Dehydroepiandrosterone Retards Atherosclerosis Formation Through Its Conversion to Estrogen
The Possible Role of Nitric Oxide

Toshio Hayashi, Teiji Esaki, Emiko Muto, Hatsuyo Kano, Yukako Asai, Navin Kumar Thakur, Daigo Sumi, Muthuvel Jayachandran, Akihisa Iguchi

Abstract—Dehydroepiandrosterone (DHEA) is speculated to have an antiatherosclerotic effect, although the mechanism of action remains unclear. The objective of the current study was to determine whether the antiatherosclerotic effect of DHEA is related to its conversion to estrogen and to define the role of nitric oxide (NO) in the antiatherosclerotic effect of DHEA. Forty-eight oophorectomized rabbits were divided into 5 groups and fed the following diets for 10 weeks: group 1, a regular rabbit diet plus 1% cholesterol (a high-cholesterol diet [HCD]); group 2, an HCD plus 0.3% DHEA; group 3, an HCD plus 0.3% DHEA and fadrozole (2.0 mg \( \cdot \) kg\(^{-1} \cdot \) d\(^{-1} \)), a specific aromatase inhibitor; group 4, an HCD plus 17\( \beta \)-estradiol (20 \( \mu \)g \( \cdot \) kg\(^{-1} \cdot \) d\(^{-1} \)); and group 5, a regular diet. Atherosclerotic lesions, lipid deposition in aortic vessels, and basal and stimulated NO release were measured in the aforementioned groups of rabbits. NO release was measured by using an NO-selective electrode as well as by measuring vascular responses and the plasma NO metabolites nitrite and nitrate. The plasma total cholesterol level was increased, but there were no significant differences in lipid profile in the 4 groups of rabbits that were fed the HCD. The area occupied by atherosclerosis in the thoracic aorta was diminished by \( \approx 60\% \) in the DHEA-treated rabbits (group 2) compared with the HCD group of rabbits (group 1); there was a corresponding 80% decrease in the estradiol group (group 4) but only a 30% decrease in the DHEA plus fadrozole group (group 3). In the aortas of rabbits from groups 1 and 3, the acetylcholine-induced and tone-related basal NO-mediated relaxations were diminished compared with those of the controls (group 5). However, these relaxations were restored in the aortas of group 2 and 4 rabbits, and an increase in NO release was observed in groups 2 and 4 compared with groups 1 and 3, as measured by an NO-selective electrode. Injection of neither solvent (20% ethanol/distilled water) nor fadrozole significantly affected the atherosclerotic area or the NO-related responses described above. We conclude that \( \approx 50\% \) of the total antiatherosclerotic effect of DHEA was achieved through the conversion of DHEA to estrogen. NO may also play a role in the antiatherosclerotic effect of DHEA and 17\( \beta \)-estradiol.

(Dearterioscler Thromb Vasc Biol. 2000;20:782-792.)

Key Words: dehydroepiandrosterone ■ atherosclerosis ■ nitric oxide ■ estrogen ■ aromatase

Dehydroepiandrosterone (DHEA) and its sulfate ester DHEA-S are weak androgens produced primarily by the adrenal gland. Although their plasma concentrations by far exceed those of any other adrenal product, their physiological roles have not yet been determined. In plasma, where the major portion of these hormones is present in the sulfate form, it is possible that DHEA-S serves as a reservoir for DHEA, since various tissues have been shown to contain steroid sulfatases.\(^1\) The peak plasma levels of DHEA and DHEA-S occur at approximately age 25 years, decrease progressively thereafter, and diminish by 95% around the age of 85 years. Epidemiological evidence has shown that adult men with high plasma DHEA-S levels are less likely to die of cardiovascular disease.\(^2\) A study indicated that administration of DHEA reduced aortic fatty streak formation and cholesterol accumulation by \( \approx 30\% \) to 40% in cholesterol-fed rabbits.\(^3\) Another report has shown a 50% reduction in aortic atherosclerosis in rabbits that underwent balloon aortic injury and were then fed a high-cholesterol diet with DHEA.\(^4\) These studies suggest that higher levels of DHEA and DHEA-S might be protective against the development of atherosclerosis.

DHEA has been shown to reduce serum LDL cholesterol levels in humans of average health,\(^5\) although no relationship was reported between the plasma cholesterol level and the beneficial effect of elevated plasma DHEA.\(^6\) Furthermore, none of these results can explain the drastic antiatherosclerotic effects of DHEA with regard to a high-cholesterol diet–induced atherosclerosis, because no remarkable lipid profile changes were observed in these kinds of animal models after DHEA treat-

Received September 22, 1998; revision accepted October 14, 1999.
From the Department of Geriatrics, Nagoya University School of Medicine, Nagoya, Japan.
Correspondence to Toshio Hayashi, Department of Geriatrics, Nagoya University School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya, 466-8550 Japan. E-mail hayashi@med.nagoya-u.ac.jp
© 2000 American Heart Association, Inc.
Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org
ment. Information regarding the receptor-mediated action of DHEA is available only in caudal epididymal spermatozoa and activated human T lymphocytes.

