Effect of Thyroid Status on Thin-Filament Ca\(^{2+}\) Regulation and Expression of Troponin I in Perinatal and Adult Rat Hearts

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There is evidence for the existence of developmental changes in expression of troponin I (TNI) in cardiac thin filaments; however, regulation of TNI expression has not been described. We tested whether thyroid state affects expression of TNI using neonatal and adult rats made hypothyroid by treatment with 6-\(n\)-propyl-2-thiouracil. Polyacrylamide gels of myofibrils from hearts of 7-, 14-, 21-, and 28-day-old animals indicated that both euthyroid and hypothyroid rats display a developmental shift toward the adult form of TNI. However, hypothyroid rats displayed a lower percentage of adult TNI at each age studied. When adult rats were made hypothyroid, the proportion of adult TNI decreased slightly. Thin-filament activity was determined from measurements of the effect of acidic pH on calcium activation of myofibrillar ATPase activity. Sensitivity to acidic pH was measured by the magnitude of shift in pCa\(_{50}\) (−log of half-maximally activating molar Ca\(^{2+}\)) between pH 7.0 and 6.5. Euthyroid rats displayed developmental increases in pH sensitivity. At 7, 14, and 28 days of development, shifts in pCa\(_{50}\) were 0.11, 0.38, and 0.43 units, respectively. Hypothyroid rats displayed less pH sensitivity with pCa\(_{50}\) shifts of 0.07, 0.21, and 0.15 units at 7, 14, and 28 days of development. Adult hypothyroid rats displayed a 0.38-unit shift in pCa\(_{50}\), whereas euthyroid adults displayed a 0.44-unit shift. Our results indicate that pH sensitivity and expression of cardiac TNI are influenced by developmental stage and hormonal status. (Circulation Research 1990;67:344–351)

Thyroid state is an important determinant of cardiac function.\(^1\) Chronic increases in thyroid hormone levels lead to cardiac hypertrophy, with increases in cardiac output, heart rate,\(^2\) and electrical excitability.\(^3\) Although these changes are undoubtedly related to changes in the peripheral vascular system and metabolic demand, there are also effects of thyroid state on the intrinsic activity of cardiac myocytes. These changes in activity are associated with a remodeling process expressed as shifts in protein isoform populations. A shift in the population of myosin heavy-chain isoforms with changes in thyroid state is the best characterized remodeling process.\(^1\) Cardiac ventricular myosin heavy chain is expressed as three isoforms—V\(_1\), V\(_2\), and V\(_3\)—consisting of homodimers and heterodimers of \(\alpha\) and \(\beta\) forms of heavy chain, which are themselves products of a multigene myosin family. The population of isoforms in the euthyroid state varies among different species,\(^4\) but in general, hyperthyroidism activates the \(\alpha\) myosin heavy-chain gene and represses the \(\beta\) myosin heavy-chain gene, resulting in a shift of myosin heavy-chain population to one in which V\(_1\) predominates. V\(_3\) is predominant in the hypothyroid state. There are also varying increases in myosin heavy-chain isoform population during cardiac development, and these changes have been shown to correlate with developmental changes in thyroid hormone levels.\(^5\)

An important question is whether the isotype population of other myofibrillar proteins is regulated by thyroid state. We have been particularly interested in the thin-filament protein troponin I (TNI), the inhibitory component of the troponin complex that is expressed as a tissue-specific variant. Previous studies indicated that TNI exists as an isotype population that is developmentally regulated.\(^6\)–\(^9\) Perinatal rat hearts demonstrate developmental changes in TNI expression,\(^8\)\(^,\)\(^9\) and these changes have been shown to be associated with a relative decrease in the inhibition of submaximal Ca\(^{2+}\)-activated myofibrillar activity by acidic pH.\(^7\) The alteration in sensitivity of myofibrillar Ca\(^{2+}\) activation to acidic pH appears to

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provide a functional correlate of expression of the adult form of TNI.

