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Dehydroepiandrosterone Protects Vascular Endothelial Cells against Apoptosis through a $G\alpha_i$ Protein-Dependent Activation of Phosphatidylinositol 3-Kinase/Akt and Regulation of Antiapoptotic Bcl-2 Expression

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The adrenal steroid dehydroepiandrosterone (DHEA) may improve vascular function, but the mechanism is unclear. In the present study, we show that DHEA significantly increased cell viability, reduced caspase-3 activity, and protected both bovine and human vascular endothelial cells against serum deprivation-induced apoptosis. This effect was dose dependent and maximal at physiological concentrations (0.1–10 nM). DHEA stimulation of bovine aortic endothelial cells resulted in rapid and dose-dependent phosphorylation of Akt, which was blocked by LY294002, a specific inhibitor of phosphatidylinositol 3-kinase (PI3K), the upstream kinase of Akt. Accordingly, inhibition of PI3K or transfection of the cells with dominant-negative Akt ablated the antiapoptotic effect of DHEA. The induced Akt phosphorylation and subsequent cytoprotective effect of DHEA were dependent on activation of $G\alpha_i$ proteins, but were estrogen receptor independent, because these effects were blocked by pertussis toxin but not by the estrogen receptor inhibitor ICI182,780 or the aromatase inhibitor aminoglutethimide. Finally, DHEA enhanced antiapoptotic Bcl-2 protein expression, its promoter activity, and gene transcription attributable to the activation of the PI3K/Akt pathway. Neutralization of Bcl-2 by antibody transfection significantly decreased the antiapoptotic effect of DHEA. These findings provide the first evidence that DHEA acts as a survival factor for endothelial cells by triggering the $G\alpha_i$-PI3K/Akt-Bcl-2 pathway to protect cells against apoptosis. This may represent an important mechanism underlying the vascular protective effect of DHEA. (*Endocrinology* 148: 3068–3076, 2007)

DEHYDROEPIANDROSTERONE (DHEA) AND its sulfated form fall sharply with aging (1), and epidemiological studies show that atherosclerotic diseases and cardiovascular mortality are inversely associated with concentrations of DHEA and its sulfated form, particularly in males (2, 3). An abundance of animal and *in vitro* studies suggest beneficial effects of DHEA on vascular endothelium and smooth muscle (4–8) and on other key mediators of atherogenesis (9–11). Human intervention study data suggest a beneficial effect on angiographic evidence of atherosclerosis (12), markers of vascular risk (13–16), and vascular function (17).

The mechanisms underlying these findings remain unknown. DHEA was considered to exert its action mainly by conversion to sex steroids (18). However, accumulating evidence shows that DHEA has vascular effects that are independent of the estrogenic effects (19–21). Recently, we (22) and others (23, 24) demonstrated a direct action of DHEA on vascular endothelial cells (ECs) *in vitro* and *in vivo* to modulate vascular function through a mechanism independent of either androgen receptor or estrogen receptor (ER).

The vascular endothelial monolayer, which separates circulating blood and peripheral tissues, plays a pivotal role in maintaining normal vascular function. Endothelial injury or loss of ECs attributable to aging-induced apoptosis contributes to the development of aging-associated vascular diseases such as arteriosclerosis (25). In the present study, we tested the hypothesis that DHEA, at physiological concentrations, may play a role as a survival factor for vascular ECs. We first examined whether DHEA protected ECs from serum starvation-induced apoptosis. We then defined the cellular mechanism of DHEA action. The results demonstrated that DHEA, at physiological concentrations, increased cell viability and protected vascular ECs against serum deprivation-induced apoptosis. Pharmacological and molecular intervention studies showed that the cytoprotective effect of DHEA was dependent on $G\alpha_i$ GTP-binding protein ($G\alpha_i$)-mediated activation of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway. Furthermore, we demonstrated that activation of Akt by DHEA increased the antiapoptotic Bcl-2

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Abbreviations: BAEC, Bovine aortic endothelial cell; Bcl-2-Luc, promoter region of the Bcl-2 gene linked to a firefly luciferase gene; CREB, cAMP responsive element-binding protein; DHEA, dehydroepiandrosterone; EC, endothelial cell; ER, estrogen receptor; FBS, fetal bovine serum; HAEC, human aortic endothelial cell; $G\alpha_i$, $G\alpha_i$ GTP-binding protein; ICI, ICI182,780; LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; NF-κB, nuclear factor κB; PI3K, phosphatidylinositol 3-kinase; PTX, pertussis toxin; 18S, ribosomal 18S RNA; TUNEL, terminal deoxynucleotidyltransferase dUTP nick-end labeling.

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promoter activity, gene, and protein expression and that neutralization of Bcl-2 by antibody transfection reduced the antiapoptotic effect of DHEA.

Materials and Methods

Reagents

17β-Estradiol and DHEA were from Steraloids (Newport, RI); ICI 182,780 (ICI) was purchased from Tocris Cookson (Ballwin, MO). Culture media and supplements were from Invitrogen (Carlsbad, CA); endothelial growth factors were obtained from Cambrex Bioscience (Rockland, ME). Akt, phospho-Akt, ERK 1/2, phospho-ERK 1/2 (Thr202/Tyr204), Bcl-2, and β-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA); nitrocellulose membranes and protein assay kit were from Bio-Rad (Hercules, CA); supersignal chemiluminescence detection system was purchased from Pierce (Rockford, IL). N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK), caspase-3 assay kit and CellTiter96, a nonradioactive methylthiazolyldiphenyl-tetrazolium-based, cell viability assay kit was from Promega (Madison, WI); cell death detection ELISA^{PLUS} and terminal deoxynucleotidyltransferase dUTP nick-end labeling (TUNEL) kits were purchased from Roche (Indianapolis, IN). A dominant-negative Akt cDNA plasmid was from Upstate (Charlottesville, VA); plasmid purification kits were from Qiagen (Valencia, CA); a pcDNA 3.1/His/*lac*Z control vector was from Invitrogen. cDNA transfection reagent was purchased from Targeting Systems (Santee, CA); pRL luciferase control vector and dual luciferase assay system were obtained from Promega; Chariot protein transfection reagent was bought from Active Motif (Carlsbad, CA). RNAeasy kit and MinElute Purification kit were from Qiagen; iScript cDNA synthesis reagents and iQ Supermix were obtained from Bio-Rad; primers were synthesized by Integrated DNA Technologies (Coralville, IA). Pertussis toxin (PTX), 2-(4-morpholinyl)-8-phenyl-4H-l-benzopyran-4-one (LY294002), protease and phosphatase inhibitors, and all other laboratory chemicals were from Sigma (St. Louis, MO). Stock solutions of steroids, at 10 mM in dimethylsulfoxide, were stored at −20 C before use.