On the other hand, it is well known that hormone replacement therapy with estradiol decreases the risk of coronary events in postmenopausal women. An abundance of epidemiological data confirms this atheroprotective effect of estradiol and has also prompted recommendations for the widespread use of estrogen replacement therapy for the primary prevention of ischemic cardiovascular disease in postmenopausal women. The antiatherosclerotic effects of estradiol were thought to be partly attributable to changes in plasma lipid levels (i.e., the increase in HDL cholesterol and decrease in LDL cholesterol). However, the contribution of these changes to the total antiatherosclerotic effect of estrogen is only ~50%, based on multiple regression analyses. The direct action of estrogen on the vessel wall has been studied vigorously over the past decade. Recently, estrogen receptors have been found in the vascular endothelium and smooth muscle cells, as well as in blood cells such as monocytes. The antiatherosclerotic effect of estrogen could be induced by a direct effect on the vessel wall, such as the inhibition of smooth muscle cell proliferation and migration or of LDL oxidation. Furthermore, estrogen was able to inhibit cholesterol accumulation in the aorta as well as in the coronary arteries of cholesterol-fed rabbits and monkeys, and this was partly explained by suppression of the arterial uptake and/or degradation of LDL. Whereas the direct antiatherogenic effect of estrogen per se on the vessel wall is poorly understood, interest has focused on the role of nitric oxide (NO) because NO has antiatherosclerotic effects such as inhibition of monocyte adherence to endothelial cells; inhibition of smooth muscle cell chemotaxis, proliferation, and relaxation; and inhibition of platelet aggregation. Estrogen has also been shown to increase endothelial NO synthase (eNOS) protein and activity and NO production by receptor-mediated mechanisms in cultured endothelial cells. It has therefore been hypothesized that the ability of estrogen to increase NO bioavailability is involved in the antiatherogenic effect of estrogen. More recently, direct evidence was shown that NO mediates the antiatherosclerotic effect of estrogen. DHEA is known to convert to estradiol in vivo. Therefore, the current study was undertaken to determine whether the antiatherosclerotic effect of DHEA is related to its conversion to estrogen and to define the role of NO in the antiatherosclerotic effect of DHEA.

Methods

Chemicals and Solutions

l-Arginine hydrochloride, acetyltachylene chloride (ACh), progesteradiolin F39, (PGF2α), calcium ionophore A-23187, hemoglobin, indomethacin, and Nω-monomethyl-l-arginine acetate (L-NMA) were all purchased from Sigma Chemical Co. Nitroglycerin (2.0 mg · kg⁻¹ · d⁻¹), an aromatase-specific inhibitor. Group 4 received the HCD supplemented with 0.3% DHEA. Group 3 received the HCD supplemented with 0.3% DHEA and a daily injection of fadrozole (2.0 mg · kg⁻¹ · d⁻¹), an aromatase-inhibitor. Group 1 received a high-cholesterol diet (regular rabbit diet with 1% cholesterol [HCD]). Group 2 received the HCD supplemented with 0.3% DHEA. Group 3 received the HCD supplemented with 0.3% DHEA and a daily injection of fadrozole (2.0 mg · kg⁻¹ · d⁻¹), an aromatase-specific inhibitor. Group 4 received the HCD plus a daily injection of 17β-estradiol (20 µg · kg⁻¹ · d⁻¹), and group 5 received a regular rabbit diet. Fadrozole and 17β-estradiol were each dissolved in 20% ethanol/distilled water. Final concentrations of fadrozole and 17β-estradiol were 10 mg/mL and 50 µg/mL, respectively. The rabbits were treated for 10 weeks under the conditions described above.

Lipid and Sex Steroid Hormone Concentration Assays

Total and free cholesterol levels were determined by using cholesterol oxidase (Wako Pure Chemical Industries, Ltd.). Triglyceride levels were measured by enzymatic techniques as described previously. HDL cholesterol was initially measured after precipitation of high-density lipoprotein cholesterol. Serum estradiol were 10 mg/mL and 50 µg/mL, respectively. The rabbits were treated for 10 weeks under the conditions described above.

Histological Evaluation of Atherosclerosis

The descending thoracic aorta was taken from the portion of the orifice at the left first costal artery (~4 cm distal to the aortic valve) to 3 cm above the portion enclosed by the diaphragm (~7 cm distal to the aortic valve). Morphometric analysis was performed as described by Weiner et al., with slight modifications. Six blocks were taken from the descending thoracic aorta of each rabbit. Each block was adjacent to another segment taken for evaluation of endothelial-dependent responses and was stained with hematoxylin-eosin to examine the endothelial lining and with van Gieson’s elastic stain to determine the surface involvement of atherosclerotic lesions (fatty streaks and fibrous plaques) and the area occupied by the atherosclerotic lesion as defined below. The first complete section of each block was projected onto a vertical surface with a projecting microscope. The contours of the lumen and the internal elastic lamina were traced, and the internal elastic lamina was digitized (PC-9801 ES, NEC) by using a graphics tablet. The mean surface involvement by atherosclerotic lesion per vessel per animal was calculated by summing all results obtained after dividing the lesion circumference by the circumference of the internal elastic lamina and then dividing the sum by the number of sections studied (n = 6 for 1 vessel). Circumferences of lesions and normal portions were defined as circumferences of internal elastic lamina where intimal thickening was observed and where normal intima was observed, respectively. The area occupied by atherosclerotic lesions was defined as the percent area bounded by the lumen and the internal elastic lamina for the ideal luminal area. The ideal luminal area was calculated from the perimeter of the internal elastic lamina.
on the assumption that the true shape of the vessel was circular and to exclude the artificial effect due to tissue fixation in 10% formalin solution. The mean area occupied by the lesions per vessel per animal was calculated by summing the areas occupied by lesions of all sections and dividing the sum by the number of sections per vessel (n=6 for 1 vessel). Data were transferred to a minicomputer (Macintosh Quadra 700, Apple, Ltd) for further analysis.