In the present study, we examined the effects of thyroid status on properties of the cardiac thin-filament proteins in the developing and adult rat heart. Our results provide evidence that the developmental expression of TNI and correlated effects of acidic pH on myofibrillar Ca\(^{2+}\) activation are altered by changes in thyroid state.

**Methods**

**Experimental Animals**

Sprague-Dawley rats were killed by either pentobarbital overdose or cervical dislocation. Euthyroid adults and pups received no treatment. To produce hypothyroid rat pups, we gave pregnant rats drinking water containing 0.06% 6-n-propyl-2-thiouracil (PTU) from 21 days gestation to 28 days postpartum. Litters were evened to 10 pups per dam, and an equal number of pups per dam were maintained throughout the experiment. We made adult (80-day-old) rats hypothyroid by giving them drinking water containing 0.06% PTU for 6 weeks. Adults were killed after 6 weeks of treatment, and pups were killed at 7, 14, 21, and 28 days postpartum. In some studies, hypothyroidism was reversed by treatment of hypothyroid pups with 150 μg/kg 3,3',5-triiodothyronine (T₃) every 48 hours from 21 to 28 days postpartum.

We used several methods to determine the effectiveness of PTU treatment to produce hypothyroid rats. For adult (120-day-old) rats, heart rates were obtained by electrocardiographic measurements of conscious rats. After 6 weeks of no treatment or treatment with PTU, heart rate for control rats was 450±30 beats/min, whereas the heart rate for hypothyroid rats was significantly reduced to 332±51 beats/min. For all rats, body and heart weights were measured and heart/body weight ratios calculated (Table 1). At the time of death, blood serum was collected for assay of the thyroid hormone thyroxine (T₄). Thyroid hormone levels increased with development of euthyroid rats but were not detectable in hypothyroid rats.

**Preparations**

Myofibrils were prepared essentially as described by Pagani and Solaro. Hearts were removed and immediately placed in ice-cold normal saline. Atria and blood vessels were trimmed away, and the hearts were minced and homogenized in 10 vol of standard buffer (2 mM MgCl₂, 60 mM KCl, and 30 mM imidazole, pH 7.0) in a Teflon-glass Thomas tissue grinder. The rest of the isolation procedure followed that described by Pagani and Solaro except that the proteolytic enzyme inhibitors (5 μg/ml leupeptin, 5 μg/ml pepstatin, and 2 mM phenylmethylsulfonyl fluoride) were added to all solutions. Protein concentration was determined by the method of Lowry et al.

Antibodies to the adult form of TNI were raised by injecting rabbits with the protein excised from polyacrylamide gels. The band used for antibody generation was identified as TNI by its Ca\(^{2+}\)-dependent removal from purified myofibrils when mixed with Sepharose–troponin C (TNC), its ability to act as a substrate for cyclic AMP–dependent protein kinase, and its comigration with purified TNI. To test for specific binding to cardiac TNI, antisera binding was tested against nitrocellulose blots of neonatal and adult rat myofibrils. Antisera demonstrated reactivity against adult cardiac TNI as well as two proteins that migrate just above adult TNI with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). These two proteins with slower migration will be referred to as thin-filament–related proteins (TFPs) because of their copurification with thin filaments.

**Procedures**

We determined the activity of adult and neonatal myofibrils from measurements of Ca\(^{2+}\)-stimulated MgATP hydrolysis by myofibrillar actomyosin as previously described. Measurements of euthyroid and hypothyroid myofibrils at pH 7.0 and 6.5 were done in parallel. Incubation conditions were 30°C, 20 mM KCl, 60 mM imidazole, 2 mM MgCl₂, 5 mM Na₂ATP, 1 mM EGTA, 0.2 mg/ml myofibrillar protein, and variable total CaCl₂ concentrations to produce pCa 7.0–4.75 as previously described. The incubation solutions were adjusted to pH 7.0 or 6.5. Reactions were initiated by addition of ATP and stopped with ice-cold 10% trichloroacetic acid. Precipitated protein was removed by centrifugation, and the supernatant fraction was assayed for inorganic phosphate (P) by the method of Carter and Karl. Myofibrillar ATPase activity–pCa relations were fitted by using a form of the A.V. Hill equation:

