Comparative effects of dehydroepiandrosterone sulfate on ventricular diastolic function with young and aged female mice

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Submitted 15 April 2005; accepted in final form 2 August 2005

Maladaptive cardiac remodeling occurs during the aging process and is associated with diastolic dysfunction in humans, rats, and mice (5, 14, 20, 21, 31). The progressive increased diastolic stiffness that occurs with age has been associated with remodeling of the extracellular matrix (ECM) (3). This increased ventricular stiffness has been linked to changes in collagens I and III, increased collagen I-to-III ratios (7, 9, 17, 18), and collagen cross-linking (2, 35). Age-related remodeling of the ECM implies alteration of cardiac fibroblast function, thereby affecting 1) the rate of collagen synthesis, 2) the level of collagen degradation by matrix metalloproteinases (MMPs), and 3) the extent of collagen cross-linking mediated by cardiac lysyl oxidase. Androgens have a conspicuous ability to alter fibroblast function during wound healing, a process affected by aging (10, 22). It therefore follows that neuroendocrine senescence may also affect cardiac fibroblast function and thereby altered diastolic function. In the context of the exceptional results of dehydroepiandrosterone (DHEA) supplementation with elderly humans in normalization of metabolic functions (27), it is important to define whether this replacement therapy also affects cardiac function.

DHEA and its sulfated derivative [DHEA(S)] are abundant adrenal steroid hormones that have been studied extensively because of their potential anti-aging effects. Although the direct physiological role of DHEA is complex, it acts as a precursor to estradiol and testosterone, whereas DHEA-S serves as a reservoir for DHEA. In humans, there is clearly an age-related decline in DHEA levels, whereas other adrenal hormones remain unchanged, suggesting that DHEA may be an important factor in the aging process. Because of the reported beneficial effects of DHEA(S) supplementation in the elderly, there is a need to determine the effect of DHEA(S) on cardiac function.

Several reports support the effects of DHEA(S) on cardiac tissues harvested from young rodents (12, 24); however, there is a lack of information regarding the DHEA(S) effects in aged models. Androgen receptors are expressed by fibroblasts (4, 10), suggesting that androgens may have a direct effect on cardiac fibroblast function. We hypothesized, therefore, that DHEA(S) affects the cardiac ECM composition through modulation of fibroblast function, resulting in altered diastolic function. The study contained herein supports an effect of DHEA(S) on myocardial gene expression, collagen composition, and age-dependent diastolic function.

MATERIALS AND METHODS

Animals. Female C57BL/6j mice, 4–5 and 20–22 mo of age, were obtained from Harlan (Indianapolis, IN). Before the study, the mice were conditioned for 1 wk in the animal facility. The young and old mice were divided into controls and the 60-day treatment regimen with DHEA(S) with 12 mice in each treatment group. Seven mice were used for left ventricular functional analysis, and at the completion of the analysis the ventricular tissues were saved for the collagen assays. The hearts from five additional mice from each group were used for real-time PCR. Five naïve mice were used for cardiac fibroblast isolation for the production of primary fibroblast cultures to support the zymography analysis. DHEA(S)-treated mice were given water supplemented with 0.1 mg/ml DHEA(S) (Sigma-Aldrich, St. Louis, MO) for 60 days. All animal studies were performed after receiving approval from our animal review committee. Guidelines for the Care and Use of Laboratory Animals [DHEW Publication No. (National Institutes of Health) 85-23, Revised 1985, Office of Science

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and Health Reports. DRR/NIH, Bethesda, MD 20205] and Principles of Laboratory Animal Care (published by the National Society for Animal Research) were followed in this study.

Analysis of left ventricular function. The Millar conductance catheter system was used to generate pressure-volume loops, as has been previously described by our laboratory (30–32). All mice were anesthetized, and a 1.4-Fr Millar conductance catheter (Millar, Houston, TX) was inserted into the apex of the left ventricle with the distal electrode positioned near the aortic root and the proximal electrode in the left ventricular apex. In vivo pressure-volume relationships were analyzed with BioBench software (National Instruments, Austin, TX) and analyzed with Pvan version 2.7 (Conductance Technologies, San Antonio, TX, and Millar).

Determination of hydroxyproline and collagen cross-linking. Hydroxyproline is an amino acid found exclusively in connective tissues and is used as a means to quantify collagen concentration. For measurement of total myocardial hydroxyproline, dried left ventricular samples were hydrolyzed in 6 N HCl for 24 h at 120°C followed by neutralization with 2.5 M NaOH. Hydroxyproline levels were then quantified by comparison with a standard colorimetric curve of transhydroxyproline (Sigma) according to the methods of Stegemann and Stadler (26).

