Dehydroepiandrosterone Activates Endothelial Cell Nitric-oxide Synthase by a Specific Plasma Membrane Receptor Coupled to Goα12,3*

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The adrenal steroid dehydroepiandrosterone (DHEA) has no known cellular receptor or unifying mechanism of action, despite evidence suggesting beneficial vascular effects in humans. Based on previous data from our laboratory, we hypothesized that DHEA binds to specific cell-surface receptors to activate intracellular G-proteins and endothelial nitric-oxide synthase (eNOS). We now pharmacologically characterize a putative plasma membrane DHEA receptor and define its associated G-proteins. The [3H]DHEA binding to isolated plasma membranes from bovine aortic endothelial cells was of high affinity (Kd = 48.7 pm) and saturable (Bmax = 500 fmol/mg protein). Structurally related steroids failed to compete with DHEA for binding. The putative DHEA receptor was functionally coupled to G-proteins, because guanosine 5′-O-(3-thio)triphosphate (GTPγS) inhibited [3H]DHEA binding to plasma membranes by 69%, and DHEA increased [35S]GTPγS binding by 157%. DHEA stimulated [35S]GTPγS binding to Goα2 and Goα3, but not to Goα1 or Goαq. Pretreatment of plasma membranes with antibody to Goα2 or Goαq, but not to Goα1, inhibited the DHEA activation of eNOS. Thus, DHEA receptors are expressed on endothelial cell plasma membranes and are coupled to eNOS activity through Goα2 and Goα3. These novel findings should allow us to isolate the putative receptor and reevaluate the physiological role of DHEA activity.

The physiological role of the adrenal steroid dehydroepiandrosterone (DHEA) is not known. There are widespread data suggesting a beneficial effect of DHEA on vascular function. Extensive epidemiologic evidence shows an inverse correlation between circulating DHEA levels and the prevalence of atherosclerotic and cardiovascular diseases (1–5). There are few human intervention studies focused on vascular outcomes of DHEA administration, and these are not of a size or duration to define whether DHEA therapy has an effect on cardiovascular morbidity or mortality. Available studies do suggest a beneficial effect on atherosclerosis (6). Studies of the short term effect of DHEA on human vascular function, using sophisticated assays of vascular function, are beginning to emerge. Williams et al. (7) showed a significant increase in flow-mediated dilatation and systemic arterial compliance in postmenopausal women taking DHEA for 3 months. DHEA reduces atherosclerosis, decreases the accumulation of cholesterol in aortic and coronary arteries (8, 9), and inhibits platelet aggregation (10) in various animal models. DHEA also affects growth factor-induced mitogenesis and proliferation of vascular smooth muscle cells (11–13). However, the molecular mechanisms by which DHEA acts to protect from atherosclerotic and cardiovascular diseases are still unknown. Furthermore, it is unclear whether the effect on vascular tissues is related to DHEA or to its metabolites, which include estradiol.

Steroid hormones are known to bind specific intracellular receptors, which function as ligand-dependent gene transcription factors (14). However, previous efforts to isolate an intracellular receptor for DHEA have failed (15–18). In contrast to this classical pathway of steroid hormone action, there are also rapid, plasma membrane-dependent, non-genomic effects of steroids in various tissues, which lead to important physiological responses (19–24). Plasma membrane-associated receptors are postulated to mediate these non-genomic actions of steroids. Functional plasma membrane binding sites have been identified for several steroids, including estrogen, vitamin D, and progesterone (25–28). However, besides the receptor for estrogen, no plasma membrane steroid receptor has yet been unequivocally identified and characterized.

We have found that DHEA stimulates nitric oxide (NO) generation within minutes from bovine aortic endothelial cells (BAEC).2 Furthermore, DHEA conjugated to bovine serum albumin (BSA) had similar effects. These cellular responses to DHEA were specific and inhibited by pertussis toxin (PTX). Taken together, these results led to the hypothesis that DHEA activates NO production in endothelial cells by a specific, plasma membrane, G-protein-coupled receptor. The aim of this study was to determine whether there is a specific plasma membrane DHEA receptor, and to pharmacologically characterize that receptor. We show, for the first time, a specific, high affinity, DHEA binding site on the plasma membrane of BAEC. Specific DHEA binding to this site was saturable and reversible. We propose this distinct site of DHEA action as a novel DHEA receptor. In addition, we show that the putative DHEA receptor was functionally coupled to G-proteins of the Goα12,3

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‡The abbreviations used are: DHEA, dehydroepiandrosterone; PTX, pertussis toxin; DHEAS, dehydroepiandrosterone sulfate; BAEC, bovine aortic endothelial cell(s); HUVEC, human umbilical vein endothelial cell(s); GTPγS, guanosine 5′-O-(3-thio)triphosphate; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; eNOS, endothelial nitric-oxide synthase; ER, estrogen receptor.

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subtypes, which mediate the activation of endothelial nitric-oxide synthase (eNOS) by DHEA in BAEC.

**EXPERIMENTAL PROCEDURES**

**Materials**—All tissue culture media and reagents were supplied by the Diabetes and Endocrinology Research Center of the University of Iowa. DHEA, DHEAS, and DHEA-17-carboxymethyl oxime-BSA complexes were obtained from Steraloids (Newport, RI). 16a-Fluoro-5-androsten-17-one (fluasterone) was a gift from Dr. Arthur Schwartz (Temple University, Philadelphia, PA). Other steroids, chemical reagents, PTX, preimmune rabbit serum, IgG, protein A-Sepharose, DEAE glass fiber filters, and assay kits for plasma membrane and cytosolic markers were from Sigma. Radiolabeled and the chemiluminescence detection kit (ECL) were obtained from Amersham Biosciences. The polyclonal rabbit antisera to Ga\(_i\) and Go\(_i\) were supplied by Santa Cruz Biotechnology (Santa Cruz, CA), and antisera specific to Go\(_i\), Go\(_s\), and Go\(_i, s\), were purchased from Calbiochem (San Diego, CA). Whatman GF/B filters were supplied by Fisher. Protein molecular weight markers, nitrocellulose transfer membranes, Dowex 50 WX-8 resin, and protein assay reagents were purchased from Bio-Rad.