### Determination of Aortic Cholesterol Content

The segment of the aortic arch (2 cm distal to the aortic valve) to the bifurcation of the left subclavian arteries was removed, weighed, minced, and homogenized in 10 volumes of sucrose-Tris buffer with a motor-driven, glass homogenizer at 0°C to 2°C. The homogenates were used for total lipid extraction. The extracted lipids were used for the examination of total cholesterol, free cholesterol, and esterified cholesterol by the method of Badimon et al. 37

### Isometric Tension Measurement

After 10 weeks of diet treatment, the rabbits were killed by exsanguination after being anesthetized with pentobarbital (50 mg/kg IV). The thoracic aortas were removed carefully to protect the endothelial lining, cleaned of adhering fat and connective tissue, and cut into 2-mm-wide transverse rings. The optimal passive load for both control and atherosclerotic aortas was determined as the initial induced submaximal tension (2.6 ± 10−6 mol/L). To investigate the tone-related release of NO from endothelium-intact aortic rings, moderate vascular tone (35% to 50% of the contractile response obtained with 122 mmol/L KCl) was induced by low PGF2α concentrations (0.8 × 10−6 mol/L). Concentration-related contractile responses to L-NMMA (1 to 100 μmol/L) were also assessed. In some experiments, indomethacin (5 μmol/L) was added to the muscle chambers for 60 minutes before the start of the experiment to rule out the contribution of prostanoids.

### Measurement of NO by an NO Electrode

The function of the NO meter (model NO501, Intermedic Co, Ltd) is the measurement of the redox current between a working electrode and a counterelectrode by using a microsensor as modified by the method of Malinski and Taha. 39 The working electrode was made of a PtIr alloy (0.2 magnetomotive Force) coated with a 3-layer membrane consisting of KCl, an NO-selective resin, and normal silicone membranes. The counterelectrode was made of carbon fiber. The KCl membrane was deposited to suppress overvoltage in the discharge of NO. The NO-selective resin was made of a nitrocellulose/polyoxynlacquer. An outer membrane was used to avoid nonspecific ionic effects and electrochemical reactions. Polargraphic current was detected with a current-voltage converter circuit. The current increased linearly from 1.5 to 305 pA when the S-nitroso-N-acetyl-dl-penicillamine (SNAP) concentration increased from 5 mmol/L to 1 μmol/L. 39 We measured ACh (1 μmol/L)–stimulated NO release in ring segments from rabbit aortas (3 mm wide) cut longitudinally and mounted on a silicone plate in the Krebs-Henseleit tissue bath that was saturated with 95% O2/5% CO2. With the use of a micropositioner (0.2-mm x y z resolution), the microsensor was placed at a 0.2-mm distance from the surface of endothelial cells in the aorta. NO release from the aorta was

<table>
<thead>
<tr>
<th>Animals</th>
<th>Total Cholesterol, mg/dL</th>
<th>Triglyceride, mg/dL</th>
<th>HDL Cholesterol, mg/dL</th>
<th>Body Weight, kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (HCD)</td>
<td>1201 ± 36†</td>
<td>165 ± 13†</td>
<td>24 ± 2</td>
<td>3.06 ± 0.07</td>
</tr>
<tr>
<td>Group 2, HCD + DHEA</td>
<td>1162 ± 26†</td>
<td>171 ± 10†</td>
<td>23 ± 3</td>
<td>3.12 ± 0.06</td>
</tr>
<tr>
<td>Group 3, HCD + DHEA + fadrozole</td>
<td>1086 ± 35†</td>
<td>110 ± 11†</td>
<td>26 ± 2</td>
<td>3.09 ± 0.07</td>
</tr>
<tr>
<td>Group 4, HCD + 17β-estradiol</td>
<td>1252 ± 27†</td>
<td>189 ± 11†</td>
<td>39 ± 2</td>
<td>3.06 ± 0.02</td>
</tr>
<tr>
<td>Group 5, regular diet</td>
<td>84 ± 8*</td>
<td>35 ± 2*</td>
<td>27 ± 3</td>
<td>3.01 ± 0.06</td>
</tr>
</tbody>
</table>

Results are presented from experiment 1. *P<0.05 vs group 1. †P<0.05 vs group 5.