Cell culture

Bovine aortic endothelial cells (BAECs) were cultured as described previously (22). Human aortic endothelial cells (HAECs) (Cambrex Bioscience) were grown in M199 medium supplemented with 2% fetal bovine serum (FBS) and EC growth factors. Before experiments, the cells were cultured in the medium supplemented with 10% charcoal-stripped serum for 24 h before being switched to serum-free medium.

Cell viability assay

BAECs were serum starved for 24 h. Cells were then exposed to either vehicle or indicated concentrations of DHEA in serum-free M199 medium for 24 h. The number of viable cells was assessed using a CellTiter 96 aqueous assay kit (26).

Caspase-3 activity assay

Cytosolic enzymatic activity of caspase-3 was measured essentially as described by the protocol of the manufacturer. The caspase-3 activity in the cell lysates was normalized to the cellular protein concentration and expressed as percentage of increase over the FBS-treated cells.

Cell apoptosis assay

For apoptosis experiments, cells were serum starved for 24 h before incubation with 5% FBS, or DHEA or vehicle in serum-free medium, for 24 h. In some experiments, cells were preincubated with PTX (100 ng/ml) for 24 h, or with LY294002 (5 μM) or ICI (100 nM) for 30 min, or with aminoglutethimide (4 μM) for 60 min in serum-free medium followed by treatment with DHEA, 17β-estradiol, or vehicle in the continued presence or absence of these inhibitors for 24 h at 37 C. Cellular apoptosis was estimated by determination of cytoplasmic histone-associated DNA fragments in cell lysates using a cell death detection ELISA^{PLUS} kit according to the protocol of the manufacturer. For quantification of apoptotic cells, cells seeded on chamber slides were fixed for 30 min in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 for 2 min on ice. The apoptotic cells were detected using TUNEL techniques as described previously (27).

Immunoblot analysis

Equal amounts of protein from cell extracts were subjected to immunoblot analysis as described previously (28, 29). Membranes were probed with antibody against phospho-Akt or Bcl-2. The immunoreactive proteins were detected by chemiluminescence. Nitrocellulose membranes were stripped and reprobed with Akt or β-actin in the case of Bcl-2. The protein bands were digitally imaged for densitometric quantitation with a software program (NIH ImageJ).

Plasmid transfection

A dominant-negative Akt cDNA plasmid and a reporter plasmid containing a promoter region of the Bcl-2 gene linked to a firefly luciferase gene (Bcl-2-Luc; a kind gift from Dr. Linda M. Boxer, Stanford University, Stanford, CA) were purified using Maxi kit by Qiagen. BAECs were grown in 24-well plates in M199 medium until 60–70% confluence. Before transfection, the medium was changed to M199 with 1% FBS. BAECs were cotransfected with 0.5 μg Bcl-2-Luc vector and 1 ng pRL reporter control plasmid per well using F-1 transfection reagent according to the protocol of the manufacturer. In some experiments, the cells were transfected with dominant-negative Akt plasmids. Transfection efficiencies were determined by cotransfecting the cells with a pcDNA 3.1/His/*lac*Z control vector. After transfection, cells were incubated with complete medium containing 20% charcoal-stripped FBS for 24 h before serum starvation for an additional 24 h. The transfected cells were then treated with DHEA or vehicle for 24 h in phenol-red-free M199 medium. Bcl-2-Luc-transfected cells were harvested in reporter lysis reagent. Luciferase activity, normalized to pRL activity in the cell extracts, was determined by using the dual luciferase reporter assay system. Akt-transfected cells were used for cell viability assay as above.

Quantitative real-time RT-PCR analysis

BAECs were exposed to vehicle or DHEA (1 nM) in M199 medium for 24 h. Total RNA was isolated from the cell lysates and was checked for quality and quantity using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Then, 1 μg total RNA from each sample was reverse transcribed to cDNA using the iScript cDNA synthesis kit. Standards were made from the purified amplicons generated from each set of primer pairs using the iQ Supermix. The PCR products were purified with the MinElute Purification kit and quantified on an agarose gel with the Precision Molecular Mass Ruler (Bio-Rad). Bcl-2 was amplified on an iCycler IQ real-time quantitative PCR system using iQ SYBR Green Supermix with ribosomal 18S rRNA (18S) as an internal control. A melting curve analysis was performed on each sample to verify that no nonspecific products were synthesized. A cDNA synthesis with no RNA template was included as a negative control to ensure that chromosomal DNA was not amplified. The primers used in quantitative real-time RT-PCR were Bcl-2 (forward, 5′-CGCATCGTGGCCTTCTTTGAGTT-3′; reverse, 5′-GCCGGTTCAGGTACTCAGTCAT-3′) and 18S (forward, 5′-CCTTCCGCGAGGATCCATTG-3′; reverse, 5′-CGCTCCCAAGATCCAACTAC-3′) (30). The Bcl-2 mRNA level was normalized to that of 18S and expressed as starting quantity of cDNA per microgram of RNA.

