Possible Association of Heart Failure Status With Synthetic Balance Between Aldosterone and Dehydroepiandrosterone in Human Heart

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Background—Aldosterone is produced not only in the adrenal gland but also in the extra-adrenal tissues, including failing human heart. This study examined the production of dehydroepiandrosterone (DHEA) in human heart and elucidated the possible physiological significance.

Method and Results—Using left ventricular tissues obtained at autopsy, reverse transcription–polymerase chain reaction followed by Southern blot analysis revealed the gene expressions of CYP17. By measuring plasma aldosterone and DHEA levels at the coronary sinuses and aortic roots during cardiac catheterization, we found that DHEA but not aldosterone was secreted from control subjects (P<0.0001 and P=0.74, respectively), whereas aldosterone but not DHEA was secreted from patients with heart failure (P<0.0017 and P=0.67, respectively). To examine the significance of DHEA, we measured myocyte cell sizes and the gene expression of B-type natriuretic peptide (BNP), using a neonatal rat cardiocyte culture system. We found that DHEA (10⁻⁸ mol/L) significantly inhibited the increase in myocyte cell sizes and BNP mRNA levels upregulated by endothelin-1 (P=0.031 and P<0.0001, respectively).

Conclusions—CYP17 gene expression and production of DHEA were demonstrated in human control heart. Also, we found that cardiac production of DHEA was suppressed in failing heart. We postulated that DHEA and/or its metabolites exert a cardioprotective action through antihypertrophic effects. (Circulation. 2004;110:1787-1793.)

Key Words: heart failure ■ hormones ■ hypertrophy ■ cardiomyopathy ■ natriuretic peptides
DHEA is the most abundantly produced adrenal steroid, and serum concentrations of its sulfate ester, DHEAS, are approximately 20-fold higher than those of any other circulating steroid hormone. Plasma levels of DHEA(S) decrease with aging, and extensive epidemiological evidence shows that there is an inverse correlation between plasma levels of DHEA(S) and the prevalence of cardiovascular disease. Furthermore, plasma levels of DHEA(S) are also decreased in proportion to the severity of heart failure, which suggests that DHEA(S) may play an important physiological role in the prevention of cardiovascular disease. Therefore, a study of a possible extra-adrenal DHEA production is an interesting theme in cardiovascular science.

This study examined the production of DHEA, aldosterone, or cortisol in human heart and elucidated the possible physiological significance of DHEA production.

## Methods

### Protocol 1

#### Left Ventricle Samples and Total RNA Extraction

Samples of left ventricular tissue were obtained at autopsy from 7 cancer patients (patients 1 through 7) for protocols 1 and 2 and 6 patients who died of heart failure (patients 8 through 13) for protocol 2, as shown in Table 1. There was no metastasis to any tissues and no apparent cardiovascular diseases affecting survival. A sample of adrenal glandular tissue as a positive control was obtained at autopsy from patient 2. Total RNA was extracted from frozen tissues with the use of TRIzol reagent (Invitrogen Corp).

### RT-PCR Analysis

Oligonucleotide primers for human CYP11A, 3β-HSD2, CYP21, CYP11B1, and CYP17 have been reported. Samples (500 ng) of total RNA were reverse-transcribed by means of the SuperScript First-Strand Synthesis System (Invitrogen Corp), using oligo(dT). The amplification protocol consisted of 40 to 50 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 seconds. The PCR products were then directly sequenced.

#### Southern Blot Analysis

PCR products of CYP17 and CYP11B1 were transferred overnight to Hybond N nylon membranes; prehybridized in 6 × SSPE, 1% SDS, 10 × Denhardt’s solution, and 100 μg/mL denatured herring sperm DNA at 60°C for 2 hours; and hybridized with 32P end-labeled oligonucleotides, as previously reported, internal to each amplified segment, at 37°C for 12 hours. Membranes were washed with 6 × SSPE and 0.1% SDS twice for 5 minutes at room temperature and twice at 37°C and then exposed to BAS-IP film (Fuji Film) for 3 hours.

