Effect of Aging and Hypertension on Myosin Biochemistry and Gene Expression in the Rat Heart

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The mechanisms by which the aged heart adapts to a superimposed pressure load such as hypertension have not been described. We therefore investigated biochemical and molecular genetic adaptations in the 24-month-old rat heart subjected to renovascular hypertension. Compared with 4-month-old rats, aging was associated with a 68% increase in left ventricular mass without any change in heart weight-to-body weight ratio, a 33% reduction in calcium-activated myosin ATPase activity, and a shift from a $V_1$ to a $V_3$ predominant myosin heavy chain (MHC) isofrom distribution. A 46% reduction in $\alpha$-MHC mRNA and a reciprocal increase in $\beta$-MHC mRNA was seen. When hypertension was superimposed, there was a further 75% increase in ventricular mass, a 63% increase in heart weight-to-body weight ratio, and a 19% reduction in myosin ATPase. Myosin isozyme distribution was further shifted to $V_3$, and the ratio of $\alpha$-MHC to $\beta$-MHC mRNA was reduced. In addition, with hypertension a significant (>50%) reduction in the mRNA level of the cardiac sarcoplasmic reticulum calcium-activated ATPase was seen. These data demonstrate that the aged myocardium is able to respond to a superimposed pressure load with a molecular genetic and protein synthetic pattern of hypertrophy analogous to that seen in younger animals. (Circulation Research 1991;68:645–652)

Aging is associated with a variety of structural, mechanical, and biochemical alterations in the heart that potentially could impair its ability to maintain an effective cardiac output. Structural alterations in rats include an increase in heart weight associated with cell loss, increased collagen deposition, and foci of fibrosis. Some papillary muscle studies have shown an age-related decrease in the rates of tension development and muscle relaxation without significant impairment in peak isometric tension development. Biochemical analyses have demonstrated a reduction in myosin ATPase activities and a shift to the $V_3$ myosin isofrom in rats as well as impairment in calcium uptake by isolated sarcoplasmic reticulum. Similar observations have been made in animal models of hypertension, which have led some investigators to characterize hypertensive cardiac hypertrophy as accelerated senescence.

Hypertension is common in the elderly, and it is generally accepted that the superimposition of a pressure overload hypertrophy on the aged heart results in a deterioration of cardiac performance. The purpose of the present study was to characterize the biochemical and molecular genetic adaptations of the aged heart subjected to the superimposed load of hypertension. Two hypotheses existed at the outset, both of which could account for deteriorated left ventricular performance of the aged hypertensive heart. The first was that the aged heart would be unable to adapt to an imposed load by altering gene expression and increasing protein synthesis. The second was that the superimposition of two qualitatively similar stimuli, aging and hypertension, would elic an additive molecular genetic and protein synthetic pattern of cardiac hypertrophy, the hallmark of which would be impaired cardiovascular function.

We chose to approach these questions using a well-defined animal model, the rat, in which clear molecular genetic, biochemical, and mechanical characteristics of both hypertension and aging have been established. Measurements included analysis of myosin heavy chain (MHC) and sarcoplasmic reticular ATPase gene expression and myosin biochemistry. Our data clearly support the second hypothesis.
Materials and Methods

Animal Models

Pathogen-free male Long-Evans (Blue Spruce Farm, Altamont, N.Y.) rats were used in the aging studies. This strain of animals was used rather than Fisher 344 rats because they are reportedly heartier and therefore were more likely to withstand the physiological stress of sustained hypertension. Animals were housed in a specialized animal facility at 21°C, with 45–55% humidity, and on a 12-hour light-dark cycle. On arrival at our facility, the aged animals were placed on modest food restriction to decrease their body weights to approximate those reported in free running animals of the same species. Subsequently, they were allowed access to food and water ad libitum. Young animals were 4 months old and old animals were 24 months old at the start of the study.

Old animals were made hypertensive by bilateral cupraphane wrapping of the kidneys under ether anesthesia. This results in a brisk perinephritis, and approximately 1–2 weeks after surgery, animals develop a sustained, high-renin hypertension. Sham-operated controls were maintained. Blood pressures were checked weekly after surgery under light ether anesthesia, and only animals whose tail-cuff pressures were greater than 150 mm Hg were considered hypertensive. Tail-cuff pressures were less than 115 mm Hg in all controls. Blood pressures were equivalent in young and old normotensive animals. Animals were maintained hypertensive for 4–5 weeks before being killed. None of the young or old animals died during the study period. In contrast, four of 11 aged hypertensive animals died before the conclusion of the study.