Antibody transfection

Antibody was delivered into BAECs using a Chariot reagent. Briefly, BAECs were grown in 24-well plates to achieve 50–60% confluence. Cells were then serum starved for 6 h before transfection. Polyclonal antibody against Bcl-2 or preimmune IgG diluted in PBS (1:250) were mixed with equal volume of Chariot dilution (7:100 in water) and then incubated at room temperature for 30 min to form complexes. Cells were sequentially overlaid with 50 μl/well Chariot/Bcl-2 or Chariot/preimmune IgG complex, 100 μl/well serum-free medium, and then incubated at 37 C. After 1.5 h, 250 μl/well medium containing 5% charcoal-stripped FBS was added to the cells, and transfection was continued for 5 h. The efficiency of antibody introduction was more than 60%, as

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determined by using IgG-labeled Alexa488. After transfection, the cells were cultured in the presence or absence of DHEA for 24 h, followed by apoptosis assay using a cell death detection ELISA.

Statistical analysis

Data were analyzed with one-way ANOVA using Prism software (GraphPad Software, San Diego, CA). Treatment differences were subjected to a Tukey's multiple comparison test or paired t test as noted in figure legends, in which $P < 0.05$ was considered significantly different. Data were expressed as mean ± SE derived from at least three independent experiments.

Results

Effect of DHEA on EC viability

Initially, we evaluated whether DHEA could promote viability of BAECs. As shown in Fig. 1A, administration of DHEA enhanced cell viability during serum deprivation. The effect of DHEA was concentration dependent, with DHEA concentrations of 0.1–10 nM inducing maximal protective effect (68.1–70.6 *vs.* 55.8% viability of the control). We further evaluated the effect of DHEA on serum deprivation-induced apoptosis by measuring cytoplasmic histone-associated DNA fragments using ELISA. As shown in Fig. 1B, incubation of BAECs in serum-free medium induced an increase of nearly 100% in apoptosis. However, coincubation of the cells with DHEA significantly reduced the extent of apoptosis attributable to serum starvation by 36–56%, depending on concentrations. To further confirm the DHEA effect, TUNEL staining was performed to directly assess the percentage of apoptotic cells in the presence or absence of DHEA. Serum deprivation increased the percentage of apoptotic cells from 4.8 to 16.4% (Fig. 1, C and D). Consistent with the results observed by ELISA, addition of DHEA significantly reduced the percentage of apoptotic cells induced by serum starvation.

The caspase proteins are critical components responsible for apoptosis (31), and caspase-3 is one of the key proteases involved in the convergence of disparate apoptotic signaling pathways. In parallel with decreased cell viability, we observed that the cellular activity of caspase-3 was markedly induced after exposure of the cells to serum-free medium for 12 h. Coincubation with DHEA or the known caspase-3 inhibitor Z-VAD-FMK significantly reduced the activation of caspase-3 to a similar extent (Fig. 1E). This further supports the concept that DHEA exerts an antiapoptotic role in ECs.

