Changes in Myosin Isoenzymes, ATPase Activity, and Contraction Duration in Rat Cardiac Muscle With Aging Can Be Modulated by Thyroxine

Mark B. Effron, Gopal M. Bhatnagar, Harold A. Spurgeon, Gualberto Ruano-Arroyo, and Edward G. Lakatta

To determine whether the relative decline in cardiac myosin isoenzyme \( V_3 \) with maturation continues progressively into senescence and whether thyroxine could reverse age-associated changes in the myosin isoenzyme profile and contraction, rats 2, 6, and 24 months old were treated with thyroxine, 6.4 mg/kg, for 7 days. Myosin isoenzymes, \( \text{Ca}^{2+} \)-myosin ATPase activities, and isometric contractile function were measured in cardiac preparations from thyroxine-treated animals and age-matched controls. Right ventricular hypertrophy did not occur with aging in controls. Thyroxine increased right ventricular weight in each age group compared to the control group. Body weight decreased by 10% in all thyroxine-treated rats. The relative right ventricular \( V_3 \) isoenzyme content progressively decreased from 75 ± 1% to 54 ± 1% and 14 ± 1% in controls at 2, 8, and 24 months, respectively, and was associated with a reciprocal increase in \( V_2 \) myosin isoenzyme. \( \text{Ca}^{2+} \)-myosin ATPase activity also progressively declined monotonically with age in the control rats from 854 ± 28 nmoI Pi/mg prot/min at 2 months to 529 ± 28 nmoI Pi/mg prot/min at 24 months. Thyroxine administration increased right ventricular \( V_3 \) at each age to 97 ± 2%, 73 ± 2%, and 59 ± 2% at 2, 8, and 24 months, respectively. A thyroxine induced increase in the \( \text{Ca}^{2+} \)-myosin ATPase activity could be detected only in the 24-month-old animals. Isometric contraction duration in thin right ventricular papillary muscles increased with age from 172 ± 4 to 180 ± 4 to 225 ± 18 ms at 2, 8, and 24 months, respectively, and was markedly shortened (p < 0.001) by thyroxine at each age so that the 24-month thyroxine value was less than the 2-month control. The maximum rate of force production did not change with age in controls and was increased by thyroxine at all ages. Thus, with aging from maturation to senescence, even in the absence of right ventricular hypertrophy, there is a profound monotonous decrease in percent right ventricular \( V_3 \) that is paralleled by a decline in \( \text{Ca}^{2+} \)-myosin ATPase activity. Also, neither the change in genetic expression of myosin protein synthesis nor prolonged contraction duration that occurs with aging is irreversible. Both can be modulated by thyroxine. (Circulation Research 1987;60:238-245)

The biochemistry and function of myocardial tissue has been shown to be markedly dependent on thyroid status.\(^1\) The hyperthyroid myocardium is characterized by a longer repolarization time of the transmembrane action potential, an increased sarcoplasmic reticulum (SR) \( \text{Ca}^{2+} \) pumping rate, an increased shortening velocity, a decrease in the isometric time to peak tension and relaxation time, elevated actin and \( \text{Ca}^{2+} \)-myosin ATPase activities, and its effect on the myosin isoenzyme distribution has not been studied. Rather, most studies that have examined the effect of exogenous \( T_4 \) administration on myofilament properties have utilized relatively young hypothyroid rats or have avoided using the rat in favor of other species that have utilized relatively young hypothyroid rats or have avoided using the rat in favor of other species that already exhibit a low percent of \( V_3 \) and relatively low ATPase activities in the euthyroid state.\(^2\) As the euthyroid rat matures, the percent of \( V_3 \) decreases while the percent of \( V_2 \) increases, and this shift is accompanied by a decrease in the ATPase ac-
tivities in a variety of myofilament preparations. With further adult aging into senescence, the ATPase activity in some but not all myofilament preparations continues to decline and the contraction duration becomes prolonged. It is unknown to what extent the percent V, myosin isoenzymes declines in the senescent rat. Furthermore, it is unknown to what extent the age-related change in the genetic expression of protein synthesis that underlies the shift in isoenzymes can be reversed. Therefore, it is unclear whether any change in the isoenzyme profile and ATPase activity that occur with aging can be reversed with thyroxine, which is effective in reversing similar changes in the relative isoenzyme content that result from the hypothyroid state in young rats. Similarly, it is not known whether the prolonged contraction duration with aging can be modified by T4.

