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Developmental changes in regulation of the Na^+, K^+-ATPase $\alpha 3$ isoform by thyroid hormone in ferret heart

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Abstract

Ferret heart expresses the $\alpha 1$- as well as the $\alpha 3$-isoform of the Na^+, K^+-ATPase. We have shown previously that the $\alpha 3$ isoform is differentially upregulated during postnatal cardiac development and that in adult ferrets expression of $\alpha 3$ is not responsive to regulation by thyroid hormone (TH). Since developmental-stage dependent effects of TH have been reported previously, the present study examined whether effects of TH on expression of the Na^+, K^+-ATPase isoforms in ferret heart is modulated during development and possible mechanisms were examined. Ferrets of different age groups were treated with TH and the relative abundance of Na^+, K^+-ATPase isoforms in ferret myocardium was determined by immunoblotting. Thyroid hormone (T3; 50 μg/100 g body weight on 3 alternating days, s.c.) increased protein levels of the $\alpha 3$ isoform, but not that of $\alpha 1$ or $\beta 1$, in myocardium of 5-day-old and 3-week-old ferrets. By contrast, in myocardium of 6- and 8-week-old ferrets T3 failed to increase protein levels of $\alpha 1$ and $\alpha 3$. To determine whether elevated plasma levels of TH during development plays a role in the transition, mature ferrets were first made hypothyroid before TH treatment. In these hypothyroid ferrets expression of the $\alpha 3$ isoform remained unresponsive to TH (T4, 0.5 mg/kg for 7 days, s.c.). The transition from TH-responsive to TH-unresponsive appears to be isoform-specific because in skeletal muscle of 8-week-old ferrets and in hypothyroid ferrets the $\alpha 2$ isoform is upregulated by TH. Finally, there appears to be functional thyroid hormone receptors throughout development because in each age group TH effectively induced expression of α-MHC in the myocardium. In conclusion, these findings demonstrate that expression of $\alpha 3$ isoform in the myocardium of newborn ferret is responsive to TH; however, the responsiveness terminates between 3- and 6-weeks of age. Neither elevated endogenous TH level nor a lack of functional thyroid hormone receptor appears to be responsible for the transition from TH-responsive to TH-unresponsive. © 1997 Elsevier Science B.V.

Keywords: ATPase, Na^+/K^+-; Isoform; Thyroid hormone; Development; Western blot; (Ferret)

1. Introduction

The sodium pump, or Na^+, K^+-ATPase, maintains the electrochemical gradient of the cardiac cell and, hence, directly and indirectly modulates the electrical and contractile activities of the heart. Na^+, K^+-ATPase consists of a transmembrane catalytic α-subunit (MW = 110 000) and a β-subunit (MW = 35 000) which is glycosylated. Four different isoforms of the α-subunit, $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\alpha 4$ and three separate isoforms of the β-subunit, $\beta 1$, $\beta 2$ and $\beta 3$, have been cloned and sequenced [1–4].

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Expression of the Na^+, K^+-ATPase isoforms in the myocardium is regulated under a variety of physiological and pathological conditions [5–9], such as during postnatal development. In rat heart, $\alpha 1$-, $\alpha 3$- and $\beta 1$-mRNAs are expressed at birth, but that $\alpha 2$-mRNA replaces $\alpha 3$-mRNA between the first and second postnatal weeks [9]. The $\alpha 3$ isoform is usually undetectable in the myocardium of adult rat [10], except in the cardiac conduction system where the presence of $\alpha 3$-mRNA has been demonstrated [11,12]. Neonatal ferret heart, on the other hand, expresses predominately the $\alpha 1$ isoform and a very small amount of $\alpha 3$ isoform. The level of the $\alpha 3$ isoform increases markedly during cardiac development [8,13] such that in myocardium of mature ferrets there is about an equal abundance of $\alpha 1$ and $\alpha 3$ isoform. These studies demonstrated for the first time a developmental regulation of $\alpha 3$ in a myocardium which expresses this isoform throughout adulthood. An understanding of the regulation of this isoform may be clinically important because human heart expresses the $\alpha 3$ as well as the $\alpha 1$ and $\alpha 2$ isoforms [10,14,15].