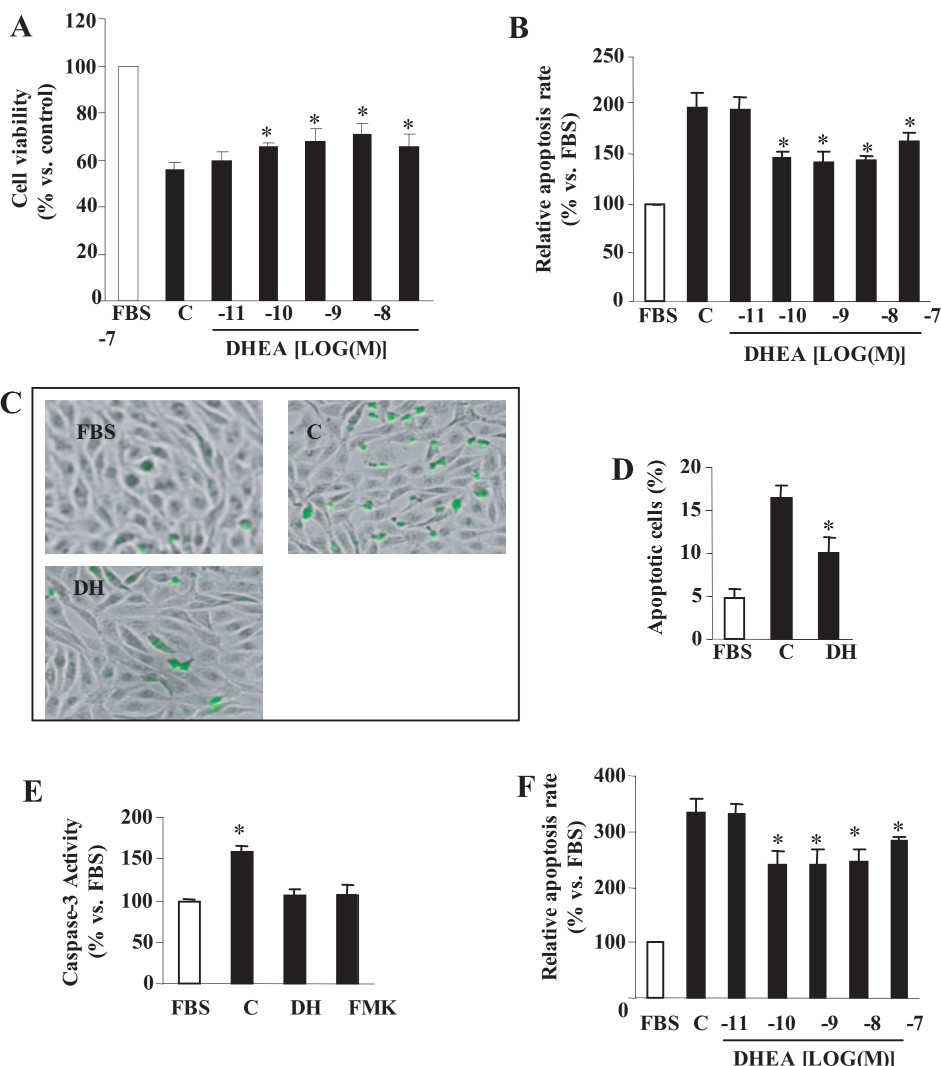

FIG. 1. DHEA protects vascular ECs against serum deprivation-induced apoptosis. A and B, BAECs were serum starved for 24 h and then treated with 5% FBS, various concentrations of DHEA, or vehicle (C) for 24 h. A, Cell viability was measured by a methylthiazolyldiphenyl-tetrazolium-based assay. B, Apoptosis was measured by ELISA. C and D, BAECs were serum starved for 24 h and then treated with 5% FBS, DHEA (DH; 1 nM), or vehicle (C). After 24 h, the cells were labeled by using a TUNEL assay. C, Representative photographs of EC apoptosis manifested as green fluorescein. D, Percentage of TUNEL-stained cells assessed under fluorescence microscope with four random views per sample examined. E, BAECs were serum starved for 24 h and then treated with 5% FBS, DHEA (DH; 1 nM), vehicle (C), or caspase-3 inhibitor Z-VAD-FMK (FMK; 10 μM) for 12 h. Caspase-3 activity in cell extracts was measured. F, HAECs were serum starved for 12 h and then treated with 5% FBS or various concentrations of DHEA for 24 h, followed by apoptosis measurements using ELISA. Data (mean ± SE) for A, B, E, and F were expressed as a percentage of values measured in FBS alone-treated cells derived from three to five separate experiments performed in triplicate; data (mean ± SE) for D were expressed as percentage of TUNEL-positive cells among all cells in a microscopic view, obtained from three independent experiments performed in duplicate. *, $P <$ 0.05 *vs.* vehicle alone-treated cells.

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Similarly, serum deprivation for 24 h induced over 200% increase in apoptosis of HAECs, but addition of DHEA reduced the serum starvation-induced apoptosis of HAECs by 21–41% (Fig. 1F), suggesting a nonspecies-specific antiapoptotic effect of DHEA.

DHEA induces Akt phosphorylation

It is well recognized that the Akt signaling pathway plays an important protective role in cell viability and apoptosis (32). To investigate the underlying mechanism by which DHEA protects against serum deprivation-induced apoptosis, we therefore evaluated the effect of DHEA on Akt activation. Incubation of BAECs with DHEA induced a rapid increase in Akt phosphorylation detected by immunoblotting (Fig. 2A). The level of Akt phosphorylation peaked at 15 min of incubation with DHEA and returned to basal level over 60 min. Dose-response studies demonstrated that DHEA induced maximum Akt phosphorylation at a concentration of 0.1 nM (Fig. 2B). Total Akt expression was unaltered by DHEA over the time course of these studies. Pharmacological inhibition of PI3K, the upstream kinase of Akt with LY294002, completely prevented the phosphorylation of Akt induced by DHEA (Fig. 2C). These data suggest that DHEA activates the PI3K/Akt signaling cascade in these cells.

PI3K/Akt-dependent effects of DHEA on cell apoptosis

Given the evidence that DHEA both enhances serum-starved cell survival and induces Akt activation, we next determined whether the activation of Akt was involved in DHEA-induced cell viability. BAECs were preincubated with LY294002, for 30 min, or transfected with plasmids containing the dominant-negative Akt cDNA. Consistent with the LY294002 effect to inhibit DHEA-induced phosphorylation of Akt (Fig. 2C), inhibition of PI3K blocked the antiapoptotic effect of DHEA (Fig. 3A). Additionally, in cells transfected with the dominant-negative Akt plasmid, the cytoprotective effect of DHEA was reduced by 43.6%, whereas transfection of cells with the control plasmids was without effect (Fig. 3B).

Gα_i-dependent effects of DHEA on Akt activation and cell viability

We have shown previously that PTX-sensitive G proteins are involved in the DHEA signaling in ECs (22). To further characterize the signaling events involved in the DHEA action in ECs, we examined whether DHEA-induced Akt activation was mediated through PTX-sensitive G proteins, which are expressed in ECs and are known to couple agonist-dependent membrane receptors to activate the Akt signaling cascade (22, 33). Preincubation of BAECs with PTX (100 ng/