### TABLE 2. Plasma Lipid Levels and Body Weights After Consumption of an HCD

<table>
<thead>
<tr>
<th>Animals</th>
<th>Total Cholesterol, mg/dL</th>
<th>Triglyceride, mg/dL</th>
<th>HDL Cholesterol, mg/dL</th>
<th>Body Weight, kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1b, HCD + solvent</td>
<td>1236 ± 43</td>
<td>169 ± 14</td>
<td>28.3 ± 3.1</td>
<td>17.4 ± 2.0</td>
</tr>
<tr>
<td>Group 2b, HCD + DHEA + solvent</td>
<td>1208 ± 35</td>
<td>173 ± 13</td>
<td>28.7 ± 2.1</td>
<td>62.8 ± 8.2</td>
</tr>
<tr>
<td>Group 5b, regular diet + solvent</td>
<td>86 ± 4</td>
<td>42 ± 7</td>
<td>32.0 ± 2.7</td>
<td>16.4 ± 2.1</td>
</tr>
<tr>
<td>Group 10, regular diet + solvent</td>
<td>1269 ± 49</td>
<td>168 ± 14</td>
<td>31.2 ± 2.5</td>
<td>18.9 ± 2.0</td>
</tr>
<tr>
<td>Group 5, regular diet</td>
<td>91 ± 5</td>
<td>37 ± 6</td>
<td>29.0 ± 2.3</td>
<td>19.3 ± 2.1</td>
</tr>
</tbody>
</table>

T-Chol indicates total cholesterol; TG, triglyceride; and HDL-Chol, HDL cholesterol.
expressed as pA per mm² of luminal surface area of rabbit aorta segment. Three picoamperes was assumed to be the amount of NO released from 10 nmol/L SNAP.

Measurement of NO₂⁻/NO₃⁻
Concentrations of nitrite and nitrate (NO₂⁻/NO₃⁻) in plasma were measured in an automated NO detector–high-performance liquid chromatography system (ENO10, Eicom Co) as previously reported.40,41 In brief, samples were collected in an automated sample injector connected to an automated NO detector. NO₂⁻ and NO₃⁻ in each sample were diluted 5 times with mobile-phase buffer (10% methanol containing 0.15 mol/L NaCl/NH₄Cl and 0.5 g/L sulfanilamide with 0.25 g/L N-naphthylethylenediamine) and were separated on a reverse-phase separation column packed with polystyrene polymer (NO-PAK, 4.6 x 50 mm, Eicom), and NO₃⁻ was reduced to NO₂⁻ in a reduction column packed with copper-plated cadmium fillings (NO-RED, Eicom). NO₂⁻ was mixed with a Griess reagent to form a purple azo dye in a reaction coil. The absorbance of the color of the product dye at 540 nm was measured by a flow-through spectrophotometer (NOD-10, Eicom). The mobile phase was delivered by a pump at a rate of 0.33 mL/min with mobile-phase buffer. The Griess reagent (0.5% sulfanilamide, 0.025% N-naphthylethylethylenediamine dihydrochloride, and 1.25% HCl) was employed to form a purple azo dye.

Measurement of cGMP
The concentration of cyclic GMP (cGMP) in homogenates of aortic tissue was determined by a specific radioimmunoassay.42 In brief, 4 aortic rings (derived from the abdominal aorta from the portion enclosed by the diaphragm to 3 cm just above the orifice of the right renal artery; wet weight each, 30 ± 1 mg) were incubated in test tubes containing Krebs-Henseleit buffer saturated with 95% O₂/5% CO₂ for 30 minutes to exclude the hypoxic effect induced during sacrifice of the rabbits. The rings were promptly frozen in LN₂ and stored at −80°C. To determine basal cGMP levels, the rings were homogenized in 1 mL of 6% trichloroacetic acid at 4°C and centrifuged at 12,000 g for 5 minutes. The supernatant was washed 4 times with 4 mL of water-saturated ethyl ether. Liquid samples were then frozen at −80°C and lyophilized overnight. The lyophilized sample was resolubilized in 1 mL of 0.05 mol/L sodium acetate buffer, and 50-µL aliquots were placed in test tubes. The cGMP contents in the samples were determined by an enzyme-linked immunoassay in a commercial assay kit (RPN226, Amersham). Solids remaining from the initial homogenization step were digested in 1 mL of 0.1N NaOH overnight, and total protein was determined by the method of Lowry et al.43

Data Analysis
Relaxation was measured as the percent decrease in tension below that evoked by PGF₂α (2.6 x 10⁻⁶ mol/L) in arterial rings. Contraction

---

**TABLE 3. Plasma Steroid Levels After Consumption of an HCD**

<table>
<thead>
<tr>
<th>Animals</th>
<th>DHEA, µg/dL</th>
<th>DHEA-S, µg/dL</th>
<th>Estrogen, pg/mL</th>
<th>Testosterone, pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1, HCD</td>
<td>15.8±2.5</td>
<td>21.5±2.4</td>
<td>14.3±1.4</td>
<td>27.4±5.4</td>
</tr>
<tr>
<td>Group 2, HCD+DHEA</td>
<td>54.7±9.1†</td>
<td>125.7±14.3†</td>
<td>34.3±5.4†</td>
<td>45.8±5.0</td>
</tr>
<tr>
<td>Group 3, HCD+DHEA+fadrozole</td>
<td>50.5±8.3†</td>
<td>136.0±31.0†</td>
<td>10.2±1.2</td>
<td>36.2±9.9</td>
</tr>
<tr>
<td>Group 4, HCD+17β-estradiol</td>
<td>16.0±1.3</td>
<td>22.8±3.4</td>
<td>59.2±5.3†</td>
<td>30.0±2.9</td>
</tr>
<tr>
<td>Group 5, regular diet</td>
<td>18.0±0.9</td>
<td>29.6±6.1</td>
<td>13.7±2.3</td>
<td>25.3±6.4</td>
</tr>
</tbody>
</table>

Results are presented from experiment 1.

