Discordant Regulation of Isoforms of Na,K-ATPase and Myosin Heavy Chain in the Hypothyroid Postnatal Rat Heart and Skeletal Muscle*

Kathleen J. Swadeiner‡§, Kevin M. McGrail‡**, and Ban-An Khaw**

(Received for publication, July 1, 1991)

During postnatal life, many contractile and electrophysiological properties of the rat heart undergo changes. Among the changes is a switch in the expression of Na,K-ATPase catalytic subunit isoforms. Thyroid hormone has been postulated to play an important role in the postnatal transformation of the heart, and its effect on myosin heavy chain isoform gene transcription is well documented. To test whether it controls Na,K-ATPase gene switching in vivo, we made neonatal rats hypothyroid by maternal treatment with methimazole. The expression of Na,K-ATPase catalytic subunit isoforms in cardiac and skeletal muscle membranes was measured with specific antibodies at time points from birth to 4 weeks of age. Postnatal changes in Na,K-ATPase isoform expression in cardiac ventricle and hind limb skeletal muscle were similar in control and hypothyroid animals. In the same hypothyroid animals, the postnatal switch from the V3 (β) isoform of myosin heavy chain to the V1 (α) isoform was blocked. The conclusion is that thyroid hormone may have a modulatory role in Na,K-ATPase gene expression, but it is not the developmental signal that dominates gene switching.

The Na,K-ATPase\(^1\) is the enzyme responsible for active transport of Na\(^+\) and K\(^+\) across the plasma membrane. In excitable cells, it establishes the ion gradients that are utilized for electrical activity, and in cardiac muscle it provides the electrochemical Na\(^+\) gradient required for Ca\(^2+\) extrusion via Na\(^+-\)Ca\(^2+\) exchange. It has two types of subunits: a catalytic subunit (α) that spans the membrane multiple times and a glycoprotein subunit (β) that spans the membrane once and is required for assembly and insertion of the α subunit into the plasma membrane. The Na,K-ATPase is the only proven receptor for the cardiac glycosides, a class of drugs with inotropic action in the heart. The cardiac glycoside-binding site resides on the α subunit. Multiple forms of the α subunit (α1, α2, and α3) are known (reviewed in Refs. 1 and 2). In the rat, these have different affinities for cardiac glycosides, resulting in biphasic inotropic responses in the heart. The control of the expression of the Na,K-ATPase isoforms is thus important for understanding the role of active ion transport in the regulation of contractility.

Postnatal maturation is characterized by several important changes in the properties of cardiac muscle (3). In the rat, the action potential plateau shortens considerably, reflecting changes in the expression of ion channels. Contractility changes as a consequence of a switch between the slow (V3 or β) and fast (V1 or α) isoforms of myosin. Autonomic innervation arrives and becomes active during the second week of life. Systemic vascular resistance increases markedly. Changes have been detected in cardiac adrenergic receptors, G proteins, troponin isoforms, and a variety of other proteins. During this time, rat pups open their eyes, and are weaned by 3 weeks of age. The expression of the α1 isoform of the Na,K-ATPase remains relatively constant during postnatal maturation (4, 5). The α3 isoform is expressed in neonatal rat hearts (6, 7), but its expression declines during the postnatal period. In a reciprocal fashion, the α2 isoform increases, replacing α3 in the young adult heart (5, 7). Both α2 and α3 have high affinity for cardiac glycosides (8) and make up about 25% of the total Na,K-ATPase in crude sarcolemma fractions from either young adult or neonatal heart (5). The physiological consequence of the switch between α3 and α2 is not known.

In rat skeletal muscle, there are two isoforms of the Na,K-ATPase with different cardiac glycoside affinities (9) shown to be α2 and α3 (10–12). During development, the α2 mRNA undergoes changes in level of expression (7, 11) that parallel the induction of α2 seen in a differentiating myogenic cell line (13).

