Thyroid Hormone Regulates Hyperpolarization-Activated Cyclic Nucleotide-Gated Channel (HCN2) mRNA in the Rat Heart

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Abstract—Thyroid hormone regulation of the cardiac pacemaker gene, the hyperpolarization-activated cyclic nucleotide-gated channel gene (HCN2), was studied in rats by Northern analysis. Thyroid hormone administration to hypothyroid rats resulted in a doubling of the HCN2/β-actin mRNA ratio. A smaller, not statistically significant, increase in HCN2 mRNA occurred when euthyroid animals were made hyperthyroid. A single large dose of L-triiodothyronine given to hypothyroid rats caused a 4.7-fold increase in myocardial HCN2 mRNA expression level and only a 2.3-fold increase in the β-actin mRNA level. Although the rat HCN2 promoter has not been cloned, we identified a consensus thyroid hormone response element in the promoter sequence of the human HCN2 gene. Therefore, the increase in rat HCN2 mRNA is likely due to L-triiodothyronine stimulation of HCN2 gene transcription. The results suggest that the regulation of heart rate by thyroid hormone may be explained, at least in part, by the positive effect of this hormone on HCN2 gene expression. (Circ Res. 1999;85:498-503.)

Key Words: HCN2 ■ heart ■ triiodothyronine ■ transcription

It is well known that changes in thyroid state alter heart rate. The mechanism for this effect is unknown, although intrinsic changes in myocardial genes and changes brought about by alterations in whole-body oxygen consumption are likely to be involved.1–4 Previous studies showed that the rate of contraction of the isolated, denervated heart5–7 or of embryonic myocardial cells in cell culture8,9 are similarly affected by thyroid hormone, indicating that thyroid hormone has an intrinsic positive chronotropic effect.

Thyroid hormone regulates the expression of mRNAs encoding several voltage-dependent potassium channels (Kv).10–13 These channels, however, are primarily responsible for the duration and shape of action potentials but not for the rate of slow diastolic depolarization.14 It is now generally accepted that pacemaker current in cardiac myocytes is related to a specific mixed sodium-potassium inward current, called If in the sinus node, rather than to deceleration of outward potassium current.15

The molecular basis for the myocardial pacemaker has been further elucidated by the cloning of cDNAs encoding the mammalian hyperpolarization-activated cyclic nucleotide-gated channels.16,17 Two mRNA isoforms of the hyperpolarization-activated cyclic nucleotide-gated channels (HCN) have been identified in myocardium, HCN2 (old names in mouse, HAC1 and BCNG-2; and in humans, IH1) and HCN4 (old name, BCNG-3). The mixed Na+/K+ current through these channels is similar to the depolarization current in the sinus node.15,17 Recent data indicate that HCN2 and HCN4 gene products might be responsible for fast and slow components of the If current.18 Moreover, HCN2 mRNA is expressed throughout the myocardium,16,17 consistent with latent pacemaker activity in all cardiac myocytes.19

We speculated that the intrinsic chronotropic effect of thyroid hormone might be explained by increases in HCN2 gene expression. To test this hypothesis, we analyzed HCN2 mRNA in rat myocardium from different thyroidal states. We found a significant positive effect of thyroid hormone on this mRNA, suggesting that this could be 1 of the mechanisms for the positive effect of thyroid hormone on heart rate.

Materials and Methods

Materials
dCTP and Gene Screen membranes were purchased from New England Nuclear. Prime-it II random hexamer labeling kit was purchased from Stratagene, and thyroxine, L-triiodothyronine (T3), and methimazole (MMI) were purchased from Sigma. Trizol was purchased from Gibco BRL.

Animals

All treatments were approved and in compliance with the requirements of the Animal Welfare Committee of the Brigham and Women’s Hospital and the Animal Care and Use Committee of the University of Pittsburgh. Adult male Sprague-Dawley rats (Harlan...
Sprague-Dawley, Indianapolis, Ind., and Charles River Laboratory, Worcester, Mass) weighing between 125 and 200 g were acclimated to a cycle of 12 hours of light/12 hours of dark and a controlled temperature. Rat chow and tap water were provided ad libitum. Hypothyroidism was induced by the addition of MMI (0.02% or 0.025%) to the drinking water. Hypothyroidism was assessed by plateau of weight and serum thyroid hormone measurements.

