Dehydroepiandrosterone-Induced Phosphorylation and Translocation of FoxO1 Depend on the Mineralocorticoid Receptor

Carsten Lindschau, Torsten Kirsch, Uwe Klinge, Peter Kolkhof, Imke Peters, Anette Fiebeler

Abstract—In humans, dehydroepiandrosterone (DHEA), with its sulfate, is the most abundant adrenal steroid, whereas the rat adrenals do not make this steroid. Circulating concentrations of DHEA sulfate lie in the millimolar range and those of DHEA in the subnanomolar range. DHEA exerts protective potential during vascular remodeling, although the underlying mechanisms of this protection are imperfectly defined. We hypothesized that physiological doses of DHEA alter signaling pathways that are of central importance for vascular integrity. We exposed human endothelial cells, vascular smooth muscle cells, and fibroblasts to DHEA (10⁻⁶ to 10⁻¹⁰ mol/L) and observed a dose- and time-dependent increase of extracellular signal-regulated kinases 1 and 2 activation. Similar results were observed in rat vascular smooth muscle cells. In addition, in rat vascular smooth muscle cells, we found altered phosphorylation and cellular translocation of the transcription factor FoxO1. Pharmacological blockade of the mineralocorticoid receptor (MR) with eplerenone or small interfering RNA-mediated MR-silencing prevented DHEA-induced extracellular signal-regulated kinase 1/2 phosphorylation and its effects on FoxO1. Of note, in a cell-based MR transactivation assay, we did not find any agonist effect of DHEA on MR activity. We conclude that DHEA induces early signaling events in vascular cells that might underlie the DHEA-mediated protection against vasculopathies. These effects are dependent on the MR, although the finding that DHEA fails to act as a direct MR agonist suggests that additional signaling proteins are involved. In this regard, DHEA may either interact with corefactors to modify MR activity or serves as a ligand for a yet unknown receptor that might transactivate the MR. (Hypertension. 2011;58:471-478.)

Key Words: dehydroepiandrosterone ■ mineralocorticoid receptor ■ smooth muscle cells ■ signaling

Dehydroepiandrosterone (DHEA), with its conjugated sulfate, is the most abundant adrenal steroid in humans, whereas rat adrenals do not make this steroid. In humans, circulating levels of DHEA sulfate are in the millimolar range and those of DHEA are in the subnanomolar range. Numerous studies have reported its protective capabilities in terms of general mortality and, in particular, in cardiovascular disease, which is characterized by progressive vascular remodeling, a leading cause of age-related morbidity and mortality. Barrett-Connor et al. demonstrated that reduced concentrations of circulating DHEA sulfate represent an independent marker for prediction of mortality in general, as well as death from cardiovascular diseases in particular. Other groups found similar results.

The mode of action of DHEA and its related signaling pathways have remained largely ill defined. During human lifetime, circulating concentrations of DHEA and DHEA sulfate decline from peak levels in the 20s to 10% to 20% in the 70s, and the incidence of inflammatory and malignant processes is inversely correlated with a decline in circulating concentrations of DHEA. Circulating hormones first contact the vascular endothelium, which plays a crucial role in vascular remodeling, both as a barrier and a signal transducer. In this regard, Dillon and colleagues have focused on DHEA-induced effects in endothelial cells and showed that DHEA induces endothelial cell proliferation and angiogenesis. Whether DHEA has similar effects on other vascular cell types has not been described so far.

We hypothesized that DHEA may have additional effects on early signaling of fibroblasts and vascular smooth muscle cells (VSMCs). In studies on human and rat VSMCs, we observed time- and concentration-dependent induction of extracellular signal-regulated kinase (ERK) 1/2 signaling by DHEA that mimics aldosterone- and angiotensin II–induced signaling effects described previously.

