Dehydroepiandrosterone (DHEA) Inhibition of Monocyte Binding by Vascular Endothelium Is Associated With Sialylation of Neural Cell Adhesion Molecule

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Anna-Maria Curatola, PhD¹, Kui Huang, PhD¹, and Frederick Naftolin, MD, PhD¹

Abstract

Rationale: Adhesion of monocytes to vascular endothelium is necessary for atheroma formation. This adhesion requires binding of endothelial neural cell adhesion molecule (NCAM) to monocyte NCAM. NCAM:NCAM binding is blocked by sialylation of NCAM (polysialylated NCAM; PSA-NCAM). Since estradiol (E2) and dihydrotestosterone (DHT) induced PSA-NCAM and decreased monocyte adhesion, in consideration of possible clinical applications we tested whether their prohormone dehydroepiandrosterone (DHEA) has similar effects. **Experimental:** (1) DHEA was administered to cultured human coronary artery endothelial cells (HCAECs) from men and women. Monocyte binding was assessed using fluorescence-labeled monocytes. (2) HCAECs were incubated with E2, DHT, DHEA alone, or with trilostane, fulvestrant or flutamide. Expression of PSA-NCAM was assessed by immunohistochemistry and Western blotting. **Results:** Dehydroepiandrosterone inhibited monocyte adhesion to HCAECs by $\geq 50\%$ ($P < .01$). Fulvestrant or flutamide blockade of DHEA's inhibition of monocyte binding appeared to be gender dependent. The DHEA-induced expression of PSA-NCAM was completely blocked by trilostane. **Conclusions:** In these preliminary in vitro studies, DHEA increased PSA-NCAM expression and inhibited monocyte binding in an estrogen- and androgen receptor-dependent manner. Dehydroepiandrosterone appears to act via its end metabolites, E2 and DHT. Dehydroepiandrosterone could furnish clinical prevention against atherogenesis and arteriosclerosis.

Keywords

atherosclerosis, heart disease, coronary, estrogen, androgen, adhesion, NCAM, PSA-NCAM

Introduction

Arteriosclerosis (arteriosclerotic vascular disease [ASVD]) remains the most common cause of death in women and men despite improved prevention and treatment. In addition, deaths from vascular complications of common diseases such as metabolic syndrome/diabetes and stroke/dementia make ASVD even more deadly as these diseases are associated with aging populations. Since wholesale treatment of the complications of ASVD is not a feasible public health option, prevention is the only reasonable approach to containing ASVD complications and deaths. Although many of the risk factors for ASVD respond to prevention, the practicalities of aiming at individual risk factors, such as hypertension, smoking, and lifestyle, are daunting. New methods are needed for the prevention of ASVD, which address common processes regardless of the differences in risk factors.

Monocyte adhesion to vascular endothelial cells is a nexus for atherogenesis; regardless of the prevalence of the usual risk factors, plaque cannot form without the presence of monocytes/

macrophages in the vascular wall, which can form foam cells/plaque. Monocyte adhesion to the vascular endothelium depends, in part, on the tethering of monocytes to endothelial cells by endothelial neural cell adhesion protein (neural cell adhesion molecule [NCAM]):monocyte NCAM binding.¹ NCAM:NCAM binding can be prevented by sialylation of NCAMs, forming polysialylated NCAM (PSA-NCAM) that obviates electrostatic bonding between NCAMs.² We showed that estrogen induces the enzymes that sialylate NCAM,³ that PSA-NCAM is increased by estrogen, and that human vascular

¹ Department of Obstetrics and Gynecology, New York University, New York, NY, USA

Corresponding Author:

Frederick Naftolin, Department of Obstetrics and Gynecology, NYU Langone Medical Center, 550 First Avenue, New York, NY 10016, USA
Email: frederick.naftolin@nyumc.org

endothelial cells express PSA-NCAM under the stimulation of estradiol (E2).⁴

We propose that prevention of monocyte adhesion to the vascular endothelium via sex steroid-induced sialylation could be a feasible method of prevention of atherogenesis/ASVD. We proved that pretreatment of human coronary artery endothelial cells (HCAECs) from both sexes with E2, testosterone (T), or DHT strongly inhibits monocyte binding (see below). However, since prevention against atherogenesis must be started before there is significant intravascular plaque,⁵ estimated at age 35 to 45, we sought a possible alternative that could furnish the effects of E2, T, or DHT without side effects that would compromise compliance by reproductively active men and women. Dehydroepiandrosterone (DHEA) is a widely used, clinically safe prohormone for estrogens and androgens. In this communication, we present preliminary findings, testing the effects of DHEA on endothelial-monocyte adhesion and induction of PSA-NCAM.