Thyroid hormone is known to increase Na^+, K^+-ATPase activity in responsive tissues [16–19] and to differentially regulate Na^+, K^+-ATPase isoforms [6,20–22]; in general, the $\alpha 2$ isoform is more sensitive to regulation by thyroid hormone than the $\alpha 1$ isoform. We reported previously that in the myocardium of adult ferrets expression of the $\alpha 3$-isoform is not responsive to thyroid hormone [23]. However, it is possible that the thyroid hormone sensitivity of the Na^+, K^+-ATPase isoforms may be altered during development. In developing rat brain, Schmitt and McDonough [24] demonstrated that thyroid hormone upregulates the α ($\alpha 1$) and $\alpha+$ ($\alpha 2$ and/or $\alpha 3$) isoforms in brain of newborn rats until postnatal day 22; beyond this age, the isoforms are unresponsive to the effects of thyroid hormone. The mechanism(s) underlying such changes remains unclear.

In the present study, we examined the effects of thyroid hormone on the expression of the subunit isoforms in ferret heart from 5-days to 8-weeks of age. The results reveal that thyroid hormone selectively increases the level of $\alpha 3$ isoform in neonatal ferret heart; however, this effect of thyroid hormone is developmental stage-dependent. The time window when $\alpha 3$ changes from thyroid hormone-responsive to thyroid hormone-unresponsive was delineated and possible underlying mechanisms were examined.

2. Materials and methods

2.1. Animals and treatments

Mature male (8 weeks old) and dated-pregnant female ferrets were purchased from Marshall Farms (North Rose, NY). Ferrets 6 weeks old and less were obtained from litters delivered by pregnant females in our animal care facility. These studies were conducted with the approval of and under the guidelines established by the Animal Research Committee of the Milton S. Hershey Medical Center. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No 85-23, revised 1985).

To induce hyperthyroidism in developing ferrets, newborns of either sex were treated with T3 (50 μg/100 g body weight, s.c.) for three alternating days [25]. T3-treatment was initiated when ferrets were at 5 days of age and at 3-, 6- or 8-weeks of age. Age-matched, control ferrets were injected with diluent (0.5 mM NaOH/0.9% NaCl). The ferrets were killed 24 h after the last injection. Small ferrets (<200 g) were decapitated and trunk blood was collected. The larger ferrets were deeply anesthetized with an anesthetic cocktail (30 mg/kg ketamine, 2 mg/kg xylazine, 0.1 mg/kg atropine and 0.05 mg/kg acepromazine, s.c. or i.m.) and blood was collected by cardiac puncture. All hearts were removed, weighed and immediately frozen in liquid nitrogen. In some ferrets, skeletal muscle was isolated from the hind limbs.

To induce hypothyroidism in mature ferrets, male ferrets (8 weeks old) were given a low iodine diet (ICN Biomedicals, Costa Mesa, CA) and 0.5% sodium perchlorate/50 mg/l methimazole in their drinking water. Two weeks after initiating treatment, blood samples were drawn periodically from the sub-clavicle vein of anesthetized ferrets to determine thyroid hormone levels (data not shown). After 8 weeks of treatment, hypothyroid ferrets were then treated with either T4 (0.5 mg/kg, s.c., daily) or diluent (1 mM NaOH/10% ethanol) for 7 days [23]. Following T4-

treatment, the ferrets were deeply anesthetized, blood was collected by cardiac puncture and the hearts were removed. The left ventricle plus septum (LV) was dissected from the right ventricle and the tissues were weighed and immediately frozen in liquid nitrogen. Mixed hindlimb and temporalis skeletal muscles were also collected. All tissues were stored at −70°C until used.

2.2. Western blotting of Na^+, K^+-ATPase isoforms

Crude membrane preparations were derived from ferret tissues as previously described [8]. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad, Melville, NY). Total protein yields from T3-treated newborn ferrets were slightly but insignificantly elevated compared to those from untreated newborns. In hypothyroid and hyperthyroid mature ferrets, there was no difference in the yields of total protein (data not shown).