### Protocol 2

#### Quantitative Real-Time RT-PCR Analysis for Human CYP17, CYP11B1, Human BNP, and Rat BNP

The following primers and fluorescent-tagged probes were used to quantify human CYP17: forward primer; 5' - GCCGTGACATCACAATGAGA-3', reverse primer; 5' - CTAAGCTGCTGCTGAAACACCTCCA-3', and probe; 5' - ATGCTGGGCCTCAATATGAAAG-3', and probe; 5' - FAM-TCGACACATGTCGAAACACCTCCA-TAMRA-3', human CYP11B1: forward primer; 5' - CAACGCTGCTGCTGAAACACCTCCA-3', reverse primer; 5' - ATGCTGGGCCTCAATATGAAAG-3', and probe; 5' - FAM-TGACACATGTCGAAACACCTCCA-TAMRA-3', human BNP; forward primer; 5' - TCTGTGCTCTCTTTGTGATCTTG-3', reverse primer; 5' - TTTTGCCCTGCAAATGTTG-3', and probe; 5' - FAM-CCTCGTGCAGCGGAGCTGAG-3', and probe; 5' - FAM-TGACACATGTCGAAACACCTCCA-TAMRA-3', human BNP, and rat BNP mRNA levels were quantified through the use of real-time RT-PCR. Real-time PCR was performed according to the manufacturer’s protocol, using an ABI PRISM 7700 Sequence Detector (Applied Biosystems).

Standard curves for human CYP17 and CYP11B1 were generated by using dilutions of plasmid DNA containing the target sequence, and a standard curve for rat BNP was generated using dilutions of samples of total RNA prepared from rat hearts. Human CYP17, human CYP11B1, and rat BNP mRNA were normalized with human and rat GAPDH, using TaqMan human GAPDH Control Reagents and TaqMan Rodent GAPDH Control Reagents (Applied Biosystems), respectively.
cells were incubated for 8 hours with anti-rat sarcomeric action-positive cells attached. 

Protocol 3
We compared the expression levels of DHEA from patients with heart failure and from control subjects during cardiac catheterization.

Patient Population and the Cardiac Catheterization Study
Blood samples were obtained from 26 patients with heart failure (11 caused by dilated cardiomyopathy, 7 old myocardial infarction, 5 hypertensive heart disease, 2 hypertrophic cardiomyopathy, and 1 mitral regurgitation) and 48 control subjects who underwent cardiac catheterization at our institution. No control subjects had coronary stenosis, left ventricular dysfunction, or left ventricular hypertrophy. Table 2 shows the characteristics of the control subjects and patients with heart failure. All medications including ACE inhibitors, angiotensin II receptor antagonists, aldosterone receptor blockers, nitrates, Ca-antagonists, adrenergic β-antagonists and diuretics were withheld for at least 4 days before the study in all patients.

Hormonal Analysis
Plasma aldosterone concentrations were measured by radioimmunoassay with the use of an SPAC-S aldosterone kit (Daichii RI). Plasma DHEA concentrations were measured by radioimmunoassay (Otsuka Assay). Plasma cortisol concentrations were measured by radioimmunoassay (Immunotech).

Protocol 4
We studied the possible effects of DHEA on cardiac hypertrophy in response to endothelin-1, measuring myocyte cell sizes and the gene expression of BNP in a neonatal rat cardiocyte culture system.

Cell Cultures
In a study conforming to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health, neonatal rat myocytes and nonmyocytes were prepared on Percoll gradient and maintained as previously described. 21 Cells were cultured for a further 12 hours before treatment in serum-free medium containing antibiotics and 0.01% BSA.

Measurement of Myocyte Cell Sizes
Immunocytochemistry for sarcomeric actin was performed to evaluate the morphological changes of the myocytes. Cardiocytes cultured on the culture slide were fixed with 3% formaldehyde for 10 minutes after prepermeabilization with 0.2% Triton X-100. Fixed cells were incubated for 8 hours with anti-rat sarcomeric action antibody α-Sr-1 (DAKO A/S), followed by treatment with a peroxidase-conjugated second antibody for visualization with 3,3’- diaminobenzidine tetrahydrochloride. Additionally, nuclei of cultured cardiac cells were stained with hematoxylin. The cell sizes of the myocytes were estimated by measuring the area of sarcomeric action-positive cells attached. 