**Cell Culture**—Bovine aortic endothelial cells (BAEC) were kindly provided by Dr. Robert Bar (Veterans Affairs Medical Center, Iowa City, IA). Human umbilical venous endothelial cells (HUVEC) were provided by Dr. Arthur Spector (University of Iowa, Iowa City, IA). Both BAEC and HUVEC were grown in M199 medium supplemented with 20% fetal calf serum, 10 ng/ml basic fibroblast growth factor, 1 mM PMSF, and 100 U/ml Penicillin and 0.05 mg/ml streptomycin. Both were cultured as described previously (29). In some experiments, cytosolic fractions were prepared by differential centrifugation (30). Briefly, cells were homogenized with a Dounce homogenizer in HES buffer (20 mM HEPES, 250 mM sucrose, 1 mM EDTA, 5 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM aprotinin, and leupeptin). Post-nuclear homogenates were collected following centrifugation at 20,000 g for 2 min, and the microsomal and cytosolic pellets were sedimented by centrifugation at 3,000 × g for 15 min. Plasma membranes from HUVEC, rat liver, heart, and kidney were isolated by sucrose gradient centrifugation (29, 31, 32). Plasma membrane purity and exclusion of contamination of the plasma membranes. In selected studies, plasma membranes were treated with DHEA (1 nM) or 17β-DHEA (10 μM) as indicated before exposure to radiographic film. The immunoprecipitated 35S-labeled G-proteins—Plasma membrane proteins (20 μg) were incubated for 25 min at 25°C in assay buffer (250 μl) containing 5 nM [35S]GTP \(\gamma\)S, 10 μM GDP, 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl\(_2\), 1 mM CaCl\(_2\), 0.6 mM EDTA, 0.1% BSA, 100 μg/ml bacitracin, 0.1 mM PMSF, and 1 μM each of leupeptin, pepstatin A, and aprotinin. In some experiments, G-protein \(\alpha\)-subunit-specific antisera was added to the reaction mixture. The plasma membranes were washed three times in a buffer containing 50 mM HEPES, pH 7.4, 100 μM NaF, 50 mM sodium phosphate, 100 mM NaCl, 1% Triton X-100, and 0.1% SDS. The final pellets were boiled in 0.5% SDS, and the immunoprecipitated 35S-labeled G-proteins were counted in a scintillation counter. Non-specific activity was determined in the presence of 50 μM unlabeled GTP\(\gamma\)S.

**Western Blotting**—Twenty μg of BAEC plasma membrane proteins and molecular weight markers were separated by 12.5% SDS-polyacrylamide gel electrophoresis as described previously (34). The proteins on the gel were transferred electrophoretically to nitrocellulose filters. The membranes were blocked at 25°C for 1 h with 5% nonfat milk and 0.1% Tween 20 in Tris-buffered saline, and then incubated overnight at 4°C with the following dilutions of specifically reacting rabbit polyclonal antisera: G\(_\alpha_i\) (1:200), G\(_\alpha_{s}\) (1:500), G\(_\alpha_{i, s}\) (1:500), and G\(_\alpha_{i, s}\) (1:1,000). The membranes were washed four times with Tris-buffered saline buffer containing 0.1% Tween 20 and then blocked for 10 min, prior to the addition of donkey anti-rabbit horseradish peroxidase secondary antibody (1:10,000). Chemiluminescence reaction was performed before exposure to radiographic film.

**NO Synthase Activation**—Endothelial NOS activity was determined by measuring the conversion of l-[3H]arginine to l-[3H]citrulline, as previously described (34–36), with minor modifications. Briefly, purified plasma membranes were reconstituted in Hepes-buffered saline solution buffer (135 mM NaCl, 1.2 mM CaCl\(_2\), 1.2 mM MgCl\(_2\), 5.0 mM KOH, 10 mM HEPES, 10 mM glucose, pH 7.4) supplemented with 100 μM/ml bacitracin, 0.1 mM PMSF, and 1 μM each of pepstatin A, leupeptin, and aprotinin. Plasma membranes (8 μg in a 100-μl volume) were incubated for 15 min at 37°C with 1.5 μCi/ml l-[3H]arginine in the presence or absence of DHEA (1 nM), and citrulline production was measured. Studies were also performed in the presence of added calmodulin (5 units/ml), β-NAPDH (2 μM/liter), tetrahydrobiopterin (2 μM/liter), flavin adenine dinucleotide (10 μM/liter), flavin mononucleotide (10 μM/liter), and CaCl\(_2\) in excess of EDTA (36). For PTK experiments, the BAEC were pre-incubated in 100 ng/ml PTX for 6 h prior to isolation of the plasma membranes. In selected studies, plasma membranes were treated with DHEA (1 nM) or 17β-estradiol (10 nM) in the

bound [3H]DHEA was determined by subtracting the nonspecific binding from the membranes incubated only with [3H]DHEA (total binding).

