Dehydroepiandrosterone Feeding Prevents Aortic Fatty Streak Formation and Cholesterol Accumulation in Cholesterol-fed Rabbit

Yadon Arad, Juan J. Badimon, Lina Badimon, Wylie C. Hembree, and Henry N. Ginsberg

The concentration of dehydroepiandrosterone sulfate (DHEA-S) in human plasma is higher than any other steroid. Recent evidence has suggested an inverse relationship between plasma DHEA levels and the development of coronary atherosclerosis in humans. We used the cholesterol-fed rabbit model to investigate whether DHEA feeding would diminish aortic fatty streak formation in this model. Fifteen New Zealand White rabbits were fed rabbit chow supplemented with 0.5% cholesterol (wt/wt). Seven animals were, in addition, fed DHEA, 0.5% of diet (wt/wt). Animals were sacrificed after 2 months, and the aortic involvement with fatty streaks was evaluated by computerized planimetry of Sudan IV-stained aortas and by chemical analysis of aortic wall lipids. Compared to controls, DHEA-fed animals had similar plasma levels of total, very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) cholesterol, corticoids, and estrogens. DHEA-fed animals had higher plasma levels of total, VLDL, and LDL triglycerides and lower HDL triglycerides than did controls. DHEA feeding resulted in 30% and 40%, respectively, inhibition of fatty streak formation by chemical analysis and planimetry. We conclude that DHEA feeding inhibits the development of aortic fatty streaks in cholesterol-fed rabbits, independent of changes in plasma total and LDL cholesterol levels or DHEA conversion to estrogens or corticoids. (Arteriosclerosis 9:159-166, March/April 1989)

Dehydroepiandrosterone (DHEA) and its sulfate ester, DHEA-S, are weak androgens produced primarily by the adrenal gland. Although their serum concentrations far exceed those of any other adrenal product, their physiological roles have not been determined. In plasma, the majority of the hormone is present in the sulfated form, the concentration of which is stable. DHEA, itself, which has a rapid turnover rate, displays variable levels during the day.1 Since tissues contain steroid sulfatases,2 it is possible that DHEA-S serves as a reservoir for DHEA. Reported effects of dietary DHEA supplementation include prevention of obesity and insulin resistance in genetically predisposed animals.3-5 A recent study indicated that administration of DHEA was associated with a reduction of body fat and plasma low density lipoprotein (LDL) cholesterol levels in healthy humans.6 Since hyperinsulinemia and central obesity,7 as well as elevated LDL cholesterol levels, are factors predisposing to increased risk of atherosclerosis, it is possible that DHEA might be antitherogenic. Indeed, in a recent study, DHEA-S levels were inversely related to subsequent death from coronary disease in men.8

The cholesterol-fed rabbit model has been used previously to study the effects of various agents on the development of aortic atherosclerosis. This model does not completely simulate the development of disease in humans because the large dietary cholesterol load raises the cholesterol concentration in very low density lipoprotein (VLDL) remnants to a larger degree than in LDL.9,10,11 Nonetheless, the cholesterol-fed rabbit has been used to study the effects of calcium channel blockers,12 propranolol,13,14 and β-blockers15,16 on atherosclerosis. The epidemiological observation relating DHEA levels to atherosclerosis in humans was extended to the cholesterol-fed rabbit model in a recent report by Gordon et al.17 In that study, aortic injury was first induced by an intra-aortic balloon. A reduction of 39% in the degree of fatty streak formation, as measured by cross-sectional plaque thickness, was demonstrated in animals fed both DHEA and cholesterol as compared to controls fed cholesterol alone for 11 weeks after injury. The balloon injury model, however, may not be relevant to human atherosclerosis in which overt injury need not occur. We now report a study of the effect of DHEA feeding on the occurrence of aortic fatty streak formation in cholesterol-fed rabbits, without induction of prior injury.
Methods

Animal Model

Adult New Zealand White rabbits (3.0±0.5 kg body weight) were housed in the Animal Research facilities of the Mount Sinai Medical Center. They were individually caged in stainless-steel wire-bottomed cages, in a room controlled at 20°±2°C temperature, 50%±10% humidity, and a 12-hour light/dark cycle. This animal care facility is accredited by the American Association for Accreditation of Animal Care, and all procedures were reviewed and approved by the Institutional Animal Care and Use Committee.