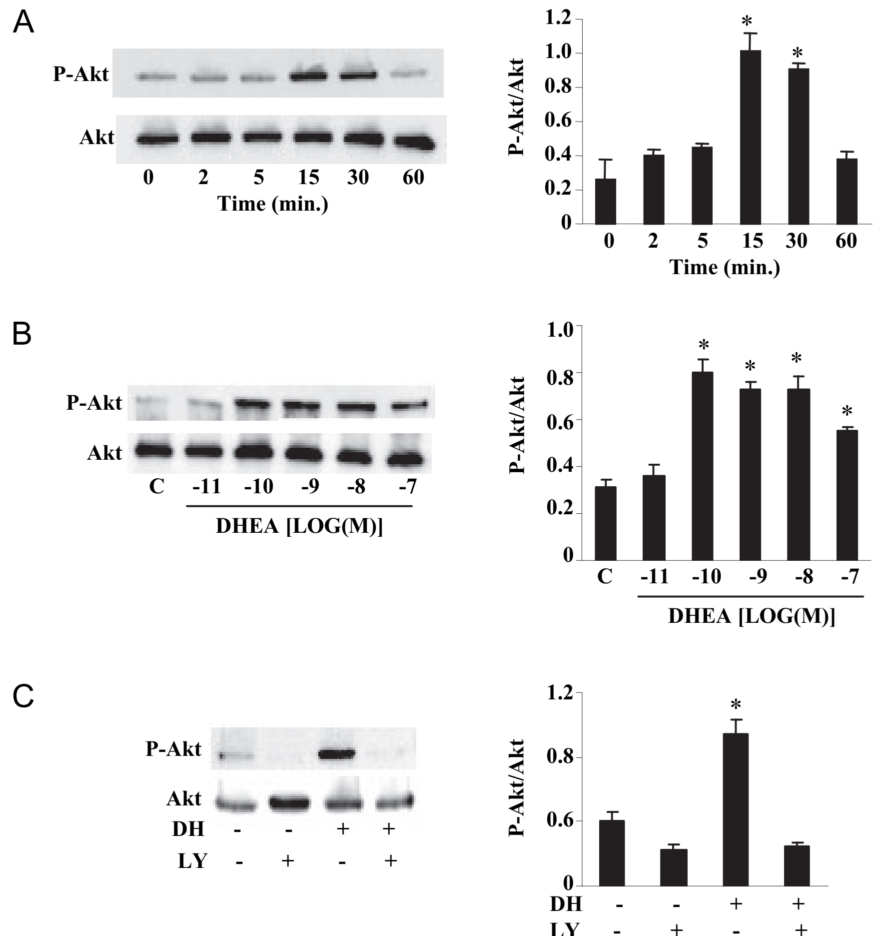

FIG. 2. DHEA stimulates Akt phosphorylation in BAECs. BAECs were incubated with 1 nM DHEA for different times (A) or with various concentrations of DHEA or vehicle (C) for 15 min at 37 C (B). C, Cells were exposed to LY294002 (LY; 5 μM) for 30 min before stimulation with 1 nM DHEA (DH) for 15 min. Phosphorylation of Akt was detected by Western blot using a phospho-specific Akt antibody (*top*), normalized to total Akt (*bottom*). The bar graphs (*right*) represent three independent experiments. *, $P < 0.05$ *vs.* vehicle alone-treated controls or LY294002 preincubated cells.

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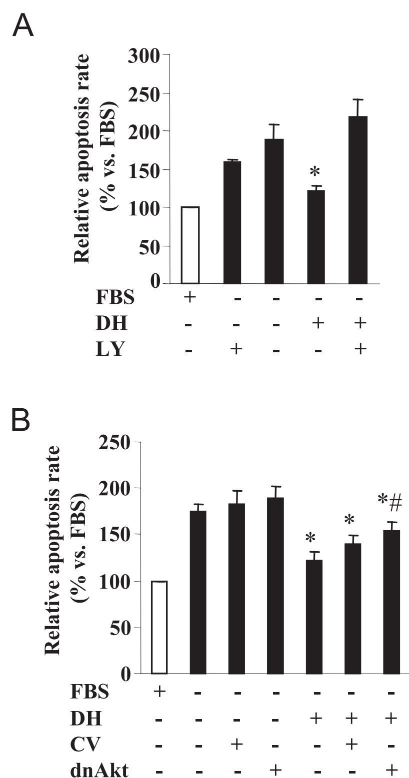

FIG. 3. The antiapoptotic effect of DHEA is PI3K/Akt dependent. A, BAECs were preincubated with LY294002 (LY; 5 μM) for 30 min or transfected with Akt dominant-negative plasmid (dnAkt) or control vector (CV) (B), followed by incubation with FBS (5%), or 1 nM DHEA (DH) or vehicle in serum-free medium at 37 C. At 24 h later, incubation was terminated for apoptosis assay. Data were expressed as mean ± SE of four separate experiments, each performed in triplicate. *, $P < 0.05$ *vs.* vehicle alone-treated control, LY294002 preincubated or Akt dominant-negative plasmid-transfected cells; #, $P < 0.05$ *vs.* DHEA alone-treated cells.

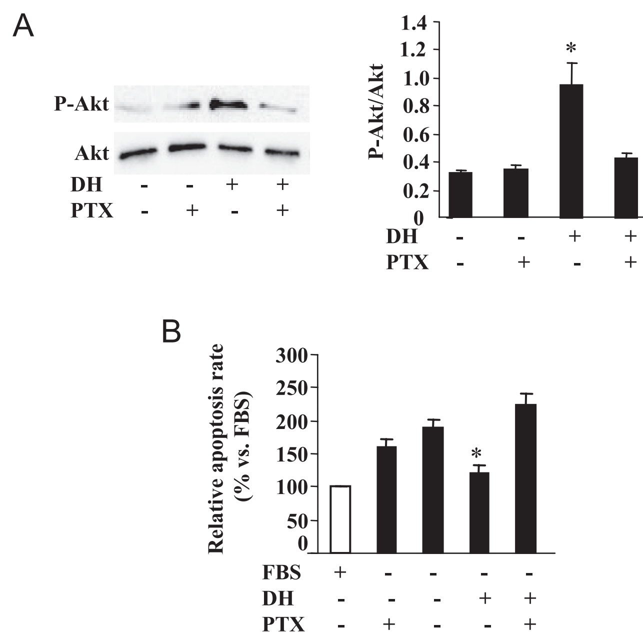

FIG. 4. DHEA stimulates Akt phosphorylation and protects BAECs against serum deprivation-induced apoptosis by a $G\alpha_i$ protein-coupled action. A, BAECs were incubated with 1 nM DHEA (DH) or vehicle for 15 min, or were preincubated with or without PTX (100 ng/ml) for 24 h and then incubated with 1 nM DHEA (DH) or vehicle for 15 min at 37 C. Phosphorylated Akt (*top*), total Akt (*bottom*), and normalized densitometry measurements (bar graph) were determined. B, BAECs were preincubated with or without PTX for 24 h in serum-free medium and then treated with 5% FBS, 1 nM DHEA (DH), or vehicle for 24 h. Apoptosis was measured by ELISA, and values were normalized as a percentage of those measured in FBS-treated cells. The data were derived from three independent experiments performed in triplicate and expressed as mean ± SE. *, $P < 0.05$ *vs.* vehicle alone-treated control or PTX preincubated cells.

ml) for 24 h did not alter the basal phosphorylation of Akt in BAECs but fully inhibited the phosphorylation of Akt induced by DHEA (Fig. 4A). Accordingly, exposure of the cells to DHEA in the presence of PTX effectively prevented the antiapoptotic effect of DHEA in ECs (Fig. 4B).