Collagen cross-linking was determined using cyanoan bromide digestion according to Woodiwiss et al. (29), which is a modification of that originally described by Mukherjee and Sen (19). Dried left ventricular samples were homogenized in PBS and then centrifuged at 4,000 g for 10 min. The resulting pellet was then washed in 2% SDS three times to remove noncollagenous proteins, each time followed by centrifugation at 4,000 g for 10 min. The 2% SDS was removed by three successive washes in PBS followed by two washes in acetone. After the second acetone wash, the resulting pellet was air-dried and resuspended in 20 mg/ml cyanoan bromide in 70% formic acid and incubated at 25°C for 24 h. After incubation, the suspension was centrifuged at 4,000 g for 20 min. The resulting supernatant was dried using a speed vacuum followed by the measurement of hydroxyproline as described above. Percent cross-linking was determined by comparing the total hydroxyproline with cyanogen bromide-soluble hydroxyproline. The data were expressed as microgram of collagen per milligram of dry heart weight, assuming that collagen contains an average of 13.5% hydroxyproline.

RNA extraction and real-time PCR. Cardiac tissues from five mice were harvested in Trizol (Invitrogen Life Technologies, Carlsbad, CA). Diluted cDNA was used for reaction with Quantitect SYBR Green PCR kit (50 μl; Qiagen), and real-time PCR was performed with SYBR Green using the Rotor-Gene RG-3000 (Corbett Research) in a 72-well rotor. Custom primers were designed using Primer3 (23) and synthesized by Integrated DNA Technologies. Primer quality (lack of primer-dimer amplification) was confirmed by melting curve analysis and gel-electrophoresis of each primer product. In each experiment, the relative amounts of mRNA for target genes were calculated from the standard curves and normalized to the relative amounts of reference gene RNA (β-actin mRNA), which were obtained from a similar standard curve. Real-time PCR primers are listed in Table 1.

Cardiac fibroblast isolation and culture. Ventricular sections from five naïve mice were minced and sequentially digested 15 times with 0.54 mg of Liberase 3 enzyme (Roche Biochemical) in 60 ml of warm sterile Krebs-Henslett buffer, and the adherent fibroblasts were plated in a T75 with 20 ml of DMEM/HEPES with 10% fetal bovine serum medium. These primary fibroblast cultures were grown to 80% confluence, and the fetal bovine serum decreased to 1% before being treated with DHEA(S). This protocol provided a cardiac fibroblast purity of >88% and used the cardiac fibroblast-specific DDR2 antibody with FACS.

Zymography. Gelatin zymography was performed using fibroblast culture supernatants. The culture medium (15 μl) was diluted 1:1 with SDS loading buffer and applied to precast 10% polyacrylamide gel Zymogram (Novex, Frankfurt, Germany). After electrophoresis at 100 V, 4°C, for 120 min, the gels were washed twice with renaturing buffer at room temperature for 30 min. Zymograms were then transferred into a developing buffer at 37°C for 12 h. After fixation and staining with Coomassie brilliant blue G-250 (0.25%), the zymograms were destained with 10% (vol/vol) acetic acid, and bands were quantified with image analysis (Bio-Rad GS-800).

Statistical analysis. ANOVA with multicomparison procedures was used to test the differences among the defined groups with SPSS version 11.5. Values obtained from treatment groups were compared with control values using the Student’s t-test. Comparable nonparametric tests (Kruskal-Wallis and the rank sum test) were substituted when tests for normality and equal variance failed. All data are reported as means ± SE.

RESULTS

Cardiac function. Figure 1 shows that DHEA(S) supplementation significantly increased the ventricular stiffness of the young mice by 41% (P = 0.05) and decreased the supplemented aged group by 56% (P = 0.032). Table 2 shows that DHEA(S) supplementation increased cardiac index by 18% (not significant) and preload recruitable stroke work (PRSW) by 26% (P = 0.039) in the aged group but had no demonstrated effect on these parameters in the young group. The rate of passive diastolic filling supports the changes observed in the ventricular stiffness parameter in the aged groups. The ventric-
ular end-diastolic volume increased by 33% and heart weight-to-body weight ratios by 31% in the treated aged group, which coincides with the decreased ventricular collagen and cross-linking in addition to a 30% increase in ventricular systolic pressure.