Experimental fatty streak formation was induced by feeding the animals 140 g of a 0.5% (wt/wt) cholesterol-rich diet daily for 60 days. The atherogenic diet was prepared by spraying normal rabbit chow (HF# 5326, Ralston Purina Company, St. Louis, MO) with cholesterol dissolved in chloroform and allowing the solvent to evaporate for 2 days.18 We previously demonstrated that feeding cholesterol to rabbits at that concentration results in 30% to 50% of the rabbit aorta being covered by fatty streaks in both male and female rabbits with no apparent sex differences18 (Badimon JJ, unpublished observation). Water was provided ad libitum.

Experimental Design

In our study, the animals received the atherogenic diet (control group) or the atherogenic diet plus 0.5% (wt/wt) DHEA (DHEA-fed group). The DHEA, which was in powder form, was incorporated into the atherogenic diet by mixing. Both groups of animals were studied simultaneously, and the animals were sacrificed 8 weeks after the initiation of the atherogenic feeding. There were seven females and one male in the control group and six females and one male in the DHEA group.

Plasma Lipid Analysis

Blood samples were collected in EDTA (1.5 mg/ml) after a 12- to 16-hour fast before starting the diet, at 20 days, at 40 days, and at sacrifice for determination of total cholesterol, high density lipoprotein cholesterol (HDL), and total triglyceride levels. Plasma was immediately obtained by low-speed centrifugation at 4°C. Aliquots were separated and kept frozen at −70°C until assayed. Cholesterol concentration in plasma was determined by using a Beckman enzymatic kit (Dry Stat Beckman Instruments Incorporated, Carlsbad, CA). Triglyceride concentration in plasma was measured using an enzymatic kit (Seragen Diagnostics, Indianapolis, IN). Prestudy HDL cholesterol concentration was measured in plasma after precipitation of apolipoprotein (apo) B-containing lipoproteins by PEG-6000.20 The HDL isolation was performed on an aliquot of fresh plasma after which the samples were kept frozen until assayed. We have observed that measurement of HDL cholesterol level by the precipitation method in severely hypercholesterolemic rabbits may be inaccurate, probably because of contamination by VLDL and LDL (data not shown). Poststudy HDL cholesterol levels are therefore reported only for HDL obtained by ultracentrifugation (below).

Preparation and Isolation of Lipoproteins

Additional blood was collected at sacrifice and separated as described above. Sodium azide was added to the freshly prepared plasma (0.01%). EDTA (0.1%) was present in all solutions to prevent hydroperoxidative degradation of lipoproteins. Lipoproteins were obtained by sequential ultracentrifugation of normal rabbit plasma by using a Beckman ultracentrifuge (model L5-65) and a 50.3 Ti Beckman rotor. VLDL (d<1.006), intermediate density lipoprotein (IDL) plus LDL (IDL+LDL, 1.006<d<1.063), and HDL (1.063<d<1.21) lipoproteins were separated by solvent density according to standard procedures, adjusting densities with KBr.21