These present studies determined 1) whether the decline in the percent V, that begins early in the rat continues progressively into senescence, 2) whether T4 could reverse age-related alterations in isoenzyme pattern and Ca2+-myosin ATPase activity, and 3) whether twitch contractile parameters that vary with age can be modulated by T4, and if so, whether this could be correlated with the changes in the isoenzyme profile and ATPase activity that occur with aging or with induction of the hyperthyroid state.

Materials and Methods

Maturational (2-month), adult (8-month), and senescent (24-month) male Wistar rats obtained from the Gerontology Research Center colony were injected intramuscularly with thyroxine (Sigma Chemical Co., St. Louis, Mo.), 6.4 mg/kg initial body weight/day for 7 days. This protocol has previously been shown to induce a marked hyperthyroid (H) state. Uninjected rats from the same cohort were used as untreated controls (C). Body weight was obtained prior to beginning the injections and just prior to study. A venous blood sample was obtained immediately prior to study and analyzed for plasma T4 concentration using an Immobeads Radioimmunoassay system (Coming Medical and Scientific, Medfield, Mass.). Animals were killed by cervical dislocation, and the heart was immediately removed and placed in oxygenated physiologic salt solution. After removal of a right ventricular papillary muscle for contractile studies, the remainder of the heart, devoid of the atria, was quickly weighed in two sections — the right ventricular free wall (RV) and the left ventricle and septum (LVS) — and used for the biochemical studies.

Biochemical Studies

Myosin Preparation. Myosin was isolated either from both ventricles or from the right ventricle by a procedure similar to that described by Nauss et al. The purified myosin fraction that precipitated 35–50% ammonium sulfate saturation was collected by centrifugation, dissolved, and stored at −20°C in 50 mM imidazole buffer, 0.5 M KCl, and 50% glycerol at pH 7.0. For assays of ATPase activity, myosin was dialyzed in 50 mM imidazole buffer containing 0.5 M KCl at pH 7.0. The purity of the myosin preparation was checked from a densitometric record of the SDS gel electrophoresis. Myosin preparations were found to be free of actin, troponin, and tropomyosin and were also without evidence of proteolytic degradation. For isoenzyme studies, myosin was dialyzed in 50 mM sodium pyrophosphate buffer containing 50% glycerol, 1% 2-mercaptoethanol, and 5 mM dithiothreitol (DTT) at pH 8.8. All procedures were performed at 4°C.

In studies examining isoenzyme distribution in selected areas of the heart, a small amount of myocardial tissue (1–10 mg) was used to obtain myosin by first placing the tissue in 50% glycerol, 1 mM sodium pyrophosphate buffer, 1 mM EDTA, 1% 2-mercaptoethanol, and 5 mM DTT at pH 8.8 for 4 hours at 0°C and then extracting the tissue overnight at 0°C in 200 μl of 50% glycerol, 50 mM sodium pyrophosphate buffer, 1 mM EDTA, 1% 2-mercaptoethanol, and 5 mM DTT at pH 8.8. The myosin-containing extract was then analyzed by electrophoresis for isoenzymes as described below.

Myosin Isoenzyme Determination. Myosin isoenzymes were separated on polyacrylamide gel using nondissociating conditions as described previously. A Pharmacia GE4 Electrophoresis unit was used in which the electrophoresis buffer (10% glycerol, 20 mM sodium pyrophosphate, 1 mM EDTA, 0.1% 2-mercaptoethanol at pH 8.8) was recirculated between the lower and upper chambers. The temperature in the lower chamber of the electrophoresis unit was maintained at 2°C. Myosin samples (2–4 μg) were loaded onto gel tubes containing 4% polyacrylamide. Electrophoresis was carried out for 18 hours at a voltage gradient of 14 V/cm. Following electrophoresis, gels were stained with Coomassie Blue R (0.04% in 3.5% perchloric acid) and destained with 25% 2-propanol and 7.0% acetic acid. The destained gels were scanned on a Kratos (Schoeffel Instrument) spectrophotometer at 560 nm. The relative isoenzyme content was then determined by weighing the paper under the curve of the isoenzyme of interest and comparing it to the weight of the total curve of the isoenzymes. The amount of the heterodimer V2 was divided equally between V1 and V3.