Three experiments were performed. In the first, a thyrotoxic state was induced in euthyroid rats by intraperitoneal injections of T3 (50 μg/100 g body weight every other day for 8 days). Two control groups were composed of age-matched euthyroid rats and hypothyroid rats (3 weeks on 0.025% MMI in drinking water). In the second experiment, rats were given 0.02% MMI in water for 5 weeks. During the last 2 weeks, they also received daily intraperitoneal injections of graded doses of T3 or l-thyroxine (T4), as previously described.20 The doses were as follows: 0, 0.25, 1, and 3 μg of T3 and 0, 0.1, 0.2, 0.5, and 1.5 μg of T4 per 100 g of body weight. Age-matched euthyroid rats served as controls. There were 6 rats in each group in the first 2 experiments. The third experiment evaluated the minimal time requirement for the T3 effect on HCN2 mRNA. MMI-treated (6 weeks) rats were given a single intraperitoneal injection of T3 (200 μg) 4, 12, and 24 hours before being euthanized. This T3 dose was previously shown to saturate all thyroid hormone receptors.21-22 Euthyroid rats were used as controls. There were 3 rats in the 4-hour response group. The remaining groups in this experiment had 4 animals. Rats were killed under pentobarbital anesthesia.

Blood was collected through exsanguination, and hearts were quickly removed and stored at −80°C until RNA extraction.

Isolation and Analysis of RNA

RNA was extracted from the ventricular myocardium using TriZol reagent according to the manufacturer’s instructions. RNA concentration was estimated from the A260 value. Total RNA (10 μg) was electrophoresed through 1% agarose, 5% formaldehyde, and 1× MOPS gel. Ethidium bromide was added to sample buffer. Osmotic transfer of RNA to Gene Screen membranes was done by Vavacene in 10× SSPE. The quality of RNA and completion of transfer were confirmed by an A260 to A280 absorption ratio, 28S to 18S ribosomal RNA band ratios on the membrane, and the absence of ribosomal RNA bands on the gels after transfer. After cross-linking, membranes were hybridized with specific probes under high-stringency conditions (50% formamide, 5× SSPE, 1× Denhardt’s reagent, and 50 μg/ml denatured salmon sperm DNA at 42°C). The membranes were washed 3 times (final wash with 0.1× SSPE and 0.1% sodium dodecyl sulfate at 42°C). Quantification of mRNA was performed using a PhosphorImager and software from Molecular Dynamics. All blots were hybridized with a β-actin probe to normalize specific mRNA signals. Several identical samples were placed on all gels to allow comparisons between them.

A mouse HCN2 cDNA fragment (~0.5 kb) was made by reverse transcription–polymerase chain reaction (RT-PCR) reaction from euthyroid mouse cortex RNA. The primer sequences AATGGGGTTGTGGGTTAATGG for RT and TTTGACAATCTCCTGGATGATG, GATGGTAACCTCAACTGCCG for PCR were derived from the mouse 3.1 kb cDNA sequence (accession number, AJ225122). The cDNA was confirmed by sequencing. Specific anti-sense primers complementary to the respective 3′-end template in the presence of [α-32P] dCTP and 3 other dNTPs.

Hormone Measurements

Plasma T4 and T3 concentrations were measured by RIA in the NIH General Clinical Research Center core laboratory of the Brigham and Women’s Hospital or by RIA kit (Coat-A-Count, Diagnostic Product Corp).

Statistical Analysis

Statistical analysis was done using a general linear model algorithm from the SPSS program, version 7.0 (SPSS Inc). Groups were compared by Tukey’s test when ANOVA within a given family of variables was statistically significant. When indicated, log transformation of dependent variables to equalize variances between groups. ANOVA tests for each mRNA, except β-actin mRNA, were highly significant (P<0.001). Groups were compared by Tukey’s test. #P<0.05 when compared with hypothyroid group; *P<0.05 when compared with euthyroid group. All significant differences between groups are shown.

Results

In the initial experiment, the level of HCN2 and α- and β-MHC mRNAs were quantified in myocardial RNA from hypothyroid, euthyroid, and hyperthyroid rats (Figure 1). Hypothyroidism resulted in a decrease of both serum T4 and T3 levels as well as in lower weight. All T3-treated rats had an increase in serum T3 concentrations above the euthyroid level. A linear relationship between thyroid state and HCN2 mRNA levels was found (P<0.001 for linear contrast), with the highest level in the hyperthyroid and the lowest in the
hypothyroid state. The difference between the hypothyroid and the euthyroid groups was greater than that between the euthyroid and the hyperthyroid groups (Figure 1). As expected, α-MHC mRNA content was significantly decreased and β-MHC mRNA significantly increased in hypothyroid myocardium. Hyperthyroidism, however, was not associated with any further increase in α-MHC mRNA or decrease in β-MHC mRNA. Paradoxically, the β-MHC mRNA was slightly, but significantly, higher in the hyperthyroid than the euthyroid group ($P=0.003$). Still, both were much lower than in hypothyroid myocardium. The explanation for the deviation from the usual pattern of response of this mRNA is unknown.