Thyroid hormone increases in serum during the postnatal period (14), and in experimental conditions it can mimic some of the phenotypic changes associated with postnatal maturation of the heart and skeletal muscle. Na,K-ATPase isoforms have been reported to be selectively regulated by thyroid hormone in adult rat heart and in skeletal muscle (15–17). Here, we examined the effect of hypothyroid status during postnatal maturation on the relative levels of expression of α1, α2, and α3 Na,K-ATPase isoforms in cardiac ventricle and of α1 and α2 in hind limb skeletal muscle. In the same animals, we documented the severity of hypothyroidism by...
the alteration in expression of cardiac myosin heavy chain isoforms.

MATERIALS AND METHODS

Animals—Pregnant Wistar rats were obtained from Charles River Laboratories, Inc., Wilmington, MA, and maintained on normal rat chow. The drinking water was supplemented with 200 mg/100 ml methimazole (Sigma) from day 15–16 of gestation, according to the protocol of Silva and Larsen (18). Methimazole passes to the pups both transplacentally and in the milk, suppressing the synthesis of thyroxine. Rats were sacrificed by decapitation (neonates) or CO2 gas (older pups), and samples of blood were collected for thyroid hormone assay. Serum thyroxine was measured by the method of Larsen (19); assays were generously performed by Dr. J. L. Leonard, University of Massachusetts Medical Center, Worcester, MA.

Sample Preparation—Cardiac ventricle and hind limb skeletal muscle were dissected from animals sacrificed at the day of birth, and at 1, 2, 3, and 4 weeks of age. To avoid the losses of Na,K-ATPase usually attending muscle plasma membrane preparation, all experiments were performed on very crude particulate preparations. Tissue was minced in 320 mM sucrose, 20 mM Tris, 1 mM EDTA (Tris salt pH 7.2, and homogenized with 50 strokes in a motor-driven Teflon-glass homogenizer. The particulate fraction was obtained by centrifuging the homogenate at 40,000 rpm for 30 min in a Beckman Ti-70 rotor. The pellet, which contains the actomyosin complex, as well as membrane fractions, was resuspended in the same buffer and stored at −70 °C.

Samples of each particulate preparation were dissolved in sodium dodecyl sulfate in volumes large enough to be distributed to several gel wells. Each sample was then quantitatively divided among different gel lanes for staining by different antibodies. This made it possible to determine the ratio of stain for different isoforms (i.e. the Na,K-ATPase α2 to α1 ratio or the myosin heavy chain β to total myosin ratio) within each master sample. This solved the technical difficulty of accurately pipetting myosin-containing samples, since myosin becomes insoluble after freezing and thawing in low ionic strength buffer. Since the different antibodies had different intrinsic sensitivities, the fraction of the master sample used for each antibody was sometimes adjusted, i.e. twice as much protein from skeletal muscle samples was used to detect Na,K-ATPase α1 as α2 (300 versus 150 μg of protein) and five times as much protein was used to detect myosin β as total myosin (50 versus 10 μg). Large amounts of total protein had to be added to detect the Na,K-ATPase subunits because of the excess of myosin. Gels were used that were 1.5 mm thick to accommodate the myosin without overloading artifacts.

Gel Electrophoresis and Immunoblotting—Methods for gel electrophoresis and blotting to nitrocellulose were as described by Felsenfeld (8). For detection of myosin isoforms, two monoclonal antibodies were used. Antibody R11-D10, (the generous gift of R. Mercer, Washington University, St. Louis). For detection of myosin isoforms, two monoclonal antibodies were used. R11-D10 is specific for V3 (β), and 2G4-2D7 recognizes both V1 and V3 isomyosins (21), and 2G4-2D7 recognizes both V1 and V3 isomyosins (21, 22). Myosin was stained with antibody 2G4-2D7 and of V3 myosin in fetal rat is well established; expression of V3 myosin in neonates, almost absent in normal animals of 4 weeks of age, and even higher in hypothyroid animals than in normal neonates. Fig. 2 shows the results of densitometric scanning. Antibody 2G4-2D7 gave much more stain/μg of protein than antibody R11-D10, but this is an antibody-specific difference. The quantitative predominance of V3 myosin in fetal rat is well established; expression of V1 myosin mRNA and protein begins before birth, and the ratio of V1

