Marked attenuation of production of collagen type I from cardiac fibroblasts by dehydroepiandrosterone

Tomoyuki Iwasaki,1,6* Koji Mukasa,1,6* Masato Yoneda,1 Satoshi Ito,1 Yoshihiko Yamada,1 Yasumichi Mori,1 Nobutaka Fujisawa,1 Toshio Fujisawa,1 Koichiro Wada,2 Hisahiko Sekihara,1 and Atsushi Nakajima1

1Division of Endocrinology and Metabolism, Yokohama City University Graduate School of Medicine, Yokohama; and 2Department of Pharmacology, Graduate school of Dentistry, Osaka University, Osaka, Japan

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Iwasaki, Tomoyuki, Koji Mukasa, Masato Yoneda, Satoshi Ito, Yoshihiko Yamada, Yasumichi Mori, Nobutaka Fujisawa, Toshio Fujisawa, Koichiro Wada, Hisahiko Sekihara, and Atsushi Nakajima.

Marked attenuation of production of collagen type I from cardiac fibroblasts by dehydroepiandrosterone. Am J Physiol Endocrinol Metab 288: E1222–E1228, 2005. First published January 18, 2005; doi:10.1152/ajpendo.00370.2004.—Dehydroepiandrosterone (DHEA) is a type of adrenal steroid. The concentrations of DHEA and its sulfate (DHEA-S) in serum reach a peak between the ages of 25 and 30 yr and thereafter decline steadily. It was reported that DHEA-S concentration in humans is inversely related to death from cardiovascular diseases. In this study, we examined the effects of DHEA on regulation of collagen mRNA and collagen synthesis in cultured cardiac fibroblasts. Treatment with DHEA (10−6 M) resulted in a significant decrease in procollagen type I mRNA expression compared with controls. This was accompanied by a significant decrease in procollagen type I protein accumulation in the medium and also a significant decrease in procollagen type I protein synthesis in the cellular matrix. Furthermore, to confirm in vitro results, we administered DHEA to Sprague-Dawley rats, which were treated with angiotensin II for 8 wk to induce cardiac damage. Procollagen type I mRNA expression was significantly decreased and cardiac fibrosis significantly inhibited in DHEA-treated rat hearts without lowering the systolic blood pressure. These results strongly indicate that DHEA can directly attenuate collagen type I synthesis at the transcriptional level in vivo and in vitro in cardiac fibroblasts.

Type I collagen is the major collagenous product of cardiac fibroblasts, representing ~80% of the total newly synthesized collagen, which is secreted into the culture medium of cultured cardiac fibroblast as procollagen type I (42). About 20% of the total collagen synthesized is type III collagen, and a small proportion is type V collagen (<5%) (34). The collagen concentration and the intermolecular cross-linking of collagen increase with age (51), as does collagen concentration (13, 51). Excessive deposition of cardiac extracellular matrix (fibrosis) has been associated with the pathological mechanical overload of heart (47). Cardiac fibrosis is associated with the three most prevalent chronic cardiovascular diseases, namely hypertension (46, 48), heart failure (45, 49), and myocardial infarction (4, 19–22, 25–27, 35). The protective effect of DHEA and/or DHEA-S against cardiovascular disease has been the subject of many studies (7, 15–17, 28), but there are no reports regarding the effect of DHEA on heart tissue and cardiac fibroblasts. Therefore, we evaluated the effect of DHEA on cardiac fibroblasts in vitro and furthermore the effect of DHEA on cardiac fibrosis on the pressure-overload model in vivo. This is the first study to demonstrate the direct attenuation of collagen gene expression of heart tissue in vivo and cultured cardiac fibroblasts in vitro by DHEA.

MATERIALS AND METHODS

Reagent. DHEA and angiotensin II (ANG II) were purchased from Sigma (St. Louis, MO). DHEA was dissolved in 99.5% ethanol before use. Cultured cardiac fibroblasts were treated with different concentrations of DHEA from 10−7 M to 10−6 M.

Primary cardiac fibroblasts isolation and culture. Preparation of adult rat cardiac fibroblasts was performed as previously described (44, 48). Confluent fourth-passage adult rat cardiac fibroblasts from 8-wk-old Sprague-Dawley rats (48) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco-BRL, New York, NY) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1-ascorbic acid 2-phosphate. Cells were maintained in DMEM supplemented with 10% charcoal/dextran-treated FBS (Hyclone, Logan, UT), 1% penicillin-streptomycin for 24 h. DHEA was dissolved in ethanol (99.5%) and administered to culture medium of cardiac fibroblasts at the concentration of 10−7 to 10−5 M. The control group was treated with vehicle (ethanol without DHEA) alone. The final concentration of ethanol in control or DHEA-treated medium was <0.1%.