**Analysis of relative mRNA expression.** The effect of cardiac ECM on ventricular stiffness is related to the relative amount of collagen types I and III, MMP activity, and collagen cross-linking. Therefore, the mRNA levels for pro-collagen I and III, MMP activity, and collagen cross-linking. Therefore, the mRNA levels for collagen types I and III, MMP activity, and collagen cross-linking were determined by normalizing with β-actin and expressed as ratios related to the young untreated control group. Table 3 shows that the relative pro-collagen Iα1, pro-collagen IIIα1, and pro-MMP-13 mRNA levels were significantly decreased in the untreated aged mice ($P < 0.05$) compared with untreated young controls. DHEA(S) supplementation significantly decreased collagenases pro-MMP-13 gene expression in the young group, whereas significantly increasing the pro-MMP-13 plus gelatinase pro-MMP-9 genes by greater than fivefold in old group when compared with the young controls.

**Cardiac ECM.** Figure 2 shows that total cardiac collagen levels, measured by hydroxyproline, were predictably increased in the aged group by 69% ($P = 0.0035$) compared with the younger control mice. DHEA(S) supplementation decreased the total cardiac collagen by 13% ($P = 0.032$) in the aged mice and increased the total collagen by 12.5% (not significant) in the young group. Moreover, Fig. 3 shows that the percentage of collagen cross-linking was increased by threefold ($P = 0.0099$) in the aged compared with the young control groups. Most striking was that DHEA(S) supplementation decreased collagen cross-linking by 70% ($P = 0.0015$) in the aged and increased cross-linking in the young by 40% (not significant). Figure 4 demonstrates that the percentage of collagen cross-linking is associated with ventricular stiffness ($P = 0.001$). These data demonstrate that DHEA(S) supplementation markedly affects the quantity and quality of cardiac collagen.

**In vitro effects of DHEA(S).** DHEA(S) was added to 80% confluent primary young and old cardiac fibroblasts at a final concentration of $10^{-6}$ and $10^{-5}$ M. After 48 h, the supernatant was removed and the MMP-2 and MMP-9 activity was analyzed with zymography as shown in Fig. 5. Comparable with the hemodynamic and collagen factors in the in vivo model, DHEA(S) decreased MMP-9 and -2 activities in the young cardiac fibroblasts and increased the MMP activities in the cardiac fibroblast harvested from the older mice. These data demonstrate that DHEA(S) has a direct affect on cardiac fibroblasts.

**DISCUSSION**

The major conclusion of this investigation is that chronic supplementation of mice with DHEA(S) affects left ventricular compliance, and aging.

| Table 2. Comparison of the hemodynamic effects of DHEA(S) supplementation related to age |
|---------------------------------|---------------|---------------|---------------|---------------|
|                               | Young         | DHEA(S)       | Old           | DHEA(S)       |
| $n$                            | 7             | 7             | 7             | 7             |
| BW, g                          | 24.6 ± 0.6    | 25.7 ± 0.9    | 31.7 ± 2.6    | 36.4 ± 3.4†   |
| HW, mg                         | 138 ± 2       | 132 ± 8       | 141 ± 6      | 185 ± 6†‡     |
| HW/BW, mg/g                    | 5.6 ± 0.1     | 5.1 ± 0.2     | 4.5 ± 0.2     | 5.3 ± 0.5     |
| Heart rate, beats/min          | 550 ± 19      | 475 ± 17*     | 555 ± 11     | 520 ± 22      |
| $P_{max}$, mmHg                | 99 ± 11       | 89 ± 4        | 73 ± 4*      | 95 ± 6†       |
| EF, %                          | 63.8 ± 2.9    | 63.8 ± 4.1    | 65.1 ± 6.5   | 70.8 ± 3.8    |
| SVI, μl/g                      | 0.46 ± 0.04   | 0.50 ± 0.03   | 0.34 ± 0.03* | 0.44 ± 0.08†  |
| CI, μl/min $^{-1}$ g$^{-1}$    | 253 ± 24      | 238 ± 21      | 185 ± 15*    | 219 ± 33      |
| PRSW, mmHg                     | 97.4 ± 7.0    | 95.0 ± 12.2   | 77.3 ± 4.6*  | 97.7 ± 6.6†   |
| Ved, μl                        | 17.4 ± 1.7    | 18.2 ± 1.8    | 15.3 ± 0.8*  | 20.3 ± 1.8†   |
| dV/dtmax, μl/s                 | 523 ± 71      | 562 ± 47     | 465 ± 62     | 712 ± 73†‡    |
| $τ$, ms                        | 4.7 ± 0.4     | 7.2 ± 0.3*    | 7.3 ± 0.3*   | 7.3 ± 0.4     |

Values are means ± SE. These real-time PCR fold changes compare the number of cycles to achieve threshold for each gene expressed. The threshold cycle number for each group is presented as the mean relative fold change compared with the young control group normalized with β-actin. *$P < 0.05$ compared with young controls. †$P < 0.05$ compared with old controls. ‡$P < 0.05$ compared with young DHEA(S) treated.