RESULTS

Hypothyroid Status during Postnatal Development—Maternal administration of methimazole was used to reduce thyroid hormone production. Silva and Larsen (18), using the same protocol, reported serum thyroxine levels approximately 10% of normal, accompanied by typical signs of functional hypothyroidism. Serum triiodothyronine levels were variable, averaging 50% of normal at 2 weeks of age, due to adaptive mechanisms that increase the deiodination of thyroxine in brain. In the present experiments, reductions in serum thyroxine levels also averaged 50% (Table I); triiodothyronine was not measured. By several criteria, the animals appeared hypothyroid. Their weight gain was delayed; they did not groom themselves normally; and there was a pronounced motor tremor and weakness.

Myosin Isoform Changes in the Heart—A similar experimental protocol utilizing propylthiouracil instead of methimazole resulted in rats that expressed only the V3 (β) isoform of myosin heavy chain at 4 weeks of age, instead of progressively changing from the V3 to the V1 (α) isoform (27). We were able to reproduce this phenomenon. Fig. 1 shows the detection of total myosin with antibody 2G4-2D7 and of V3 myosin alone with antibody R11-D10. The expression of V3 myosin was high in neonates, almost absent in normal animals of 4 weeks of age, and even higher in hypothyroid animals than in normal neonates. Fig. 2 shows the results of densitometric scanning. Antibody 2G4-2D7 gave much more stain/μg of protein than antibody R11-D10, but this is an antibody-specific difference. The quantitative predominance of V3 myosin in fetal rat is well established; expression of V1 myosin mRNA and protein begins before birth, and the ratio of V1


table 1

<table>
<thead>
<tr>
<th>Age in weeks</th>
<th>Control</th>
<th>Hypothyroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>26 ± 2  (2)</td>
<td>ND (4)</td>
</tr>
<tr>
<td>1</td>
<td>67 ± 2  (2)</td>
<td>ND (4)</td>
</tr>
<tr>
<td>2</td>
<td>38 ± 2  (2)</td>
<td>14 ± 9 (4)</td>
</tr>
<tr>
<td>3</td>
<td>31 ± 5  (2)</td>
<td>4 ± 3 (3)</td>
</tr>
<tr>
<td>4</td>
<td>37 ± 4  (2)</td>
<td>ND (3)</td>
</tr>
<tr>
<td>Average</td>
<td>40 ± 15 (10)</td>
<td>ND (3)</td>
</tr>
</tbody>
</table>

FIG. 1. Monoclonal antibody detection of the V3 (β) myosin isoform. Crude particulate preparations containing myosin heavy chains were electrophoresed in sodium dodecyl sulfate on 5% polyacrylamide gels and transferred to nitrocellulose. Two identical blots were prepared and stained with different antibodies. In the left blot, antibody R11-D10 stained the 3 myosin isoform that is present in newborn cardiac ventricle (0), virtually absent at 4 weeks of age in control animals (4), and present at elevated levels at 4 weeks in hypothyroid animals (4H). In the right blot, antibody 2G4-2D7 stained total myosin in all three lanes; this antibody is intrinsically more sensitive. The molecular weight markers (Mr) were from Bio-Rad (prestained, high molecular weight range); the indicated (relative) molecular weights are in kilodaltons. Myosin (presumably derived from skeletal muscle) was also a molecular weight marker. It (and some evident breakdown products) were stained by the antibody 2G4-2D7.
Control of Na,K-ATPase and Myosin Isoform Expression

![Graph](image)

**Fig. 2.** Myosin heavy chain β (V3) isofrom changes during postnatal life. Electrophoretic blots of myosin from control and hypothyroid rat hearts were stained with the anti-myosin antibodies illustrated in Fig. 1 and quantitated by scanning densitometry. The proportion of myosin heavy chain β-specific stain decreased progressively in the controls (■), whereas it was markedly elevated and remained so in the hypothyroids (○).

