyroid rats. The increase in AC activity in hypothyroid cardiomyocytes measured in vitro is consistent with a maintenance of normal catecholamine sensitivity despite the reported decrease in β-adrenergic receptor expression.[1,2,16] Determining whether this level of AC activity is maintained in vivo would require the isolation of adult myocytes from the otherwise heterogeneous population of cardiac cells.[27]

The current observation that thyroid hormone modulates type V and type VI AC isoform expression in the intact heart provides a novel perspective to reevaluate the role of altered catecholamine responsiveness in the cardiac manifestations of thyroid dysfunction. The coordinate induction of AC mRNA and enzyme activity in the hypothyroid ventricle suggests that cardiac AC isoforms may be subject to transcriptional regulation by thyroid hormone. However, the decrease in AC activity in the hyperthyroid heart was not accompanied by a significant decrease in steady-state levels of type V or VI AC transcripts, suggesting that posttranscriptional mechanisms could also be involved. The thyroid hormone–induced changes in AC activity observed in this study may result from the direct regulatory effects of thyroid hormone on the heart or indirect hemodynamic or humoral changes induced by hyperthyroidism or hypothyroidism in the intact animal.[13]

The present studies indicate that an analysis confined to the changes in β-adrenergic receptor expression is insufficient to ascertain the role of catecholamines as mediators of thyroid hormone–dependent effects on cardiac autonomic responsiveness. Overexpression of β-adrenergic receptors in a transgenic mouse model resulted in an increase in heart rate similar to that found in thyrotoxicosis.[4,20] The ability of propranolol to consistently decrease but not completely normalize the tachycardia suggests that there is an increase in adrenergic tone in the absence of altered catecholamine sensitivity in hyperthyroidism.[1,4] This latter point is emphasized in a recent study that failed to show any change in cardiac β-adrenergic sensitivity in a primate model of hyperthyroidism.[6] The issue of whether

propranolol exerts clinically beneficial effects via nonadrenergic pathways such as changes in vascular resistance or systemic hemodynamics remains unresolved.[4,17]

In summary, studies of thyroid disease states indicate that it is important to consider all 3 components, the β-adrenergic receptor, G-coupled protein, and catalytic subunit expression, in assessing adrenergic responsiveness of target tissues. These data also allow us to conclude that thyroid hormone–induced modulation of cardiac AC enzyme isoforms is an important regulator of cAMP production in the heart.[28]

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