Dehydroepiandrosterone Reverses Systemic Vascular Remodeling Through the Inhibition of the Akt/GSK3-β/NFAT Axis

Sébastien Bonnet, PhD; Roxane Paulin, MS; Gopinath Sutendra, MS; Peter Dromparis, BSc; Melanie Roy, MS; Kristalee O. Watson, BSc; Jayan Nagendran, MD, PhD; Alois Haromy, BSc; Jason R.B. Dyck, PhD; Evangelos D. Michelakis, MD

Background—The remodeled vessel wall in many vascular diseases such as restenosis after injury is characterized by proliferative and apoptosis-resistant vascular smooth muscle cells. There is evidence that proproliferative and antiapoptotic states are characterized by a metabolic (glycolytic phenotype and hyperpolarized mitochondria) and electric (downregulation and inhibition of plasmalemmal K⁺ channels) remodeling that involves activation of the Akt pathway. Dehydroepiandrosterone (DHEA) is a naturally occurring and clinically used steroid known to inhibit the Akt axis in cancer. We hypothesized that DHEA will prevent and reverse the remodeling that follows vascular injury.

Methods and Results—We used cultured human carotid vascular smooth muscle cell and saphenous vein grafts in tissue culture, stimulated by platelet-derived growth factor to induce proliferation in vitro and the rat carotid injury model in vivo. DHEA decreased proliferation and increased vascular smooth muscle cell apoptosis in vitro and in vivo, reducing vascular remodeling while sparing healthy tissues after oral intake. Using pharmacological (agonists and antagonists of Akt and its downstream target glycogen-synthase-kinase-3β [GSK-3β]) and molecular (forced expression of constitutively active Akt1) approaches, we showed that the effects of DHEA were mediated by inhibition of Akt and subsequent activation of GSK-3β, leading to mitochondrial depolarization, increased reactive oxygen species, activation of redox-sensitive plasmalemmal voltage-gated K⁺ channels, and decreased [Ca²⁺]. These functional changes were accompanied by sustained molecular effects toward the same direction; by decreasing [Ca²⁺], and inhibiting GSK-3β, DHEA inhibited the nuclear factor of activated T cells transcription factor, thus increasing expression of Kv channels (Kv1.5) and contributing to sustained mitochondrial depolarization. These results were independent of any steroid-related effects because they were not altered by androgen and estrogen inhibitors but involved a membrane G protein–coupled receptor.

Conclusions—We suggest that the orally available DHEA might be an attractive candidate for the treatment of systemic vascular remodeling, including restenosis, and we propose a novel mechanism of action for this important hormone and drug. (Circulation. 2009;120:1231-1240.)

Key Words: apoptosis • mitochondria • muscle, smooth • peripheral vascular disease • remodeling • vasculature

Dehydroepiandrosterone (DHEA) is a steroid hormone and testosterone analog used extensively and safely in humans for a variety of diseases.1,2 Several studies have shown that patients with more cardiovascular risk factors and higher burdens of vascular disease have lower plasma levels of DHEA than patients with fewer cardiovascular risk factors.3 Nonetheless, a direct role of DHEA in cardiovascular diseases has been challenged,4 although animal studies using exogenous DHEA showed a significant improvement in vascular diseases like pulmonary hypertension and atherosclerosis5,6; however, the mechanism remains unknown. Because DHEA is a steroid, most of the research identifying the mechanism(s) responsible for the actions of DHEA has focused on cytosolic/nuclear steroid (both estrogen and androgen) receptors.7–9 However, several studies have demonstrated in many cell types, including vascular smooth muscle cells (VSMCs), that the action of DHEA also occurs independently of both estrogen and androgen receptors (ER and AR) activation.10–12 Interestingly, a cytosolic/nuclear receptor with high affinity for DHEA has not been identified.
although DHEA binding and modulation of membrane-associated proteins (including G protein–coupled receptors [GPCRs] in endothelial cells) have been suggested; in addition, DHEA has been shown to inhibit the insulin growth factor axis, which promotes proliferation and migration within the vascular wall.\(^{13}\)

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Indeed, DHEA decreases cell proliferation and promotes apoptosis in a variety of cell types, including cancer cells, VSMCs, and endothelial cells.\(^{10–12,14}\) In cancer, the antiproliferative and proapoptotic effects of DHEA appear to be mediated by inhibition of 3-phosphoinositide–dependent kinase/Akt (PI3K/Akt) signaling.\(^{14}\) PI3K/Akt signaling mediates both proliferative and antiapoptotic events\(^{15}\) downstream of growth factors such as platelet-derived growth factor (PDGF), which is involved in the development of vascular remodeling in systemic vascular restenosis.\(^{16}\) PI3K/Akt has various downstream targets such as Bad and mTOR, but more importantly, it phosphorylates and inactivates glycogen-synthase-kinase-3β (GSK-3β),\(^{16}\) which has been shown to regulate in part VSMC proliferation.\(^{15}\) By inhibiting the binding of hexokinase on the mitochondrial voltage-dependent anion channel (VDAC), Akt/GSK-3β can increase the mitochondrial membrane potential (ΔΨm) and thus promote the closure of the voltage-sensitive mitochondrial transition pore, inhibiting apoptosis.\(^{17}\) Another means by which GSK-3β can regulate proliferation and apoptosis is by inhibiting the translocation of the nuclear factor of activated T cells (NFAT) transcription factor into the nucleus,\(^{18}\) where it regulates the expression of several genes involved in proliferation and apoptosis in vascular diseases\(^{19,20}\) and cancer.\(^{21}\) Therefore, a PI3K/Akt-dependent NFAT activation could explain the sustained proliferative and antiapoptotic phenotype of VSMCs observed in vascular remodeling after vascular injury.\(^{19}\)