Subunits of Na^+, K^+-ATPase were resolved by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) according to the method of Laemmli [26] with slight modification as previously described [27]. For analysis of the α-subunit isoforms, equal amounts of crude membrane were electrophoresed in 5% acrylamide gels. For analysis of the β-subunit, equal amounts of crude membrane preparations were deglycosylated with *N*-Glycosidase F (Boehringer Mannheim, Indianapolis, IN) for 18 h at 37°C [28], incubated for 10 min at 60°C and electrophoresed in 7.5% acrylamide gels. Gels were electrophoretically transferred to Immobilon-P membrane (Millipore, Bedford, MA). Portions of some of the membranes were stained with coomassie blue or Colloidal Gold Total Protein Stain (Bio-Rad, Melville, NY) to verify equal loading and an efficient transfer of samples. The membranes were blocked in a Tris-buffered-saline solution (10 mM Tris, 150 mM NaCl, pH 7.4) containing 0.2% Tween-20 and subsequently incubated with the αN antibody. αN is an antibody specific for the α-subunit isoforms and stains the ferret isoforms equally well [8]. In one experiment anti-α2 antiserum (Upstate Biological, NY) was used to detect the α2 isoform in ferret skeletal muscle. To detect the β1 isoform, membranes were blocked in the same buffer containing 5% non-fat dry milk and then incubated with anti-β1 antiserum (Upstate Biological, Lake Placid, NY). The anti-α2 and anti-β1 antibodies have been shown to be specific for the ferret isoforms [8,29]. Bound antibodies were detected with ^{125}I-labeled goat anti-rabbit IgG (DuPont-New England Nuclear, Boston, MA). Blots were exposed to multiple X-ray films, for 16–48 h, to insure that autoradiographic signals were within the linear range of the film. For each subunit isoform, scanning densitometry was performed on autoradiograms using a Molecular Dynamics laser densitometer calibrated against a standard density scale (Sunnyvale, CA) and the Quantity One data analysis program (Protein Databases, Huntington Station, NY). Intensities of the bands are expressed in relative density units. Fig. 1 demonstrates that signal intensities of the subunit bands, up to 30 μg/lane, is a linear function of the amount of protein loaded.

2.3. Analysis of myosin heavy chain isoforms

For analysis of the myosin heavy chain (MHC) α- and β-isoforms, tissues homogenates were prepared as previously described [29] and the isoforms were resolved by gradient (4–9%) SDS-PAGE as described by Esser et al. [30]. Gels were stained with silver (Bio-Rad, Melville, NY) to visualize the isoforms and photographed.

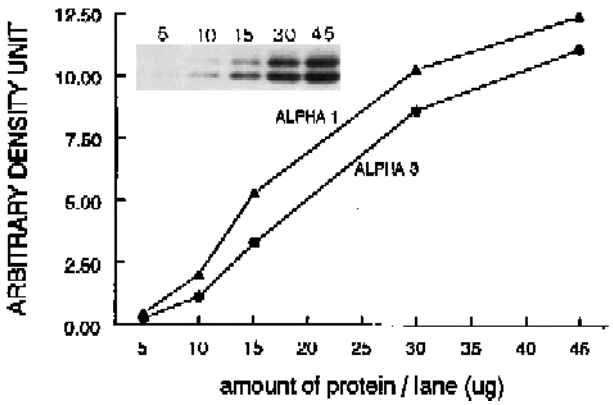

Fig. 1. Linearity of autoradiographic signals. Increasing concentrations (5, 10 15, 30 and 45 μg/lane) of a crude membrane preparation from ferret heart were subjects to SDS-PAGE in 5% acrylamide gel. Abundance of α1 and α3 isoforms was determined by Western blotting with αN and [^{125}I]-IgG, as primary and secondary antibodies, respectively, and the blot was exposed to X-ray file. Intensities of autoradiographic signals were determined for the detected bands, corresponding to the α1 and α3 isoforms, by density scanning.

2.4. Determination of serum thyroid hormone levels

GammaCoat [^{125}I]T3 and [^{125}I]T4 radioimmunoassay kits (INCSTAR Corporation, Stillwater MN) were utilized to quantitate serum levels of tri-iodothyronine (T3) and L-thyroxine (T4). The assays were performed according to the manufacturer's instructions.

2.5. Statistical analysis of data

Results are expressed as means ± standard error (SE). The Student's unpaired *t*-test was performed on non-replicating data. Two-way analysis of variance (ANOVA) with repeated measures was utilized to analyze multiple blots of the same experiment in order to increase the power of the statistical analysis.

3. Results

3.1. Effects of thyroid hormone on isoform expression in newborn ferrets

Newborn ferrets (5 days of age) were treated with a pharmacological dose of T3 (50 μg/100 gm body weight, s.c.) on 3 alternating days. Treated ferrets showed significant increases in cardiac growth; no change in body weight was evident between control and treated ferrets (Table 1). The ratio of heart weight/body weight increased 29% over control newborns. Serum T3 levels, measured in two treated newborns, increased almost 8-fold compared to two controls.