Myosin ATPase Activity Determination. Myosin K+—Ca2+—ATPase assays were carried out in a final volume of 2 ml containing 50 mM imidazole, 500 mM KCl, 5 mM ATP, 10 mM CaCl2, and 10 mM NaN3 at pH 7.0. Following a 10-minute incubation at 25°C the reaction was terminated by the addition of 0.5 ml (10% (w/v) trichloroacetic acid (TCA) solution. The assay mixture was kept on ice for 10 minutes and then centrifuged at 2,000g in a clinical centrifuge. Inorganic phosphate in the supernatant in the steady state was measured according to the method of Taussky and Short.

Protein Concentration Measurement. Protein concentrations were determined by the method of
Chambers in parallel. Right ventricular muscles were used because they were thin, discrete muscles that tolerated a superfused bath well and exhibit characteristic age changes in contractile function. The muscles were bathed at 29°C in a modified Krebs solution with a mM concentration of NaCl 119, KCl 5.0, NaHCO₃ 25, KH₂PO₄ 1.2, MgSO₄ 1.2, CaCl₂ 1.0, and dextrose 10 buffered with 95% O₂ and 5% CO₂ resulting in pH 7.4 as previously described. The muscles were stimulated 24/min by pulses of 0.5-millisecond duration immediately prior to sacrifice (crosshatched bar — control, open bar = hyperthyroid). T₄ vs. control at any age, p < 0.001; 2- vs. 8- or 24-month control, p < 0.002; 8- vs. 24-month control, p < 0.005.

**Statistics**

All results are expressed as mean ± standard error of n muscles in the control and treated groups. The statistical significance of age and thyroid status was determined by a least-squares means analysis of variance and linear regression analysis. The level of statistical significance was taken as p < 0.05.

**Results**

T₄ treatment for 7 days caused at least a tenfold increase in plasma T₄ over control at each age (Figure 1). Body and heart weights are listed in Table 1. Prior to T₄ treatment, there was no difference in body weight within each age among animals chosen for T₄ or control. After T₄ treatment, rats weighed approximately 10% less than their age matched controls. Right ventricular hypertrophy did not occur with aging in control hearts. T₄ significantly increased both the right and left heart weight and heart weight relative to body weight at all ages.

Representative pyrophosphate gels and densitometric scans of myosin from RV at each age and thyroid status are shown in Figure 2. A marked decrease in the relative content of V₁ occurred with increasing age in untreated animals (Figure 3A). The average percent RV V₁ declined progressively with age from 75 ± 1% in 2-month-old animals to 54 ± 1% in 8-month-old

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**Table 1. Body and Heart Weights From Rats of Different Ages and Thyroid Status**

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TS = thyroid status, C = control, T = hyperthyroid, N = number, BW = body weight, LVS = left ventricle + septum weight, RV = right ventricle weight, and CSA = papillary muscle cross-sectional area.

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Lowry et al using bovine serum albumin as the standard.

**Contractile Studies**

A right ventricular papillary muscle was placed horizontally in an isometric superfused muscle chamber as previously described. One muscle from each of 4 animals could be studied simultaneously in 4 muscle chambers in parallel. Right ventricular muscles were used because they were thin, discrete muscles that tolerate a superfused bath well and exhibit characteristic age changes in contractile function. The muscles were obtained from rats of different ages and thyroid status, with BW = body weight, LVS = left ventricle + septum weight, RV = right ventricle weight, and CSA = papillary muscle cross-sectional area.

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**Statistical Significance**

The statistical significance of age and thyroid status was determined by a least-squares means analysis of variance and linear regression analysis. The level of statistical significance was taken as p < 0.05.

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**Figure 1.** Plasma thyroxine (T₄) concentration obtained immediately prior to sacrifice (crosshatched bar = control, open bar = hyperthyroid). T₄ vs. control at any age, p < 0.001; 2- vs. 8- or 24-month control, p < 0.002; 8- vs. 24-month control, p = NS.
animals to 14 ± 1% at 24 months. Because the values of $V_1$ and $V_3$ were derived by adding half of the value of $V_2$ to each, the increase in $V_3$ with age was the reciprocal of the decrease in $V_1$ (results not shown). No differences were observed in the percentage of $V_1$ among the right ventricular free wall, left ventricular free wall, right ventricular papillary muscle, or left ventricular papillary muscle within an age group (Figure 4).