\[
\text{percent ATPase} = \left(\frac{[\text{Ca}^{2+}]}{[\text{K}+\text{Ca}^{2+}]^n}\right) \times 100
\]
where \([Ca^{2+}]\) is the free calcium concentration, \(K\) is a compound association constant, and \(n\) is the Hill coefficient. \(K\) and \(n\) were obtained by regression of the linearized form of the Hill equation.

Profiles of myofibrillar proteins were determined using SDS-PAGE. Myofibrils of 7-, 14-, 21-, and 28-day-old rats were centrifuged, and the pellet was resuspended in 10 vol of relaxing solution (10 mM EGTA, 2 mM Mg\(^{2+}\), 5 mM MgATP, 12 mM creatine phosphate, 0.04 mg/ml creatine phosphokinase, 60 mM imidazole, pH 7.0, and 1% Triton X-100 and proteolytic enzyme inhibitors) for 30 minutes at 2° C. Myofibrils were then centrifuged and resuspended in relaxing solution without Triton, centrifuged again, and resuspended in gel sample buffer. SDS-PAGE was performed by the procedure of Laemmlli, and gels were run in duplicate. One gel was stained with 0.1% Coomassie blue for visualization of myofibrillar proteins, and the relative amount of each protein was determined by densitometric scans using an Ultra-scan XL (Pharmacia LKB Biotechnology, Piscataway, N.J.). The relative amount of TNI was determined in relation to tropomyosin or the TFps. Percent adult TNI was obtained by dividing the area of the TNI band by the total area (TNI+TFps or TNI+tropomyosin). Because percent data fit a binomial rather than a normal distribution, percent of adult form was converted using the arcsine transformation, \(p'=\text{arcsin} \frac{p}{2}\), for statistical analysis. The duplicate gel was transferred electrophoretically to nitrocellulose as described by Towbin et al for immunoblot analysis.

**Results**

**Thin-Filament Properties of Adult Myofibrils**

We first present results of experiments with adult rats in which we measured the effect of hypothyroidism on the expression of adult TNI and on the pH sensitivity of myofibrillar Ca\(^{2+}\) activation. As illustrated in Figure 1 (top panel), the activation of ATPase activity by Ca\(^{2+}\) at pH 7.0 or 6.5 was similar for myofibrils from hypothyroid and euthyroid adult rat hearts. As expected from the relative increase in the fraction of \(V_1\) in the myosin isoform population, hypothroid myofibrils had an reduced maximum ATPase activity. To more clearly show the effect of acidic pH on Ca\(^{2+}\) activation, data from Figure 1 (top panel) were normalized to maximum ATPase activity for hypothyroid (Figure 1, middle panel) and euthyroid (Figure 1, bottom panel) adult myofibrils. With a decrease in pH from 7.0 to 6.5, the half-maximally activating Ca\(^{2+}\) concentration (\(pCa_{50}\)) was shifted to the right by 0.44 units in euthyroid and by 0.38 units in hypothroid adult myofibrils. This difference was not, however, statistically significant.

When isolated cardiac myofibrils were analyzed by SDS-PAGE, both euthyroid and hypothyroid rats contained a predominance of the adult form of cardiac TNI. No significant differences in expression of adult TNI were observed between euthyroid and hypothyroid rats.