For growth study, adult rat cardiac fibroblasts were seeded in 96-well plates (10,000 cells/well) in DMEM supplemented with 10% ethanol. Marked attenuation of production of collagen type I from cardiac fibroblasts by dehydroepiandrosterone

DEHYDROEPIANDROSTERONE (DHEA) and its sulfate (DHEA-S) are the most abundant adrenal steroids in humans, having serum concentrations on the order of 10−8 and 10−6 M, respectively (37, 38). The concentrations in serum reach a peak between the ages of 25 and 30 yr and thereafter decline steadily, so that, by age 60, serum concentrations are only 5–10% of corresponding values in young adults (32). This age-dependent decline has recently been clinically linked to age-related illnesses such as atherosclerosis (2), obesity, diabetes (10), aging (44), some forms of cancer (33), and cardiovascular system disease (5). The cardiac fibroblast is the most abundant cell type present in the myocardium and is mainly responsible for the deposition of extracellular matrix. Important components of cardiac extracellular matrix include structural and adhesive proteins such as collagen and fibronectin.

* T. Iwasaki and K. Mukasa contributed equally to this article.

Address for reprint requests and other correspondence: A. Nakajima, Division of Endocrinology and Metabolism, Yokohama City Univ. Graduate School of Medicine, 3-9 Fuku-ura, Kanazawa-ku, Yokohama 236-0004, Japan (E-mail: nakajima-tky@umin.ac.jp).
FBS, 1% penicillin-streptomycin, and l-ascorbic acid 2-phosphate. After 1 day, cells were transferred to DMEM supplemented with 10% charcoal/dextran-treated FBS and 1% penicillin-streptomycin and allowed to grow for 24 h before being incubated with either vehicle or DHEA (10^{-7} to 10^{-5} M) for 24 or 72 h. Growth was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (11). Cell numbers were measured colorimetrically by using the Cell Counting Kit (Dojindo, Kumamoto, Japan) and an ImmunoMini NJ-2300 (NJ InterMed, Tokyo, Japan) at a test wavelength of 450 nm.

Northern blot hybridization and competitive reverse transcriptase-PCR. Total RNA was isolated from cultured fibroblasts and heart tissue using Isogen (Nippon Gene, Toyama, Japan). The amount of RNA was measured spectrophotometrically, and then the RNA was dissolved in RNase-free water. Northern blot hybridization was performed; the RNA from each sample was electrophoresed on a 1% agarose-formaldehyde gel with 1× MOPS as a running buffer, transferred onto a nitrocellulose membrane (Amerham, Piscataway, NJ), and cross-linked in a UV cross-linker. The membrane was then subjected to prehybridization for 1 h, followed by hybridization at 68°C for 2 h. Hybridization and washing were performed according to Sambrook et al. (36a). The same membrane was used sequentially for multiple comparisons were done by ANOVA. A P value of <0.05 was taken as statistically significant. A greater significance was assumed for P < 0.001.

RESULTS

Effect of DHEA on procollagen mRNA expression in cultured cardiac fibroblasts. The effects of DHEA (10^{-7} and 10^{-6} M) on collagen synthesis were examined by Northern blot analysis. Treatment of adult fibroblasts with DHEA for 24 h decreased the expression of mRNA for procollagen type I (α1), the major fibrillar collagen form in the rat heart (Fig. 1, A and B). The cells treated with 10^{-6} M DHEA showed a 76.8 ± 6.3% decrease in procollagen type I (α1) mRNA expression compared with untreated control cells (P = 0.037 vs. control). On the other hand, the expression of mRNA for procollagen type III was not different between controls and DHEA-treated cultures (Fig. 1, A and C).

Time course of collagen type I protein accumulation and cell proliferation after DHEA exposure in cultured cardiac fibroblasts. To determine the time course of inhibition of collagen type I protein accumulation in the medium by DHEA, we measured the accumulation of collagen type I protein in the medium of cells at 6, 12, 24, and 72 h by Western blotting. DHEA at 10^{-6} M inhibited the accumulation of collagen type I at 24 h and at 72 h after exposure to DHEA. At 24 h, the control measurement was 66.4 ± 4.50% (vs. 72-h control), and the value for cells treated with 10^{-6} M DHEA was 28.7 ± 7.65% (P = 0.0054 vs. 72-h control). At 72 h, the control measurement was 99.9 ± 10.2% (vs. 72-h control) and the value for cells treated with 10^{-6} M DHEA was 56.5 ± 3.00% (P = 0.0066 vs. 72-h control; Fig. 2, A and B).