**[3S]GTP\(\gamma\)S Binding Assay**—Before the assays were performed, all drugs were di-
DHEA Plasma Membrane Receptor

**TABLE I**

Marker enzyme analysis of endothelial cell plasma membranes

BAEC were homogenized and plasma membranes prepared as described under "Experimental Procedures." Marker enzymes were analyzed in the post-nuclear homogenate and in the purified membrane preparations. Values are presented as mean ± S.E. of quadruplicate determinations.

| Enzyme                  | Homogenate | Plasma membranes |
|-------------------------|------------|------------------|
| 5'-Nucleotidase         | 0.4 ± 0.1  | 5.2 ± 0.2        |
| (units/mg protein/min)  |            |                  |
| Alkaline phosphatase    | 137 ± 8    | 2046 ± 71        |
| (units/mg protein)      |            |                  |
| Lactate dehydrogenase   | 1012 ± 11  | 64 ± 3           |
| (units/µg protein)      |            |                  |

absence or presence of the estrogen antagonist ICI 182,780 (1 µM) for 15 min, without added calmodulin or enzyme cofactors. The reaction was terminated by the addition of HEPES buffer, pH 5.5, containing EDTA (5 mM/liter), and solutions were immediately applied to a column terminated by the addition of HEPES buffer, pH 5.5, containing EDTA.

The reaction was carried out at 37 °C for 30 min, without added calmodulin or enzyme cofactors. The reaction was terminated by the addition of HEPES buffer, pH 5.5, containing EDTA (5 mM/liter), and solutions were immediately applied to a column terminated by the addition of HEPES buffer, pH 5.5, containing EDTA.

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In a separate experiment, eluted with 2 ml of HEPES buffer.

A sample of eluate in Biosafe N/A® scintillation mixture was counted by liquid scintillation. Nonspecific activity was determined by the addition of excess L-arginine (10 µM) and represented ~10% of total activity. Endothelial NOS activation by the known agonist bradykinin (1 µM) was also tested, as a positive control. In individual experiments, each treatment condition was repeated in duplicate and all findings were repeated in four independent studies.

In additional experiments, purified plasma membranes were preincubated with buffer alone, nonimmune rabbit IgG, or antibodies against G α 1 , G α 2 , or G α 3 (1:2,000). Endothelial NOS activation by DHEA (1 nM) or bradykinin (1 µM) was evaluated as outlined above.

**Statistical Analysis**—Binding data of [3H]DHEA were analyzed by nonlinear regression using the Prism GraphPad program (GraphPad Software, San Diego, CA). Affinity (K d ) and maximum binding capacity of [3H]DHEA were calculated from nonlinear curve fitting. Results were analyzed using a one-site model because this was superior to other models tested. All other data were subjected to one-way analysis of variance using the General Linear Model procedure of SAS®, and significant differences were subjected to Duncan’s multiple comparison test at 5% probability. All values in each study were derived from at least three separate experiments and expressed as mean ± S.E.

**RESULTS**

Plasma Membrane Purity—Plasma membrane fractions from BAEC were isolated by homogenization and sucrose gradient centrifugation as previously described (29). The activities of plasma membrane-specific enzymes, 5'-nucleotidase and alkaline phosphatase, were 13–15-fold higher in the plasma membrane preparations than in the post-nuclear homogenate (Table I). The activity of the cytosolic marker, lactate dehydrogenase, was negligible in the plasma membrane preparations compared with that in the postnuclear homogenate fractions. These data confirm that the plasma membrane samples had little contamination with non-membrane proteins.

**Binding of [3H]DHEA to BAEC Plasma Membranes**—The association kinetic data showed that the binding of [3H]DHEA to plasma membrane fractions occurred rapidly at 4 °C and pH 7.4 (Fig. 1), reaching a plateau at 5 min of incubation and remaining essentially stable for 90 min. To determine the affinity constant, we incubated each sample (6 µg of protein in 250 µl) with increasing concentrations of [3H]DHEA, from 5 pm to 2 nm in the absence or presence of 10 µM unlabeled DHEA. The saturation binding curve and Scatchard analysis of the data indicated a single high affinity membrane binding site, with a K d = 48.7 ± 4.6 pm and B max = 500.3 ± 8.7 fmol/mg protein (Fig. 2).

To determine whether high affinity DHEA binding sites exist in tissues other than the vascular endothelium, we isolated plasma membranes from rat liver, kidney, and heart. There was no specific binding detected in rat kidney (Fig. 3). The binding activity was higher in heart than in liver, but was only 39.8 and 23.5%, respectively, of the specific binding activity observed in BAEC plasma membranes. Binding to the heart and liver plasma membranes reached saturation at 45 min of incubation and remained constant to 60 min (data not shown). These results suggest that the high affinity binding sites for DHEA are expressed in a differential tissue-specific manner and may not be expressed in all tissues. Furthermore, HUVEC plasma membranes express approximately the same degree of

**FIG. 1.** Kinetics of [3H]DHEA binding to BAEC plasma membranes. Plasma membranes (8 µg) were incubated with [3H]DHEA (1 nM, specific activity 60 µCi/mmol) in Tris-HCl buffer, pH 7.4, at 4 °C in a total volume of 1 ml for the indicated times. Aliquots of 50 µl, in duplicate, were rapidly filtered through Whatman DEAE glass fiber filters, at each time point. The filters were washed five times with 1 ml of cold Tris-HCl buffer and placed in scintillation vials, and bound radioactivity was determined by liquid scintillation counting. Nonspecific binding was determined in the presence of 10 µM unlabeled DHEA, which was subtracted from total binding data. Data represent the means of five separate experiments.