**ATPase Activity and Troponin I Expression in Neonatal Myofibrils**

In a second set of experiments, we measured effects of thyroid state on TNI expression during
neonatal development of the rat heart. Pilot studies indicated that significant shifts in TNI isofrom population and changes in acidic pH sensitivity of Ca\(^{2+}\) activation of the neonatal myofibrils could be measured in euthyroid hearts between 7 and 28 days. We therefore studied this age range in matched groups of hypothyroid and euthyroid rat pups. Figure 2 (top panel) shows results of measurements of ATPase activity--pCa relations at pH 7.0 and 6.5 for myofibrils prepared from 7-day-old euthyroid and hypothyroid hearts. Maximum ATPase activities for the euthyroid neonatal myofibrils are lower than those of the adult preparations, as expected from the preponderance of Vn isomyosin at this age. Hypothyroidism was associated with a further reduction in the maximum myofibrillar ATPase activity in the 7-day-old rats. An important feature of the data in Figure 2 (top panel) is that regardless of thyroid state, a reduction in pH had a smaller effect on pCa\(_{50}\) for neonatal myofibrillar activation in comparison with the adult (Figure 1). This is illustrated more clearly in Figure 2, in which we replotted the ATPase activity for hypothyroid (Figure 2, middle panel) and euthyroid (Figure 2, bottom panel) myofibrils as percent of maximum activity at each pH. Although the reduction in pH caused a greater rightward shift in pCa\(_{50}\) for 7-day-old controls (0.11 units) in comparison with hypothyroid preparations (0.07 units), this difference was not statistically significant.

Figure 3 (top panel) shows results of ATPase measurements on 14-day-old euthyroid and hypothyroid rat myofibrils. Maximum myofibrillar ATPase activity of 14-day-old controls at pH 7.0 was significantly greater than maximum ATPase activity of the hypothyroid myofibrils. At 14 days of neonatal developement, hypothyroidism was associated with a significant decrease in the sensitivity of Ca\(^{2+}\) activation to acidic pH. This is illustrated in Figure 3 (middle and bottom panels), which shows normalized ATPase-pCa curves for 14-day-old myofibrils. With a reduction in pH from 7.0 to 6.5, the shift in pCa\(_{50}\) was 0.38 units for euthyroid 14-day myofibrils and 0.21 for hypothyroid myofibrils. This difference was statistically significant (p<0.05) as determined by analysis of variance (ANOVA).

At 28 days of age, hypothyroidism had the greatest effect on maximum ATPase activity and sensitivity of Ca\(^{2+}\) activation to acidic pH (Figure 4, top panel). Maximum ATPase activity of controls was significantly greater than maximum activity of hypothyroid myofibrils. Normalized ATPase-pCa curves for 28-day-old myofibrils are shown in Figure 4 (middle and bottom panels). Euthyroid myofibrils showed a 0.43-unit shift in pCa\(_{50}\) with acidic pH, and hypothyroid myofibrils showed a 0.15-unit shift in pCa\(_{50}\). This difference in pH sensitivity was highly significant (p<0.025).

Figure 5 summarizes the age dependence of the effect of acidic pH on myofibrillar Ca\(^{2+}\)-activated ATPase activity in neonatal myofibrils prepared from euthyroid and hypothyroid rat hearts. For controls, there was an age-dependent increase in pH sensitivity. For hypothyroid myofibrils, the pH sensitivity increased between 7 and 14 days of age, followed by a decrease in pH sensitivity between 14 and 28 days of age. When pCa\(_{50}\) shifts from all hypothyroid
FIGURE 3. Comparison of the effect of acidic pH on myofibrillar ATPase activity for hypothyroid and euthyroid 14-day-old rats (n=5). ●, Euthyroid rats, pH 7.0; ○, euthyroid rats, pH 6.5; ●, hypothyroid rats, pH 7.0; ○, hypothyroid rats, pH 6.5. Top panel: ATPase activity vs. pCa. Pi, inorganic phosphate. Middle panel: Percent maximum ATPase activity vs. pCa for hypothyroid rats. Shift in the half-maximally activating Ca\(^{2+}\) concentration (pCa\(_{50}\)) from pH 7.0 to 6.5, 0.21 units. Bottom panel: Percent maximum ATPase activity vs. pCa for euthyroid rats. Shift in pCa\(_{50}\) from pH 7.0 to 6.5, 0.38 units. The difference in shift in pCa\(_{50}\) was statistically significant (p<0.05).