Animals were killed by bilateral thoracotomy under ether anesthesia, and the hearts were excised, the great vessels and atria were trimmed, and the ventricles were weighed. The ventricles then were divided into three sections. The apical third was immediately frozen in liquid nitrogen and subsequently stored at −70°C for RNA analysis; the middle third was placed in buffered 3.7% formalin for pathological examination; and the basal third was placed in iced 50% glycerol containing 50 mM KCl and 10 mM KH2PO4 (pH 7) and then stored at −70°C before preparation of myosin extracts.

Contractile Protein Biochemistry

Cardiac myofibrils were isolated and purified with Triton X-100 according to the method of Solaro et al. Gradient sodium dodecyl sulfate (SDS) gels (5–16%) of purified myofibrils were run to confirm the integrity of the purified proteins. Proteolytic enzyme inhibitors—phenylmethylsulfonyl fluoride (0.2 mM), pepstatin (1 μg/ml), leupeptin (1 μg/ml), Na-p-tosyl-L-lysine chloromethyl ketone (0.1 mM) — were included in the isolation mixture. Calcium-activated myosin ATPase in myofibrils was assayed at 30°C in 0.3 M KCl, 50 mM Tris-Cl (pH 7.6), 10 mM CaCl2, 5 mM ATP, and 5 mM sodium azide. The reaction was initiated by the addition of substrate and was terminated after 10 minutes with 1.0 ml cold 10% trichloroacetic acid. Results are expressed as micromoles P per milligram per minute.

Myosin isozymes were analyzed by electrophoresis on polyacrylamide gels under nondissociating conditions at 2°C as reported by d’Albis et al. Five to seven micrograms of crude myosin extract was layered on each gel and run at a constant voltage gradient of 14 V/cm for 20–22 hours. The gels then were stained with Coomassie blue, and densitometric scans were recorded at 605 nm on an E-C apparatus. The semiquantitative estimate of each isoenzyme was calculated from the height of each peak using a Model 3390 Hewlett-Packard integrator (Palo Alto, Calif.). The relative contribution of V2 was equally distributed between the other two isozymes.

RNA Purification and Hybridization

Total RNA was purified from tissue sections of either heart or liver that had been preserved at −70°C using a modification of the method of Chirgwin et al. Briefly, the tissue was homogenized with a polytron in 4 M guanidinium thiocyanate, 1 M β-mercaptoethanol, 100 mM Tris-Cl (pH 7.6), and 50 mM EDTA and then was centrifuged through a 5.7 M CsCl cushion at 175,000×g for 18 hours. Pellets then were resuspended in TE, were extracted with phenol:chloroform and chloroform, and were precipitated and washed with ethanol. RNA was stored as an ethanol precipitate at −20°C. Aliquots of the RNA then were either size-fractionated on 1.0% agarose gels in MOPS–formaldehyde and transferred or were spotted onto Genescreen membranes (New England Nuclear, Boston) for subsequent Northern or quantitative slot blot analysis according to standard techniques. cDNA and oligonucleotide probes were hybridized according to previously described protocols at either 65°C (cDNA) or 10–12°C below the calculated Tm (oligos). cDNA probes were labeled with 32P by random primer (Pharmacia LKB Biotechnology, Piscataway, N.J.) and oligonucleotides by polymerase kinase. In all hybridizations, 5×105 cpm/ml were used with the exception of those involving the 18S rRNA probe, which were done with 10-fold less counts per minute. Filters were washed in 2× SSC/0.2% SDS at hybridization temperatures. A sample of rat liver RNA was present on all filters to confirm the specificity of the cardiac-specific probes. Slot blots all contained two concentrations of RNA (5 and 10 μg), and quantitative densitometry of all blots was done on multiple exposures using a laser.
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densitometer (Quantimet 970, Cambridge Instruments, Inc., Buffalo, N.Y.) to ensure that measurements were made in the linear range.