Figure 1. Expression of steroidogenic enzyme mRNA in human heart and adrenal gland. Samples (500 ng) of total RNA were subjected to RT-PCR, after which amplified products were detected by gel electrophoresis on 2% agarose gels (A), followed by Southern blot analysis (B). M indicates φ X Hae III digest; N, cardiac tissue without RT as negative control. Lanes 1 to 7: Cardiac tissues from patients numbered 1 to 7, respectively. P indicates adrenal glandular tissue as positive control.

Materials
Endothelin-1 and DHEA were purchased from Sigma Chemical Co.

Statistical Analysis for All the Study Protocols
Statistical analysis was performed with the use of Wilcoxon signed rank test and unpaired t test. Also, 1-way ANOVA followed by the multiple comparison methods of Scheffe was used for statistical analyses. Data were expressed as mean±SEM. Values of P<0.05 were considered significant.

Informed Consent and Animal Care
Written informed consent was obtained for each study protocol from each patient and/or his or her family where appropriate, and the ethics committee at our institution approved the protocol. Also, the institutional committee on animal care at Kumamoto University approved this study.

Results

Protocol 1
Expression of CYP17 mRNA in Human Cardiac Tissue
As shown in Figure 1A, the expressions of CYP11A, 3β-HSD2, CYP21, and CYP17 mRNA were detected in left ventricular and adrenal tissues obtained at autopsy from patients free of cardiovascular diseases, using RT-PCR analysis (PCR cycles: CYP11A; 40, 3β-HSD2; 40, CYP21; 40, CYP17; 50, CYP11B1; 50), but that of CYP11B1 was not detected in any of the left ventricular tissues. The specificity of the amplified products was confirmed by direct sequencing of gel-purified bands (data not shown). In addition, CYP17 gene expression was confirmed by Southern blot analysis (Figure 1B), although the bands were weak in RT-PCR.
Figure 2. Determination of CYP17 mRNA levels using real-time RT-PCR. A, Standard curve for human CYP17 was generated using dilutions of plasmid DNA (100 pg, 10 pg, 1 pg, 10^{-3} pg, 10^{-4} pg, 10^{-5} pg, and 10^{-6} pg) containing the target sequence. B, CYP17 gene amplification plots (ΔRn versus cycle) used to determine the quantity of initial template in cardiac and adrenal glandular tissue samples. C, Standard curve for human CYP11B1 was generated using dilutions of plasmid DNA (100 pg, 10 pg, 1 pg, 10^{-3} pg, 10^{-4} pg, 10^{-5} pg, and 10^{-6} pg) containing the target sequence. D, CYP11B1 gene amplification plots used to determine quantity of the initial template in cardiac and adrenal glandular tissue samples. †Cardiac samples; *adrenal sample. Initial amounts of template were determined by interpolation from a standard curve, using observed critical threshold cycle (Ct), which is defined as the cycle at which the fluorescence becomes detectable above background.

Protocol 2

Quantitative Analysis of CYP17 and CYP11B1 in Human Heart and Adrenal Gland

Because we found gene expressions of CYP17 in protocol 1, we then proceeded to protocol 2. We compared the expression levels of CYP17 mRNA in left ventricle and adrenal gland tissues by using real-time RT-PCR. Figure 2B shows a typical amplification pattern of the CYP17 transcripts. In this analysis, we succeeded in detecting the CYP17 gene, although the amount of CYP17 mRNA in left ventricle tissue was determined to be approximately 2×10^5 times lower than in adrenal gland. On the other hand, we could not detect the gene expression of CYP11B1 by this method, as shown in Figure 2, C and D.

Figure 3 shows a summary of results of the expression levels of CYP17 and BNP mRNA between the non–heart failure and heart failure groups. The levels of BNP were significantly higher in the heart failure group than in the non–heart failure group (P<0.01). However, CYP17 expression was not statistically different between the two groups.

Protocol 3

Comparison of Hormonal Levels

Cardiac index was significantly lower and left ventricular end-diastolic pressure and pulmonary capillary wedge pressure were significantly higher in patients with heart failure than in control subjects (Table 2).