To determine whether a dose-response relationship exists in the thyroid hormone-dependent alteration of myocardial HCN2 mRNA content, hypothyroid rats were treated with vehicle or with increasing doses of T3 or T4. The thyroid state was confirmed by serum thyroid hormone radioimmunoassay. As in the initial experiment, myocardial HCN2 mRNA was significantly decreased in hypothyroid rats compared with euthyroid rats (Figures 2C and 2G). Increasing T3 doses administered to hypothyroid rats caused a significant rise in myocardial HCN2 mRNA (Figure 2C). There was, however, no significant difference between the effects of the various T3 doses, indicating that the lowest dose produced maximal effect. Increasing T4 doses induced a significant, dose-dependent increase in HCN2 mRNA, again to a level not different from that in euthyroid rats (Figure 2G). The changes in the α- and β-MHC mRNAs confirmed the well-recognized positive and negative effects, respectively, of thyroid hormone on expression of those mRNAs (Figures 2A, 2B, 2E, and 2F). Similarly, all T3 doses, but only the highest T4 dose, resulted in maximal growth, as assessed by weight gain, with no significant differences between any of the T3 groups (Figures 3A and 3B). In the T4-treated groups, a clear dose-response relationship existed in growth velocity, with significant differences between all T4 doses (Figure 3B).

Hyperthyroidism caused a reduction of the heart weight to body weight ratio (H/B ratio) (hypothyroid rats: H/B ratio=3.015·10^{-3}, n=12; euthyroid rats: H/B ratio=3.440·10^{-3}, n=6; $P<0.05$ by $t$ test). Treatment with graded T3 doses caused a significant linear increase in H/B ratio (Figure 3C). In contrast, T4 treatment did not cause a significant increase in H/B ratio, which was consistent with the milder biological effect of these relatively small T4 doses (Figure 3D).

In the third experiment, we examined the time course of the myocardial HCN2 mRNA response to thyroid hormone. A single high T3 dose (200 μg) was injected intraperitoneally at various time intervals 24 hours before being euthanized. This dose was designed to cause rapid and complete saturation of all thyroid hormone receptors. A significant increase in both myocardial HCN2 and α-MHC mRNAs and a decrease in β-MHC mRNA occurred within 4 hours (Figures 4 and 5). By 24 hours, all mRNAs had reached normal levels. Interestingly, we noted an increase in myocardial β-actin mRNA content as well. The ratio of specific β-actin mRNA content to the 28S ribosomal band (as assessed by ethidium bromide staining of the gel) increased 2.3-fold after 24 hours ($P<0.01$ for difference between the hypothyroid and either the 12-hour or 24-hour group). Similarly, a small increase was also seen in the level of GAPDH mRNA (Figure 4). In addition, the H/B ratio also normalized 24 hours after a single T3 dose (hypothyroid versus 24-hour group, $P<0.05$; euthyroid versus 24-hour group, $P=0.82$), which indicated a rapid physiological and transcriptional response to thyroid hormone.

**Discussion**

These results are the first demonstration that myocardial HCN2 mRNA content is thyroid hormone–dependent. The
time course and direction of the thyroid hormone–induced effect is similar to that of α-MHC mRNA. Specifically, HCN2 mRNA is decreased in hypothyroid myocardium relative to that of euthyroid and hyperthyroid tissues. Although the overall pattern of response of these 2 mRNAs is the same, differences exist in the magnitude of the changes. The average HCN2 mRNA content in hypothyroid myocardium was 50% of normal, whereas α-MHC mRNA was 5% of normal in the same rats (P < 0.001 for both t-tests when all euthyroid and hypothyroid rats were pooled). The differences in HCN2 and α-MHC mRNA content between euthyroid and hyperthyroid animals are not significant (Figure 1). Thus, the major portion of the thyroid hormone–dependent increases in these 2 mRNAs in the rat occurs between the hypothyroid and euthyroid states.

In nonworking, isolated, perfused hearts of rats and other species, hypothyroidism and hyperthyroidism have been consistently associated with a decrease or increase, respectively, in heart rate.23,24 However, in a working heart model, the transition from the euthyroid to the hyperthyroid state in rats was not associated with any further chronotropic effect, which was similar to the effect on HCN2 and α-MHC mRNA.25,26 If the human HCN2 mRNA gene responds in the same way as that of the rat, one would expect that HCN2 dependent increases in these 2 mRNAs in the rat occurs between the hypothyroid and euthyroid states.
mRNA is not responsible for the tachycardia of hyperthyroidism. However, the regulation of gene expression by thyroid hormone varies between species, and humans may be different from rats in this respect. For example, β-MHC had only a minimal response to depletion of thyroid hormone in 1 well-documented human subject. The regulation of heart rate by thyroid hormone is complex. Some cardiovascular effects of hyperthyroidism can be induced by T3 and likely accounts for the T3-dependent increase in rat HCN2 mRNA. This mRNA is not responsible for the tachycardia of hyperthyroidism. However, the regulation of gene expression by thyroid hormone varies between species, and humans may be different from rats in this respect. For example, β-MHC had only a minimal response to depletion of thyroid hormone in 1 well-documented human subject.