*P<0.05 vs group 1.
†P<0.05 vs group 5.
in response to L-NMA (1 to 100 μmol/L) was measured as the percent increase in tension above that evoked by PGF2α (0.8×10^{-6} mol/L) in arterial rings. Data are expressed as mean±SEM. Means were compared by ANOVA with repeated measurements. When a significant F value was found, Scheffe’s test for multiple comparisons was used to identify differences among groups. A level of P<0.05 was considered statistically significant.

Results
Status of Lipids and Sex Steroid Hormones
There were no significant differences in total serum protein among the 4 experimental groups and the control group over the course of the study (data not shown). The addition of 1% cholesterol to the diet (groups 1, 2, 3, and 4) increased total cholesterol and triglyceride levels but had no significant effect on total protein or HDL cholesterol compared with the baseline values (Table 1). Body weight was not changed among the 5 groups (Table 1). The application of DHEA or 17β-estradiol, in addition to the 1% cholesterol in the diet, did not affect the plasma lipid levels. In experiment 2, injection of solvent into group 1, 2, and 5 rabbits did not affect the lipid levels significantly (Table 2). Injection of fadrozole into the HCD or the regular-diet rabbit group also had no significant effect (Table 2). The plasma concentrations of DHEA and the sex steroid hormones as metabolite products are shown in Table 3. No significant increase in testosterone was demonstrated, although 17β-estradiol was increased by DHEA treatment (group 2) but not by DHEA plus fadrozole treatment (group 3). DHEA and DHEA-S levels increased significantly after DHEA treatment, with or without fadrozole. Injection of solvent into group 1, 2, and 5 rabbits (groups 1b, 2b, and 5b) and injection of fadrozole into group 1 and 5 rabbits had no significant effect on the data described above. Fatty livers were observed in groups 1, 2, 3, and 4; however, no liver function abnormality was detected in the plasma samples from these groups (data not shown). Figure 1 shows the metabolite map of DHEA.

Histological Evaluation of Atherosclerosis and Assays for Tissue Cholesterol Content
Histological examination of the thoracic aorta revealed that there were more atheromatous lesions in group 1 than in groups 2, 3, 4, or 5. The area of atherosclerosis in the thoracic aortas of the
DHEA group (group 2) was reduced by ~60%, the estradiol group (group 4) by 80%, and the DHEA plus fadrozole group (group 3) by only 30% when compared with group 1 (HCD group; Figure 2). Total and esterified cholesterol concentrations in the vessels showed the same tendency as that of atherosclerotic area. Free cholesterol was increased by feeding with the HCD (groups 1, 2, 3, and 4), without any significant differences among these 4 groups (Figure 3). Injection of solvent into group 1, 2, and 5 rabbits and injection of fadrozole into group 1 and 5 rabbits had no significant effect on the above-mentioned parameters (Table 2).

Endothelium-Dependent and -Independent Relaxation
In all experimental groups, the endothelium-dependent vasodilator ACh and A-23187 (data not shown) produced concentration-dependent relaxation of precontracted aortic rings with an intact endothelium (Figure 4). No significant differences in ACh-induced, endothelium-dependent relaxation were observed among the aortic rings obtained from the normocholesterolemic group (group 5) or from the hypercholesterolemic animals administered DHEA only (group 2) or 17β-estradiol (group 4). The magnitude of relaxation of the aortic rings from the hypercholesterolemic animals without DHEA (group 1) or with DHEA plus fadrozole (group 3) was significantly diminished compared with the aortic rings from normolipidemic animals (group 5). The endothelium-independent vasodilator NTG produced a concentration-dependent relaxation in precontracted, endothelium-denuded aortic rings. There were no significant differences among the 5 groups in terms of the relaxation response to NTG in the

---

**Figure 3.** The content of total, free, and esterified cholesterol in the rabbit aortic arch. Lipid contents were measured from the aortic arch of rabbits fed an atherogenic diet (group 1), an atherogenic diet plus DHEA (group 2), an atherogenic diet plus DHEA and fadrozole (group 3), an atherogenic diet plus 17β-estradiol (group 4), or a regular diet (group 5). Error bars indicate SEM. These data are from experiment 1.