Figure 4 shows aldosterone, DHEA, and cortisol concentrations in plasma samples obtained from the aortic root and coronary sinus regions in control subjects and patients with heart failure. In control subjects, levels of DHEA were significantly higher at the coronary sinus than the aortic root region (CS: 0.90±0.06×10^{-8} mol/L versus Ao; 0.80±0.05×10^{-8} mol/L, P<0.0001), whereas there were no significant differences in the aldosterone levels in the two regions (CS: 1.73±0.14×10^{-8} mol/L versus Ao: 1.71±0.13×10^{-8} mol/L, P=0.74) and in the cortisol levels in the two regions (CS: 38.89±1.93×10^{-8} mol/L versus Ao: 38.33±1.91×10^{-8} mol/L, P=0.97). On the other hand, in patients with heart failure, levels of aldosterone were significantly higher at the coronary sinus than the aortic root region (CS: 2.32±0.27×10^{-8} mol/L versus Ao: 2.01±0.25×10^{-8} mol/L, P=0.0017), whereas there were no significant differences in the DHEA levels in the two regions (CS: 0.66±0.06×10^{-8} mol/L versus Ao: 0.65±0.07×10^{-8} mol/L, P=0.67) and in the cortisol
TABLE 3. Myocyte Cell Sizes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Area, μm²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1160±50</td>
<td></td>
</tr>
<tr>
<td>ET-1 10⁻⁹ mol/L</td>
<td>1525±59</td>
<td>P&lt;0.0001 vs control</td>
</tr>
<tr>
<td>DHEA 10⁻⁹ mol/L+</td>
<td>1341±34</td>
<td>P=NS vs control, ET-1 10⁻⁹ mol/L</td>
</tr>
<tr>
<td>P=0.031 vs ET-1 10⁻⁹ mol/L</td>
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Cardiocytes in culture were treated with vehicle (control), ET-1 (10⁻⁹ mol/L), or DHEA (10⁻⁹ mol/L) + ET-1 (10⁻⁹ mol/L). After 48 hours incubation, cell size of myocytes was estimated by measuring the area of sarcomeric action-positive cells by computed image analyzer. Data are mean±SEM (sizes of 20 cells for each group were measured) for 2 separate experiments.

levels in the two regions (CS; 40.46±2.90×10⁻⁶ mol/L versus Ao; 40.81±3.43×10⁻⁶ mol/L, P=0.82). The value of DHEA/aldosterone in the coronary sinus was significantly lower in patients with heart failure than in control subjects (0.390±0.056 versus 0.637±0.054, P=0.0048).

Protocol 4

Effects of DHEA on Myocyte Cell Sizes and BNP mRNA in Rat Cardiocytes

To examine the effect of DHEA on rat cardiocytes, cells were incubated in the presence or absence of DHEA added 12 hours before treatment with endothelin-1 for 48 hours.

As shown in Table 3, endothelin-1 (10⁻⁹ mol/L) significantly increased myocyte cell sizes (P<0.0001), and DHEA (10⁻⁹ mol/L) significantly inhibited the increase in cell sizes (P=0.031, compared with the levels stimulated by endothelin-1 alone) upregulated by endothelin-1.

As shown in Figure 5, endothelin-1 (10⁻⁹ mol/L) significantly increased BNP mRNA levels (1.51±0.11-fold: P=0.0016, compared with control), and DHEA (10⁻⁹ and 10⁻⁶ mol/L) significantly inhibited BNP mRNA levels (P<0.0001 and P<0.0001 respectively, compared with the levels stimulated by endothelin-1 alone) upregulated by endothelin-1.

Discussion

In protocol 1, using left ventricular tissue samples collected from autopsies, we examined gene expressions for the synthesis of DHEA and cortisol. CYP11A, 3β-HSD2, CYP21, CYP11B1, and CYP17 are required to produce DHEA and cortisol. The expression of CYP11A, 3β-HSD2, and CYP21 were clearly demonstrated in all left ventricular tissue samples. This result is in agreement with those of previous reports. As for CYP17, we were able to demonstrate its expression, although the bands were weak compared with those of the other three enzymes. However, direct sequencing and Southern blot analysis reinforced the expression in left ventricular tissues.