**FIG. 2.** Saturation binding of [3H]DHEA to BAEC plasma membranes. BAEC plasma membranes (2 µg) were incubated with increasing concentrations of [3H]DHEA for 15 min in Tris-HCl buffer, pH 7.4, at 4 °C. Aliquots of 100 µl, in duplicate, were rapidly filtered through Whatman DEAE glass fiber filters, washed five times with 1 ml of cold Tris-HCl buffer, placed in scintillation vials, and counted. Nonspecific binding, determined in the presence of 10 µM unlabeled DHEA, was below 10% of total binding at saturation. Specific binding represents total minus nonspecific binding. Data are representative of four different experiments. Inset shows a Scatchard analysis of [3H]DHEA binding to the BAEC membranes.
DHEA Plasma Membrane Receptor

Specifically Bound [3H]DHEA (fmol/mg protein) for BAEC, HUVEC, Heart, Liver, and Kidney. Values are expressed as mean ± S.E. of four separate experiments.

Fig. 3. Specific binding of [3H]DHEA to plasma membranes of BAEC, HUVEC, rat heart, liver, and kidney. Values are expressed as mean ± S.E. of four separate experiments.

[3H]DHEA binding as do BAEC (616.3 ± 89.5 fmol/mg protein (HUVEC) versus 490.9 ± 45.2 fmol/mg (BAEC)). Thus, the ability of bovine vascular endothelial cells to bind DHEA is not species-specific.

Displacement Studies—We determined the specificity of the [3H]DHEA binding sites in plasma membranes using unlabeled DHEA, or other related steroids, at concentrations from 1 pM to 1 μM. As shown in Fig. 4, unlabeled DHEA inhibited binding of [3H]DHEA (0.1 nm) to plasma membranes in a concentration-dependent manner, with a complete inhibition at 1 μM. The monophasic competition binding curve suggests that [3H]DHEA interacts with a single population of binding sites in the plasma membrane preparations. Fluasterone had a very low affinity for the [3H]DHEA binding site, only slightly inhibiting the [3H]DHEA binding to plasma membranes. All other steroids tested, including DHEAS, 17α-hydroxyprogrenolone, androstenedione, 17β-estradiol, and testosterone, failed to displace [3H]DHEA from its binding site at concentrations up to 1 μM, demonstrating that the plasma membrane binding site for [3H]DHEA was highly specific.

Effect of GTPγS on [3H]DHEA Binding to Plasma Membranes—We demonstrated previously² that the DHEA-stimulated NO release from BAEC is plasma membrane-dependent and sensitive to PTX. This result, along with the above findings, suggests the possible coupling of putative membrane-bound DHEA receptors to G-proteins of the Gᵢₒ family. To test this hypothesis, we examined the binding of [3H]DHEA to plasma membranes in the presence or absence of 50 μM GTPγS, a hydrolysis-resistant GTP analogue. As shown in Fig. 5A, the specific binding of [3H]DHEA to plasma membranes was reduced by 69% after preincubation with GTPγS for 30 min. These data showed that the binding of [3H]DHEA to membranes was sensitive to GTPγS, which indicates coupling of membrane-bound DHEA receptors to G-proteins. Consistent with our previous findings, pre-incubation of the plasma membranes with PTX inhibited specific [3H]DHEA binding by 85% (Fig. 5B).

Effect of DHEA on [35S]GTPγS Binding—Ligand-regulated GTPγS binding to G-proteins is a sensitive method for examining the coupling of G-proteins to a receptor (37). To confirm that the putative DHEA receptors were coupled to G-proteins, we examined the effect of DHEA on [35S]GTPγS binding to G-proteins in plasma membranes. As shown in Fig. 6A, DHEA increased the specific binding of [35S]GTPγS by 157% over basal binding activity. Kinetic studies showed that DHEA stimulated specific [35S]GTPγS binding in a time-dependent manner, reaching maximum specific binding at 15 min (Fig. 6B). Specific binding declined to zero by 2 h of incubation (data not shown). The stimulation of [35S]GTPγS binding was dependent on DHEA concentration between 1 μM and 1 μM, with a maximal increase of 72% at 10 nM DHEA and an EC₅₀ of 43 μM (Fig. 6C). There was no significant difference in DHEA-stimulated [35S]GTPγS binding between the 0.1, 1, and 10 nM DHEA concentrations. Pre-incubation of the plasma membranes with PTX reversed the DHEA-stimulated increase in [35S]GTPγS binding (Fig. 6D).

We solubilized membrane proteins with the nondenaturing detergent, Triton X-100 (1%), to investigate whether soluble DHEA receptors retain ligand binding affinity and functional coupling to G-proteins. Solubilization with Triton X-100 yields ~65% of membrane-bound proteins. In binding studies with soluble membrane proteins, DHEA actively and specifically bound to detergent-solubilized receptor, as illustrated in Fig. 7A. Furthermore, DHEA enhanced the [35S]GTPγS binding to G-proteins in the solubilized protein extract (Fig. 7B), indicating that intact and functional receptor-G-protein complex was solubilized. Taken together, these results provide evidence that the plasma membranes have putative DHEA receptors, which are coupled to G-proteins.