Morphological Evaluation of Fatty Streak Formation

At sacrifice, the rabbits were anesthetized with ketamine plus Rompun (5 and 35 mg/kg, respectively, given intramuscularly). The carotid artery was cannulated, and the animals were exsanguinated. After laparotomy and exposure of the aorta, the animals were killed by an overdose of anesthetic, and the aortas were perfused in situ with 0.2 M phosphate-buffered saline containing papaverine (60 mg/500 cc) at 37°C to avoid vasconstriction. The aortas were removed intact from the aortic arch to the iliac bifurcation and were stripped of all adventitial debris. Thereafter, they were cut lengthwise into two halves through a dorsal and ventral incision. Care was taken to dissect between the dorsal bifurcations so that the flow dividers were evenly split between the two halves. One half of each aorta was fixed in 10% buffered formalin, and the other half was processed for lipid analysis. The fixed aortas were stained with Sudan IV for visualization of sudanophilic plaques. After staining, the aortas were pinned open to flatten them. A template was then made for each aorta by tracing the outlines of the aorta and the atheromatous lesions on a clear plastic sheet. Each template was magnified (×2) and photographed. Morphometric measurements of the percentage of total aorta covered with lipid deposits was determined by computerized planimetry (Numonic 124 EM Image analyzer). Each of three blinded observers prepared a template for each aorta, and each then performed planimetric measurements on his own templates, as well as on those prepared by the other two observers. Intra-observer variation was less than 1%.

Since atherosclerotic lesions form at specific sites, such as the bifurcation points of branching vessels, we alternated between choosing the right or left half of each aorta for biochemical or morphological analysis, so as to diminish the effect of differences between the two halves and negate possible artifacts due to unequal sectioning.

Biochemical Analysis of Aortic Wall and Liver

Aortas (half) were homogenized at 4°C in 5 ml of 0.13 M Tris HCl (pH 7.4), 0.1% EDTA, and 0.01% NaNO2 by using a Polytron homogenizer. The total lipids were extracted from the homogenates in 10 volumes of chloroform/methanol (2:1; vol/vol) containing 0.001% butylated hydroxytoluene as an antioxidant. A second extraction...
Table 1. Plasma Steroid Levels after High-Cholesterol Diet

<table>
<thead>
<tr>
<th>Animals</th>
<th>DHEA (μg/dl)</th>
<th>Estrogen (pg/ml)</th>
<th>Corticoids (μg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=8)</td>
<td>13.7±14.9</td>
<td>122.1±24.9</td>
<td>1.3±0.7</td>
</tr>
<tr>
<td>DHEA-fed (n=7)</td>
<td>75.9±105.5</td>
<td>146.3±42.1</td>
<td>2.2±1.6</td>
</tr>
<tr>
<td><em>p values</em> (Control vs. DHEA-fed)</td>
<td>p=0.09</td>
<td>p=0.2</td>
<td>p=0.2</td>
</tr>
</tbody>
</table>

*A log transformation of the plasma DHEA levels was performed for statistical analysis.

DHEA=dehydroepiandrosterone.

Table 2. Plasma Lipid Levels before and after High-Cholesterol Diet

<table>
<thead>
<tr>
<th>Animals</th>
<th>Total cholesterol</th>
<th>Triglycerides</th>
<th>HDL cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=8)</td>
<td>Before</td>
<td>After</td>
<td>Before</td>
</tr>
<tr>
<td></td>
<td>43.3±10.2</td>
<td>816.4±298.2</td>
<td>79.0±2.8</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.00*</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>DHEA-fed (n=7)</td>
<td>55.0±2.3</td>
<td>964.3±258.3</td>
<td>70.6±15.5</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.001*</td>
<td>p&lt;0.005*</td>
<td>19.6±5.1</td>
</tr>
<tr>
<td>*p value (Control vs. DHEA-fed)</td>
<td>NS</td>
<td>NS</td>
<td>p&gt;0.005</td>
</tr>
</tbody>
</table>

Values are mg/dl, means±SD. *p value before versus after high-cholesterol diet.

Statistical Analysis

Results between the two groups were compared by using Student's t test and the nonparametric Wilcoxon sum-rank test. Differences between groups that reached significance by that test were considered truly significant only if they were also significant by the Wilcoxon sum-rank test and only if no skewness was present (skewness is a measurement of deviation from normal distribution). If skewness was present, the differences were considered statistically significant only if skewness could be eliminated by exclusion of "outliers" while maintaining significance. The Wilcoxon sum-rank test was used to confirm significance because this test does not assume a normal distribution (which may be a problem with a small sample size). DHEA levels were compared using the t test on the natural log of the DHEA concentration. Differences were considered significant if p<0.05.

Results

No differences between groups were observed in total food intake, weight gain, hematocrit, or platelet count.