**Fig. 3.** Na,K-ATPase α subunit isofrom changes in the cardiac ventricle during postnatal life. Electrophoretic blots of crude particulate fractions from control and hypothyroid rat hearts were stained with Na,K-ATPase α1-, α2-, and α3-specific antibodies and scanned densitometrically. The data are expressed as α2/α1 or α3/α1 stain ratios, since α1 expression remained constant. ●, α2/α1, controls; □, α2/α1, hypothyroids; ○, α3/α1, controls; ●, α3/α1, hypothyroids. In both controls and hypothyroids, α3/α1 levels dropped and α2/α1 levels increased with comparable time courses. Representative immunoblots showing the changes in isoform stain in control animals have been published previously (5).

Na,K-ATPase and Myosin Isoform Expression

The data in Fig. 3 were pooled from densitometric scans of many different immunoblots, and because of the large number of samples, control and hypothyroid samples were generally not on the same blots. Although changes in staining intensity between samples on a given blot were easily quantitated, day-to-day differences in total staining intensity made it impossible to compare the final levels of isoform expression of control and hypothyroid samples. Additional immunoblots were prepared in which the levels of α2 and α1 expression and their ratios at 4 weeks of age could be compared directly. α1 levels, expressed per milligram of crude membrane protein, did not change significantly with age or thyroid status (data not shown). There was a reduction in the level of α2 expression in hypothyroid rat hearts amounting to about 30% of that in age-matched controls. The actual data (expressed as the ratio of staining intensity, α2/α1 +/- S.D.) were 0.524 +/- 0.03, n = 4, for normal animals, and 0.355 +/– 0.04, n = 6, for hypothyroid animals. Thus, a reduction in thyroid hormone does affect α2 expression quantitatively, although it does not prevent the developmental gene switch.

**Skeletal Muscle Na,K-ATPase**—Hind limb skeletal muscle was examined for the expression of α1 and α2 Na,K-ATPase isoforms at the same time points from birth to 4 weeks of age. An increase in level of expression of α2 was seen, accompanied by a gradual decline in the level of α1. Fig. 4 shows immunoblots of samples obtained from control animals. By 4 weeks of age in controls, the preponderance of stain for α2 resembled that reported previously for adult rat hind limb muscle (12).

For skeletal muscle, there were no convincing differences between control and hypothyroid animals. Quantitative detection of α1 was difficult because of its low abundance in this tissue (12). When multiple immunoblots were scanned (six separate blots for each antibody, five to six samples/time point), the pooled data suggested that the α2/α1 ratio in hypothyroid animals was about 3-fold higher in controls than in hypothyroids. This was due to comparable amounts of α2 stain but barely detectable α1 stain in some blots of control samples. When samples from control and hypothyroid 3- and 4-week-old animals were electrophoresed and stained on the same blots, however, no statistically significant difference was detected in the absolute level of either α1 or α2. The actual data (expressed as the ratio of staining intensity, α2/α1 +/- S.D.) were 2.44 +/- 0.43, n = 4, for normal animals and 1.94 +/– 0.62, n = 7, for hypothyroid animals. Consequently, the α2/α1 ratios presented in Fig. 5 (taken from the pooled data) were corrected for equivalent α1 levels. Skeletal muscle from both hypothyroid and control rats underwent changes in Na,K-ATPase isoform levels with the same time course.

**DISCUSSION**

**Myosin Heavy Chain Control by Thyroid Hormone**—Investigation of the expression of myosin heavy chain isoforms has been previously detected with two monoclonal antibodies (McK1 against α1; McR2 against α2) and a peptide-direced antisera against α3 (5). In normal animals, α1 was found to be relatively constant during postnatal maturation, whereas α3 declined and α2 increased. Fig. 3 shows the densitometric analysis of isoform expression in normal and hypothyroid animals. The data are expressed as ratios of stain for α3/α1 or α2/α1 to correct for any differences in sample loading. In contrast to the remarkable effect of low thyroid hormone levels seen for myosin isoforms in the same animals (Fig. 2), there was little effect on the progress or time course of the switch between α3 and α2 Na,K-ATPases.