With $T_4$ treatment, the percent of $V_1$ increased significantly at all ages (Figure 3A) with a simultaneous decrease in percent of $V_3$ (not shown). The resulting percent $V_1$ with $T_4$ treatment at each age reached the control level of the preceding younger age group. Thus, within the $T_4$-treated animals, while the percent $V_1$ still differs in 2 vs. 8 months and 8 vs. 24 months ($p < 0.001$), the magnitude of the difference between 8 and 24 months is substantially reduced compared to controls, and when considered across the entire age span, the monotonic decline with age was no longer statistically significant (Figure 3 legend).

The Ca$^{2+}$-myosin ATPase activity in control animals showed a progressive monotonic decrease with increasing age (Figure 3B). The magnitude of this decline (38%) from 2 to 24 months was substantially less than the shift in the $V_1$ isoenzyme measured over the same age range. $T_4$ reversed the age related decrease in ATPase activity in hearts from the 24-month animals which, in the controls, showed the largest Ca$^{2+}$-myosin ATPase activity decrement due to age (Figure 3B). However, $T_4$ did not appreciably increase Ca$^{2+}$-myosin ATPase activity in the younger age hearts and actually decreased it slightly (9%) in the 2-month hearts. Thus, the monotonic age-related decline in Ca$^{2+}$-ATPase activity in controls was not maintained after treatment with $T_4$. Rather, in the $T_4$ hearts the Ca$^{2+}$-ATPase activity remained fairly constant across age (Figure 3B).

The isometric contraction parameters of right ventricular papillary muscles are shown in Table 2. Resting tension did not significantly differ among groups. $dT/dt$ in the control animals did not change with age. DT increased slightly from 2 to 24 months although this change did not reach statistical significance. However, TPT (Figure 3C) and CD significantly increased with age in the control animals and RTW increased between 8 and 24 months. $T_4$ treatment increased $dT/dt$ at each age and the magnitude of this change was least in the 2-month group (25% vs. 50% in 8- or 24-month animals). The most impressive contractile changes
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with T₄ occurred in the twitch duration parameters. TPT, RT, and CD all decreased to values less than that of the 2-month-old control rats, which had the shortest contraction times of the control animals (Figure 3C and Table 2). Among the T₄ groups, prolongation of the twitch duration with age was abolished except for TPT (Figure 3C), which still exhibited a small but significant increase with age.

Discussion

The present results demonstrate a profound reduction of the percent V₁ myosin isoenzyme from 75% at 2 months to 14% in the 24-month-old rat. This marked shift to the predominantly V₃ myosin form is similar to that which is present in other animals early in life33,34,45 and is also comparable to that in myocardi- um of young rats with induced hypothyroidism18 or diabetes.47,48 The magnitude of the decline in V₁ in senescence in the present study, however, is substantially greater than that reported to occur secondary to pressure overload of the ventricle in younger rats.49,50

While it may be argued that the 24-month-old rat may have hypothyroidism, the difference in the plasma T₄ between 8 and 24 months is small and not significant although plasma T₄ decreased 50% between 2 and 8 months (Figure 1). Yet, a substantial difference in percent V₁, as well as in contractile properties, occurred between 8 and 24 months. Thus, the decrease in T₄ might be argued to occur secondary to pressure overload of the ventricle in younger rats.49,50
The specific stimulus for the decrease in the percent of genetic expression of the myosin ATPase isoenzyme, reversed to some extent under certain circumstances. Changes in genetic expression that occur with aging, T4 treatment has not been previously reported in studies not concerned with aging. This result suggests the changes in genetic expression that occur with aging, whatever their cause, are not fixed and may be reversed to some extent under certain circumstances.

In the present study, there was no difference in myosin isoenzyme distribution between the right and left ventricle (Figure 4). There appears to be no consensus as to whether a difference exists in the isoenzyme profile between the two chambers, since some studies show identical profiles in the two ventricles, while others show V3 greater in the LV than the RV. Distribution of isoenzymes between the ventricles was also shown to vary with the species studied.