Plasma levels for total DHEA, estrogens, and corticoids after the high-cholesterol diet are shown in Table 1. While there was a trend toward higher total DHEA levels in DHEA-fed animals compared to controls, the difference between the two groups did not reach statistical significance (p=0.09) due to considerable individual variability and to a small rise in total DHEA levels even in control animals (data not shown). There were no differences in estrogen or in corticoid levels between the two groups after the high-cholesterol diet.

Plasma lipids are presented in Table 2. Both groups responded to the high-cholesterol diet with similar, marked
increases in plasma total cholesterol concentration compared to the prestudy levels (control rabbits from a mean of 43.3 mg/dl to 816.4 mg/dl, DHEA-fed rabbits from a mean of 55.0 mg/dl to 964.3 mg/dl). HDL cholesterol levels remained unchanged in both groups (controls from a mean of 24.4 mg/dl to 19.4 mg/dl, DHEA-fed rabbits from a mean of 19.6 mg/dl to 18.4 mg/dl). There were no differences in plasma total cholesterol or HDL cholesterol concentrations between the two groups either before or after the cholesterol feeding. Plasma lipid levels drawn at 20 and 40 days after initiation of the diets demonstrated similar rates of rise in plasma cholesterol between the two groups (data not shown). Plasma triglyceride levels in the two groups before the high-cholesterol diet were also comparable (see Table 2). Although the triglyceride level of the control group did not change after the high-cholesterol diet, the triglyceride levels of the DHEA-fed animals rose significantly (from a mean of 70.6 to 128.2 mg/dl) compared to the prestudy triglycerides of either group, and to the poststudy triglycerides of the control group (p<0.01 for all three).

We also studied the lipid distribution among the different classes of plasma lipoproteins after the high-cholesterol diet (Table 3). Both groups showed similar high concentrations of cholesterol in both VLDL and IDL+LDL, compared to the prestudy total cholesterol. After the high-cholesterol feeding, there were no differences between the two groups in cholesterol content in any of the lipoprotein subfractions. However, both the VLDL and IDL+LDL fractions from the plasma of DHEA-fed animals contained significantly more triglycerides than did those fractions from the plasma of the control animals. In contrast, HDL of DHEA-fed animals contained significantly less triglyceride than that of control animals.

Aortic involvement with fatty streaks, as determined by computerized planimetry of Sudan IV-positive areas, is shown in Figure 1. Fatty streak involvement occurred mainly at branch points of the aortic arch and the thoracic aorta. The abdominal aorta was much less involved. Comparison of the mean fatty streak involvement of the aortas, expressed as a percentage of the total aortic area covered by fatty streaks, showed a statistically significant 40% reduction of plaque area in the DHEA-fed animals versus controls (34% in the control groups vs. 19% in the DHEA-treated group, p<0.01).

To verify this reduction in fatty streak involvement by an independent method, we performed a chemical analysis of lipids extracted from the aortic wall (Figure 2). This examination showed a statistically significant 30% reduction of total cholesterol deposition in the aortas of DHEA-fed animals as compared to the control animals (controls 12.7 mg/g tissue, DHEA-fed 8.9 mg/g tissue, p<0.02). In contrast, no differences were found in phospholipid or triglyceride deposition in the aortas of DHEA-fed animals versus controls.

The content of lipid in livers from treated and control rabbits is shown in Table 4. The livers from DHEA-fed animals had a slightly but significantly higher total cholesterol content (control 18.1 mg/g tissue, DHEA-fed...
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22.1 mg/g tissue, p<0.01). Similar to the finding in the aortas, no difference was found in hepatic phospholipid content between the two groups.

Discussion

This study examined the effect of DHEA feeding on the development of aortic fatty streaks in cholesterol-fed rabbits. Using two independent objective methods for assessing aortic involvement with fatty streaks, we found that DHEA feeding was protective against the development of fatty streaks in the cholesterol-fed rabbit. After a high-cholesterol diet, DHEA-fed animals developed 40% less aortic infiltration with fatty streaks, as determined by computerized planimetry of the aortic wall compared to control animals. Their aortas also contained 30% less cholesterol compared to control animals. While we used only one half of each aorta for either determination, there was a direct correlation between the area covered with fatty streaks and the magnitude of aortic cholesterol deposition in the same animal (not shown), demonstrating that the effects observed were not due to artifacts of the dissection process.

