Aldosterone Antagonism Fails to Attenuate Age-Associated Left Ventricular Fibrosis

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Collagen accumulates disproportionately in cardiac remodeling induced by hypertension and associated with advancing age. Spironolactone (Spiro), an aldosterone antagonist, attenuates the accumulation of collagen induced by hypertension. It was hypothesized that Spiro would attenuate the age-associated increase in percent collagen in the heart. Female Fisher 344 rats at 3 months (Y), 12 months (M), and 21 months (O) of age were treated with Spiro (30 mg/kg/d) or vehicle (Veh) for 2 months, yielding six groups: Y-Veh, Y-Spiro, M-Veh, M-Spiro, O-Veh, and O-Spiro. Hearts were harvested for immunoblotting, RNA blotting, and biochemical analysis. Percent collagen in the left ventricle and septum was greatest in the oldest rats. Spiro did not significantly attenuate the age-associated increase in collagen fraction or the age-associated increases in expression of atrial natriuretic factor and β-myosin heavy chain messenger RNA. Chronic aldosterone antagonism does not attenuate the age-associated increase in collagen fraction in the female Fisher 344 rat heart.

INTERSTITIAL fibrosis is a hallmark of cardiac hypertrophy in heart failure (1–3), hypertension, and other pathological conditions (4,5), as well as normal aging (6–8), but is absent from hearts in which the stimulus for hypertrophy is exercise training (9–11) or hyperthyroidism (12). Diastolic dysfunction related to fibrosis is observed in patients with hypertension and hypertrophic cardiomyopathy, and is particularly prevalent in elderly persons (13). Age is the leading risk factor for congestive heart failure (CHF), but the biological mechanisms responsible are not clear. Normal aging is associated with a modest accumulation of extracellular matrix, increased collagen cross-linking, and increased myocardial stiffness, but the regulation is complex and is not completely understood (9,10,14,15). For example, with advancing age there is elevated cardiac expression of some matrix component messenger RNAs (mRNAs) such as fibronectin, but not others, such as collagen (6–8).

Extracellular matrix in heart tissue is composed primarily of collagen proteins, although a number of other proteins, such as fibronectin and osteopontin, play important roles in the complex structure of the interstitial space. Fibrillar Type I and Type III collagens make up the vast majority of the collagen protein in the heart, and provide the raw materials for the connective tissue “skeleton” of the heart (16,17). Collagen proteins assemble into a network of epimysial, perimysial, and endomysial fibers. These collagenous fibers function to maintain the alignment of myocytes and blood vessels, organize myocytes into bundles, and transduce force from myocytes to the chamber. Collagen fibers also provide passive stiffness, which prevents excessive diastolic enlargement and contributes to the Starling effect. Fibrillar collagens are large trimeric molecules that form triple helices and contain amino acid sequences that bind specific myocyte and fibroblast receptors (integrins), as well as other extracellular matrix molecules, such as fibronectin. Generally, Type I collagen predominates in the left ventricle (LV), although in the fetus and neonate Type III collagen levels are high and may exceed levels of Type I. Type III collagen is considered “youthful” collagen; with aging there is a gradual shift toward more Type I and less Type III collagen (18–20). Significantly, during periods of rapid tissue growth and collagen accretion, the Type III collagen fraction increases and the Type I fraction decreases (16). Such an increase in Type III collagen accretion is observed in several models of cardiac hypertrophy and is considered evidence of connective tissue remodeling of the myocardium.

Excessive fibrosis is a major determinant of impaired stiffness and pumping capacity in hypertrophied hearts. Its disproportionate accumulation accounts for a ventricular dysfunction that first appears during diastole, subsequently involves systole, and ultimately accelerates progression to CHF (16). Treatments that ameliorate the devastating effects of CHF and slow the progression to irreversible failure and death include agents that antagonize the action of aldosterone by blocking the mineralocorticoid receptor (MR), but treatments that reverse the negative functional and fibrotic characteristics of heart failure have not been identified (21). Furthermore, the mechanisms by which MR antagonists work are not entirely clear, and the impact of age on MR antagonism is virtually unknown.