The data in Fig. 3 were pooled from densitometric scans of many different immunoblots, and because of the large number of samples, control and hypothyroid samples were generally not on the same blots. Although changes in staining intensity between samples on a given blot were easily quantitated, day-to-day differences in total staining intensity made it impossible to compare the final levels of isoform expression of control and hypothyroid samples. Additional immunoblots were prepared in which the levels of α2 and α1 expression and their ratios at 4 weeks of age could be compared directly. α1 levels, expressed per milligram of crude membrane protein, did not change significantly with age or thyroid status (data not shown). There was a reduction in the level of α2 expression in hypothyroid rat hearts amounting to about 30% of that in age-matched controls. The actual data (expressed as the ratio of staining intensity, α2/α1 +/- S.D.) were 0.524 +/- 0.03, n = 4, for normal animals, and 0.355 +/– 0.04, n = 6, for hypothyroid animals. Thus, a reduction in thyroid hormone does affect α2 expression quantitatively, although it does not prevent the developmental gene switch.

**Skeletal Muscle Na,K-ATPase**—Hind limb skeletal muscle was examined for the expression of α1 and α2 Na,K-ATPase isoforms at the same time points from birth to 4 weeks of age. An increase in level of expression of α2 was seen, accompanied by a gradual decline in the level of α1. Fig. 4 shows immunoblots of samples obtained from control animals. By 4 weeks of age in controls, the preponderance of stain for α2 resembled that reported previously for adult rat hind limb muscle (12).

For skeletal muscle, there were no convincing differences between control and hypothyroid animals. Quantitative detection of α1 was difficult because of its low abundance in this tissue (12). When multiple immunoblots were scanned (six separate blots for each antibody, five to six samples/time point), the pooled data suggested that the α2/α1 ratio in hypothyroid animals was about 3-fold higher in controls than in hypothyroids. This was due to comparable amounts of α2 stain but barely detectable α1 stain in some blots of control samples. When samples from control and hypothyroid 3- and 4-week-old animals were electrophoresed and stained on the same blots, however, no statistically significant difference was detected in the absolute level of either α1 or α2. The actual data (expressed as the ratio of staining intensity, α2/α1 +/- S.D.) were 2.44 +/- 0.43, n = 4, for normal animals and 1.94 +/– 0.62, n = 7, for hypothyroid animals. Consequently, the α2/α1 ratios presented in Fig. 5 (taken from the pooled data) were corrected for equivalent α1 levels. Skeletal muscle from both hypothyroid and control rats underwent changes in Na,K-ATPase isoform levels with the same time course.
implicated thyroid hormone in the postnatal maturation of the heart (29). Characterization of the heavy chains revealed that there are two separate gene products whose expression varied according to the age of the animals. In the rat, the V3 (β) isoform was expressed in neonates, the V1 (α) isoform predominated in young adults, and a mixture was found in older adults (27, 28). During the postnatal period, making the animals hypothyroid prevented the switch from V3 to V1 (27). Serum levels of thyroid hormone change considerably during the postnatal period. Plasma levels of thyroxine start increasing at 5 days and peak between 12 and 20 days of life; triiodothyronine levels rise later (12 days) and peak at 20–28 days (14, 27, 30). The change in myosin isoform content does not quantitatively parallel changes in serum thyroid hormone levels, but it could be argued that it is a sensitive early indicator of those changes. It was ultimately shown that the transcription rates of myosin heavy chain genes fused to chloramphenicol acetyltransferase and transfected in fetal heart cells were affected by thyroid hormone (31), and cis-acting transcription elements have been described (32). In skeletal muscle, however, it has been demonstrated that the effects of thyroid hormone are very tissue-specific, exhibiting both positive and negative control of given myosin genes (33). Muscle-specific trans-acting factors are thought to be responsible for the differences. The present understanding is that both transcriptional and post-transcriptional events are subject to multiple and overlapping controls.