There was no effect of DHEA on total plasma, VLDL, IDL+LDL, or HDL cholesterol concentrations. The high-cholesterol diet resulted in similar increases of total cholesterol, VLDL cholesterol, and IDL+LDL cholesterol in both groups, whereas HDL cholesterol did not change in either group. In contrast, DHEA feeding resulted in an increase in total, VLDL, and IDL+LDL triglyceride levels and a decrease in HDL triglycerides compared to controls. DHEA feeding also resulted in increased liver content of cholesterol compared to controls. Finally, DHEA feeding had no effect on plasma estrogen or corticoid concentrations.

These data are consistent with the recent report by Gordon et al.,17 that DHEA feeding caused a 39% reduction of aortic involvement with fatty streaks in rabbits fed cholesterol after aortic injury. It is possible, however, that the observed results in that study were due to DHEA modification of postinjury processes.

We chose to look at a model that utilized high-cholesterol feeding without induction of aortic injury because we felt that this model more closely resembles the disease process in humans. The cholesterol-fed rabbit model has limitations as a model for human disease because: 1) the rise in cholesterol is, to a large degree, due to accumulation of β-VLDL as previously described,9,10,11 2) feeding diets high in cholesterol for short periods of time may not resemble the chronic human atherosclerotic process, and 3) most of the lipid deposition in the aorta in this model is in the form of cholesteryl ester-enriched fatty streaks,24,25,26 which are presumed, but not proven, to be the precursors of the more complicated fibrous plaques seen in humans in association with myocardial infarction. The cholesterol-fed rabbit, however, has been used previously to study the effects of calcium channel blockers, probucol, and β-blockers on the development of fatty streaks. The extent of reduction in fatty streak formation seen with those agents ranged from zero27,28 to over 90%29 for calcium channel blockers, from 40% to 60% for probucol,13,14 and from 20% to 40% for some, but not all, β-blockers.15,16 The effects of DHEA feeding that we observed in this study were similar to those seen with those other agents. However, in contrast to those agents, DHEA is a naturally occurring hormone, suggesting that the effects we observed may be more relevant physiologically to human atherosclerosis.

The cholesterol-fed rabbit model has also been used to study the effects of other naturally occurring hormones on the development of atherosclerosis. Glucocorticoids have been found to reduce aortic cholesterol accumulation and aortic 3H-thymidine uptake in cholesterol-fed rabbits.30,31 In humans, however, elevated cortisol levels are associated with increased atherosclerosis.32 It has also been shown that estrogens reduce the development of fatty streaks in cholesterol-fed rabbits.33,34 This may indeed be relevant to human atherosclerosis, since estrogens are thought to be protective against atherosclerosis in females.35 In our study, however, there were no differences in either plasma corticoids or plasma estrogen levels between the two groups.
The amount of DHEA that we fed the animals was large and was chosen because of the proven metabolic effects of the hormone in similar doses in previous investigations in animals. Unfortunately, the assay we used to measure DHEA in rabbits cannot distinguish between the base and the sulfated form in plasma. However, DHEA-S constitutes the majority of the hormone measured by this assay due to its higher concentration. Although there was a trend toward higher DHEA levels in DHEA-fed animals, the difference did not reach statistical significance. We did not observe an association between the blood levels of the hormone and the extent of fatty streak involvement in the aortas. It is possible that the large variation in the blood levels of DHEA that we observed was due to intra-animal variations in kinetics of gastrointestinal absorption or metabolism of DHEA, or to the effects of other factors, such as stress, which might alter the plasma concentration of DHEA. Such factors might confound an analysis of the relationship between DHEA blood levels and the prevention of atherosclerosis but not the analysis of the overall antiatherogenic effects of DHEA, which may relate more to mean daily concentrations of the hormone. The rise in plasma DHEA that we observed was also smaller than that seen in humans after a smaller dose. This may have resulted from different levels of sulfatase activity in rabbits versus humans. The above notwithstanding, plasma levels of total DHEA in the treated animals were well within the range seen in normal humans.