The antiapoptotic effect of DHEA is independent of the ER or conversion to estradiol

There is evidence that some of the long-term biological effects of DHEA are attributable to conversion of the hormone into estrogens (18). In addition, previous studies have shown that 17β-estradiol also can activate Akt in ECs (34). However, incubation of the cells with an excess amount of the ER antagonist ICI blocked the cytoprotective effect of estradiol but failed to block the cytoprotective effect of DHEA (Fig. 5A). Consistent with this, the presence of ICI was effective in blocking the estrogen-induced Akt phosphorylation but did not cause a significant change in the DHEA activation of Akt (Fig. 5B). To further confirm that the antiapoptotic effect of DHEA in ECs is not attributable to its conversion to estradiol by aromatase, we assessed the effect of DHEA (1 nM) on cell apoptosis and Akt activation in the presence and absence of the selective aromatase inhibitor aminoglutethimide (4 μM). The concentration of aminoglutethimide selected was based on other studies that demonstrated significant aromatase inhibition in ECs at this concentration (35). DHEA significantly inhibited serum deprivation-induced apoptosis (Fig. 5C), and this effect of DHEA was similar to that observed in the presence of aminoglutethimide. Consistent with this result, inhibition of aromatase had no effect on DHEA-induced Akt phosphorylation in ECs (Fig. 5D). These data indicated that the antiapoptotic effect of DHEA did not involve metabolism to estradiol or ER-dependent signaling mechanisms.

The antiapoptotic effect of DHEA is mediated by Akt-dependent expression of Bcl-2

To elucidate the downstream target for DHEA-activated Akt, we first determined whether DHEA enhanced the expression of the antiapoptotic protein Bcl-2. As shown in Fig. 6A, serum deprivation suppressed the Bcl-2 protein level in ECs. Addition of DHEA reversed the serum deprivation-impaired Bcl-2 protein expression by 52%. Inhibition of PI3K with LY294002 attenuated this DHEA effect by 54% (Fig. 6B). DHEA directly increased Bcl-2 promoter activity, as determined by a Bcl-2 promoter-driven luciferase reporter assay (Fig. 6C), indicating that DHEA may regulate Bcl-2 expression at the transcriptional level. To confirm this, real-time quantitative RT-PCR was used to determine whether DHEA

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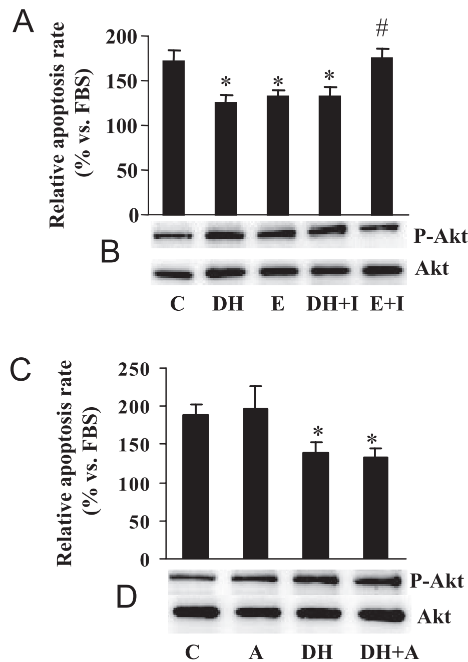

FIG. 5. The antiapoptotic effect of DHEA is independent of ER or conversion to estradiol. Serum-deprived BAECs were preincubated with or without the specific ER antagonist ICI (I; 100 nM) for 30 min (A and B) or the selective aromatase inhibitor aminoglutethimide (A; 4 μM) for 60 min (C and D). The cells were then treated with DHEA (DH; 1 nM), 17β-estradiol (E; 1 nM), or vehicle (C) in the continued presence or absence of inhibitors at 37 C. Incubation was either terminated after 15 min to determine Akt phosphorylation (B and D) or continued for 24 h to determine cell apoptosis by ELISA (A and C). Data were expressed as mean ± SE of observations from three separate experiments, each performed in triplicate. *, $P < 0.05$ *vs.* vehicle alone-treated cells; #, $P < 0.05$ *vs.* 17β-estradiol alone-treated cells.

had an effect on mRNA expression in serum starved BAECs. As shown in Fig. 6D, exposure of ECs to 1 nM DHEA increased the Bcl-2 mRNA level by 47% over the control. Furthermore, we examined whether DHEA modulates Bcl-2 transcription through the PI3K/Akt signaling pathway. The Bcl-2 promoter-transfected cells were treated with DHEA in the presence or absence of LY294002 for 24 h. Consistent with its effect on DHEA-induced Bcl-2 protein expression, inhibition of the PI3K/Akt signaling by LY294002 inhibited the DHEA-induced promoter activity by 48% (Fig. 6E).

Finally, we tested whether Bcl-2 mediates the DHEA effect on apoptosis. We delivered Bcl-2 antibody into the BAECs with a protein delivery reagent that has been successfully used in previous studies (36) and further validated in our preliminary experiments. As shown in Fig. 6F, transfection of the cells with Bcl-2 antibody attenuated the antiapoptotic effect of DHEA by 43%, whereas preimmune IgG had no significant effect on blocking DHEA-induced cytoprotection, indicating that the antiapoptotic effect of DHEA was at least partially mediated by Bcl-2 protein through activation of Akt.

Discussion

Vascular ECs, which not only serve as a biological barrier separating circulating blood and peripheral tissues but also secrete various vasoactive substances, play a pivotal role in maintaining normal vascular function. Aging is known to be the major risk factor for the development of atherosclerosis. On a cellular level, enhanced EC apoptosis is associated with aging (25, 37) and may be an important factor that initiates the pathogenesis of aging-associated vascular disease such as atherosclerosis (38). Indeed, many studies have demonstrated that endothelial apoptosis is increased in atherosclerotic lesions compared with normal vessels (38, 39). In this study, we demonstrated that DHEA, at physiological concentrations, inhibited serum deprivation-induced apoptosis of both bovine and human vascular ECs. This suggests that DHEA may be a prosurvival factor for the vascular endothelium, providing a critical antiapoptotic environment to protect the vascular endothelial monolayer against apoptosis and thereby maintaining endothelial integrity and function. Based on these data, it is interesting to speculate that the rapid decline of DHEA with aging may contribute to the age-associated increase in apoptosis of ECs and thereby the development of atherosclerosis.