Methods

Cell Culture
Human umbilical vein endothelial cells were isolated from umbilical cords, as described earlier. Cells were cultivated in EGM-2
medium (Lonza, Vervier, Belgium) and stimulated with 0.25 mg/mL of 8-(4-chlorophenylthio)-cAMP sodium salt (pCPT-cAMP, Sigma-Aldrich, Munich, Germany) for 72 hours to obtain a tight monolayer. The Hannover Medical School Ethics Committee approved the study, and written informed consent was obtained. Primary aortic human VSMCs (Promocell, Heidelberg, Germany) were grown in medium as recommended by the suppliers. Human fibroblasts were isolated from fresh skin punch biopsies from healthy volunteers after approval from the local authorities. Cell culture techniques are routinely used in our laboratory and have been described elsewhere. Aortic VSMCs were isolated from Wistar rats, as described previously. We followed all of the requirements of the American Physiological Society, and local authorities approved the studies. We used passages 3 to 8 after phenotyping by staining VSMCs for α-actin (DAKO) and desmin (Boehringer-Mannheim). The following substances were used: DHEA (Sigma), PD98059 (Calbiochem), eplerenone (Pfizer), aldosterone (Climafia), and 12-O-tetradecanoylphorbol-13-acetate (Sigma).

**Transfection of Cells**
Cells were treated with DHEA (10−7 mol/L). Eplerenone (10−5 mol/L) was used to specifically block the mineralocorticoid receptor (MR). All of the experiments were performed under 24-hour serum-free conditions. For small interfering RNA (siRNA) experiments, cells were nucleofected with specific siRNA (Dharmacon) directed against MR or nontargeting siRNA following the manufacturer’s protocol (Amaxa). The following sequences were used as designed and provided by Dharmacon: MR-directed siRNAs: CCAAGUCAU-UGCAOUGUAA, CGCAAUAUCUCAAACUC, GUGAA-GAGGCCUCUAACUC, and CUAUGAGAGCUGGAAAGCA; siSTABLE nontargeting siRNA control and siSTABLE cyclophilin B siRNA served as controls. Three days after transfection, cells were stimulated as described.

**Immunocytochemistry**
Confocal microscopy was performed as described previously. Fifty to 80 cells at each treatment point from ≥3 independent experiments were examined by 2 blinded investigators. Quantification was done with ImageJ. The subcellular regions were outlined manually, and mean fluorescence intensities were obtained for the regions delineated with data presented as mean fluorescence intensity in each cell area. Rabbit and mouse antiphospho-ERK1/2 (Cell Signaling; 1:200), FoxO1 (Cell Signaling; 1:200), and pFoxO1 (Cell Signaling; 1:200) antibodies were used in the studies.

**Western Blot**
The following primary antibodies were used: polyclonal ERK1/2 (Cell Signaling; 1:1000), phospho-ERK1/2 (Cell Signaling; 1:1000), FoxO1 (Cell Signaling; 1:50), and pFoxO1 (Cell Signaling; 1:100). Peroxidase-conjugated secondary antibodies were purchased from Dianova (1:10 000). Blots were developed with a chemiluminescent substrate and visualized digitally with a ChemiSmart apparatus (PqLab). Three to 6 different cell stimulation experiments were performed and quantified for each protocol. For quantification, the intensity of the control was defined as 100% and intensity of other bands was calculated accordingly.

**Cell Compartment Analysis**
Separation of cytoplasmic and nuclear compartments was performed and quantified for each protocol. For quantification, the intensity of the control was defined as 100% and intensity of other bands was calculated accordingly.

**Cell-Based Transactivation Assays**
The generation of stable steroid hormone receptor cell lines, their cultivation, and respective assay procedure have been described in Fagart et al. Briefly, stable MR, glucocorticoid receptor (GR), androgen receptor (AR), progesterone receptor (PR), and estrogen receptor (ER)-expressing CHO-K1 cell lines were generated. On the day of the experiment, compounds were given in 8 dilutions to the cells, followed by the relevant EC50 concentration of each agonist. After an incubation time of 5 to 6 hours, luciferase activity was determined with a luminescence detecting video camera system. The GraphPad Prism Software (version 3.02, GraphPad Software, Inc, San Diego, CA) was used for curve fitting and calculation of EC50 values. The EC50 values from the luciferase assay were determined in ≥2 independent experiments performed in duplicate and are given as mean ± SEM.