The mechanism of action of DHEA in preventing fatty streak formation in the cholesterol-fed rabbit is not known. DHEA feeding to animals modifies several parameters that are considered to be risk factors for atherosclerosis. Thus, it has been shown that DHEA feeding to animals that are genetically predisposed to diabetes and obesity (e.g., the BL/6 mouse and the Zucker rat) will prevent obesity, reduce parametral and peritoneal (central) fat, and prevent the development of insulin resistance and overt diabetes. We have no data to suggest similar effects during our short-term feeding protocol. DHEA feeding to animals has also been shown to retard the development of carcinogen-induced tumors and to reduce the spontaneous development of breast cancer in genetically predisposed animals. Whether this antiproliferative activity may extend to the effects of growth factors, such as platelet-derived growth factor, in the arterial vessel wall is unknown.

It is possible that the antiatherogenic effect of DHEA in our study was related to the changes observed in lipoprotein triglyceride concentrations. Nordaasgaard et al. demonstrated that larger triglyceride-enriched VLDL may not pass through the endothelium, thus rendering them nonatherogenic. However, in that study, the VLDL triglyceride levels were much lower than those in our study. Furthermore, polyacrylamide gradient gel electrophoresis of the plasmas of our rabbits failed to show a difference between the groups in terms of mean β-VLDL size, partly because of the wide distribution of VLDL particle sizes in each rabbit after cholesterol feeding (data not shown). Conversely, triglyceride-rich LDL are thought to be associated with increased risk for developing atherosclerosis. In our study, however, the IDL+LDL of both groups contained similar, high levels of cholesterol, whereas "triglyceride-enriched LDL" are usually small, relatively cholesterol-poor particles.

We also found that DHEA feeding led to a slightly higher cholesterol content in the liver. The implication of this finding is unclear at this time, particularly in view of the similar plasma total cholesterol levels in the two groups of rabbits.

Regardless of the mechanism involved, our data may be of interest in relation to previous observations concerning DHEA and DHEA-S levels in humans. These include the demonstration that both DHEA and DHEA-S levels, as well as their responses to adrenocorticotropic hormone and corticotropin-releasing hormone, fall with age in both men and women, so that the levels of these androgens at age 70 are only 10% of the peak at age 25. Age-related decline is not seen in other steroids. DHEA levels have been found to be inversely related to LDL cholesterol concentrations in preterm infants and in anencephalic fetuses who are hypoadrenal. In addition, the 24-hour urinary DHEA-S levels were inversely related to blood cholesterol concentrations in industrial workers. DHEA feeding to humans resulted in no change in serum cholesterol in one study but was associated with decreased LDL cholesterol levels in another study.

While all of the above-noted associations support the epidemiological data indicating that DHEA-S levels were inversely correlated to subsequent death from atherosclerosis in humans, the one published study of DHEA levels in patients undergoing coronary angiography did not demonstrate differences in plasma DHEA concentrations between patients with positive and those with negative angiograms. On the other hand, we (Arad Y, Littenberg B, and Kluger J, unpublished data) and others have found higher DHEA levels in patients with negative coronary angiograms compared to patients with angiographically demonstrated coronary disease.

In summary, we have demonstrated that dietary DHEA supplementation significantly reduces the formation of Sudan IV-stainable fatty streak lesions and the accumulation of cholesterol in the aortas of cholesterol-fed rabbits. This finding, together with previous studies in both animals and humans, suggests an antiatherogenic activity for DHEA, DHEA-S, or one of their metabolites. Further studies of the action of DHEA on cells of the arterial wall seem warranted.

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Body fat distribution and hyperinsulinemia as risk factors for diabetes and cardiovascular disease. 

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Improved methods for deter-


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