Two DNA and two oligonucleotide probes were used. The DNA probes included a 225 base pair EcoRI fragment of the cDNA specific for the human slow (cardiac) sarcoplasmic reticular ATPase (courtesy of D. MacClennan). This region of the gene shows virtual sequence homology with the rat cDNA,17 and we have confirmed by genomic Southern analysis that it recognizes only a single gene under the hybridization conditions used. The second DNA probe is specific for 18S rRNA18 and was used to normalize all data. The two oligonucleotides used were both 24 base fragments specific for unique regions in the 3' untranslated regions of the rat α-MHC and β-MHC genes19,20 and have been shown in previous studies to be gene specific. The α sequence was 5'-CAACGGCGAGGC-TCTTYCTGCTGG, and the β sequence was 5'-CTC-CAGGTCTCAGGGCTTCACAGG.

**FIGURE 1.** Calcium-activated myosin ATPase activity in myofibrils and myosin isoenzyme distribution in young, old, and old hypertensive (HTN) animals. Data are expressed as mean±SEM of seven to 11 studies. All measurements in all three groups of hearts are statistically distinct (p<0.05).

**FIGURE 2.** Representative gradient sodium dodecyl sulfate slab gel (5–16%) and scans of purified myofibrils from young (Y), old (O), and old hypertensive (OH) hearts. The major contractile proteins, myosin heavy chain (MHC), light chains 1 and 2 (LC1, LC2), actin, and troponin T (TnT) are indicated.
kits (Pantex Corp., Santa Monica, Calif.) that we have previously validated for use with rat plasma.

Statistical Analysis

When more than two groups were compared, results were submitted to analysis of variance (ANOVA) and the mean square error within groups then was used in a Newman-Keuls multiple comparison test to evaluate differences between any two groups.\(^{21}\) Data expressed as percent were subjected to a logarithmic transformation before ANOVA. When two groups were compared, a two-tailed \(t\) test was used. Significance is reported at the 0.05 level.

Results

Table 1 shows body and heart weight data for three groups of animals: 5-month-old controls (young), 25-month-old normotensives (old), and 25-month-old hypertensives (old hypertensives). Aging was associated with a significant increase in heart and body weights, both of which were nearly double those seen in young animals. Heart weight-to-body weight ratios fell slightly with aging. In contrast, in the aged animals made hypertensive, there was a further 40% increase in heart weight associated with a slight decline in body weight so that the ratio of these two measurements was significantly increased relative to both young and normotensive old animals.

Histological examination of six to 12 hearts from each of these three groups was performed and confirmed that the increase in heart weight in part reflected myocyte hypertrophy. Hypertrophy (0–1+) and interstitial fibrosis (0–1+) were seen in the young hearts, whereas a significant degree of hypertrophy (1–2+) and fibrosis (1–2+) were seen in the hearts of the old normotensive animals. The hearts of all hypertensive old animals were evaluated by the blinded reader as showing a marked increase in both hypertrophy (3+) and in fibrosis (2–3+). No significant differences in interstitial edema were seen in any of the three groups.

Myosin ATPase activities and isomyosin distribution were measured in cardiac myofibrils. Figure 1 shows the results of this contractile protein biochemical analysis. The calcium-activated ATPase activity of myosin in myofibrils was 33% depressed in old versus young hearts and was further depressed by 19% in the hearts of old hypertensive animals. A similar trend was seen in isomyosin distribution. \(V_1\) myosin accounted for more than 95% of the total in young hearts, but with aging this percentage fell to 63%, and with age plus hypertension to 49%. A reciprocal increase in the percent of \(V_1\) myosin was seen. That this decrease in \(V_1\) and increase in \(V_3\) reflected an isomyosin shift and not a differential loss of the \(V_1\) isoform was demonstrated by SDS gel electrophoresis of myofibrils (Figure 2), which established that myosin accounted for a constant percentage of total cellular protein in all three groups and, further, that the ratio of MHC to actin did not change with aging or hypertension.
To determine whether the observed changes in myosin protein expression reflected changes at the mRNA level, Northern gel analysis of total RNA purified from the hearts of young, old, and old hypertensive animals was performed. Figure 3 demonstrates MHC probe specificity and also qualitative changes in the levels of α-MHC and β-MHC mRNAs in each experimental group. This figure demonstrates that α-MHC mRNA is predominant in the hearts of young rats and that β-MHC mRNA increases and α-MHC mRNA decreases with aging. To make quantitative measurements, RNA slot blots were hybridized, and multiple densitometric scans were obtained. Figure 4 shows data from six to 12 hearts normalized to 18S rRNA in each group. In the young animals, α-MHC predominates by approximately 2 to 1, whereas in old animals there is a 90% increase in β and a parallel decrease in α myosin gene mRNA relative to that of 18S so that the ratio of the two is reversed. With hypertension, a further 68% increase in β and a 48% decrease in α is seen.