Na,K-ATPase Isoform Control by Thyroid Hormone—An increase in ion transport capacity accompanies the many metabolic enhancements produced by elevated levels of thyroid hormone. Biosynthesis of the Na,K-ATPase has long been believed to be under its control (34). Thyroidectomy decreases and administration of thyroid hormone to normal or hypothyroid animals increases cardiac Na,K-ATPase activity (35–39), accompanied by changes in sensitivity to digitalis toxicity (40). Similar effects are seen in skeletal muscle (41–43). Most reports have been in agreement that the increase is due to an increase in the number of pump sites without change in their enzymatic characteristics or ouabain affinity.

Several recent studies have documented that thyroid hormone has differential effects on Na,K-ATPase α isoform expression in heart and skeletal muscle. Haber and Loeb (17) showed in adult rat diaphragm that the Na,K-ATPase activity with high affinity for ouabain is affected much more than that with low affinity; the high affinity form is presumably α2, and the low affinity form, α1. Ng et al. (44) showed that two electrophoretically different α subunits in ferret heart were differentially affected: in this species, the higher affinity and slower migrating α(+) form was unaffected, and the α (presumably α1) form was increased by thyroid hormone administration in the heart, but not in the kidney. In the rat, α2 mRNA and protein was preferentially affected both in the heart and in skeletal muscle (15, 16). In the present experiments, quantitative changes were seen in the level of expression of α2 in the heart by 4 weeks of age, which confirmed the prior observations made on adult rat heart. Thyroid hormone thus appears to be a modulatory factor for Na,K-ATPase gene expression. The data do not indicate whether its effects are mediated through thyroid receptor action directly on Na,K-ATPase gene transcription rates, or whether the hormone acts through changes in ion concentrations or in other proteins.

In primary cultures of rat cardiac myocytes grown in defined medium, addition of thyroid hormone to the medium was sufficient to mimic the normal postnatal change in myosin isoforms (45). Recent experiments by Orlowski and Lingrel (4) showed that in defined medium without thyroid hormone, neonatal rat cardiac myocytes spontaneously lost the ability to express either α2 or α3 Na,K-ATPase isoforms. Addition of thyroid hormone induced the expression of the mRNAs for both α2 and α3. It was also shown that addition of dexamethasone to cardiac myocytes in defined culture medium induced α2 mRNA alone, and in combination with thyroid hormone, it induced α2 but suppressed the expression of α3 mRNA, mimicking the adult phenotype. The plausible suggestion was made that since these two hormones differentially regulate the expression of the α genes, they may be important physiological regulators in vivo (4).

The hypothesis tested here was whether during the postnatal period, similar gene transcription mechanisms specify the choice of Na,K-ATPase and myosin isoforms. Available data on the time courses of myosin and Na,K-ATPase isoform changes are not encouraging. Cardiac myosin isoform mRNA changes appear to precede Na,K-ATPase isoform mRNA changes significantly (7, 28), although it could be argued that quantitatively higher thyroid hormone levels are required for one set of genes than for the other, or that mRNA half-lives are significantly different. Plasma levels of corticosteroids, like thyroid hormone, change markedly during postnatal life (46), but their levels do not begin to rise until 12 or more days postnatal, peaking on day 24, which is after the switch in Na,K-ATPase isoform mRNA expression at 7–12 days (7). Enzymes known to be sensitive to corticosteroid induction (jejunal lactase and sucrase) rise immediately after.Serum corticosteroid levels peak 5 days postnatal.

When rats were made hypothyroid during the period of postnatal maturation, changes were seen in the expression of Na,K-ATPase isoforms in both cardiac muscle and skeletal muscle that occurred on schedule. This clearly indicates that other developmental or physiological signals dominate the choice of Na,K-ATPase isoform gene expression.

Acknowledgments—We would like to thank Heather Shutt, Sima Farahi, and Don Gibbons, Jr., for their excellent technical assistance. Generous help was provided by Dr. Robert Mercer (Washington University, St. Louis) who contributed an antiserum, and Dr. Jack L. Leonard (University of Massachusetts, Worcester) who performed thyroxine assays.

REFERENCES

Control of Na,K-ATPase and Myosin Isoform Expression

773