Immunoprecipitation of [35S]GTPγS-labeled G-proteins—We subsequently determined whether DHEA receptors were specifically coupled to Giᵢₒ proteins. Plasma membranes were incubated with or without DHEA in the presence of [35S]GTPγS. [35S]GTPγS-labeled G-proteins were immunoprecipitated with antisera directed against specific α-subunits, following detergent extraction. The activity of [35S]GTPγS in the resulting immunoprecipitate was determined and served as an indicator of G-protein activation. Incubation of the plasma membranes with DHEA (1 nM) for 25 min at 25 °C produced a significant increase in [35S]GTPγS binding to Goᵢ₂ (from 1.05 ± 0.13 pmol/mg protein to 2.69 ± 0.53 pmol/mg protein) and Goᵢ₃ (from 1.86 ± 0.37 pmol/mg protein to 3.17 ± 0.56 pmol/mg protein) (Fig. 8). However, DHEA did not affect [35S]GTPγS binding to Goᵢ₁ and Goᵦ. In DHEA-treated plasma membranes, Goᵢ₁ and Goᵦ accounted for approximately 53 and 42%, respectively, of total stimulated α-subunit GTP binding activities. Immunoblotting analysis revealed that Goᵢ₂ and Goᵢ₃ predominated in the BAEC membranes, with little Goᵢ₁ (Fig. 9). There was no Goᵦ expressed in the plasma membranes. Taken to-
together, these findings provide the first evidence that putative DHEA plasma membrane receptors are selectively coupled to G\(_{i2}\) and G\(_{i3}\).

Endothelial NO Synthase Activation by DHEA—We have recently demonstrated that DHEA (0.1–10 nM) maximally stimulated NO production in intact endothelial cells. Based on the immunoprecipitation studies above, we hypothesized that the putative DHEA receptors activate eNOS through G\(_{i2}\) and G\(_{i3}\), to which they are coupled. To test this hypothesis, we measured eNOS activity in purified plasma membranes, in response to DHEA and in the presence of G\(_{i}\) subtype antibodies. In the absence of added calmodulin and cofactors, basal eNOS activity was low but detectable in membranes (1.37 pmol of citrulline/mg of protein). Under the same conditions, DHEA (1 nM) and the known eNOS agonist bradykinin (1 nM) increased eNOS activity by 66 and 65%, respectively, compared with basal levels (Fig. 10A). The addition of calmodulin, calcium, and co-factors greatly increased the basal activity of eNOS. DHEA (1 nM), but not bradykinin, enhanced this activity by 42% (Fig. 10B). In the presence of calmodulin, calcium, and eNOS co-factors, DHEA stimulated eNOS activity in a concentration-dependent manner between 1 pm and 1 \(\mu\)M, with a maximal increase of 78.3 ± 9.3% at 10 nM DHEA and an EC\(_{50}\)

![Fig. 5. Effect of GTP\(_{\gamma}\)S or pertussis toxin on \(^{35}\)S]DHEA binding activity in BAEC plasma membranes. A, membranes (8 \(\mu\)g of protein) were preincubated with, or without (Control), GTP\(_{\gamma}\)S (50 \(\mu\)M) in Tris buffer, pH 7.4, for 30 min. \(^{35}\)S]DHEA (0.1 nM) was then added and incubated at 4 °C for 15 min. Non-specific binding was determined in the presence of excess unlabeled DHEA (10 \(\mu\)M), and specific binding represents total minus non-specific binding. Data are means ± S.E. from four separate experiments, each performed in duplicate, \(\ast\), \(p < 0.05\) versus control. B, membranes (8 \(\mu\)g of protein) were preincubated with 100 ng/ml PTX or vehicle for 6 h. The membranes were then incubated at 4 °C for 15 min with 0.1 nM \(^{35}\)S]DHEA, in Tris-HCl buffer, pH 7.4. Non-specific binding was determined in the presence of excess unlabeled DHEA (10 \(\mu\)M), and specific binding represents total minus non-specific binding. Data are means ± S.E. from six experiments, each performed in duplicate. \(\ast\), \(p < 0.05\) versus control.

![Fig. 6. Effect of DHEA on \(^{35}\)S]GTP\(_{\gamma}\)S binding to BAEC plasma membranes. A, activation of G-proteins in endothelial cell plasma membranes by DHEA. BAEC membranes (10 \(\mu\)g) were incubated with 10 \(\mu\)M GDP, 0.5 nM \(^{35}\)S]GTP\(_{\gamma}\)S, in the presence or absence of 1 \(\mu\)M DHEA in Tris-HCl buffer at 25 °C for 15 min. \(\ast\), \(p < 0.05\) versus control. B, time course of the effect of DHEA on \(^{35}\)S]GTP\(_{\gamma}\)S binding to G-proteins. Membrane incubation was performed with added \(^{35}\)S]GTP\(_{\gamma}\)S (0.5 nM) in the presence or absence (Basal), of DHEA (1 nM). C, concentration-dependent effect of DHEA on \(^{35}\)S]GTP\(_{\gamma}\)S binding activity. Membranes were incubated with \(^{35}\)S]GTP\(_{\gamma}\)S (0.5 nM) in the absence or presence of 10 pm to 10 \(\mu\)M DHEA for 30 min. The same concentrations of vehicle were added into control samples. D, effect of PTX on DHEA-induced \(^{35}\)S]GTP\(_{\gamma}\)S binding to BAEC plasma membranes. Purified plasma membranes (10 \(\mu\)g) were isolated from BAEC, pretreated with 100 ng/ml PTX or vehicle for 6 h, and incubated with \(^{35}\)S]GTP\(_{\gamma}\)S (0.5 nM) in the absence or presence of 1 \(\mu\)M DHEA for 15 min. Following incubation, aliquots of 100 \(\mu\)l, in duplicate, were transferred onto Whatman CF/B filters. The filters were rapidly rinsed three times with 1 ml of cold Tris-HCl buffer under vacuum, placed in scintillation fluid, and counted. Non-specific binding was determined in the presence of 10 \(\mu\)M unlabeled GTP\(_{\gamma}\)S. Specific binding represents total binding minus non-specific binding. Data for all graphs are representative of four different experiments and expressed as mean ± S.E. \(\ast\), \(p < 0.05\) versus basal.
FIG. 7. Specific DHEA and GTPγS binding in soluble membrane proteins. A, binding of [3H]DHEA to solubilized BAEC plasma membrane proteins. Plasma membranes were solubilized in buffer containing 1% Triton at 4°C, with stirring for 1 h, and supernatants were collected following centrifugation at 110,000 × g for 45 min. Binding assays were performed as described above. Data represent four experiments, determined in duplicate. B, DHEA stimulates the binding of [35S]GTPγS to solubilized BAEC membrane protein. Solubilized membrane proteins (8 μg) were incubated with 10 μM GDP, 0.5 mM [35S]GTPγS, in the presence or absence (Control) of 1 nM DHEA in Tris-HCl buffer at 25°C for 15 min. Following incubation, aliquots of 100 μl, in duplicate, were transferred onto Whatman CF/B filters. The filters were rapidly rinsed three times with cold Tris-HCl buffer under vacuum, placed in scintillation fluid, and counted. Nonspecific binding was determined in the presence of 10 μM unlabeled GTPγS. Specific binding represents total binding minus nonspecific binding. Data are representative of four different experiments. *, p < 0.05 versus control.