In cell proliferation assays, we found that cell proliferation slightly increased by 108, 107, and 104% after 24 h and 113, 109, and 105% after 72 h of treatment with DHEA at 10^{-7}, 10^{-6}, and 10^{-5} M, respectively (Fig. 2, C and D).

Effect of DHEA on procollagen type I protein accumulation in cultured cardiac fibroblasts. The effects of DHEA (10^{-7} and 10^{-6} M) on collagen accumulation in the medium of...
cultured cardiac fibroblasts were examined. Treatment of primary cultured adult fibroblasts with $10^{-6}$ M DHEA for 72 h decreased the accumulation of procollagen type I in the medium detected by Western blotting. The decrease in procollagen type I protein accumulation after treatment with DHEA was 65.3 $\pm$ 14.1% ($P = 0.029$ vs. control; Fig. 3, A and B).

**Effect of DHEA on procollagen type I protein synthesis in cultured cardiac fibroblasts.** The effects of DHEA ($10^{-7}$ and $10^{-6}$ M) on collagen synthesis were examined by Western blotting. Treatment of adult fibroblasts with DHEA for 72 h decreased the synthesis of procollagen type I. Compared with untreated controls, the decrease in procollagen type I protein synthesis by $10^{-6}$ M DHEA was 63.5 $\pm$ 8.12% ($P = 0.0451$ vs. control; Fig. 4, A and B).

**Effect of DHEA on cardiac fibrosis in the rat model of pressure-overload.** Variation in the systolic blood pressure (SBP) of the animals is indicated in Fig. 5A. The SBP gradually increased over time in both groups, but there was no significant difference between the control and the DHEA-treated rats. The plasma levels of DHEA were $0.0166 \pm 0.0023 \times 10^{-6}$ M (control rat) and $1.11 \pm 0.35 \times 10^{-6}$ M (DHEA-treated rat). As shown in Fig. 5B, DHEA administration induced a 62 $\pm$ 0.32% ($P = 0.032$ vs. control) decrease in procollagen type I mRNA expression. However, there was no significant change in heart-to-body weight ratio between the control (3.58 $\pm$ 0.075 mg/kg body wt) and the DHEA-treated rats (3.49 $\pm$ 0.064 mg/kg body wt). Results of the image analysis are seen in Fig. 5C, showing that procollagen type I mRNA expression was significantly lower in the DHEA-treated group than in the
control group. Microscopic evaluation of the heart tissue in the chronic administration is seen in Fig. 6, A and B, showing that the Masson-Trichrome-stained areas of the DHEA-treated rats and the fibrotic area (stained in blue) were 43% less than those of the control. Figure 6 C shows the quantification of the image analysis of the blue area in both groups. Chronic administration of DHEA caused a significant decrease in the cardiac fibrotic area.

DISCUSSION

It was reported that DHEA and DHEA-S plasma levels decrease with age (1, 43, 50). DHEA-S can be converted readily to DHEA, presumably the active moiety of DHEA-S, by steroid sulfatases that are present in all tissues studied to date. Most of the clinical studies measured DHEA-S rather than DHEA because of the very high turnover of DHEA (5); this high turnover is a characteristic of a biologically active hormone (30).

In the present study, using hypertensive rat and rat primary cardiac fibroblast cells, we have demonstrated that the beneficial effect of DHEA is related to the attenuation of collagen type I synthesis in vivo and in vitro. We have demonstrated that DHEA dramatically reduces collagen type I mRNA expression, collagen type I synthesis in heart tissue and cultured cardiac fibroblasts, and collagen type I protein accumulation in the medium of these cells.

Although the heart’s primary function as a pump depends mainly on the cardiomyocyte, its structural and functional integrity depends largely on the nonmyocyte fibroblast. The fibroblast is, in fact, the predominant cell type in heart and plays a major role in the deposition of the extracellular matrix, of which collagen is a major component (1, 18, 19). Cardiac fibroblasts are known to synthesize fibronectins, vitronectin, collagen types I, III, and V, and collagenases, among many other extracellular matrix and extracellular matrix-related proteins (6, 42). Collagen type I is the major collageneous product of cardiac fibroblasts, representing 80% of total newly synthesized collagen (42), which is secreted into the culture medium as procollagens. About 20% of the total collagen synthesized is collagen type III, and a small proportion is collagen type V (<5%). We investigated procollagen type I accumulation in the medium of cultured cells, which mainly reflect collagen turnover. Because collagen type III is associated with the transition from compensated to decompensated hypertrophy (29), the expression of collagen type III mRNA was not different between controls and DHEA-treated cultures. These results suggest that DHEA could not affect the chronic state of hypertrophy. We strongly speculate that the inhibitory effect of DHEA on collagen synthesis is mainly at the transcriptional level. In support of this, we clearly demonstrated a decrease in cardiac fibroblast proteins and mRNA levels.
the RNA expression levels by Northern blot hybridization, and we also found that the inhibition occurred 24 h after DHEA administration. We propose that the inhibition is mediated at the transcriptional level; however, there may be other possibilities, such as nongenetic mediated pathways (39).