Because we obtained positive data for gene expression of CYP17 from human hearts, we then compared the expression levels of CYP17 mRNA from human heart between patients who died of heart failure (heart failure group) and those who were free of cardiovascular disease (non-heart failure group) in protocol 2. In the analysis, we succeeded in detecting the gene expression of CYP17 by real time RT-PCR in both heart failure and non-heart failure groups. The amount of CYP17 mRNA expressed in left ventricle was found to be ≈2×10⁻⁶ times lower than in adrenal gland. However, because the heart is 30- to 50-fold larger than the adrenal gland, the total mass of CYP17 mRNA in the heart is probably at least 0.01% of that in the adrenal gland. However, we could not detect a difference in degree of gene expression between the heart failure and non-heart failure groups. Nonetheless, we still hypothesized a possible decrease in DHEA production in heart failure and then proceeded to protocol 3. Interestingly, we were able to obtain a significant difference in secretion of DHEA between the heart failure and control groups, as shown in Figure 4; we demonstrated cardiac DHEA production in control heart, whereas its production was decreased in failing heart. Even in the control group, the amount of DHEA synthesized by the heart may be too small to serve any endocrine function, and it may act as an autocrine/paracrine manner within healthy heart.

The results of protocol 2 and 3 seems not to agree; we did not find any difference in CYP17 gene expression between the heart failure and non-heart failure groups in protocol 2, but we found a significant difference in secretion of DHEA between the heart failure and control groups in protocol 3. At present, we cannot clearly explain the reason, but we hypothesize as follows. Cardiac DHEA production could depend on not only CYP17 mRNA level but also the stability of CYP17 mRNA and 17,20-lyase activity. CYP17 has two enzyme activities, 17α-hydroxylase and 17,20-lyase; 17α-hydroxylase reaction leads to cortisol, and the subsequent 17,20-lyase reaction leads to DHEA. These are regulated by redox partners such as cytochrome P450 reductase and cytochrome b₅. Cytochrome P450 reductase activity significantly decreases in aged adrenal glands; however, the cytochrome b₅ contents do not change between young and aged.
adrenal glands.\textsuperscript{37} The decrease in P450 reductase activity causes a specific decrease in 17,20-lyase activity without affecting 17α-hydroxylase activity. It is thus possible that 17,20-lyase activity would be suppressed in failing heart. Many neurohormonal factors including angiotensin II and aldosterone are activated in heart failure\textsuperscript{5,28} and may increase radical oxidative species,\textsuperscript{29} contributing to a decrease in 17,20-lyase activity.

It is interesting to see a synthetic balance of aldosterone and DHEA between patients with heart failure and control subjects in protocol 3; DHEA is produced in the healthy heart, but it decreases as heart failure advances and aldosterone production is upregulated in turn. The pattern of aldosterone secretion is in agreement with our earlier report.\textsuperscript{3} Although this phenomenon is considered to be a result of heart failure or oxidative status, it may be one factor that further advances heart failure. Indeed, in this study, we found an antihypertrophic effect of DHEA by measuring not only myocyte cell sizes but also the gene expression of BNP, a sensitive marker of cardiac hypertrophy and heart failure.\textsuperscript{23} Endothelin-1 is known to stimulate cardiac hypertrophy in cultured rat cardiocytes.\textsuperscript{32} DHEA significantly inhibited BNP mRNA levels upregulated by endothelin-1 in a dose-dependent manner from 10\textsuperscript{-9} to 10\textsuperscript{-6} mol/L. It should be noted that the concentration of 10\textsuperscript{-8} mol/L was at a level similar to the circulating hormonal levels of DHEA shown in this study. These results suggested that a small dose of DHEA produced in the heart could act as a cardioprotective agent against neurohormonal factors stimulating cardiac hypertrophy in an autocrine/paracrine manner.