**Table 3. Comparison of the gene expression effects of DHEA(S) supplementation related to age**

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>DHEA(S)</th>
<th>Old</th>
<th>DHEA(S)</th>
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<td>$n$</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
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<td>Pro-Collagen Iα2</td>
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<td>−1.1 ± 0.07</td>
<td>−1.5 ± 0.04*</td>
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<tr>
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<td>−1.1 ± 0.01</td>
<td>−1.3 ± 0.06</td>
<td>−1.4 ± 0.0*</td>
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<tr>
<td>Pro-MMP-9</td>
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<td>1.5 ± 0.08</td>
<td>1.7 ± 0.08</td>
<td>5.4 ± 0.2*</td>
</tr>
<tr>
<td>Pro-MMP-13</td>
<td>1.0</td>
<td>−3.0 ± 0.06*</td>
<td>−8.0 ± 0.01*</td>
<td>5.4 ± 0.3*‡</td>
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</table>

Values are means ± SE. These real-time PCR fold changes compare the number of cycles to achieve threshold for each gene expressed. The threshold cycle number for each group is presented as the mean relative fold change compared with the young control group normalized with β-actin. *$P < 0.05$ compared with young controls. †$P < 0.05$ compared with old controls. ‡$P < 0.05$ compared with young DHEA(S) treated.
The primary focus of this study was to compare the effects of DHEA(S) supplementation on cardiac MMP and collagen gene expression, ECM composition, and left ventricular stiffness in young and old mice. The control groups demonstrated an age-related increase in left ventricular stiffness and ventricular collagen, which is consistent with our previous work and that of others (9, 20, 31). This increased left ventricular stiffness is supported by a decreased rate of passive diastolic filling and a decrease in end-diastolic volume in the aged control group. Sixty days of DHEA(S) supplementation modified the diastolic function, as determined with left ventricular stiffness and end-diastolic volume, in the aged group toward that of the younger control group values. Paradoxically, this treatment decreased diastolic function in the younger group. It has been suggested that this age-related paradox is through increasing the availability of DHEA in the aged with DHEA(S) and in contrast with DHEA(S) supplementation of the young mice, the DHEA(S) acts as an antagonist to DHEA (J. M. Howard, personal communication). Therefore, we report that there is an age-related response to DHEA(S), where DHEA(S) appeared to decrease cardiac diastolic function in the young and increase diastolic function in the aged.

The doses of DHEA(S) for the in vivo and in vitro portions of this study were similar to those reported by others. Similar increases in DHEA serum levels were achieved with DHEA(S) doses ranging from 0.01 to 0.3 mg/ml (6, 8). Iwasaki et al. (12) demonstrated that 0.3 mg/ml of DHEA(S) significantly reduced ANG II-induced cardiac fibrosis and also demonstrated a direct effect of DHEA(S) on collagen gene expression with primary cultured cardiac fibroblasts. The in vitro concentrations of DHEA(S) used by others to treat fibroblasts ranged from $10^{-8}$ to $10^{-5}$ M (12, 16), which is comparable to our reported concentrations of $10^{-6}$ and $10^{-5}$ M. Therefore, our in vivo and in vitro doses of DHEA(S) appear to be appropriate, as supported by the literature.

We determined whether collagen cross-linking is correlated with ventricular stiffness. The young mice had 26% cross-linked collagen compared with 82% in the old group. The extent of cardiac collagen cross-linking has been previously associated with age (35). With DHEA(S) treatment in the older mice, the cross-linked collagen content decreased to 29% and was associated with a twofold decrease in ventricular stiffness. Other reports confirm this relationship through the use of chronic administration of the collagen cross-linking inhibitor, β-aminopropionitrile (13) and suggested that further reduction in myocardial collagen cross-linking is an important mechanism contributing to left ventricular dilation in heart disease (29). The DHEA(S)-mediated reduction in ventricular stiffness, and corresponding increase in end-diastolic volumes, occurred without the total cardiac collagen being decreased to that of the young control mice. Therefore, the cardiac collagen cross-linking appears to be a significant contributor to passive diastolic performance.

We report a nominal decrease in collagen gene expression in the younger treated mice and a significant decrease in the older treated group. These findings are consistent with the report that DHEA(S) supplementation decreased collagen gene expression, collagen type I levels in vitro, and ANG II-induced cardiac fibrosis in vivo (12). However, our data suggest that the primary determinant of cardiac collagen content is MMP activity.