Cardiac fibrosis is associated with the three most prevalent chronic cardiovascular diseases, namely hypertension (46, 48), heart failure (45, 49), and myocardial infarction (4, 19–22, 25–27, 35). The extracellular collagen matrix appears to play a major role in left ventricular remodeling (4, 19–22, 25–27, 35, 45, 46, 48, 49). A common factor in these diseases, and in aging, is the chronic pathological myocardial fibrosis (16, 45, 46, 48, 49), which contributes to progressive left ventricular dysfunction, heart failure, disability, and death. Marked attenuation of fibrosis by DHEA might be protective to heart failure, disability, and death.

The cardiac collagen concentration increases with age (13, 51). The collagen type I fibers become densely packed and thick (13). The mechanism for the increase of myocardial collagen fiber content is the loss of myocytes, which are postmitotic cells and are replaced as they die (31). The loss of myocytes could explain the accumulation of collagen in the walls of the ventricles. Another mechanism for collagen accumulation with age may be inhibition of collagen degradation (12, 36), but the factor responsible for the increase of myocardial collagen fiber content is at present unknown. We propose that the decrease of DHEA with aging may influence cardiac fibrosis.

Since Barrett-Connor et al. (5) reported that DHEA-S might be protective of cardiovascular disease (CVD), many reports about the effect of DHEA and/or DHEA-S on CVD have been published. The incidence of heart failure has increased in the elderly population (9), and Anker et al. (3) reported that DHEA levels were decreased in male patients with chronic heart failure. Moriyama et al. (28) reported that the plasma levels of DHEA-S are decreased in heart failure patients in proportion to

![Fig. 5. Effect of DHEA on cardiac fibrosis in the rat model of pressure-overload. A: variation in systolic blood pressure (SBP) of the animals are shown. SBP gradually increased over time in both groups, but there was no significant difference between control and DHEA-treated rats. B: quantification of procollagen type I mRNA in heart tissue of rats treated with DHEA. Data are presented as percent change from control; means ± SE of 6 experiments. *P < 0.05 vs. control. C: competitive RT-PCR showing decreased procollagen type I mRNA with DHEA treatment.](http://ajpendo.physiology.org/)

![Fig. 6. Microscopic evaluation of heart tissue in chronic administration. Masson-Trichrome-stained area of DHEA-treated rats and fibrotic area (stained in blue) was 43% less (A) than that of control (B). C: quantification of image analysis of the blue area in control and DHEA-treated groups. Chronic administration of DHEA caused a significant decrease in cardiac fibrotic area. *P < 0.05 vs. control.](http://ajpendo.physiology.org/)
the severity of chronic heart failure. From our experiments, we hypothesize that DHEA may inhibit the decrease in ventricular function by inhibiting the production of collagen type I of cardiac fibroblasts. In the MITT assay, DHEA stimulated cell proliferation weakly, but DHEA did not inhibit adult rat cardiac fibroblasts growth. Therefore, it is unlikely that the reduction of collagen by DHEA resulted from a decrease in fibroblast proliferation.

It has been reported that serum concentrations of DHEA in humans range from $10^{-9}$ to $10^{-7}$ M, and when given as an oral supplement the typical dose of DHEA is 25–50 mg/day (8, 14, 23). The concentration of DHEA that we used in this study was $10^{-7}$ to $10^{-6}$ M; this may be high relative to that in humans. However, several reports have indicated that this concentration is not excessive in rats (23, 40, 41). Factors such as pharmacokinetics and sensitivity may explain the difference in concentration effects between humans and rats. Further investigation is necessary to determine whether the normal clinical concentrations of DHEA are effective in treating cardiac fibrosis in humans.

In conclusion, the present results suggest that DHEA attenuates collagen type I mRNA expression, collagen type I protein accumulation, and collagen type I protein synthesis in vivo and in vitro. DHEA inhibits the decrease in ventricular function by attenuating the production of collagen type I of cardiac fibroblasts.

REFERENCES

metalloproteinases associated with aging and hypertension in the rat heart. 