The abundance of α3 isoform increased more than 2-fold in the myocardium of T3-treated newborn ferrets, compared to controls (control = 0.89 ± 0.08, T3 = 2.01 ± 0.26, in arbitrary density units, $P < 0.001$ by ANOVA; Fig. 2). By contrast, abundances of the α1- and β1-isoforms were unaltered (α1: control = 2.50 ± 0.09, T3 = 2.24 ± 0.18; β1: control = 7.49 ± 0.47, T3 = 8.11 ± 0.53). These results demonstrated for the first time that expression of the α3 isoform in myocardium of newborn ferrets is upregulated by thyroid hormone.

Table 1
Effects of thyroid hormone treatment on newborn ferrets

Measurements	Control ($n = 4$)	T3-treated ($n = 4$)
Initial body weight (g)	17.4 ± 2.4	18.5 ± 1.6
Final body weight (g)	39.7 ± 3.6	39.5 ± 1.8
Heart weight (mg)	208 ± 49	266 ± 11
HW/BW (mg/g × 1000)	5.2 ± 0.4	6.7 ± 0.3 *
T3 (ng/ml)	0.95 ($n = 2$)	7.4 ($n = 2$)

Values are shown as MEAN ± SE.
* $P < 0.05$ by Student's *t*-test.

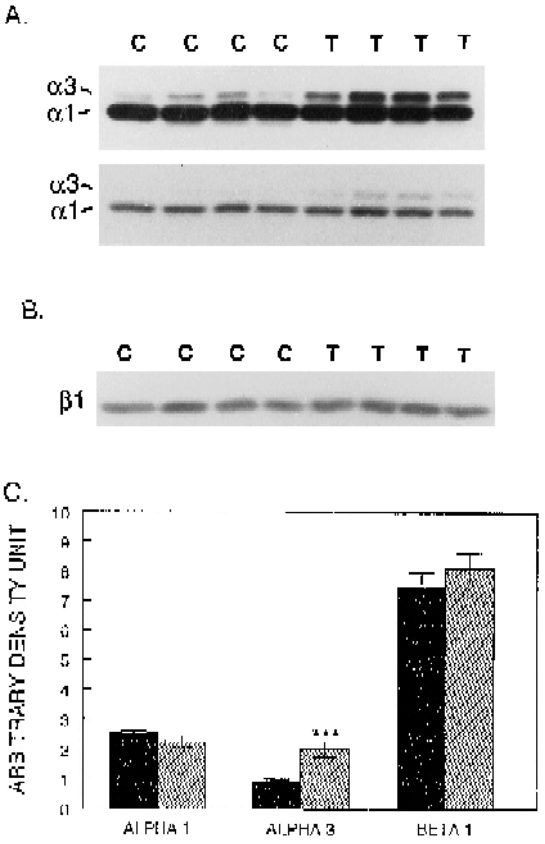

Fig. 2. Expression of Na^+, K^+-ATPase isoforms in the myocardium of newborn ferrets treated with thyroid hormone. (A) Crude membrane preparations of cardiac tissue (15 μg/lane) were subjected to Western blotting analysis using αN and [^{125}I]-anti-IgG as primary and secondary antibodies, respectively. Top: A typical autoradiogram showing α1 and α3 isoforms in heart of control (C) and T3-treated (T) ferrets. Bottom: Lighter exposure of the above autoradiogram to show more clearly the abundance of the α1 isoform. (B) For analysis of the β-subunit, the same crude membrane preparations (12 μg/lane) were first treated with *N*-glycosidase F, as described in Section 2. (C) Density scanning was performed on duplicate autoradiograms from each experiment and results are expressed as relative density units. Vertical lines represent the standard error of the mean. ** $P < 0.001$ by ANOVA. Solid bar: Control ($n = 4$); hatched bar: T3-treated ($n = 4$).

3.2. Developmental stage-dependent changes in thyroid hormone responsiveness of the ferret α3 isoform

Our earlier report demonstrated that in the cardiac muscle of mature ferrets expression of the α3 isoform was unresponsive to regulation by thyroid hormone [23]. Thus, the results presented above suggest a possible developmental stage-specific transition in thyroid hormone responsiveness of the α3 isoform. To identify this critical transition period, ferrets of increasing ages (3-, 6- and 8-week-old) were treated with T3 (50 μg/100 g body weight, s.c.) on 3 alternating days. Body weight of 3-week-old control ferrets increased significantly during the course of the experiment whereas that of 3-week-old treated ferrets increased only slightly (Table 2). This T3 effect on body weight was evident in the other age groups as well; treated ferrets gained less weight than controls. There was no significant change in heart weight in any of the three age groups examined. Serum T3 levels were dramatically increased in 3- and 6-week-old ferrets treated with T3 compared to age-matched controls (T3 levels were not measured in 8-week-old ferrets).