Spironolactone is a competitive antagonist of the MR that has been available for decades. It is relatively selective for the MR, but does possess some progestational and antiandrogenic properties that produce unwanted side effects in
a subset of patients (22). A clinical trial demonstrated efficacy of spironolactone therapy in human patients with heart failure (23), but an understanding of the mechanisms by which spironolactone exerts its effects requires additional animal model studies. Aldosterone production is elevated in failing ventricles of humans (24), and spironolactone reduces the levels of collagen fragments in the plasma (25), suggesting that spironolactone works, at least in part, by reducing fibrosis of the myocardium. Past and recent data from animal models support and amplify this concept (26,27). Spironolactone inhibits the production of collagen by cultured cardiac fibroblasts (28). In animal models of hyperaldosteronism and hypertension, spironolactone therapy is effective in preventing or attenuating myocardial fibrosis (29,30). Another rat study showed that spironolactone and angiotensin-converting enzyme inhibitors interact to reduce fluid volume, and suggests that fluid reduction may at least partially explain the benefits of MR antagonism in humans (31). Aldosterone antagonism with a new, slightly more selective agent, eplerenone, reduces or prevents fibrosis in the viable myocardium after myocardial infarction in rats and improves the outcomes of patients with LV dysfunction after myocardial infarction (32,33). Despite the remarkable success of MR antagonism in treating heart failure patients, there is much to learn about the mechanisms of aldosterone signaling and the effectiveness of aldosterone antagonism in various subpopulations of patients, as pointed out recently (34,35). In particular, the effects of age on the efficacy of MR antagonists are not known, but at least one clinical study suggests that MR antagonism may be less effective in the elderly population (33). To our knowledge, there are virtually no data regarding age-associated changes in the local cardiac aldosterone signaling system. Thus, there is a need to obtain basic information on important components required for the postulated effects of mineralocorticoid excess on the fibrosis of advancing age in the heart.

Collectively, these data strongly suggest that the current lack of information regarding age-associated differences in the cardiac aldosterone system and the impact of age on efficacy of MR antagonists should be addressed by basic and clinical studies. The female Fisher 344 (F344) rat has served as a model to study the mechanisms of these changes in the heart and their implications for the response of the heart to physiological and pathological challenges (9,10,14,36–39). The hypothesis that aldosterone antagonism by spironolactone would attenuate the disproportionate age-associated increase in collagen accumulation was tested. The effects of spironolactone on the expression of two genes that typically exhibit age-associated increases in the heart, atrial natriuretic factor, and β-myosin heavy chain (β-MHC) were also studied.

Materials and Methods

Animals
Specific pathogen-free female Fisher 344 (F344) rats were obtained from the National Institute on Aging-sponsored colony at Harlan Industries (Indianapolis, IN). Rats were obtained at 3 (young; Y), 12 (middle-aged; M), and 21 (old; O) months of age and were treated with spironolactone (Spiro; 30 mg/kg/d) or vehicle (Veh; vegetable oil) orally for the next 2 months. Groups were as follows: Y-Veh ($n = 6$), Y-Spiro ($n = 6$), M-Veh ($n = 6$), M-Spiro ($n = 6$), O-Veh ($n = 8$), and O-Spiro ($n = 6$). Rats were housed 2–3 per cage and fed a standard laboratory diet 5001 (PMI Nutrition International, LLC, Richmond, IN) and water ad libitum, and were maintained on a 12-hour light/dark cycle. All animal protocols were approved by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan. All animal protocols conformed to the Guiding Principles in the Care and Use of Animals of the American Physiological Society. At designated times, the LVs, septum, and right ventricles (RVs) were quickly dissected free, weighed, and rapidly frozen by clamping withongs cooled to the temperature of liquid nitrogen. Tissues were stored at −80°C for subsequent analysis.

RNA Blotting

RNA blotting was performed as described previously with modifications (35,36). RNA was isolated from the LV. Ten micrograms of total RNA were size-fractionated by electrophoresis through 1% agarose gels, transferrred electrophoretically at 5 V/cm to nylon (Nytran-SPC; Whatman, Inc., Florham Park, NJ) membrane, and hybridized with 32P-radiolabeled probes overnight at 68°C for complementary DNA probes and 42–45°C for oligonucleotide probes using PerfectHyb Plus (Sigma, St. Louis, MO). Hybridization intensity was quantified with a Personal PhosphorImager FX (Bio-Rad, Hercules, CA). Signals visualized on a computer screen were identified by position relative to 18S and 28S ribosomal RNA migration, delineated by rectangles, and quantified after background subtraction. Each blot was subsequently stripped and reprobed. The signal from each sample was normalized to the signal obtained with an oligonucleotide specific for the 3′-untranslated region of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (40). The probe for β-MHC was an oligonucleotide described previously (41). A complementary DNA probe for atrial natriuretic factor (ANF) was synthesized from a template (41) by the random prime method (Promega, Madison, WI).

Plasma Aldosterone

Plasma aldosterone levels were measured by radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA).

Collagen Type III Immunoblotting

Portions of the frozen LV were processed for immunoblotting as described (42). The relative levels of collagen Type III protein were measured using a monoclonal antibody directed at collagen Type III (Santa Cruz Biotechnology, Santa Cruz, CA). Signals were quantified using enhanced chemiluminescence.

Hydroxyproline Assay

Hydroxyproline concentration was measured colorimetrically as described previously (10). Collagen concentration was calculated assuming that collagen weighs 7.25 times hydroxyproline and has a molecular weight of 300,000 daltons.
Wet weight to dry weight ratio of the LV did not differ among age groups or in response to Spiro treatment, indicating that there were no effects of age or Spiro treatment on hydration status. RV weight was increased with advancing age (p < .001), but not by Spiro treatment.