Materials and Methods

Cell Culture

Human coronary artery endothelial cells were purchased from Clonetics (Lonza, Allendale, New Jersey). Two different clones were used, one obtained from a female donor and another from a male donor. The genders were confirmed by polymerase chain reaction (PCR) for SRY RNA. Cells were cultured on gelatin-coated plates in Endothelial Basal Medium-2 (EBM-2) (Lonza) supplemented with growth factors, according to the recommended protocol from the manufacturer. The HCAECs used in this study were between passage 3 and 6. When the monolayers were ~70% confluent, the test compounds were added for 24 hours. By the time the monocytes were added, the HCEAC monolayer was ~90% confluent.

Monocytes. Human acute monocytic leukemia cells (THP1; ATTC, Manassus, Virginia) were incubated with 1 μ mol/L 12-O-tetradecanoylphorbol-13-acetate phorbol ester overnight at 37°C to transform them to monocytes.⁶

Administration of test compounds. To avoid estrogen-like effects of phenol red, test HCAEC cultures were incubated in EBM phenol red-free medium (Lonza). In these experiments, the growth supplement was 5% dextran-coated charcoal-exposed fetal calf serum (FCS), using FCS from the same batch. The FCS was added 24 hours before the addition of test compounds. The following compounds were used: 17 β -E2 (Sigma, St Louis, Missouri), 5 α -androstan-17 β -ol-3-one (dihydrotestosterone [DHT]; Sigma), and DHEA (Steraloids, Inc Newport, Rhode Island). In each experiment, following 24-hour incubation with phenol red-free EBM supplemented with 5% charcoal/dextran-treated FCS, the test compounds were added for 24 hours. In some experiments, the HCEACs were pretreated with fulvestrant (Sigma) or flutamide (TCI America,

Portland Oregon) for 1 hour before the addition of sex steroids. Sex steroids were administered 24 hours before the adhesion assay. Dosages are described in the Results section.

Monocyte-HCAEC adhesion assay. Monocytes were labeled with diluted LeukoTracker solution (Cell Biolabs, Inc, San Diego, CA) for 60 minutes at 37°C, according to the manufacturer's instructions. The monocytes were then washed 3 times with serum-free medium, added to the monolayers of untreated or hormone-treated HCAECs, and incubated for 60 minutes at 37°C. At the end of the incubation, the culture medium was carefully removed, the cultures were rewashed 3 $\times$ with phosphate-buffered saline (PBS), and fixed with 4% formalin solution. 4',6-Diamidino-2-phenylindole (DAPI) staining illuminated the nuclei of the monocytes. The number of adherent monocytes was determined in fields that had confluent endothelial cells. The monocytes were studied at $\times 20$ and $\times 40$ using an EVOS fluorescence microscope (AMF, Bothell, Washington).

Immunofluorescence staining. HCEACs at a density of 2×10^5 /mL were seeded in gelatin (1 mg/mL)-coated multiple-chamber glass slides. Following treatments, each chamber was washed twice with PBS. The cells were then fixed in 4% paraformaldehyde (in PBS) for 10 minutes. The chambers were rinsed twice in PBS and the cells were permeabilized by incubation for 5 minutes at 25°C in 0.5% Triton X-100. The cells were then rinsed twice again with PBS and blocked with a solution containing 1% bovine serum albumin in PBS for 1 hour at 25°C. Immunofluorescence staining was carried out by incubation for 1 hour with primary antibody at room temperature, followed by washing in PBS and incubated for 45 minutes with fluorophore-labeled secondary antibody. After incubation, the coverslips were rinsed with PBS for 3 additional times and covered with coverslips in mounting fluid for fluorescence microscopy using the Evos instrument. Cells were observed at $\times 20$, $\times 40$, and $\times 60$, using the DAPI nuclear staining to identify the HCAECs and Leukotracker to identify the monocytes.