Several factors protect cells from apoptosis by activating the PI3K/Akt signaling pathway, leading to the inhibition of the apoptotic signaling cascade (40–42). We showed that DHEA stimulated a rapid Akt phosphorylation in vascular ECs, with a maximal activation at 15 min incubation, and the relative magnitude of the response declined after 30 min. Consistent with the dose-response relationship for the cytoprotective effect of DHEA, the activation of Akt by DHEA was also maximal at concentrations of DHEA between 0.1 and 10 nM. These concentrations are well within the physiological plasma concentrations of DHEA in adult humans. Our data further demonstrated that PI3K is a proximal element of Akt activation because inhibition of PI3K completely blocked DHEA-mediated activation of Akt. These findings provide the first evidence that DHEA activates a critical prosurvival protein kinase, Akt, in ECs. Indeed, the antiapoptotic effect of DHEA was inhibited by either a specific inhibitor of PI3K or by expression of a dominant-negative mutant of Akt. These data further confirmed that PI3K and Akt are crucial signaling components in the signal transduction pathway leading to endothelial survival induced by DHEA.

Although accumulating evidence indicates that DHEA has vascular effects that are independent of the estrogenic effects (19, 20, 22, 23), it may also exert its biological effect through conversion into estradiol (18), which has been shown to induce the PI3K/Akt pathway through the plasma membrane ER in ECs (34). However, the antiapoptotic effect of DHEA in ECs appears to be independent of estrogens. First, we found that there was no detectable estradiol in the conditioned culture media after 24 h incubation in the presence of DHEA and ECs (data not shown). This is consistent with data recently published by others (20). Second, inhibition of aromatase, the final enzyme involved in conversion of DHEA to estrogen, did not inhibit the effects of DHEA on Akt or apoptosis. Third, our data demonstrated that an ER antagonist completely blocked the antiapoptotic effect of estrogen but failed to reverse this action by DHEA. Consistent with this result, we (21, 22) and others (23) clearly show that the acute effect of DHEA on endothelial nitric oxide synthase

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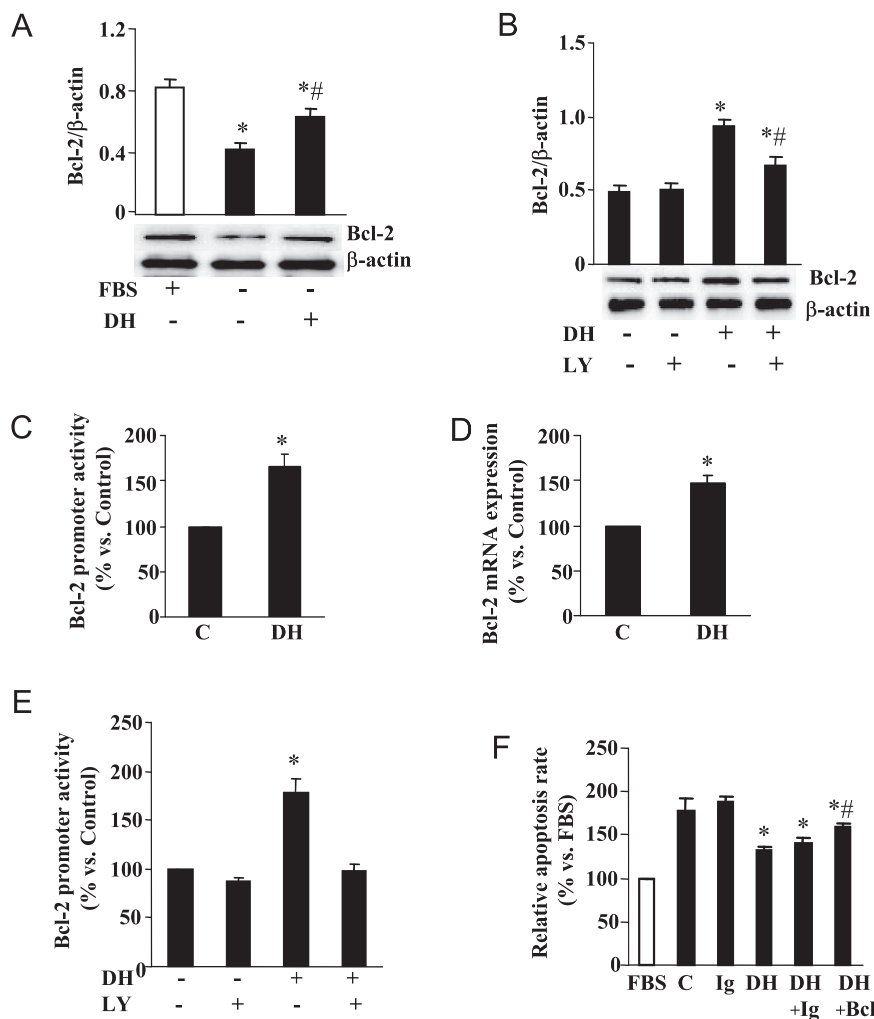