DISCUSSION

Based on our previous studies, we hypothesized that DHEA binds to specific receptors on the surface of endothelial cells to activate intracellular G-proteins and eNOS. To test this hypothesis, we used assays of radioligand binding, ligand-dependent G-protein activation, and eNOS activity. The principal findings of this study are that BAEC membrane preparations contained a specific, saturable, high affinity DHEA binding site. The binding site for DHEA had features of a G-protein-coupled receptor. DHEA binding was linked to activation of G-proteins (specifically G_{i2} and G_{i3}) and was inhibited by incubation with nonhydrolyzable GTP analogs or PTX. Binding of DHEA to solubilized plasma membrane preparations was associated with activation of eNOS within those solubilized fragments, and specific G_{i2} and G_{i3} antibodies inhibited this activation. Taken together, we show for the first time strong evidence for a high affinity DHEA receptor in endothelial cells. Furthermore, we show that this putative receptor is expressed on plasma membranes, is G-protein-coupled, and activates endothelial cell NOS. This new information will form the basis for efforts to isolate a specific DHEA receptor. The findings may also allow us to develop a unifying mechanism for the physiological actions of DHEA.

The [3H]DHEA binding activity in plasma membranes was differentially expressed, in a tissue-specific manner. Plasma membranes from rat liver and heart expressed ~30% of the specific DHEA binding of BAEC membranes, whereas rat kidney membranes had no detectable binding. These results, demonstrating quantitative differences in expression of functional
membranes. The conversion of L-[^3H]arginine to L-[^3H]citrulline was measured after 5 min incubation with buffer alone (Control), 1 nM DHEA or 10 nM 17β-estradiol (E), alone, 1 nM DHEA alone, 10 nM 17β-estradiol (E) alone, or with combinations of DHEA and ICI 182,780 or 17β-estradiol and ICI 182,780, as indicated. Values are mean ± S.E., n = 4–6. *, p < 0.05 versus basal or ICI 182,780; §, p < 0.05 versus 17β-estradiol + ICI 182,780.

FIG. 12. Effect of estrogen receptor antagonist on DHEA-stimulated eNOS activity. The conversion of L-[^3H]arginine to L-[^3H]citrulline was measured in purified plasma membranes incubated for 15 min in the absence of eNOS enzyme cofactors or calmodulin. Membranes were incubated with buffer alone, 1 μM ICI 182,780 (ICI) alone, 1 nM DHEA alone, 10 nM 17β-estradiol (E) alone, or with combinations of DHEA and ICI 182,780 or 17β-estradiol and ICI 182,780, as indicated. Values are mean ± S.E., n = 4–6. *, p < 0.05 versus control or unrelated Ig.

The high level of expression in HUVEC suggests that the human cardiovascular system may be an important target of the membrane-initiated effects of DHEA.

The results from ligand competition experiments demonstrated that the binding of [^3H]DHEA to plasma membranes was highly specific. Closely related steroid structures, DHEAS, androstenedione, 17α-hydroxyprogrenenolone, testosterone, and 17β-estradiol, did not compete with [^3H]DHEA for binding at any concentrations tested. Fluasterone (38) had a low affinity for the plasma membrane DHEA binding site, displacing 30% of the [^3H]DHEA binding at a concentration of 1 μM.

DHEA and DHEAS differ only in the substitution of a sulfate group for the 3β-hydroxy group. The absence of DHEAS competition for DHEA binding suggests that the 3-position of the A ring may be an important component of the functional group for this receptor. Therefore, it seems that similar absences of 3β-hydroxyprogrenenolone to displace the binding of [^3H]DHEA cannot be explained on the basis of this structural difference at the 3-position. Other structural differences between DHEA and these steroids must also contribute to the specificity of DHEA binding to the plasma membranes.
shape of the steroid, all of which are important for plasma membrane binding (39). In previous studies, we have found that DHEA-17-carboxymethyl oxime and BSA-conjugated DHEA-17-carboxymethyl oxime compete with [3H]DHEA for plasma membrane binding to BAEC. These agents are also as potent as native DHEA in stimulating NO production from BAEC. This suggests that receptor binding of the ligand tolerates large added groups at the 17-position. This property may be useful in designing affinity ligands to isolate the putative receptor.