**Statistics**
Statistical significance was calculated by Kruskal-Wallis rank test using PASW Statistics 18 (Chicago, IL). Where human donors were used, cells were from ≥3 different donors. A value of P<0.05 was considered statistically significant.

**Results**

**DHEA Induces Phosphorylation of ERK1/2 in Vascular Cells**
To confirm findings of others and to validate our experimental setting, we first studied different human vascular cells for their early response to DHEA by determining phosphorylation of the mitogen-activated protein kinases ERK1/2 at 2 different concentrations. All 3 of the human cell types, namely, endothelial cells (human umbilical vein endothelial cells), VSMCs, and fibroblasts, responded in a concentration- and time-dependent manner to DHEA, demonstrating that all 3 of the cell types show biological responses on stimulation with DHEA (Figure 1). To investigate the principal mechanisms of DHEA-induced signal transduction in more detail, we used rat aortic VSMCs. First, we determined whether DHEA induced comparable effects on activation of ERK1/2 in those cells and observed a time-dependent phosphorylation in rat VSMCs (Figure 2). Thus, in the subsequent experiments, we focused our studies on rat VSMCs and investigated key components of DHEA-induced signaling cascades. In our system, DHEA concentrations as low as 100 nmol/L significantly increased ERK1/2 activation with a peak in phosphorylation at 1 nmol/L of DHEA.

**DHEA Induces Phosphorylation and Cellular Translocation of FoxO1**
The status of ERK phosphorylation is central for the activity of numerous downstream proteins; among them is the transcription factor FoxO1. FoxO1 belongs to the family of forkhead transcription factors. It plays a role in vascular homeostasis and, thus, represents a relevant factor in the pathophysiology of vasculopathies (reviewed in Reference15). To evaluate the possible responsiveness of FoxO1 to DHEA, we stimulated aortic VSMCs with 100 nmol/L of DHEA and observed a time-dependent response involving early phosphorylation and cellular translocation (Figure 3A). To confirm DHEA-induced phosphorylation and translocation of FoxO1, we next performed a cell compartment separation with subsequent analysis of protein levels. Similar to the results of the immunocytochemical analysis, we found increased protein phosphorylation of FoxO1 at 15 minutes (Figure 3B). Blocking mitogen-activated protein kinase kinase 1, an upstream kinase of ERK1/2, by PD98059 did not inhibit DHEA-induced effects on FoxO1 in cells, even after exposure for longer time points (Figure 3C).

There have been numerous attempts to find a receptor that binds DHEA and transmits its signal. Studies of DHEA...
signaling via classical receptors have focused primarily on androgen and estrogen receptor interactions because of the structural similarities of DHEA and androgenic steroids. There have been no reports on the effects of DHEA on the MR. However, with the elucidation of the multiple roles for MR, in addition to its classic role regarding salt and water balance, we set out to determine effects of DHEA on MR signaling. We silenced the MR with specific siRNA, repeated the stimulation with DHEA, and analyzed phosphorylation of ERK1/2. Successful silencing of the MR was confirmed by immunohistochemistry (Figure S1, please see the online Data Supplement at http://hyper.ahajournals.org). Scrambled non-
specific siRNA, aldosterone (1 nmol/L), and 12-O-tetradecanoylphorbol-13-acetate stimulation were used to assess non-specific activations. MR-silencing abrogated DHEA (1 nmol/L)-induced ERK1/2 phosphorylation (Figure 4A; for this experiment we chose 15 minutes of DHEA stimulation to look at a similar time point compared with stimulation by aldosterone). Blocking the MR with the selective antagonist eplerenone (10\(\mu\)mol/L) had a similar effect on DHEA-induced ERK1/2 phosphorylation (Figure 4B). MR silencing also prevented DHEA (1 nmol/L)-induced phosphorylation of FoxO1 (Figure 4C), whereas scrambled siRNA had no effect (Figure S2).