When 3-week-old ferrets were treated with T3, the α3 isoform increased 1.7-fold (control = 1.20 ± 0.09, T3 = 2.07 ± 0.21 in relative density units; $P < 0.01$ by *t*-test), but the α1 isoform was unaltered (control = 3.97 ± 1.25, T3 = 4.09 ± 1.26; Fig. 3). In contrast, levels of both the α1- and α3-isoform were unchanged, compared to controls, when T3-treatment was started at 6 weeks of age (α1: control = 2.82 ± 0.85, T3 = 3.14 ± 0.73; α3: control = 2.00 ± 0.24, T3 = 2.08 ± 0.17). Furthermore, qualitatively no obvious changes in either the α1 or α3 isoform could be observed in 8-week-old ferrets treated with TH. These data suggest that α3 expression is responsive to thyroid hormone regulation until sometime between 3- and 6-weeks of age. Abundance of β1 was not examined in this experiment because thyroid hormone did not alter β1 levels in newborn ferrets in the above experiment (see Section 4).

In skeletal muscle, by contrast, substantial increases in the α-subunit isoforms, α2 in particular, can be detected in 8-week-old ferrets treated with T3, compared to control ferrets (α2: control = 1.04, T3 = 1.68; α1: control = 0.375, T3 = 0.447 ($n = 2$); Fig. 4). It should be noted that we demonstrated previously that ferret skeletal muscle does not express the α3 isoform [8].

3.3. Regulation of MHC isoforms by thyroid hormone

To determine whether a lack of functional thyroid hormone receptor in myocardium is responsible, at least in part, for the transition from thyroid hormone-responsive to thyroid hormone-unresponsive, relative abundance of the myosin heavy chain (MHC) isoforms, known to be regulated by thyroid hormone, was examined in cardiac muscle homogenates. Fig. 5 shows that in each age group α-MHC is undetectable in control cardiac muscle; however, its expression is induced by T3 treatment. This result suggests the presence of functional thyroid hormone receptors in the myocardium of these ferrets.

Table 2
Effects of thyroid hormone treatment on 3-, 6- and 8-week-old ferrets

	Initial body weight (g)	Final body weight (g)	Heart weight (g)	T3 (ng/ml)
3-wk-old (C) ($n = 5$)	93.6 ± 5.5	123.9 ± 8.1 [a]	0.60 ± 0.03	1.1 ± 0.1
3-wk-old (T) ($n = 5$)	95.3 ± 5.4	110.4 ± 7.2	0.69 ± 0.05	4.7 ± 0.8 **
6-wk-old (C) ($n = 5$)	164.3 ± 16.0	231.8 ± 26.6	1.17 ± 0.14	1.2 ± 0.1
6-wk-old (T) ($n = 5$)	158.7 ± 20.7	199.7 ± 39.2	1.11 ± 0.30	5.2 ± 1.3 *
8-wk-old (C) ($n = 2$)	620 ± 30	672 ± 5	2.68 ± 0.10	—
8-wk-old (T) ($n = 2$)	607 ± 37	659 ± 62	2.39 ± 0.01	—

(C): control; (T): thyroid hormone treated. Values are shown as mean ± SE.
[a] Significantly different from initial body weight $p < 0.05$.
* $p < 0.05$.
** $p < 0.005$.
*** $p < 0.001$ by Student's *t*-test.