Cell lysate preparation. After the treatment, the cells were washed with ice-cold PBS, scraped from the plate, lysed with a 1 $\times$ RIPA (Radio-Immunoprecipitation Assay) buffer (containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.5 [Tris-buffered saline, TBS], 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1 mmol/L Na orthovanadate, 10 μ g/ml leupeptin, 10 μ g/mL aprotinin, PMSF (Phenylmethylsulfonyl fluoride) and 1% Triton) at 4°C for 30 minutes, and then clarified by centrifugation at 14 000 rpm for 15 minutes. The supernatants were transferred to fresh tubes and kept at -70°C .

Western blot analysis. The cell lysates (≥ 20) were loaded on 10% gel and subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to a nitrocellulose membrane using a transblot apparatus (Bio-Rad, Hercules, California). The membrane was blocked against non-specific binding overnight in TBS with 5% nonfat dry milk and

Figure 1. Monocyte adhesion following pretreatment of HCAECs with sex steroids. The * signifies comparison with the untreated control; *P* values are next to the box. HCAECs indicates human coronary artery endothelial cells.

Figure 2. Monocyte adhesion following pretreatment of HCAECs with sex steroids or sex steroids and receptor modulators. The * signifies comparison with the untreated control; *P* values are next to the box. HCAECs indicates human coronary artery endothelial cells.

0.1% Tween 20 at 4°C. The primary antibody was added in a dilution of 1:1000 and incubated at room temperature for 2 hours, then washed 3 times with 1× TBS containing 0.1% Tween20. Secondary antibody was added and incubated at room temperature for 1 hour. Immunoreactivity was detected by enhanced chemiluminescence (Pierce, Rockford, Illinois).

Statistical Analysis

All monocyte binding data reported is selected from studies that were performed in triplicate at least 2 times. Data were analyzed using the Student *t* test. Results are presented as the mean $\pm$ SD. A *P* value $< .05$ is considered significant.

Results

Monocyte Binding by HCAECs

Monocytes were exposed to HCAEC monolayers and the non-adherent cells were washed off. The results were compared to untreated HCAEC control incubations. All reported data are from triplicate experiments. Representative figures are shown for each group of experiments.

Estradiol, DHT, and DHEA. In all studies, E2, DHT, and DHEA were strong inhibitors of monocyte binding to HCAECs. At maximally effective doses, all 3 compounds caused $\geq 50\%$ decrease in adherence, with $P < .01$. (Figures 1 and 2.)

Effect of hormone receptor modulators. Fulvestrant blocked the effect of E2, and flutamide blocked the effect of DHT. The blockades were not complete, usually reaching $\sim 50\%$ reversal of the inhibition. However, this was usually sufficient to make the results indistinguishable from untreated controls (Figure 2).

Effect of gender. HCAECs from men and women responded to E2, DHT, and DHEA by binding fewer monocytes ($P \leq .05$ vs control hormone-treated cultures). Interestingly, in male HCAECs, the effect of flutamide was stronger than fulvestrant; while in female HCEACs, the effect of fulvestrant was stronger than that of flutamide (Figure 2).

Expression of PSA-NCAM by Sex Steroid-Exposed HCAECs

The HCAECs were cultured in the presence of test compounds and then prepared for immunohistochemistry and Western blotting using a specific anti-PSA-NCAM antibody. Estradiol, DHT, and DHEA all induced the expression of PSA-NCAM.

Figure 3. Human coronary artery endothelial cells after 24 hours of treatment with DHEA (10^{-5} mol/L), DHT (10^{-6} mol/L), or estradiol (10^{-8} mol/L). The cell nuclei are labeled with DAPI (blue) and the PSA-NCAM is labeled with anti-PSA-NCAM (red). DHEA indicates dehydroepiandrosterone; DHT, dihydrotestosterone; DAPI, 4'-6-diamidino-2-phenylindole; PSA-NCAM, polysialylated neural cell adhesion molecule.