DHEA Does Not Directly Stimulate the MR

Given that the MR appears crucial to DHEA-induced signaling, we determined in recombinant Chinese hamster ovary cells whether there is a direct ligand-receptor interaction. We, therefore, investigated possible direct agonist activities of DHEA in comparison with control steroids in a platform of cell lines stably expressing different steroid hormone receptors. We found no agonist activity of DHEA in these MR, GR, AR, PR, or ER-\(\alpha\) transactivation assays and a very weak partial agonist activity in the cell-based ER-\(\beta\) assay. Figure 5 shows the results for MR and ER-\(\beta\).

Discussion

DHEA has been demonstrated to modulate multiple inflammatory disorders, including vasculopathies. Many studies of its mode of action have demonstrated that DHEA may lower cytokine-induced effects, but there have been only a few reports describing direct actions of DHEA on signaling pathways. In our studies, we have observed that DHEA exhibits rapid aldosterone-mimicking effects: DHEA not only induces phosphorylation of mitogen-activated protein kinase and FoxO1, but, most interestingly, the MR is crucial for transmitting these effects of DHEA.

Given that both protective and detrimental effects of DHEA have been published in different experimental settings of vascular injury in vivo and in vitro, we studied DHEA-induced modulation of early signaling in primary culture of endothelial cells, smooth muscle cells, and fibroblasts and thereby assessed phosphorylation of the mitogen-activated protein kinase ERK1/2 as an early marker of biological
response. In contrast to many previously published studies, we observed an early upregulation of ERK1/2 phosphorylation already at subnanomolar and, thus, physiological DHEA levels. This finding suggests that DHEA specifically modulates the complex signaling networks, in which activity of ERK1/2 represents a key issue. For instance, activation of ERK1/2 affects signaling pathways associated with cellular proliferation and, thus, modulates inflammation and vasculopathy (reviewed in Reference 17). In all of the human cells studied, we observed a time- and dose-dependent effect of DHEA. This is in accordance with findings of Liu et al, who previously described induction of ERK1/2 phosphorylation in response to DHEA in endothelial cells. The effect occurred within 15 minutes and was a prerequisite for DHEA-induced endothelial proliferation and formation of capillary tubes. We observed in our study similar direct effects of DHEA in both VSMCs, the major cell type during (re)stenosis of arterial vessels, as well as on fibroblasts. This observation supports the well-described pluripotency of DHEA in different settings. The underlying mechanisms and their consequence remain to be clarified.

To shed more light on DHEA-induced signaling, Chen et al. investigated endothelial cells and observed DHEA-induced phosphorylation of the forkhead transcription factor FoxO1, which depends on ERK1/2. In addition, these authors demonstrated that a DHEA-induced signaling cascade via FoxO1 leads to increased expression of the vasoconstrictor endothelin 1. The same group showed in a previous study that relatively high concentrations (100 nmol/L) of DHEA led to a significant stimulation of endothelial NO synthase and release of NO within 5 minutes from bovine aortic endothelial cells. Therefore, a model was drawn of different DHEA signaling pathways leading either to vasodilation via phosphatidylinositol 3-kinase and endothelial NO synthase or vasoconstriction via mitogen-activated protein kinase kinase/FoxO1-endothelin 1. Abid et al. found that active FoxO inhibited proliferation, and the authors concluded that FoxOs might be a therapeutic target in vasculopathic states. We identified MR to be crucial for transmitting the signaling response to DHEA (Figure 6). The possibility that DHEA signals via the MR without being a direct MR ligand appears peculiar but is not inconsistent with the published literature. A specific receptor for DHEA has never been identified despite intensive research efforts from several groups (reviewed in Reference 16). Previous studies have identified agonist, as well as antagonist, activities of DHEA on AR and ER. One reason for the observed conflicting results likely lies in the different experimental