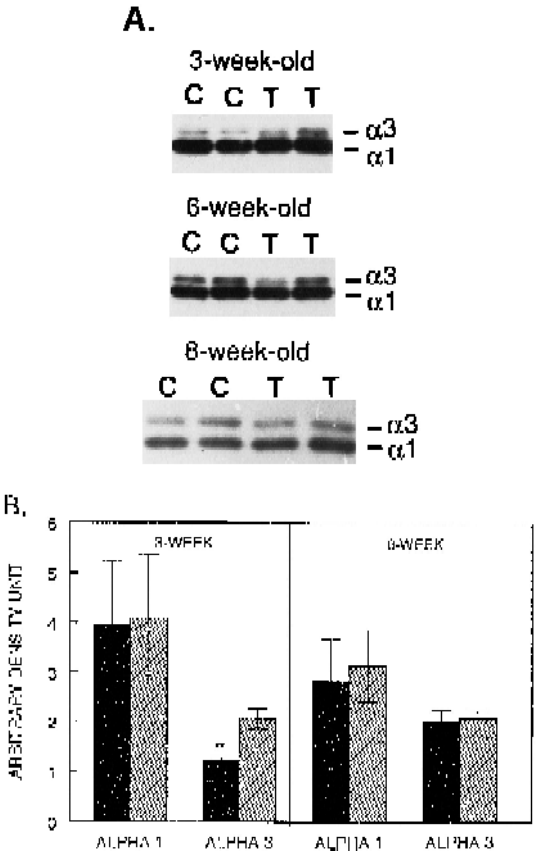

Fig. 3. Expression of Na^+, K^+-ATPase isoforms in myocardium of 3-, 6- and 8-week-old ferrets treated with thyroid hormone. (A) Crude membrane preparations of cardiac tissue (15 μg/lane) were subjected to Western blotting analysis using αN antibody as described in Fig. 2 (for 3- and 6-weeks old ferret, $n = 5$; for 8-week old ferret, $n = 2$). A typical autoradiogram shows α1 and α3 isoforms in myocardium of control (C) and T3-treated (T) ferrets. (B) For ferrets at 3- and 6-weeks of age, results are expressed as relative density units ($^*P < 0.05$ by Student's *t*-test). Solid bar: control ($n = 5$); hatched bar: T3-treated ($n = 5$). Over-exposed autoradiograms are shown to reveal the presence of the α3 isoform.

3.4. Expression of Na^+, K^+-ATPase isoforms in hypothyroid and hyperthyroid mature ferrets

We also postulated that elevated plasma levels of endogenous thyroid hormone in mature ferrets, in comparison to the low levels in newborn ferrets [31], may have masked the response of α3 to exogenous thyroid hormone. Therefore, we examined whether reduced levels of endogenous thyroid hormone in mature ferrets would allow the α3 isoform to respond to thyroid hormone.

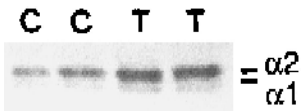

Fig. 4. Effects of thyroid hormone on the expression of Na^+, K^+-ATPase isoforms in skeletal muscle. Crude membrane preparations of skeletal muscle from 8-week-old ferrets (20 μg/lane) were subjected to Western blotting analysis using the αN antibody as described in Fig. 2. C: control; T: T3-treated ($n = 2$).

Male ferrets (approximately 8 weeks old) were made hypothyroid as described in Section 2. Eight weeks after treatment, serum T4 levels of these ferrets were less than 0.2 ng/dl; euthyroid ferrets have serum T4 levels of 3–4 ng/dl (data not shown). Half of the hypothyroid ferrets were made hyperthyroid by treating with T4 (0.5 mg/kg, s.c., daily) for 7 days. At the end of the T4-treatment period, serum T4 levels were higher than 20 ng/dl. No discernible differences were observed in body weight (hypo = 850 ± 79 g; hyper = 925 ± 46 g) or heart weight (hypo = 3.73 ± 0.16 g; hyper = 3.68 ± 0.10 g) between the hypothyroid and hyperthyroid ferrets at the end of this short treatment period.

In hyperthyroid mature ferrets, abundances of both α1- and α3-isoforms were unchanged, compared to hypothyroid ferrets (α1: hypo = 1.03 ± 0.15, hyper = 1.13 ± 0.13; α3: hypo = 0.73 ± 0.13, hyper =

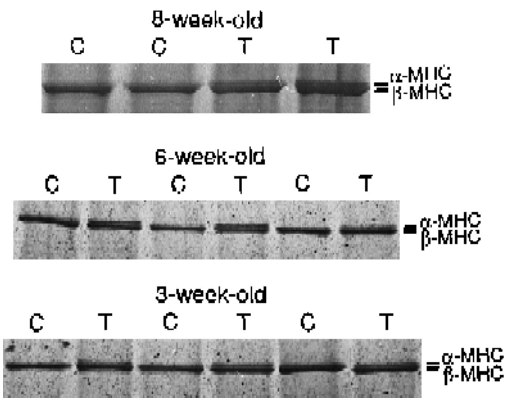

Fig. 5. Effects of thyroid hormone on expression of myosin heavy chain (MHC) isoforms in the myocardium of T3-treated ferrets at different ages. Cardiac tissue homogenates (2 μg/lane) were subjected to SDS-PAGE in 4–9% gradient gels. Gels were stained with silver to visualize the α- and β-isoforms of MHC. Samples are from control (C) and T3-treated (T) ferrets at 3-, 6- and 8-weeks of age.