FIG. 6. The antiapoptotic effect of DHEA is mediated by antiapoptotic Bcl-2 protein through PI3K/Akt. A, BAECs were serum starved for 24 h and then treated with FBS (5%), DHEA (DH; 1 nM), or vehicle (C) for 24 h. B, BAECs were serum starved for 24 h and then preincubated with LY294002 (LY; 5 μM) for 30 min, followed by addition of DHEA (DH; 1 nM) or vehicle (C) for 24 h. The level of Bcl-2 in the treated cell extracts was measured by Western blot and normalized to β-actin content. C, BAECs were cotransfected with Bcl-2 reporter construct and pRL-cytomegalovirus plasmids. After serum starvation for 12 h, cells were treated with DHEA (DH; 1 nM) or vehicle (C) for 24 h. Firefly luciferase activity, normalized to Renilla luciferase activity, in the cell extracts was expressed as percentage of the vehicle-treated control. D, BAECs were treated with DHEA (DH; 1 nM) or vehicle (C) for 24 h. The mRNA level of Bcl-2 was measured by real-time RT-PCR and normalized to 18S. Values were expressed as percentages of control. E, Bcl-2 promoter-transfected cells were preincubated with LY494002 (LY; 5 μM) or vehicle for 30 min and then in the presence or absence of DHEA (DH; 1 nM) for 24 h, followed by luciferase activity assay as above. F, BAECs were serum starved for 24 h and then transfected with antibody against Bcl-2 (Bcl) or preimmune IgG (Ig). Five hours later, cells were treated with DHEA (DH; 1 nM) or vehicle (C) for 24 h. Cell apoptosis analysis was then performed using ELISA. Data were expressed as mean ± SE of observations from three (A, B, and F) or four (C, D, and E) separate experiments, each performed in triplicate. *, $P < 0.05$ *vs.* vehicle alone- or preimmune IgG-treated cells; #, $P < 0.05$ *vs.* DHEA alone-treated cells.

activation is not prevented by antagonism of ER or androgen receptor. Indeed, we demonstrated previously that estradiol does not compete for the DHEA membrane binding sites on vascular ECs (22). This observation has been extended recently to sympathoadrenal medulla cells (43). Collectively, these data support the concept that DHEA acts through a novel mechanism, which is different from the effects of estradiol, to regulate vascular endothelial function.

The best characterized mechanism of steroid hormone action involves ligand interaction with intracellular receptors and initiation of gene transcription (44). There is also increasing evidence for rapid, plasma membrane-dependent actions of steroid hormones in various cell types (45–56). The rapid onset of Akt activation by DHEA supports the notion that DHEA may act on plasma membranes to initiate the Akt signaling. Indeed, we pharmacologically characterized a specific cell surface DHEA receptor on vascular ECs that is coupled to $G\alpha_i$ proteins (22). We showed in the present study that the DHEA-induced Akt activation and antiapoptotic action were completely blocked by pretreatment of the cells with PTX, which uncouples $G\alpha_i$ proteins from their cognate G protein-coupled receptors. Our finding that a rapid DHEA induction of Akt and subsequent cellular events are PTX sensitive is entirely consistent with the concept of a specific plasma membrane DHEA receptor. Data in recent publications by others are also consistent with our findings (20, 23). How this endothelial Akt pathway interacts with PTX-sensitive $G\alpha_i$ proteins and the putative DHEA receptor remains to be elucidated.

Bcl-2 has been known to play a critical role in promoting cell survival. Our data showed that Bcl-2 protein was a downstream effector of Akt in the DHEA signaling cascade, because inhibition of Akt activation blocked the DHEA-induced Bcl-2 expression. Apparently, DHEA regulates Bcl-2 at the transcriptional levels, because exposure of the cells to DHEA increased Bcl-2 promoter activity and mRNA level. Although our data show that DHEA may regulate Bcl-2 expression via the PI3K/Akt pathway, the detailed molecular mechanism of this action is unclear. The promoter region of the Bcl-2 gene contains the cAMP responsive element and nuclear factor κB (NF-κB)-sensitive motifs, and Bcl-2 is directly up-regulated by the transcriptional factors cAMP responsive element-binding protein (CREB) and NF-κB (26). In the present study, we demonstrated that DHEA stimulated the Bcl-2 promoter activity via the PI3K/Akt pathway. Akt has been found to be an upstream regulator of CREB and NF-κB (26, 57). Recent studies indicate that DHEA activates CREB and NF-κB in neuronal cells (43). Taking these results

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together, it is tempting to speculate that DHEA may protect ECs against apoptosis through up-regulation of Bcl-2 expression by a transcriptional effect mediated through activation of Akt and possibly involving CREB and NF-κB. This hypothesis needs additional investigation. Nevertheless, the role of Bcl-2 in the cellular response to DHEA is important, because neutralization of Bcl-2 by its antibody significantly attenuated the protective effect of DHEA in ECs.

There are trends, which do not reach statistical significance, to dose-dependent biphasic effects of DHEA, seen in Figs. 1, A, B, and F, and 2B. Biphasic effects of steroids are well described (58), and there are many potential mechanisms for the concentration-dependent biphasic effects of DHEA. These include dose-dependent effects on plasma membrane fluidity (59), ion channel regulation (60), differential recruitment of specific G proteins (61), or related proteins (62) to the putative G protein-coupled DHEA receptor, effects of DHEA on other intracellular receptors (*e.g.* peroxisome proliferator-activated receptor) resulting in proapoptotic effects (63), or increased levels of potentially proapoptotic metabolites of DHEA [*e.g.* testosterone (64)]. It will be important to determine the concentration-dependent effects of DHEA to understand the potential effects or side effects of DHEA *in vivo*. However, it should be noted that human trials of DHEA have most frequently resulted in high physiological plasma concentrations of 20–40 nM (65, 66) rather than concentrations of 100 nM or greater. In our *in vitro* studies reported here, these concentrations of DHEA increase endothelial Akt activation and decrease apoptosis.

In summary, we provided data showing the ability of DHEA to inhibit apoptosis in ECs under serum deprivation conditions, suggesting that DHEA may act as a survival factor in these cells. We further demonstrated that the cytoprotective effects of DHEA were ER independent but were mediated through the $G\alpha_i$-PI3K/Akt-Bcl-2 module, thereby defining a novel mechanism of this DHEA action in vascular ECs. These findings potentially provide a basic mechanism underlying the physiological effects of DHEA in the vasculature.

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