**Figure 4.** Cumulative concentration-response curves to ACh during contraction evoked by PGF2α (2.6×10⁻⁶ mol/L) in the thoracic aorta of rabbits fed an atherogenic diet (group 1), an atherogenic diet plus DHEA (group 2), an atherogenic diet plus DHEA and fadrozole (group 3), an atherogenic diet plus 17β-estradiol (group 4), or a regular diet (group 5). Relaxation is expressed as a percent decrease in tension from the contraction evoked by PGF2α (2.6×10⁻⁶ mol/L) alone. These data are from experiment 1. Data are shown as mean±SEM. *Significant difference vs group 5 at P<0.05.
aortic rings (Figure 5). Inhibition of NOS by L-NMA (100 μmol/L) led to a contractile response in the aortic rings precontracted with PGF$_2$α (Figure 6). This contractile response was concentration dependent, and its magnitude was decreased in endothelium-intact aortic rings obtained from HCD-fed rabbits (group 1) when compared with rings obtained from control rabbits (group 5). The contractile response of aortic rings from atherosclerotic rabbits treated with DHEA or 17β-estradiol (group 2 or group 4) did not differ significantly from that of control animals (group 5). The contractile response of animals treated with DHEA plus fadrozole (group 3) was almost the same as that of untreated, atherosclerotic rabbits (group 1). Preincubation of the aortic rings with indomethacin did not affect endothelium-dependent relaxation, indicating that prostanoids did not alter the effect of DHEA in endothelium-dependent relaxation. Injection of solvent into group 1, 2, and 5 rabbits (groups 1b, 2b, and 5b) or fadrozole treatment alone for group 1 or 5 rabbits did not significantly affect the above-mentioned results (data not shown).

## Measurement of NO by NO Electrode

The release of NO from normolipemic rabbit aortas (group 5) and from hypercholesterolemic rabbit aortas (groups 1, 2, 3, and 4) was determined by using an NO-selective electrode. The release of NO from endothelial cells of each aorta stimulated by 1 μmol/L ACh is shown in Figure 7 and Table 4. NO release in the groups treated with 17β-estradiol or DHEA increased only in comparison with that in the group of HCD rabbits (group 1); however, NO release in the group treated with DHEA plus fadrozole (group 3) was not increased. Preincubation of aortic rings with L-NMA abolished the NO release stimulated by ACh (data not shown).
DHEA has been shown to have an antiatherosclerotic effect in animal models, although its mechanism of action is not yet known. Although its plasma concentration usually far exceeds that of any other sex steroid hormone, little information is available on its direct action in blood vessels. We focused on the metabolite of DHEA as the candidate responsible for the antiatherosclerotic effect, since DHEA converts to estrogen, a major metabolite that is well known for its antiatherosclerotic effect. Fadrozole (CGS 16946A) was developed recently as an anti–breast cancer drug and is 1 of the strongest specific aromatase inhibitors. Its potency as an aromatase inhibitor is 400 times stronger than that of an aromatase inhibitor is 400 times stronger than that of a physiological concentration of 17β-estradiol. Fadrozole treatment inhibited 50% of the antiatherosclerotic effect of DHEA, although it did not have a direct effect on atherosclerosis. The antiatherosclerotic effect of DHEA in the current study may be due to its conversion to estrogen, because inhibition of aromatase (estrogen-converting enzyme) by fadrozole reduced by 50% the antiatherosclerotic effect of DHEA.

The plasma estradiol concentration was increased by DHEA treatment, although testosterone concentration was not significantly increased. Epidemiological and pharmacological studies suggest that even a low plasma concentration of estradiol can achieve an antiatherosclerotic effect. Our previous reports suggested that the rate of progression of atherosclerosis was much slower in female than in male rabbits and that an identical HCD led to a similar increase in serum lipoprotein concentrations. This suggests that a higher concentration of plasma estrogen in female rabbits (basal plasma estradiol concentration of 23.5±5.5 pg/mL) than in male rabbits (8.4±8.3 pg/mL) under control conditions might cause this greater basal release of NO in females in the initial stages of atherosclerosis. However, the precise mechanism by which endogenous estrogen inhibited the progression of atherosclerosis was not fully addressed, because the plasma estradiol concentration level was identical in males and females after 10 weeks of HCD-induced hyperlipidemia. It has been shown that a considerable part of the plasma lipid–independent, antiatherogenic effect of estrogen is mediated through its effect on endothelial NO in cholesterol-fed rabbits. Based on these studies, to attain a physiological plasma estradiol concentration in this study, we treated the rabbits with 20 μg·kg⁻¹·d⁻¹ of 17β-estradiol (group 4) and compared its antiatherosclerotic effect with the HCD group (group 1), the HCD plus fadrozole group (group 3), and the HCD plus DHEA group (group 2), in comparison with that of the HCD group (group 1), was reduced by 80%; that of the estradiol group (group 4) was reduced by 78%; and that of the DHEA plus fadrozole group (group 3) was reduced by only 30%. The antiatherosclerotic effect of DHEA was almost the same as that of a physiological concentration of 17β-estradiol. Fadrozole treatment inhibited 50% of the antiatherosclerotic effect of DHEA, although it did not have a direct effect on atherosclerosis. The antiatherosclerotic effect of DHEA in the current study may be due to its conversion to estrogen, because inhibition of aromatase (estrogen-converting enzyme) by fadrozole reduced by 50% the antiatherosclerotic effect of DHEA.