Because an intracellular steroid hormone receptor for DHEA has not been identified, the molecular mechanisms of DHEA are still to be clearly described, despite evidence suggesting beneficial cardiovascular effects.\textsuperscript{30} However, recent reports have shown that there are specific DHEA-biding sites in cardiovascular tissues\textsuperscript{31,32} and that this putative receptor is present in rat heart.\textsuperscript{32} DHEA probably is converted to testosterone or 17β-estradiol in the heart,\textsuperscript{25,33} and furthermore, specific receptors for testosterone and 17β-estradiol are present in the heart.\textsuperscript{34} Thus, it is unclear whether DHEA directly exerts its effects or if it acts after conversion to these hormones. Also, our preliminary data showed that testosterone or 17β-estradiol each significantly inhibited mRNA expression of BNP upregulated by endothelin-1, suggesting that the effect of DHEA may depend on, at least in part, its metabolites, testosterone or 17β-estradiol (data not shown). Aromatase, which metabolizes testosterone to 17β-estradiol,\textsuperscript{34} is reported to be present in rat cardiocytes.\textsuperscript{34} Even in the presence of an aromatase inhibitor, DHEA still significantly inhibited mRNA expression of BNP, indicating that the effect of DHEA does not depend entirely on conversion to 17β-estradiol. However, it is unclear whether DHEA or testosterone has the stronger antihypertrophic effect. At present, we can conclude that DHEA and/or its metabolites directly exert an effect on the heart; the effect of the conversion from DHEA to testosterone or 17β-estradiol in the heart might be involved in antihypertrophic effects. Also, the direct intracellular signaling cascade of DHEA has been unconfirmed as yet; thus, investigation is required.

Cortisol is released from the zona fasciculata of adrenal glands and plays an important role in the stress response.\textsuperscript{35,36} The plasma levels of cortisol increase with aging, contrasting with those of DHEA.\textsuperscript{35,36} It is still unclear whether cortisol or CYP11B1, the key enzyme catalyzing the final step in the synthesis of cortisol, is expressed in human heart. In the present analysis, we could not detect the expression of CYP11B1 by using RT-PCR followed by Southern blot analysis and real-time RT-PCR in human ventricles. We also could not detect any significant increase at the coronary sinus as compared with that at the aortic root. We thus concluded that neither cortisol nor CYP11B1 was expressed in the heart. These results are in agreement with previous reports.\textsuperscript{7,8} However, the examination of whether there is any exception for cardiac cortisol production is required.

In this study, we were able to demonstrate CYP17 expression in human heart, even though others have reported that CYP17 is not expressed in heart.\textsuperscript{7,8} In the present study, the bands of CYP17, which were subjected to 50 cycles of RT-PCR, were very weak but were detectable; however, we were able to confirm them by direct sequencing and Southern blot analysis. Furthermore, the levels of CYP17 mRNA were quantified by real-time RT-PCR, which is more sensitive than conventional RT-PCR and other methods of detecting mRNA.\textsuperscript{37}

It may seem difficult to assess the relative significance or contribution of in situ production of DHEA or in situ expression of CYP17 in human heart compared with larger amounts in systemic circulation because of low levels of CYP17 expression. At present, we do not have a complete answer; however, a similar discussion with regard to aldosterone has been carried out. Although the expression of aldosterone synthase gene CYP11B2 is very small in human failing heart, we certainly detected it by modified real-time PCR.\textsuperscript{9} Interestingly, there also is a report that the tissue levels of aldosterone were 17 times as high as circulating levels.\textsuperscript{3} Several explanations for this cardiac concentration effect are possible. For instance, aldosterone degradation may be slower in cardiac tissue than in plasma or may be segregated intracellularly once produced and/or locally delivered into the extracellular space instead of being released into plasma. This in situ synthesis of a biologically active hormone might thus result in a far greater concentration within tissue than that which could be achieved via the blood stream; supporting a putative physiological role. Possibly, the same situation as this might be applicable to DHEA, although this is only speculation on our part.

It would also be of interest to know the effect of DHEA on myocardial and vascular inflammation because perivascular inflammation has been shown to precede aldosterone-induced myocardial fibrosis.\textsuperscript{38} Although this study is important to clarify the pathogenesis of myocardial inflammation and fibrosis, we have no data about it at present. This kind of study should be undertaken in the future.

In summary, DHEA, secreted from the heart, might directly or after conversion to its metabolites exert a cardioprotective action. Also, it may be possible that reduced cardiac
DHEA production causes a worsening of status during heart failure. The cardiac steroid-genesis of small quantity would be important; however, its significance needs to be continuously discussed in detail in the future.

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