0.65 ± 0.08) (Fig. 6), nor was the abundance of $\beta 1$ (hypo $= 5.54 \pm 0.34$, hyper $= 6.68 \pm 0.52$). Thus, in the myocardium of hypothyroid adult ferrets expression of the Na^+, K^+-ATPase subunit isoforms is not responsive to thyroid hormone.

The T4-treatment was effective, however, in inducing molecular changes in ferret heart; expression of α-MHC was induced in the myocardium of hyperthyroid ferrets, compared to hypothyroid controls (Fig. 7A). In addition, in the skeletal muscle of hypothyroid ferrets the level of the $\alpha 2$ isoform decreased dramatically and T4-treatment elevated the $\alpha 2$ isoform to a level similar to that of euthyroid ferrets (Fig. 7B). These data again demonstrated the selective unresponsiveness of the $\alpha 3$ isoform to thyroid hormone in ferret myocardium.

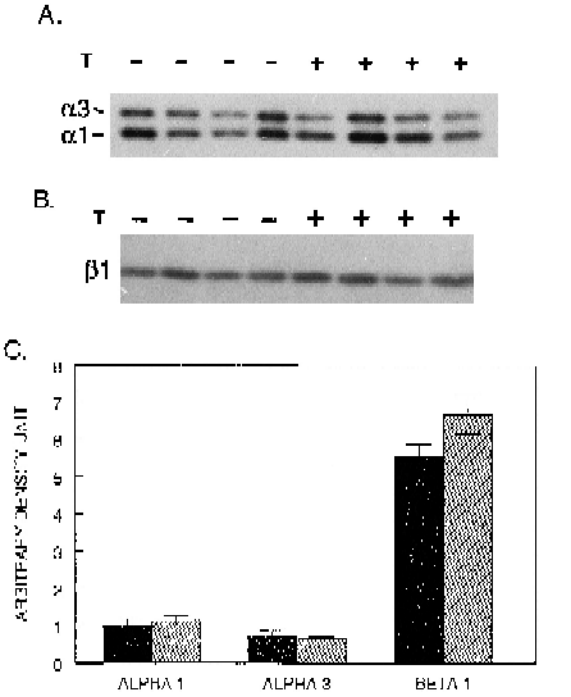

Fig. 6. Expression of Na^+, K^+-ATPase isoforms in myocardium of hypothyroid and hyperthyroid mature ferrets. (A) Crude membrane preparations of cardiac tissue (20 μg/lane) from hypothyroid (−) and hyperthyroid (+) ferrets were subjected to Western blotting analysis using αN antibody as described in Fig. 2. (B) For analysis of the β-subunit, the protocol is the same as that described in Fig. 2 using $\beta 1$-specific antibody. (C) Density scanning was performed on autoradiograms of four and two independent blots for analysis of α-isoform and $\beta 1$-isoform, respectively. Results are expressed in relative density units. Solid bar: control ($n = 4$); hatched bar: T4-treated ($n = 4$).

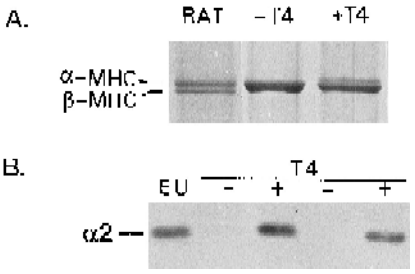

Fig. 7. Thyroid status on myosin heavy chain isoforms in myocardium and on the Na^+, K^+-ATPase $\alpha 2$ isoform in skeletal muscle of mature ferrets. (A) Left ventricular tissue homogenates from ferret and rat (3 μg/lane) were subjected to SDS-PAGE in 4–9% gradient gels. Gels were stained with silver to visualize the α- and β-isoforms of MHC (left lane: control rat heart tissue; center lane: hypothyroid ferret; right lane: hyperthyroid ferret). (B) Crude membrane preparations of ferret skeletal muscle (20 μg/lane) were subjected to Western blotting analysis using the $\alpha 2$-specific antibody and [^{125}I]-anti-IgG as primary and secondary antibodies, respectively. Typical autoradiogram shows $\alpha 2$ isoform in skeletal muscle of hypothyroid (−), hyperthyroid (+) and age-matched control (EU) mature ferrets.