Agonist-stimulated [35S]GTPγS incorporation into G-protein α-subunits has been widely used as evidence of G-protein coupling to receptors. Using this method, we found that DHEA stimulated [35S]GTPγS incorporation into G-protein α-subunits in a concentration- and time-dependent manner. Specifically, DHEA maximally stimulated [35S]GTPγS binding at 10 nM, although the differences in effect between 0.1 and 10 nM DHEA did not reach statistical significance. At these concentrations DHEA consistently induced the highest production of NO from BAEC in previous studies. Activation of eNOS in the presence of calcium, calmodulin, and eNOS co-factors was also maximal at 10 nM DHEA (Fig. 10C). Furthermore, the Kp for DHEA binding (48.7 pm) is close to the EC50 for DHEA-induced [3H]DHEA binding (43 pm) and the EC50 for eNOS activity (87 pm), suggesting that these processes are functionally linked. The DHEA-induced binding of GTPγS and activity of eNOS showed similar biphasic dose-response curves, with a decreased responsiveness at DHEA concentrations greater than 10 nM. The similarity of the concentration dependence curves for GTPγS binding and eNOS activity again support a link between these two processes. The DHEA-stimulated binding of [35S]GTPγS increased over time, reaching a maximum at 15 min and then declining to zero at 2 h of incubation. This suggested that DHEA catalytically increased the rate of GTPγS binding at relatively early time points, but did not alter the maximal nucleotide binding at equilibrium. These results are consistent with other studies of G-protein-coupled receptors showing ligand-dependent induction of GTP binding to G-proteins (40–43). Furthermore, we found that the addition of GTPγS potently inhibited [3H]DHEA binding. This again confirmed the functional interaction of receptor and G-protein (44), because binding of GTP or its analogues to the G-proteins reduces the agonist affinity of the G-protein-coupled receptor by uncoupling the G-protein from its receptor (45). This inhibitory effect of GTP, or its nonhydrolyzable analogues, on the high affinity binding of agonist to its plasma membrane receptor is observed for most other G-protein-coupled receptors (46–49). Therefore, these analyses of G-protein coupling provided strong evidence that the binding sites for [3H]DHEA were responsible for G-protein activation.

The G1 subfamily of G-proteins consists of at least six different subtypes, including four PTX-sensitive isoforms (Goα, Gq11, Gq2, Goq3). However, Gq3 protein was absent in BAEC, in agreement with previous observations (50–52). Using the agonist-stimulated [35S]GTPγS binding assay and antibodies raised against distinct epitopes of Goα1, Goα2, Goα3, and Goα4 subunits, we determined that only Goα2 and Goα3 were coupled to the putative DHEA membrane receptor. Consistent with these data, we showed that binding of DHEA or [35S]GTPγS to plasma membranes was inhibited by PTX.

DHEA caused acute activation of eNOS in isolated plasma membranes from endothelial cells. These findings demonstrate that DHEA directly acts on the plasma membrane to activate eNOS and stimulate NO production, as we previously observed in the intact BAEC (2). The attenuation of DHEA-induced eNOS activity by Goα2 and Goα3 antisera in isolated plasma membranes confirms our previous data that these two isoforms of Goα mediate the DHEA stimulation of eNOS. However, these experiments do not determine whether α- or βγ-G-proteins are specifically responsible for the signaling to eNOS. The mechanism of activation of eNOS by DHEA is unknown. One possibility is that DHEA activates a tyrosine kinase such as Src by a Goα-dependent (53) or Gβγ-dependent (54) mechanism. This may then activate eNOS through a mechanism involving phosphoinositide 3-kinase and Akt kinase (28, 55). We are continuing to evaluate this hypothesis. It is also noteworthy that the Triton-solubilized membrane preparations retained high affinity binding to [3H]DHEA and functional association with G-proteins. Thus, it seems likely that the solubilized receptors and related G-proteins were still preserved in a functionally stable configuration with this solubilization protocol. This property should provide a major advantage for the subsequent affinity purification of DHEA receptors.

Although animal and human studies have demonstrated the beneficial effects of DHEA on the cardiovascular system (1–4, 8–13), the mechanism of DHEA action is not known. Nitric oxide is a potent vasodilator and has other beneficial effects in the vasculature, including inhibition of monocyte chemotaxis and adhesion to endothelial cells (56, 57), regulation of smooth muscle cell proliferation (58), as well as inhibition of platelet aggregation (59). By showing an effect of physiological concentrations of DHEA on endothelial cell NO production, our results propose a mechanistic basis for the physiological actions of DHEA in the vascular system. How the effects that we demonstrate are associated with the epidemiological studies showing an inverse correlation of cardiovascular disease and DHEA levels remains to be studied.

Both DHEA and its sulfated metabolite, DHEAS, have been shown to have multiple actions in different tissues and organs. Many of these actions are reported at pharmacological concentrations, or with more prolonged incubations than we have used in our studies (60). In these situations the effects of lipophilic steroids on the plasma membrane (61–63) or the direct effects of intracellular steroid (64) may be of greater importance than in our study. Furthermore, many of the effects of high circulating levels of DHEA in vivo cannot be differentiated from the effects of DHEA metabolites, including estradiol or testosterone. Our data do not negate any of the mechanisms proposed for pharmacologic concentrations of DHEA, but we propose this high affinity ligand-receptor interaction as a potential mechanism for physiological concentrations of DHEA. The circulating plasma concentration of DHEA peaks at ~16 nM in the third decade. The steroid is also bound to plasma components, including proteins, e.g. albumin and high density lipoprotein (65, 66). Thus, the circulating free hormone concentration would be lower than the total DHEA levels. The cellular effects that we show, between 0.1 and 10 nM, are well within the range of physiological concentrations in humans.