Figure 3. Dehydroepiandrosterone (DHEA) induces phosphorylation and translocation of FoxO1. Rat vascular smooth muscle cells were stimulated with DHEA (10^{-7} mol/L) for 15 to 60 minutes. A, Shown are representative micrographs of phospho (p)FoxO1 immuno- stainings in rat vascular smooth muscle cell (VSMC). The diagrams depict quantification of the pFOXO1 immunofluorescence, with * indicating significant differences (P<0.05) vs control conditions. B, Stimulation of rat VSMC with DHEA results in a rapid translocation of FoxO1 into the nucleus, as determined by Western blot analysis using pFoxO1-specific antibodies. *Significant differences (P<0.05) to protein phosphorylation compared with controls. C, Pretreating rat VSMC with the mitogen-activated protein kinase kinase (MEK) 1 inhibitor PD98059 (10^{-5} mol/L) did not prevent DHEA-induced phosphorylation of FoxO1 completely but led to a significant reduction of the stimulated protein. *P<0.001 vs DHEA-treated cells.
settings used. In addition, and as already pointed out by Chen et al.,21 further interactions to other steroid receptors possibly exist (also summarized by Webb et al.,25 among others). Alternatively, DHEA may signal through a receptor/activator complex, thereby affecting cellular function. For instance, regarding the estrogen receptor, 60 different coactivators and 20 corepressors, as well as protein interactions with caveolins, G proteins, mitogen-activated protein kinases, and receptor tyrosine kinases, have been described (reviewed in Reference26). Cofactors of the MR are also well described, although almost all of them represent general coregulators without specific function toward MR.27 Hultman et al.28 showed that the MR strongly interacts with peptides derived from steroid receptor coactivator 1a and peroxisome proliferator-activated receptor-γ coactivator 1α and β. Interestingly, steroid receptor coactivator 1 has been shown to be required for ligand-specific activity of tamoxifen.29 In contrast, the RNA polymerase II elongation factor eleven-l9259 has been identified as a selective coactivator of the MR.30 To what extent the interaction between DHEA and its putative receptors is regulated via such cofactors remains to be analyzed. However, the assumption of a simple ligand-receptor binding interaction appears to be insufficient to account for our findings.

According to our data, the MR is centrally involved in DHEA-induced processes and is thereby integrated into general mechanisms of physiological vascular remodeling in vivo. Ongoing studies are focusing on its particular role in this regard. Several in vivo studies have pointed toward involvement of DHEA during cardiovascular events. In heart failure, for example, Nakamura et al.31 suggested an inverse relation between the MR ligand aldosterone and DHEA in the heart as one possible mechanism for fatal outcome. They demonstrated decreased DHEA levels in the failing heart associated with increasing aldosterone levels, suggesting a possible link between these 2 steroids. This hypothesis has...
been supported by studies of Bonnet et al.32 The authors reported that DHEA reverses vascular remodeling with decreasing proliferation and increasing smooth muscle cell apoptosis in vivo and in vitro. In preliminary observations, we found a stimulatory effect of DHEA on expression of the MR. The role of the MR and its classic ligand aldosterone in vascular remodeling has been studied extensively. For instance, Bodary et al33 demonstrated that blockade of aldosterone effects after carotid artery injury improved vascular texture in a mouse model, whereas aldosterone usually aggravates vascular remodeling. Although the impact of DHEA on MR expression and function has to be studied in more detail, a possible modulation of MR expression by DHEA might support some effects of DHEA.