FIGURE 4. Comparison of the effect of acidic pH on myofibrillar ATPase activity for hypothyroid and euthyroid 28-day-old rats (n=5). ●, Euthyroid rats, pH 7.0; ○, euthyroid rats, pH 6.5; ●, hypothyroid rats, pH 7.0; ○, hypothyroid rats, pH 6.5. Top panel: ATPase activity vs. pCa. Pi, inorganic phosphate. Middle panel: Percent maximum ATPase activity vs. pCa for hypothyroid rats. Shift in the half-maximally activating Ca\(^{2+}\) concentration (pCa\(_{50}\)) from pH 7.0 to 6.5, 0.15 units. Bottom panel: Percent maximum ATPase activity vs. pCa for euthyroid rats. Shift in pCa\(_{50}\) from pH 7.0 to 6.5, 0.43 units. The difference in shift in pCa\(_{50}\) was highly significant (p<0.025).

(7–28-day-old) pups were compared with those of all euthyroid pups using ANOVA, the differences in pCa\(_{50}\) shifts were statistically significant (p<0.05).

Figure 6 shows analysis of neonatal control and hypothyroid heart myofibrils of 7–28-day-old rats by SDS-PAGE. The protein profiles were identical when compared in dephosphorylated preparations and preparations incubated with cyclic AMP and cyclic AMP-dependent protein kinase. During development, expression of adult rat cardiac TNI increases. The relative amount of adult TNI at various ages in the neonatal period is illustrated in Figure 7. When
percent of adult TNI for all of the hypothyroid rats (7–28-day-old) was compared with that of all of the euthyroid rats (7–28-day-old) using ANOVA, this difference was statistically significant (p<0.05). When hypothyroid and euthyroid rats were compared with each other at individual ages using ANOVA, the difference between 21-day-old hypothyroid and euthyroid rats was significant (p<0.05), and the difference between 28-day-old hypothyroid and euthyroid rats was significant (p<0.025). These results were the same whether the amount of TNI was expressed in relation to tropomyosin or to the TFPs. The difference in abundance of adult TNI in euthyroid and hypothyroid hearts at 28 days was associated with a difference in effect of acidic pH on myofilament Ca\(^{2+}\) activation (Figure 5). An exception to this trend was a lack of significant difference in the relative amount of adult cardiac TNI expressed in euthyroid and hypothyroid rat pups at day 14, despite a significant difference in effect of acidic pH (Figure 5).

To test if the effects of PTU on expression of TNI isoforms could be reversed by treatment with thyroid hormones, a group of hypothyroid rats was treated with T\(_3\) from 21 to 28 days of age. Figure 8 shows the percent of adult TNI in these preparations obtained from scans of two SDS-PAGE gels. It can be seen that the hypothyroid pups treated with T\(_3\) have a greater percentage of adult TNI than the hypothyroid pups, which did not receive T\(_3\). The difference between the PTU-treated (hypothyroid) rats and the PTU+T\(_3\)-treated rats was statistically significant.
Moreover, we have shown cross-react muscle of TNI. These investigators also showed that with development, a variant of TNI was expressed that shared properties of immunoreactivity and relative mobility with slow muscle TNI. The polyclonal antibody used in our study indicated a decrease in expression of adult TNI but did not cross-react with slow or fast skeletal muscle TNI. Our antibody did, however, cross-react with proteins that we have termed TFPs. Moreover, we have shown in previous studies that the TFPs bind tightly to the thin filament. Yet we do not have definitive evidence as to the identity of the TFPs, although they share some properties of TNI. In addition, it is apparent that whatever neonatal forms of TNI are present, they are able to act together with TNC, troponin T (TNT), and other thin-filament proteins to confer Ca\(^{2+}\) sensitivity to myofilaments. In previous studies, myofilaments from 1-day neonatal rat ventricles, which contain only 5–10% adult TNI, showed force and ATPase rate Ca\(^{2+}\) sensitivity essentially the same as that of the adult.