Thyroid hormone has been shown to regulate MHC gene expression in a variety of animal models.22 We therefore measured plasma T3, T4, and TSH levels. These data are shown in Table 2. T3 levels were all within the normal range, T4 levels were depressed to an equivalent degree in both old and old hypertensive animals, and TSH levels were within normal limits in all three groups.

Data for the cardiac sarcoplasmic reticular ATPase mRNA are shown in Figures 5 and 6. Because the DNA probe was obtained from a human cDNA library, Northern gel analysis of RNA purified from human and rat ventricles and from rat liver is shown in Figure 5. The sarcoplasmic reticular ATPase mRNA is of similar size in human and rat and is not present in liver. Of interest, this mRNA appears to be more abundant in rat than in human. The data in Figure 6, as in Figure 4, are derived from densitometric scans of slot blots made from multiple hearts in each experimental group. With age alone, no change in gene expression of the sarcoplasmic reticular ATPase is seen. With the superimposition of hypertension, however, a reduction of greater than 50% in the level of mRNA for this gene product is seen.

**Table 2. Thyroid Hormone Levels**

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Old</th>
<th>Old-HTN</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3 (60–200 mg/dl)</td>
<td>73±9*</td>
<td>35±7</td>
<td>21±3</td>
</tr>
<tr>
<td>T4 (4–11 μg/dl)</td>
<td>5.4±0.2</td>
<td>4.6±0.3</td>
<td>4.1±0.3</td>
</tr>
<tr>
<td>TSH (1–6 IU/ml)</td>
<td>...</td>
<td>2.4±0.2</td>
<td>2.4±0.1</td>
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</table>

Values are mean±SEM of seven to 11 studies. Normal values are indicated in parentheses. Htn, hypertensive; T3, triiodothyronine; T4, thyroxine; TSH, thyroid stimulating hormone.

*P<0.05 vs. old.

**Figure 4. Myosin heavy chain (MHC) gene expression with aging and hypertension.** Data are expressed as the ratio of MHC/18S gene expression (see "Materials and Methods") and are the mean±SEM of seven to 11 studies. Arbitrary optical density units are plotted along the ordinate. Comparisons between young and old and between old and old hypertensives (Old-HTN) were all p<0.05.

**Discussion**

The major findings of this study can be divided into two areas. The first is a description of the morphological, biochemical, and molecular genetic sequelae of aging in a rat model, and the second is an analysis of how the aged rat heart adapts to a superimposed pressure load.

Our description of the aged rat heart is consistent with data from several other groups working with a variety of animal models and humans.1,23 An age-
related increase in heart mass associated with a progressive decline in isolated muscle performance and in exercise capacity has been seen in a variety of species.\textsuperscript{1,23–26} We and others previously have described a decrease in myosin ATPase activities and a shift toward the V\textsubscript{3} myosin isoform in the aged heart.\textsuperscript{5,6} One unique contribution of the present study, however, is the description of the molecular genetic adaptations that underlie these biochemical alterations. There are clear shifts in MHC mRNA levels in the aged heart, from \(\alpha\) to \(\beta\) predominant, suggesting that the regulation of the myosin isoforms is transcriptional or posttranscriptional and is analogous to that associated both with hypothyroidism and with the cardiac adaptation to a superimposed pathological load.\textsuperscript{27,28} Somewhat surprising was the absence in the aged heart of a significant change in the level of mRNA for the cardiac sarcoplasmic reticular calcium ATPase, as aging has been associated with alterations in active relaxation and with depressed sarcoplasmic reticular uptake of free calcium.\textsuperscript{7,25,29} While the previous determinations were done in different strains at slightly different ages, our data suggest that these biochemical and physiological observations cannot be explained solely by alterations in mRNA levels. Indeed, other investigators have suggested that the coupling of ATP hydrolysis to calcium transport by the sarcoplasmic reticulum may be altered with aging independent of its calcium-activated ATPase.\textsuperscript{30} It is also conceivable that the relative rates of protein synthesis and degradation of the calcium ATPase may be altered in aged animals.