There are extensive data showing that steroid hormones interact with intracellular receptors to modulate nuclear gene transcription and subsequent protein synthesis (14). In keeping with this paradigm of steroid hormone action, specific intracellular DHEA binding activities have been demonstrated by many (15–18), although not all (67), research groups. Specific high affinity DHEA binding has been found in cytosolic, nuclear, and whole cell assays in hepatocytes, melanoma, and lymphoid cells of various species. Although the expression levels of these putative DHEA receptors are similar to our results, the dissociation constants are 50–1,400 times higher than in...
the present study (Table II). At this time, researchers have been unable to isolate proteins supporting these intracellular DHEA binding activities. Thus, there is no known intracellular steroid hormone receptor for DHEA. Furthermore, we have not been able to identify a DHEA binding site in BAEC cytosolic protein under the conditions of our assay. DHEA has also been shown to interact with known intracellular steroid hormone receptors. Nephew et al. (68) showed interaction of DHEA with a cloned human estrogen receptor in a yeast system. However, the $K_d$ for binding was 15.8 ± 3.4 μM, well above the potential circulating DHEA concentration. Thus, it is unlikely that the physiological effects of DHEA are mediated by the intracellular estrogen receptor. Besides their classical “genomic” effects, characterized by latency of action (69), rapid and non-genomic effects of steroids have been widely recognized and characterized (69–72). These membrane-initiated steroid actions appear to be transmitted by specific membrane-bound receptors, which subsequently activate intracellular signaling to regulate cellular function. Our results suggest that DHEA acts at a plasma membrane site to activate intracellular signaling.

Our discovery of a putative plasma membrane receptor for DHEA does not negate the possibility of also finding an intracellular receptor for this hormone. All of the major steroid hormones, where plasma membrane receptors have been proposed, have well-characterized intracellular receptors. A coordinated cellular mechanism of action, with initial rapid, membrane-dependent activity and subsequent longer-term, genomic activity, has been postulated for mineralocorticoids and other steroid hormones (73–75).

The plasma membrane estrogen receptor (ER) has been described and characterized. The membrane receptor appears to be structurally identical to the intracellular ERα and -β (76). Ligand binding of the receptor activates G$i$ proteins and eNOS (77). In these ways the plasma membrane ER resembles the DHEA receptor that we describe. However, estradiol does not inhibit binding of DHEA to the DHEA binding site. Furthermore, the ER antagonist, ICI 182,780, blocks the action of estradiol on eNOS in plasma membrane preparations, but does not alter the effect of DHEA in the same system. We have also found that tamoxifen blocks the effect of estradiol to stimulate NO production in cultured BAEC, but does not inhibit the action of DHEA, again suggesting that these steroids are not functioning through the same receptor. Because the ER does not have the classical seven-transmembrane-spanning structure of a G-protein-coupled receptor, Wyckoff et al. (77) have hypothesized that the ER may be interacting with another “classical” G-protein-coupled receptor to initiate its signaling. A similar mechanism may be postulated for the DHEA receptor. The similarity of the activities of estradiol and DHEA raises the possibility that both the DHEA receptor and the ER may signal by interacting with a common G-protein-coupled receptor. It will be important to determine whether the DHEA receptor is interacting directly with G-proteins or is interacting with a G-protein-coupled receptor. It will also be important to determine whether the cell surface-initiated activities of DHEA and estradiol are entirely the same in endothelial cells or whether they have some differences.

The significance of our findings is that they establish a mechanistic basis for the physiological actions of DHEA, although the detailed signaling pathways involved in receptor-mediated DHEA action remain to be elucidated. Based on our results, we are now in a position to isolate the putative plasma membrane receptor for DHEA. The data on vascular endothelial effects of DHEA raise the possibility of developing non-metabolized DHEA receptor ligands to evaluate their effects on vascular function in vivo. Furthermore, our results are another example of the plasma membrane-initiated, non-genomic activity of steroid hormones. This property has been demonstrated for many other steroids. However, the finding with DHEA is novel, because it has no known intracellular receptor, unlike other steroids with partially or fully characterized membrane receptors. These findings expand the repertoire of signaling pathways that have been associated with plasma membrane-initiated actions of steroids and with DHEA.

In summary, we present evidence for a putative specific DHEA plasma membrane receptor on bovine aortic endothelial cells. This receptor is functionally coupled to the G$i$ protein subfamily and primarily to G$\alpha_{i2}$ and G$\alpha_{q3}$ subtypes. Activation of these G$\alpha$-proteins mediates the effect of DHEA on eNOS. This finding has a fundamental impact on our interpretations of the biological actions of DHEA. The signal transduction pathway associated with the ligand-receptor interaction requires further investigation. The receptor purification, characterization, and cloning are necessary to fully understand the physiological significance of DHEA.

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DHEA Plasma Membrane Receptor

Table II
Comparison of DHEA receptor characteristics

| Citation | Cell type | Subcellular fraction | $K_d$ (nM) | $B_{max}$ (fmol/mg) |
|----------|-----------|----------------------|------------|---------------------|
| (*) BAEC | PM        | 0.05                 | 500        |
| (17) Rat liver | C         | 2.3                  | ~150       |
| (18) Mouse melanoma | C         | 622                  |            |
| (19) Human T-lymphoid | WC      | 7.4                  | 144        |
| (20) T-cell hybridomas, mouse T-lymphocytes | WC, C, N | 1.4–5.5              | 189–7,244 sites/cell |

$K_d$ is the dissociation constant, and $B_{max}$ is the maximum binding capacity.
Dehydroepiandrosterone Activates Endothelial Cell Nitric-oxide Synthase by a Specific Plasma Membrane Receptor Coupled to Gαi2,3
Dongmin Liu and Joseph S. Dillon

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