The progressive decline in myosin Ca2+-ATPase activity from 854 ng/mg prot/min at 2 months to 529 ng/mg prot/min in the 24-month rat paralleled the decline in percent V3 (Figure 5A). Although an age-related decrease in actomyosin ATPase has been noted previously,9,30-32 the extent to which myosin Ca2+-ATPase activity declined in senescence has not been previously determined. The present results show that the magnitude of this depression in the 24-month-old control rat, like that of percent V2, myosin isoenzyme is comparable to that in hypothyroid10,23,24 and diabetic rats47-50 of a younger age. In the 24-month-old rat, T4 increased the Ca2+-ATPase activity to the level present in the young control rat. In 2- and 8-month-old rats, T4, in contrast to its effect on the myosin isoenzyme shift to V3, did not increase Ca2+-ATPase. That Ca2+-ATPase activity could not be increased by thyroid treatment in young rats has previously been noted.1,2,24 Failure of T4 to stimulate myosin ATPase in the 2-month-old rat is probably because the activity is near maximal in younger animals, coupled with a rather sensitive assay of the isomyosin forms.

Changes in the isometric twitch parameters with age observed in the euthyroid animals in the present study are similar to those reported previously with aging over the broad age span.13,23,31,33 Whether T4 could alter contractile function in rats as young as 2 months or as old as 24 months was previously unknown. The present results show that T4 treatment decreased TPT, RT, and CD at each age to values less than that of the 2-month-old control group, which had the shortest tim-
ing parameters of the control animals. $T_s$ also increased $dT/dt$ in each age group, with the most impressive changes occurring in the two older age groups.

Simultaneous comparison of the anatomic, biochemical, and functional measurements of the present study permit a perspective on the relationships among these variables. Since relative right ventricular mass did not increase with age in the control state and since, as measured in right ventricular tissue, the percent $V_s$ markedly decreased and TPT increased with age, the cardiac mass per se cannot be a determinant of the age-related change of either parameter with age in the control group. In the control state, the myosin Ca$^{2+}$-ATPase activity varied inversely with TPT across the age span and paralleled the age-related decline in the percent $V_s$ (Figure 5). This is in agreement with directionally similar changes observed in these properties among various previous studies$^{18,32,33}$ as well as differences in the velocity of shortening in the isotonic twitch with adult aging.$^{31}$ The inverse correlation between contraction duration parameters, i.e., TPT and CD, and Ca$^{2+}$-ATPase activity across the age span does not necessarily indicate a tight coupling between the two since the time course of myoplasmic free [Ca$^{2+}$]$^{3,34}$ SR pumping rates,$^{35}$ and the transmembrane action potential$^{33,35}$ also vary with age. Similarly, the substantial increase in Ca$^{2+}$-ATPase activity in the 24-month-old animals accompanying the reduction in TPT in response to $T_s$, may not be solely attributable to changes in myosin ATPase activity as $T_s$ also modulates other cell mechanisms that determine these parameters, among which is modulation of Ca$^{2+}$ transport at the sarcoplasmic reticulum (SR)$^{36-37}$ and sarclemma.$^{58}$

While the present results demonstrate a correlation between the percent $V_s$ and TPT across age in control and in $T_s$-treated muscles (Figure 6), the $T_s$ groups do not lie on the same line relating the two parameters as do the control groups but are shifted downward. This downward shift may indicate $T_s$ modulation of other aspects of cell Ca$^{2+}$ metabolism in addition to an effect at the myofilament level. Thus, it appears that when myosin isoenzymes in a given muscle type differ due to changes such as age or thyroid status, excitation-contraction coupling mechanisms also differ.$^{39}$ In this regard, the cornerstone argument for the functional correlate of myosin ATPase, i.e., that the contraction duration (TPT in particular) among muscle types varies inversely and the velocity of shortening varies directly with the myosin ATPase activity,$^{40}$ may require a caveat since recent evidence indicates that the rate of SR Ca$^{2+}$ pumping, as well as myosin ATPase activity and isoenzyme profile, differs between fast and slow muscle types.$^{61}$

**Figure 6.** TPT as a function of percent $V_s$. Symbols and lines as defined in Figure 3. Slopes of least-squares linear regression analyses of TPT on $V_s$ were control $= -0.43$, $r = 0.999$, $p < 0.01$: hyperthyroid $= -0.21$, $r = 0.991$, $p < 0.01$. 

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**Key Words** - aging • Ca++-myosin ATPase activity • cardiac muscle contraction • hyperthyroid state • myosin isoenzymes
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Circ Res. 1987;60:238-245
doi: 10.1161/01.RES.60.2.238

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