Levels of Plasma and Cardiac Aldosterone

Levels of plasma aldosterone did not differ significantly as a function of age (Figure 1). Administration of Spiro for 2 months increased plasma aldosterone levels similarly in each age group (p < .05). There was a main effect for Spiro treatment to decrease cardiac aldosterone levels (p < .05) and a tendency for the effect of Spiro to be greatest in the oldest group (Figure 2; p < .10).

Percent Collagen in Heart Tissues

Age-associated increases in interstitial fibrosis and increases in collagen gene expression have been reported previously (8–10,36) and likely contributed to increased myocardial stiffness and diastolic dysfunction with aging (39). In general, the present findings are consistent with previous findings, except that percent collagen in the LV...
free wall of young rats was somewhat higher than observed previously in this strain of rat. There was a main effect of age on the fractional composition of collagen in the LV, which when expressed as a percentage, was higher in the old compared to the middle-aged groups (Figure 3A). There was an interactive effect of age and Spiro treatment on LV percent collagen \( (p < .10) \), suggesting that Spiro treatment tended to exacerbate the age-associated increase in percent collagen in the oldest rats. Consistent with previous studies \((9,10)\), there was a main effect of age on percent collagen in the septum with the collagen fraction increasing progressively as a function of age (Figure 4). Spiro treatment had no significant effect on the age-associated increase in collagen content in the septum.

**Collagen Type III Levels**

There were no significant differences among the groups in Type III collagen of the LV measured by Western blotting (Figure 3, B and C). Using the hydroxyproline data in Figure 3A as a measure of total collagen and collagen III immunoblotting data from Figure 3C, the relative levels of collagen Type I in the LV were estimated (data not shown). Based on these estimates, the ratio of Type I to Type III collagen increased in the LV of female F344 rats with advancing age as previously observed in male rats of other strains \((18–20)\). This finding strongly implies that differences in levels of Type I collagen account for the observed age-associated changes in total collagen (Figure 3A), and that Spiro treatment did not significantly influence age-associated changes in total collagen or the inferred changes in Type I/Type III collagen ratio.

**Gene Expression**

To determine whether Spiro treatment altered the expression of other cardiac genes known to change with age, the level of ANF and \(\beta\)-MHC mRNA were also studied. ANF mRNA expression was increased with advancing age as observed previously \((44)\), but did not differ with Spiro treatment (Figure 5). \(\beta\)-MHC mRNA expression also increased with advancing age, but was not affected by Spiro treatment (Figure 6). When normalized to 18S, the ANF and \(\beta\)-MHC mean data exhibited a pattern similar to the GAPDH-normalized data and lead to identical conclusions (data not shown).
DISCUSSION

The present study was conducted on female F344 rats because they exhibit both age-associated accumulation of collagen in the heart and diastolic dysfunction of the LV (9,10,36,39). The main finding of this study is that long-term (2 months) treatment of Spiro does not decrease the modest disproportional increases in collagen fraction of the LV of the heart that occur with advancing age. Measuring total collagen with the hydroxyproline assay and subsequently measuring Type III collagen by immunoblotting permitted the assessment of changes in Type I collagen (Type I/Type III ratio), because hydroxyproline is essentially a measurement of both Type I and Type III collagen. Type III collagen is essential for collagen cross-linking, so any changes in measured Type III collagen would have implied changes in hydroxylsylpyridinoline cross-links, the major nonreducible cross-link in the heart. Because Type III collagen abundance would be expected to exhibit changes before they became obvious in Type I collagen (16,19,20), the finding that collagen Type III levels were unaffected by Spiro treatment (Figure 3, B and C) suggests that the degree of collagen cross-linking is unlikely to be altered by the 2-month Spiro treatment and underscores the lack of Spiro effect on age-associated fibrosis (45). Because the extracellular matrix interacts intimately with myocytes, the expression of myocyte-specific genes that are commonly upregulated with aging and hypertrophy were studied. Similar to the lack of a Spiro effect on age-associated fibrosis, Spiro treatment did not attenuate the age-associated increases in ANF or β-MHC mRNA (Figures 5 and 6). Given the pronounced effects that 2 months of Spiro treatment exhibited in preventing fibrosis induced by hypertension and hyperaldosteronism (29,30), the present findings suggest that age-associated increases in cardiac fibrosis are caused by mechanisms distinct from those that lead to fibrosis in response to hypertension and hyperaldosteronism.