**Discussion**

DHEA treatment, although testosterone concentration was not significantly increased. Epidemiological and pharmacological studies suggest that even a low plasma concentration of estradiol can achieve an antiatherosclerotic effect. Our previous reports suggested that the rate of progression of atherosclerosis was much slower in female than in male rabbits and that an identical HCD led to a similar increase in serum lipoprotein concentrations. This suggests that a higher concentration of plasma estrogen in female rabbits (basal plasma estradiol concentration of 23.5±5.5 pg/mL) than in male rabbits (8.4±8.3 pg/mL) under control conditions might cause this greater basal release of NO in females in the initial stages of atherosclerosis. However, the precise mechanism by which endogenous estrogen inhibited the progression of atherosclerosis was not fully addressed, because the plasma estradiol concentration level was identical in males and females after 10 weeks of HCD-induced hyperlipidemia. It has been shown that a considerable part of the plasma lipid–independent, antiatherogenic effect of estrogen is mediated through its effect on endothelial NO in cholesterol-fed rabbits. Based on these studies, to attain a physiological plasma estradiol concentration in this study, we treated the rabbits with 20 μg·kg⁻¹·d⁻¹ of 17β-estradiol (group 4) and compared its antiatherosclerotic effect with the HCD group (group 1), the HCD plus fadrozole group (group 3), and the HCD plus DHEA group (group 2), in comparison with that of the HCD group (group 1), was reduced by 80%; that of the estradiol group (group 4) was reduced by 78%; and that of the DHEA plus fadrozole group (group 3) was reduced by only 30%. The antiatherosclerotic effect of DHEA was almost the same as that of a physiological concentration of 17β-estradiol. Fadrozole treatment inhibited 50% of the antiatherosclerotic effect of DHEA, although it did not have a direct effect on atherosclerosis. The antiatherosclerotic effect of DHEA in the current study may be due to its conversion to estrogen, because inhibition of aromatase (estrogen-converting enzyme) by fadrozole reduced by 50% the antiatherosclerotic effect of DHEA.

**Measurement of NO2-/NO3-**

Plasma concentrations of NO (the sum of [NO2⁻] and [NO3⁻]) were increased in groups 2 and 4 compared with those in groups 1, 3, and 5 (Table 4).

**Measurement of cGMP**

In homogenate samples of rabbit aortas, animals treated with DHEA (group 2) or 17β-estradiol (group 4) showed an increased level of cGMP compared with those of hypercholesterolemic animals (group 1) or animals treated with DHEA plus fadrozole (group 3; Table 4).

**TABLE 4. NO and NO-Related Product Levels After Consumption of an HCD**

<table>
<thead>
<tr>
<th>Group</th>
<th>NO release by (NO electrode: nmol·L⁻¹·mm⁻² of aorta with 1 μmol/L Ach stimulation)</th>
<th>Plasma NO, HPLC, µmol/L</th>
<th>Tissue cGMP by RIA, µmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1, HCD</td>
<td>15.5±0.7†</td>
<td>100.7±7.8</td>
<td>0.255±0.026</td>
</tr>
<tr>
<td>Group 2, HCD + DHEA</td>
<td>21.4±0.7*</td>
<td>127.3±9.2*</td>
<td>0.321±0.03*</td>
</tr>
<tr>
<td>Group 3, HCD + DHEA + Fadrozole</td>
<td>16.3±0.8†</td>
<td>100.5±5.5</td>
<td>0.251±0.034</td>
</tr>
<tr>
<td>Group 4, HCD + 17β-Estradiol</td>
<td>23.3±0.9*</td>
<td>140.2±12.7*</td>
<td>0.372±0.034*</td>
</tr>
<tr>
<td>Group 5, Regular Diet</td>
<td>24.5±0.6*</td>
<td>91.6±3.3</td>
<td>0.234±0.028</td>
</tr>
</tbody>
</table>

HPLC indicates high-performance liquid chromatography; RIA, radioimmunoassay. For the measurement by NO electrode, 1 nmol/L is equivalent to the amount of NO released after 1 nmol/L SNAP (=0.3 pA). [NO2⁻]+[NO3⁻]. Results are presented from experiment 1.

*P<0.05 vs group 1.
†P<0.05 vs group 5.
effect with that of DHEA (group 2). The increase in plasma estrogen level in group 2 may partially account for the antiatherosclerotic effect of DHEA, which was partially abolished by concomitant treatment with fadrozole. DHEA can be converted into many steroids other than estrogens, including a number of more potent androgens.47 There was no significant increase in testosterone or pregnanediol (data not shown) in the fadrozole-treated group (group 3). However, the effect of testosterone on atherosclerosis remains controversial.48,49 In response to aromatase, rabbit testes have been shown to secrete DHEA and convert it to testosterone and testosterone-related metabolites, including estrogen.50 We used oophorectomized rabbits to establish a simple endocrinological model, although previous experiments on DHEA have either disregarded the sex of the rabbits or used males only.3,4 Therefore, we suggest that these studies can be applied to assessing the effect of DHEA on male rabbits, although more specific investigations will be necessary. Because fadrozole appeared to have no direct effect on HCD-induced atherosclerosis in this study, the possibility is remote that the slight fluctuation in testosterone level or that of other steroids due to fadrozole treatment affected the formation of atherosclerotic lesions.