There is indirect evidence that differences in pH and Ca\(^{2+}\) sensitivity between adult and neonatal heart myofilaments are largely, if not entirely, due to shifts in the isoform population of TNI. First, our past studies showed that neither adult nor neonatal heart preparations demonstrate the presence of thick-filament–related Ca\(^{2+}\) activation. Moreover, experiments with reconstituted preparations showed that the effect of acidic pH on cardiac myofilaments is a thin-filament–related phenomenon. These experiments were done with preparations containing the same thick filaments derived from skeletal muscle but with either adult or neonatal thin filaments. Both preparations showed the same differential sensitivity to acidic pH as the native myofibrils. Thus, although thick-filament proteins change with development, shifts in isoforms of thick-filament proteins do not appear important with regard to Ca\(^{2+}\) activation per se or to the effect of acidic pH on cardiac myofibrillar Ca\(^{2+}\) activation. Evidence suggests that the change in thin filaments that is responsible for the differential sensitivity to acidic pH is a shift in TNI isoform population and not other troponin components. First, although there are direct effects of acidic pH on Ca\(^{2+}\) binding to TNC, there is no evidence for the existence of TNC isoforms in developing heart. Second, although there are shifts in TNI isoform population with development in the rat heart, the isoform population of TNT is unaffected by thyroid state. Moreover, the inhibition of acidic pH on Ca\(^{2+}\) binding to regulatory sites on cardiac and skeletal TNC was increased about 10-fold, whether in the TNI–TNC complex or in the TNI–TNC–TNT complex. The mechanism for this increase in effect of acidic pH appears to be a reduction in the affinity of TNI for TNC. This is important in that addition of TNI to TNC alters the affinity of TNC by 10-fold. The importance of the role of isoform population of TNI is also indicated by the results presented here.

What is the functional significance of the shift in TNI isoform population in the intact heart? Prenatal mammalian hearts function under different intracellular and extracellular environmental and loading conditions than the adult heart, and it is not surprising that fetal forms of regulatory proteins might confer differences in myofilament Ca\(^{2+}\) regulation. Indeed, compared with the adult heart, neonatal rat hearts show differences in sensitivity of force development to changes in extracellular Ca\(^{2+}\) and pH.
That this might be due in part to shifts in TNI isoform population and changes in myofilament response to Ca\(^{2+}\) was shown in studies by Solaro et al\(^7\) of intact muscle preparations microinjected with the bioluminescent protein aequorin for determination of intracellular Ca\(^{2+}\) transients. Neonatal rat heart preparations responded to hypercapnic acidosis with a smaller initial drop in force than was the case with the adult hearts. The acidosis had either little effect or actually increased the peak of the Ca\(^{2+}\) transient, and thus, the difference is likely to be due to a difference in myofilament Ca\(^{2+}\) sensitivity. This was confirmed in vitro by treating the same preparations with detergent to produce skinned-fiber preparations. At pH 7.0, the pCa–force relation of skinned fibers from 1-day neonates was shifted to the left of the relation for adults, and a drop to pH 6.5 caused a bigger rightward shift in the adult skinned fibers than in the neonatal fibers.

In summary, the results of this study indicate that throughout development, thyroid status affects both the expression of adult rat heart TNI and the pH sensitivity of myofibrillar Ca\(^{2+}\) activation. In the normal euthyroid rat, expression of the adult form of TNI and myofibrillar sensitivity to acidic pH increases dramatically during early postnatal development. Chronic hypothyroidism, however, delays expression of these adult characteristics.

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References


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