The stimulus for the development of these molecular and biochemical adaptations in the aged rat heart is not clear. Thyroid hormone is known to modulate isomyosin expression in the heart,\textsuperscript{22,27,28} and there was a significant decrease in T\textsubscript{3} levels in both aged groups. Low T\textsubscript{3} levels have been described with aging by others and are felt to reflect impaired peripheral conversion of T\textsubscript{4}.\textsuperscript{31} Because alterations in T\textsubscript{4} and TSH were not seen and because resting oxygen consumption was not decreased (data not shown), it is likely that the animals in our study were clinically euthyroid, and therefore the reduced T\textsubscript{3} levels probably are not significant. Other hormones, such as testosterone, insulin, and norepinephrine, that have been shown to influence isomyosin distribution were not measured but have not been reported to be altered with aging. There was no difference in tail-cuff blood pressure between the old animals and a parallel group of young animals, although age-related changes in vascular compliance might have imparted a mild systolic load not reflected in our blood pressure measurements.

At the outset, we hypothesized that the aged heart would be unable to adapt to the additional load of hypertension with a further increase in mass. This
hypothesis derived from a number of studies that demonstrated diminished rates of protein synthesis with aging and from our previous data that showed a relatively minor hypertrophic response (when contrasted to that seen in younger animals) to renovascular hypertension in 24-month-old Fisher 344 rats. Our present data show that the aged rat heart with a left ventricular mass of more than twice that seen in younger animals can adapt to the superimposed load of hypertension with the development of significant cardiac hypertrophy. Hypertension increased left ventricular mass by approximately 40% versus its age-matched cohort, which is analogous to the hypertension-associated increase in cardiac mass seen in young animals. This, coupled with the ability of the aged heart to restructure its myosin isozyme content in response to hypertension, argues against an age-associated impairment in gene expression and protein synthesis, although one might speculate that the aged hypertensive hearts with a mass nearly three times that of young animals had reached the limits of cellular hypertrophy.

At the level of gene transcription and translation, the aged heart actively develops both genetic and biochemical adaptations in response to the imposed load of hypertension. With hypertension, there is a further shift in MHC mRNA levels, with an increase in \( \beta \)-MHC and a decrease in \( \alpha \)-MHC mRNA. This results in an increase in \( V_1 \) myosin and a marked decrease in myosin ATPase activity, which has been correlated with a decrease in indexes of cardiac contractile performance in a variety of animal models. In addition, the marked decrease in sarcoplasmic reticular calcium ATPase mRNA levels seen in the aged animals with hypertension suggests a molecular basis beyond that seen with aging alone for the impairment in uptake of calcium by the sarcoplasmic reticulum and the decline in the rate of active relaxation. Both of these biochemical processes might contribute to the decline in the global left ventricular performance that has been described in the aged hypertensive rat heart. The \( V_1 \)-to-\( V_2 \) ratio in the aged hypertensive hearts is among the lowest reported in rat and is in fact more similar to that observed in larger mammals and humans, demonstrating a remarkable plasticity in gene expression in this species. The phenotypic similarity in myosin isoform expression between old hypertensive rats and humans suggests that their molecular genetic responses to additional pathologic loads also might be similar; in fact, several investigators have shown impaired calcium handling in myopathic human hearts, which certainly could reflect decreased sarcoplasmic reticular calcium ATPase mRNA.

The sarcoplasmic reticular ATPase data in the present study also complement the recent observations of de la Bastie et al, who have shown no decline in cardiac sarcoplasmic reticular ATPase mRNA levels in young rats subjected to mild systolic overload with only mild hypertrophy. However, rats subjected to severe loads with more significant hypertrophy showed a decline in sarcoplasmic reticular ATPase mRNA similar to that seen in the aged hypertensive animals in the present study. Similar data have been reported by Nagai et al in hypertrophied rabbit myocardium. Thus, the heart may respond to stresses in a stepwise fashion, initially by altering the expression of members of the MHC multigene family and then subsequently by altering the expression of other genes, such as sarcoplasmic reticular ATPase, which are involved in the regulation of excitation–contraction coupling.

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