At a first glance, our findings of comparable effects of DHEA and aldosterone during early signaling events in cell culture seem contrary to the opposite role of the 2 hormones in vivo. In fact, aldosterone, as well as angiotensin II and cortisol, induces early signaling in a comparable manner, and all of them have been described to be involved in vascular remodeling.34 However, the distinct effects of aldosterone and DHEA in more complex conditions clearly show that ERK signaling may be just one player within a complex regulatory system. Thus, current knowledge on DHEA signaling clearly points to a signaling network. In a nutshell, regarding the inability of DHEA to directly activate the MR, it seems feasible that DHEA might rather act as an activator that is necessary to transactivate other receptors and that this transactivation modulates the vasculature in either a protective or detrimental mode. Furthermore, inappropriate redox or salt status of the cellular environment (as described for cortisol35) may also determine the direction of the response to DHEA in terms of modulating vascular homeostasis. The present studies in the cardiovascular system thus represent an initial step toward understanding the mechanisms of action of DHEA.

**Perspectives**

We here described a possible model for the DHEA-ERK1/2-FoxO1-MR axis in human and rat VSMCs. Nevertheless, further studies are necessary that might shed more light on the question of how the differing dose and time responses observed here translate into regulation of downstream events in the context of circulating levels of DHEA decreasing with age. Answers to these questions may determine, in the clinical setting, who will profit from DHEA substitution or blockade. In this regard, we need to focus on more individual markers to define the appropriate patient group that may benefit from DHEA modulation.

**Acknowledgments**

MR-detecting antibodies were a kind gift from Celso Gomez-Sanchez. We thank Petra Quass, Michaela Beese, and Kristin Wyss for their excellent technical assistance.

**Sources of Funding**

The German Federal Ministry for Education and Research (BMBF) supported A.F. and U.K. (BN9829).

**Disclosures**

None.

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478 Hypertension September 2011


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Carsten Lindschau, Torsten Kirsch, Uwe Klinge, Peter Kolkhof, Imke Peters and Anette Fiebeler

Hypertension. 2011;58:471-478; originally published online July 11, 2011; doi: 10.1161/HYPERTENSIONAHA.111.171280

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Dehydroepiandrosterone-induced phosphorylation and translocation of FoxO1 depend on the mineralocorticoid receptor

Carsten Lindschau\textsuperscript{1*}, Torsten Kirsch\textsuperscript{1*}, Uwe Klinge\textsuperscript{2}, Peter Kolkhof\textsuperscript{3}, Imke Peters\textsuperscript{1}, Anette Fiebeler\textsuperscript{1}

\textsuperscript{1}Nephrology Section, Department of Medicine, Hannover Medical School, Germany
\textsuperscript{2}Department of Surgery, University Hospital RWTH Aachen, Germany
\textsuperscript{3}Global Drug Discovery, Cardiology Research, BAYER Schering Pharma, Wuppertal, Germany

*CL and TK contributed equally

Short title: DHEA, smooth muscle cells, and signaling

Correspondence:
Anette Fiebeler
Carl-Neuberg-Strasse 1
30625 Hannover, Germany, Tel: +49 511 5326319, Fax: +49 511 552366
Email: Fiebeler.a@mh-hannover.de
**Figure S1.** siRNA-mediated knockdown of mineralocorticoid receptor (MR) protein expression in vascular smooth muscle cells (right panel). Non-targeting siRNA served as control (left panel).

**Figure S2.** Knockdown of the MR by siRNA successfully prevented DHEA-induced phosphorylation of ERK1/2 in rat VSMC. Shown are representative micrographs of pERK1/2 stainings three days after nucleofection of the cells with MR-specific siRNA. Non-targeting siRNA served as control.
MR-Expression in rat VSMC

Suppl. Figure 1

MR-Expression

Non-targeting siRNA

MR-targeting siRNA
Effects of MR silencing on DHEA-induced ERK 1/2 phosphorylation

Ctr | control RNA (non-targeting) + DHEA | siMR + DHEA