A direct action of estrogen on the arterial wall is important for a considerable part of the plasma lipid-independent, antiatherogenic effect of DHEA. Estrogen seems to inhibit cholesterol accumulation and intimal hyperplasia, independent of changes in plasma lipoproteins in ovariectomized cynomolgus monkeys and rabbits fed an atherogenic diet.21–23 Suppression of the arterial uptake of DHEA in ovariectomized cynomolgus monkeys and rabbits fed an atherogenic diet has been shown.24–27 Suppression of the arterial uptake and/or degradation of LDL by estrogen may explain these effects.23 However, these mechanisms are not able to work when the endothelial cell layer is seriously damaged.51 Estrogen can normalize abnormal vasomotor responses in atherosclerotic coronary arteries of cholesterol-fed monkeys.52 From these results, one might speculate that endothelium-derived NO may play an important role in the antiatherosclerotic effect of estrogen. NO released from eNOS was shown to regulate blood flow and suppress a number of processes involved in atherosclerosis.24–27 Several studies have shown that enhancing arterial NO synthesis retards the progression of atherosclerosis53,54 and that inhibiting NOS in vessels promoted the progression of atherosclerosis.55 Estrogen has been reported to increase eNOS activity by an endothelial receptor–mediated system in vitro.28 We speculate that the ability of estrogen to increase NO bioavailability is involved in the antiatherogenic effect of estrogen, especially in the initial stages of a cholesterol diet–induced atherosclerosis.30 More recently, Holm et al31 directly showed that inhibition of NOS reduced the antiatherosclerotic effect of estrogen. Thus, our attention was attracted to the role of NO in the antiatherosclerotic effect of DHEA. The aortas of DHEA-treated animals (group 2) showed restoration of the ACh-induced and tone-related basal NO-mediated relaxation, whereas it was diminished in the aortas of group 1 rabbits. The vascular response in animals treated with DHEA plus fadrozole (group 3) was not reversed significantly. The increase in plasma NO\textsubscript{3} and NO\textsubscript{2} concentrations and tissue cGMP levels in DHEA-treated rabbits showed the increasing effect of an NO-dependent mechanism. This was supported by increases in basal NO release with the improvement of a tone-related basal NO response in the arteries. These effects were diminished in the group treated with DHEA plus fadrozole (group 3). The present observations suggest that DHEA retards atherosclerosis formation by means of an NO-dependent system, which may be partially related to the conversion of DHEA to estrogen. The level of cGMP was increased in the aortas of atherosclerotic rabbits (group 1) compared with control rabbits (group 5). These observations are consistent with a previous report that the action of NO was decreased, but the release of NO and O\textsuperscript{2} decreased, in atherosclerosis.56 DHEA has been reported to induce a consistent and reversible morphological change in cultured endothelial cells derived from human umbilical vein.57 Ultrastructurally, multilamellar lysosomal lipid structures were formed, and these changes were thought to be related to the effect of peroxisomes.57 DHEA is known to induce peroxisomes, peroxisome-associated enzymes, and microsomal enzymes in the liver of rats and mice.58 Because the effects of hypolipidemic drugs such as clofibrate, which is known as a weak peroxisome proliferator, appear to be sex dependent in rats, DHEA may have some effects on the plasma lipid profile through the peroxisomes or related enzymes.58 However, the likelihood of such an effect is small, because DHEA treatment did not change the plasma lipid profile in the current study; it seemed to act on the vessels directly.

The binding activity of DHEA has been shown to be higher in an activated human T-lymphocyte line in vitro.8 This bound portion may play a role as a receptor, although that report did not show a change in function caused by DHEA binding.8 DHEA was also reported to antagonize the suppressive effects of dexamethasone on T- and B-lymphocyte proliferation in mice.39 T lymphocytes are known to be present and to play a certain role in atherosclerosis60,61; however, that role has not been fully established. Although the population and distribution of T cells in an atherosclerotic lesion after DHEA treatment did not show any change in our preliminary observations, the possibility of an antiatherosclerotic effect of DHEA through the receptor-mediated system of activating T lymphocytes remains to be elucidated.

Many possible mechanisms may be involved in the atheroprotective effect of DHEA, although certainly 1 of these is the conversion of DHEA to estrogen, and NO seems to play a specific role in the antiatherosclerotic effect of both estrogen and DHEA. The contribution of estrogen to the atheroprotective effect of DHEA was ≈50% in our study of an HCD-induced atherosclerosis in a rabbit model.

Acknowledgments

This study was supported in part by grant-in-aid No. 09470166 from the Japanese Ministry of Education. We also thank Norie Kametsuta and Yuriko Kato for their excellent technical assistance.

References


Dehydroepiandrosterone Retards Atherosclerosis Formation Through Its Conversion to Estrogen: The Possible Role of Nitric Oxide
Toshio Hayashi, Teiji Esaki, Emiko Muto, Hatusyo Kano, Yukako Asai, Navin Kumar Thakur, Daigo Sumi, Muthuvel Jayachandran and Akihisa Iguchi

Arterioscler Thromb Vasc Biol. 2000;20:782-792
doi: 10.1161/01.ATV.20.3.782
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/20/3/782

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/