Figure 4. Western blots comparing the effects of DHEA (10^{-6} mol/L), trilostane (10^{-5} mol/L), and estradiol (10^{-8} mol/L) on ir-PSA-NCAM expression by HCAECs. The lower bar graph shows the comparative optical density of the molecular size of PSA-NCAM. HCAECs indicates human coronary artery endothelial cells; DHEA, dehydroepiandrosterone; PSA-NCAM, polysialylated neural cell adhesion molecule.

Using immunohistochemistry, it could be seen that the expression was not global; only 10% to 20% of HCAECs showed expression of immunoreactive (ir) material. As described previously, the expression was seen to be in the extracellular domain of the molecule (data not shown).^{3,4} Selected, comparable examples of the staining are shown in Figure 3. However, since immunohistochemistry is not quantitative, Western blotting was performed.

Western blotting showed that DHEA (10^{-6} mol/L) increases ir-PSA-NCAM to levels similar to E2 (10^{-8} mol/L). Trilostane (10^{-5} mol/L) blocked ir-PSA-NCAM to the point that there was no difference between DHEA plus trilostane and the untreated control HCAECs, $P > .05$ (Figure 4).

Discussion

Pretreatment of HCAECs with DHEA reduced the adherence of monocytes by $\geq 50\%$ compared to untreated controls. The effect of DHEA was shown in HCAECs from both women and men and is comparable to that of E2 or DHT. In addition, flutamide blocked DHEA inhibition using HCAECs from men, and fulvestrant did the same using HCAECs from women. However, the degree of blockade by the receptor modulators is variable, implying that it is premature to infer the presence of true gender differences in response to DHEA. Of greatest interest, PSA-NCAM induction by DHEAS was blocked by the enzyme inhibitor trilostane that blocks the conversion of DHEA into androgens and estrogens. Although more extensive testing including dose-response curves must be done, these

results indicate that DHEA-induced inhibition of monocyte-endothelial binding could require DHEA's conversion into its androgen and estrogen metabolites. In fact, pretreatment of HCAECs with E2 and DHT was followed by increased expression of PSA-NCAM.

Taken together, these preliminary studies indicate that DHEA could be useful for clinical prevention of atherogenesis/ASVD in individuals who begin treatment during the reproductive period.⁷

It is well accepted that DHEA is a prohormone that acts via its end metabolites.^{8,9} The main source of DHEA is the adrenal gland. Its secretion is independent of gonadotrophins, though it seems sensitive to sex steroids as follows: During pregnancy, DHEA is secreted by the fetal adrenal cortex and is hydroxylated at C16 which allows DHEA to be the main source of estriol.¹⁰ The secretion of DHEA falls after birth until there is a rise at puberty.¹¹ The levels of DHEA are relatively stable until they fall during the approach of reproductive senescence.¹² The latter has been the indication for clinical use in older men and women in whom DHEA or its sulfoconjugate DHEAS have shown anabolic effects. These long-term clinical trials of DHEA/DHEAS were free of serious side effects.¹³

In Conclusion, DHEA is an effective in vitro blocker of monocyte binding to vascular endothelial cells. Our preliminary results are consistent with this effect due to the induction of NCAM-sialylating enzymes that lead to the production of PSA-NCAM. Dehydroepiandrosterone is as effective as its metabolites E2 and DHT in inducing sialylation of NCAM, but the production of PSA-NCAM is halted by the enzyme blocker that obviates the conversion of DHEA to E2 or DHT. Administering E2 or DHT to normal individuals during the reproductive period for prevention of atherogenesis raises question about feasibility and drug tolerance; but this is not the case for DHEA or DHEAS. However, the possibility of clinical trials must await further in vitro studies and positive results during in vivo animal testing for DHEA-inhibited entry of monocytes into the vascular wall and the formation of atheroma plaque.

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Declaration of Conflicting Interests

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