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The major difference between the young and aged groups was a markedly decreased expression of the collagenase protein.
MMP-13 in the aged group compared with the younger group. This decreased pro-MMP-13 expression corresponded with the increased cardiac collagen content in the aged mice. It has been suggested that collagen is more sensitive to collagenase degradation in the absence of cross-linking (13). Therefore, these findings confirm those of others suggesting that the accumulation of collagen may be due to a decrease in degradation by MMP-13 and also increased cross-linking (7, 9). The gene expression for the MMPs was markedly increased with DHEA(S) treatment in the aged mice, which is supported with our zymography analysis for MMP-2 and -9. Moreover, the collagenase genes were increased in aged mice treated with DHEA(S) with only minor changes in the pro-collagen I and II gene expression. It is likely that the concentration of cross-linked cardiac collagen may be a contributory factor in the overall cardiac collagen concentration. This is supported by Woodiwiss et al. (29), who reported that, despite increased MMP activity, collagen concentrations can increase in the presence of a high degree of cross-linking.

The young treated group was originally included to serve as a control group. We did not expect to see DHEA(S) affect this group. According to our data, the proposed mechanism for the increased ventricular stiffness is an increased cross-linking and decreased MMP activity. When the cross-linking of the young untreated with the young treated were compared, the level of significance was $P = 0.076$ with a doubling of the percentage of cross-linked cardiac collagen. The gene expression of MMP-13 was decreased by threefold with 60 days of treatment in the young mice, and MMP-2 and -9 were markedly decreased in the in vitro study with 48 h of treatment. The time constant of isovolumic relaxation ($\tau$) increased from $4.7$ to $7.2$ ms ($P < 0.01$) in the young with DHEA treatment. $\tau$ may have increased because of an increase in ventricular stiffness due to the increased cross-linking and collagen content. Also, $\tau$ is primarily affected by SERCA2a and phospholamban function via calcium cycling and, therefore, suggests that DHEA(S) may have affected the state of phospholamban phosphorylation as reviewed by Yang et al. (33). Our laboratory previously showed that the increased $\tau$ in the aged is due to decreased phosphorylated phospholamban (34). In summary, the data related to the young treated mice are consistent with the mechanism that increased cross-linking and decreased MMP activity leading to increased ventricular stiffness.

It has been shown that DHEA is capable of directly decreasing collagenase activity in dermal fibroblasts (16), whereas others have shown DHEA to increase collagenase activity (24). We demonstrated a dose-dependent decrease in MMP-2 and -9 activities on primary cardiac fibroblasts with DHEA(S) at similar concentrations. Moreover, it has been demonstrated that DHEA decreases collagen accumulation in vitro in cardiac fibroblasts (12, 24). These reports do support our observation that DHEA(S) and DHEA have a direct effect on the collagen and MMP synthetic pathways, and, therefore, there is a direct effect of DHEA and DHEA(S) on cardiac fibroblasts, which may be a potential mechanism contributing to the alterations of collagen composition as reported in this study.

Our report that selective immune modulation of lymphocyte function significantly alters ECM composition and diastolic function in the absence of cardiac injury, inflammation, and increased wall stress is important in the context of DHEA(S)-mediated ECM remodeling (36). It is established that DHEA(S) induces immunomodulation through a macrophage pathway (4, 15). Therefore, immune modulation induced by DHEA(S) also may be a contributory factor in the cardiovascular effects observed with DHEA(S). It is well established that the immune system undergoes changes during aging resulting in immunosenescence. It also has been shown extensively that DHEA(S) is capable of restoring the immune function in aged mice (1, 11, 25, 28). Even though we demonstrated a direct effect of DHEA(S) on cardiac fibroblasts, this evidence does not exclude the possibility that DHEA(S) is also mediating cardiac remodeling activities via the immune system.

In conclusion, we found that treatment with exogenous DHEA(S) increased left ventricular diastolic function in old mice and conversely reduced diastolic function in young mice.
This cardiac effect appears to be related to the percentage of ventricular collagen cross-linking in addition to MMP expression and activity. Because of the pleiotropic activities of this androgen precursor, a mechanism for these activities may not be clearly defined; however, in the aged population, it appears to be efficacious. These data suggest that future studies to determine the efficacy DHEA(S) in models of age-related diastolic heart failure are warranted.

GRANTS

This study was supported by the American Heart Association Grant 0455575Z, the Steinbronn Heart Failure Research Award, and by Grant 5018 from the Arizona Disease Control Research Commission.

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