4. Discussion

The present study demonstrates, for the first time, that expression of the Na^+, K^+-ATPase $\alpha 3$ isoform in ferret myocardium is responsive to thyroid hormone in newborn but not in mature ferrets. By treating ferrets of increasing age with thyroid hormone, it is revealed that a transition in $\alpha 3$ expression from thyroid hormone-responsive to thyroid hormone-unresponsive occurs sometime between 3- and 6-weeks of age. Thus, these results demonstrate a developmental stage-dependent regulation of the $\alpha 3$ isoform by thyroid hormone. Developmental changes in thyroid hormone responsiveness in the expression of the Na^+, K^+-ATPase have been reported previously in rat brain by Schmitt and McDonough [24], although the underlying cellular mechanism(s) has not been elucidated.

The transition from thyroid hormone-responsive to thyroid hormone-unresponsive could be due to increases in endogenous concentrations of thyroid hormone, to changes in the population of thyroid hor-

mone receptors, to terminal differentiation of responsive tissues, or to alterations in co-factors that may be required for thyroid hormone actions [32]. In the present study, we examined the first two possibilities. Our data show that $\alpha 3$ expression in the myocardium of hypothyroid mature ferrets remained unresponsive to T4-treatment, suggesting that increased endogenous levels of thyroid hormone is unlikely to be responsible for the transition. On the other hand, thyroid hormone treatment induced expression of α-MHC in ferret myocardium in each age group, indicating that there are functional thyroid hormone receptors in the myocardium of these ferrets. However, with the recent demonstration of subtypes of thyroid hormone receptors [33], we cannot exclude the possibility that these receptor subtypes could play a role in the differential regulation of cardiac genes.

Our result demonstrates that the transition in thyroid hormone responsiveness is not a uniform response in the ferret. Results from euthyroid (Fig. 4) and hypothyroid (Fig. 7) adult ferrets showed that expression of the α-subunit isoforms in skeletal muscle, $\alpha 2$ in particular, is sensitive to thyroid hormone status. At present it is unclear whether the transition is simply an intrinsic property of the $\alpha 3$ isoform or the tissue in which it is expressed also plays a role.

In the present study, levels of the $\alpha 1$ isoform remained unaltered in cardiac muscle of hyperthyroid ferrets. This finding appears to differ from our previous study [23] in which long-term T4-treatment of euthyroid adult ferrets increased the number of low-affinity ouabain binding sites ($\alpha 1$). The difference could be attributed to the presence of thyroid hormone-induced cardiac hypertrophy in our previous study [23], which has been shown to increase expression of $\alpha 1$ isoform [29]. It is also interesting to note that thyroid hormone treatment increased the abundance of the α-subunit without simultaneously increasing that of the β-subunit. Neither in newborn (Fig. 2) nor in adult ferrets (Fig. 6) is expression of $\beta 1$ responsive to thyroid hormone. These results suggest that elevated expression of $\beta 1$ is not required for increased expression of the $\alpha 3$ isoform and that $\beta 1$ may be expressed in excess and combines with increased amounts of $\alpha 3$ without concomitant de novo synthesis [20]. Alternatively, the possibility cannot be excluded that a yet unidentified β-subunit isoform is expressed in ferret myocardium.

Finally, our results suggest the possibility that thyroid hormone could play a role in the upregulation of $\alpha 3$ during cardiac development. The developmental stage when expression of the $\alpha 3$ isoform is responsive to thyroid hormone roughly coincides with the postnatal upsurge in thyroid hormones. To determine if thyroid hormone is required for upregulation of the $\alpha 3$ isoform during development, we attempted to produce hypothyroid newborn ferrets but so far without success; hypothyroid newborns all died before 3-weeks of age. Thus, at present it cannot be determined whether thyroid hormone is a true physiological regulator of $\alpha 3$ isoform during development.

In summary, our results demonstrate that in the myocardium of newborn ferrets expression of the $\alpha 3$ isoform is responsive to regulation by thyroid hormone. A transition in responsiveness to thyroid hormone occurs between 3- and 6-weeks of age rendering the myocardium unresponsive to thyroid hormone. Neither endogenous levels of thyroid hormone in mature ferrets nor the lack of functional thyroid hormone receptors appears likely to be responsible for the transition.

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