The lack of a Spiro effect raises the question of whether the Spiro treatment was effectively absorbed after oral administration. The markedly elevated levels of plasma aldosterone observed in Spiro-treated rats of each age group (Figure 1) and the significant effect of Spiro on cardiac aldosterone levels (Figure 2) provide indirect evidence that the Spiro was appropriately absorbed. Furthermore, the same dose of Spiro (30 mg/kg/d) administered in the same manner for 6 months to aorta-constricted rats was effective...
in reducing the percent collagen in the heart (our unpublished observations). Moreover, Brilla and coworkers (29,30) used a lower dose of Spiro (20 mg/kg/d) to effectively prevent cardiac fibrosis induced by either hyperaldosteronism or hypertension. Thus, it is unlikely that the failure of Spiro treatment to prevent cardiac fibrosis in the present study is due to insufficient bioavailability of the drug. Due to the relatively slow turnover of collagen, the possibility that a longer course of Spiro treatment might significantly reduce age-associated cardiac fibrosis cannot be ruled out by the present findings.

The effect of Spiro treatment to induce a small (3%–9%) but significant increase in LV weight was unexpected and was most evident in the oldest group (Table 1), indicating that Spiro treatment exacerbates age-associated cardiac hypertrophy. To our knowledge, no previous studies have reported a hypertrophic effect of Spiro treatment on the intact heart. In contrast, in rats with low-aldosterone hypertension, Nagata and coworkers (46) reported that Spiro treatment attenuates LV hypertrophy, without an antihypertensive effect. Exposure of cultured neonatal rat cardiac myocyte to aldosterone leads to myocyte hypertrophy mediated by genomic effects of the MR that are blocked by aldosterone antagonism (47,48). At present, no explanation for this hypertrophic effect of Spiro treatment on the LV is apparent, but it is noteworthy that Spiro treatment did not have a similar hypertrophic effect on the RV free wall (Table 1). The finding that Spiro induced a small but significant increase in LV mass was surprising. Although the magnitude of cardiac hypertrophy induced by Spiro in the present study was quite modest, it was statistically significant, and although the magnitude of hypertrophy did not differ by age, it was numerically greatest in the oldest group. In fact, if the oldest group were not included in the study, the cardiac hypertrophy would not have been statistically significant, and would therefore have escaped detection. Thus, modest cardiac hypertrophy may be a side effect of chronic Spiro treatment. To our knowledge, there are no published reports in which Spiro treatment causes cardiac hypertrophy; therefore, the mechanism is unknown. Because aldosterone induced cardiac myocyte hypertrophy in culture, it might have been predicted that Spiro would decrease, rather than increase LV mass. It is noteworthy that chronic Spiro treatment induced a large compensatory increase in circulating levels of aldosterone, and it is possible that circulating aldosterone induced the observed increase in LV mass by an MR-independent mechanism.

The two major limitations of the present study are that a treatment period longer than 2 months might be required to observe an effect of Spiro on the relatively long-lived collagen protein and the limited number of rats (n = 6–8) per group. The former limitation should be considered in light of the observations of Brilla and coworkers (29,30) that 2 months of Spiro treatment was effective in preventing fibrosis induced by hyperaldosteronism and hypertension, observations that were used to guide the design of the present study. The limitation imposed by the small number of rats per group is mitigated by the 2 × 3 design and the statistical robustness of the two-factor ANOVA procedure. The conclusions are bolstered by the fact that there are no obvious trends in the data from the Spiro groups to suggest that Spiro was having the hypothesized effect to attenuate the age-associated increase in collagen accumulation. Instead, with the exception of the collagen fraction in the septum, most trends are in the opposite direction (e.g., Figure 3A and C). Thus, the conclusion that 2 months of Spiro treatment did not favorably influence the age-associated increases in the percent collagen in the rat ventricle can be stated with confidence.

Another potential limitation is that peripheral blood pressure was not measured in the present study. Therefore, the possibility that Spiro may have exerted effects by modifying blood pressure cannot be ruled out. For example, when Spiro is administered at a high dose (200 mg/kg/d) to hypertensive rats, it has a modest effect to lower blood pressure (49). Based on studies of normotensive rats, however, administration of 30 mg/kg/d of Spiro would not be expected to influence blood pressure in normotensive rats (30). Indeed even a much higher dose of Spiro (200 mg/kg/d) failed to significantly alter blood pressure in normotensive 22-month-old rats (50). Thus, it seems unlikely that daily treatment of 30 mg/kg/d Spiro for 2 months influenced the blood pressure of the rats in the present study.

Because a disproportionate number of elderly patients suffer from CHF, cardiac fibrosis, and diastolic dysfunction (51), and because aldosterone antagonism is an important feature of pharmacotherapy for CHF, these findings have potential clinical implications: They suggest that aldosterone antagonism may be less effective at combating fibrosis and heart failure in elderly persons, a possibility also suggested by a clinical study of aldosterone antagonism (33). Further study of the effectiveness of aldosterone antagonism in heart failure models of younger and older animals is warranted.

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