It is well recognized that T3 stimulates the transcription of the rat α-MHC gene, which contains a potent thyroid hormone response element in the promoter. The mechanism for the thyroid hormone–dependent increase in rat HCN2 mRNA is not known. In this regard, however, and pertinent to the potential effect of T3 on human myocardial HCN2, is the fact that the entire human HCN2 gene, including its promoter, has been cloned as a part of human chromosome 19 p11.3 (accession numbers, AC005559 and AC005577). Although the location of the transcriptional start site has not been precisely mapped, we noted the presence of a canonical direct repeat thyroid hormone response element (GGGTTACcctAG-GTCA) within 2 kb from the most 5′ portion of the human cDNA sequence (accession number, AF065164). This strongly suggests that transcription of the human HCN2 gene will be stimulated by T3 and likely accounts for the T3-induced augmentation in rat HCN2 mRNA.

The regulation of heart rate by thyroid hormone is complex. Some cardiovascular effects of hyperthyroidism can be modulated by β-adrenergic blockers. In some studies in rats, the density of β-adrenergic receptors was increased in hyperthyroid and decreased in hypothyroid myocardium. Thyroid hormone also regulates the shaker-related potassium channel mRNA level in rat myocardium. Blockade of these channels can increase the duration and change the shape of the action potential, and it can indirectly decrease heart rate.

In 5 of these channels (Kv1.2, Kv1.4, Kv1.5, Kv2.1, and Kv4.2), only the level of the mRNA for Kv1.5 was reduced by hypothyroidism and restored by thyroid hormone treatment in rat hearts. Two other channels (Kv1.2 and Kv1.4) showed opposite regulation of mRNA by thyroidal state. In cultured rat neonatal myocytes or in early postnatal rat heart, T3 treatment enhanced the expression of Kv4.2 and Kv4.3 and decreased expression of Kv1.4.

If we assume that changes in HCN2 mRNA in pacemaking tissue are similar to those seen in the ventricular myocardium, the increase in HCN2 mRNA observed in our experiments can, at least in part, explain the intrinsic effect of thyroid hormone to increase heart rate.

As previously described and confirmed in our experiments, the effects of thyroid hormone on rat heart hypertrophy are apparent across the spectrum of thyroid states (Figure 3). For example, the H/B ratio continuously increases from the hypothyroid to the hyperthyroid state. This contrasts with the regulation of known thyroid hormone–responsive genes in rat myocardium in which the effect of thyroid hormone is primary seen in the transition from the hypothyroid to the euthyroid state. It also indicates that this physiological response to thyroid hormone might have a different molecular mechanism.

The significant increase in β-actin mRNA in myocardium after a single dose of T3 in hypothyroid rats was somewhat surprising (Figures 4 and 5). In the long-term hypothyroid or hyperthyroid rats (the first 2 experiments), differences in β-actin mRNA between groups were not significant (experiment 1) or had no consistent pattern (experiment 2). Thyroid hormone rapidly increases total cellular mRNA. For example, a 70% increase in total polyadenylated RNA synthesis in the liver occurs shortly after a single T3 dose. Similar studies of the short-term T3 effect on total or polyadenylated RNA in the hypothyroid or euthyroid heart have not been reported, but the increase in β-actin mRNA may reflect such generalized effects. In addition, the level of GAPDH mRNA also increased significantly 24 hours after T3 injection (P<0.001; Figure 4).

It should be noted that the effect of T3 on HCN2 and MHC mRNAs is corrected for changes in β-actin mRNA (Figure 5). Accordingly, if the effect on β-actin mRNA is specific, then the true increase in HCN2 is underestimated.

Opposite to what one would expect, the β-MHC mRNA level was slightly increased and the α-MHC level was slightly decreased (but not significantly) in the hearts of euthyroid rats given T3 for 8 days. This effect is present with or without normalization with β-actin. This cannot be explained by the lower serum T3 concentrations at the time of death because these levels were still above 12 nmol/L (about 12-fold the euthyroid level) at that time. We have no explanation for this deviation from the expected results. Nonetheless, the β-MHC mRNA was much higher in the hypothyroid hearts than in the euthyroid or hyperthyroid hearts.

In summary, we demonstrated that the levels of mRNA encoding the hyperpolarization-activated cyclic nucleotide-gated channel (HCN2) parallel thyroid state, which is consistent with a potential role for this gene in the thyroid hormone effect on heart rate.

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