We have previously shown that DHEA reverses pulmonary hypertension in part by a redox-dependent activation of K\(^+\) channels in pulmonary artery smooth muscle cells.\(^{5}\) Because K\(^+\) channels regulate VSMC membrane potential (Em), they can control the opening of the voltage-dependent calcium channels; downregulation/inhibition of K\(^+\) channels leads to an increase in [Ca\(^{2+}\)]\(\text{cyt}\), (leading to contraction and proliferation by activation of transcription factors like NFAT), and an increase in [K\(^+\)]\(\text{cyt}\), and thus suppression of apoptosis because K\(^+\)\(^{4}\), tonically inhibits caspases.\(^{19}\)

We aimed to explore the potential therapeutic effect of DHEA as a novel treatment of vascular remodeling after injury. On the basis of preliminary observations, we hypothesized that the effects of DHEA in human VSMCs are independent of steroid receptors and involve activation of K\(^+\) channels and inhibition of the Akt and NFAT axis, which could promote a decrease in the proliferation/apoptosis index in the vascular wall and reverse proliferative vascular remodeling. We used a number of models ranging from human carotid artery smooth muscle cells (hCASMCs) in vitro, ex vivo human vessels (saphenous vein [SV] grafts) in tissue culture, and the standard in vivo rat carotid injury model, as well as pharmacological and molecular dissection of our proposed pathway. We showed that this orally available, nontoxic, inexpensive drug effectively reverses vascular remodeling after carotid injury in vivo or exposure to growth factors like PDGF in vitro. Thus, we propose that DHEA could be a potential novel therapy for vascular remodeling and restenosis after injury. In addition, our work provides new insight and clarifies several aspects of the vascular effects of this readily available over-the-counter drug that has remained elusive.

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**Methods**

All experiments were performed with the approval of the University of Alberta and Laval University Animal and Human Ethics committees.

**Cell Culture**

hCASMCs (>6 passages) were grown in high-glucose DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic (Gibco, Invitrogen, Burlington, Ontario, Canada). Before treatment, VSMCs were placed for 24 hours in DMEM plus 0.1% serum. PDGF-BB (30 ng/mL) and DHEA or BSA-DHEA (5 to 100 μmol/L) was administered for 48 hours. DHEA was prepared in ethanol (~0.1% final) as previously described.\(^{3}\) Proper control experiments were performed with ethanol and did not show any effects.

**SV Graft in Tissue Culture**

We used undistended, nonvaricosed SVs harvested from 8 patients who had undergone surgery for ischemic heart disease. SVs were typically cut into 3 pieces. The first piece was kept for 5 days in 10% FCS and then fixed and stained with hematoxylin and cosin to measure the wall thickness before any remodeling processes. The second and the third pieces were cultured and exposed to PDGF (30 ng/mL) for 5 days to promote remodeling in the absence (piece 2) and presence (piece 3) of DHEA. The veins were then fixed and stained to measure the wall thickness compared with piece 1.

**Adenoviral Infection**

Proliferation of the virus and infection of VSMCs were performed as previously described.\(^{22}\)

**Confocal Microscopy/Immunofluorescence, Immunoblotting, Electrophysiology**

All experiments were performed as previously described.\(^{19,21}\)

**Rat Carotid Artery Balloon Injury**

Male Sprague-Dawley rats (300 g) were used. Under anesthesia, a neck incision was made. A 20-mm section of the right common carotid artery was isolated and temporarily occluded to prevent retrograde blood loss. After proximal arteriotomy, the lumen was flushed with heparinized saline, and a 2F Fogarty embolectomy catheter was introduced to perform an antegrade balloon inflation (2 atm) along a 15-mm common carotid artery segment. The arteriotomy was closed with 8–0 Prolene sutures, and perfusion was restored. One week after surgery, treated rats received 3 mg · kg\(^{-1}\) · d\(^{-1}\) DHEA (Calbiochem, San Diego, Calif) via their drinking water for 1 week. By measuring the amount of the water consumed by each rat, we were able to adjust and deliver the planned total daily dose for the treated rats.

**Vascular Doppler and Ultrasound Techniques**

Doppler imaging and ultrasound were performed as previously described.\(^{19}\)
Averaged data are mean±SEM. Normality of our data was assessed by the Shapiro-Wilk normality test. All our data were normally distributed ($P>0.05$). For comparison between 2 means, we used unpaired Student t test. For comparison between >2 means, we used 1-way ANOVA followed by Tukey-Kramer tests. $P<0.05$ was considered as significant.

Results
DHEA Decreases hCASMC Proliferation Through Inhibition of the Akt/GSK3β Axis Independently of Both ER and AR but Through a Plasma Membrane GPCR

Using immunofluorescence for proliferating cell nuclear antigen and confocal microscopy, we demonstrated that DHEA (100 μmol/L) decreased PDGF-induced hCASMC proliferation, essentially to control levels (Figure 1A). This effect was associated with a reversal of activation in the Akt-GSK3β axis because DHEA decreased the ratio of phosphorylated (P) Akt and P–GSK-3β expression over their total (nonphosphorylated) forms measured by immunoblots (Figure 1A).

Adenoviral infection of hCASMCs with a constitutively active myr-Akt1 (but not with the empty green fluorescent protein–only virus) or treatment with SB216763 (a GSK3β inhibitor)23 completely blocked the action of DHEA (Figure 1A). These results suggest that inhibition of Akt signaling mediates the effects of DHEA on hCASMC proliferation. We then studied whether the effects of DHEA on hCASMCs were independent of nuclear steroid receptor signaling and involved a membrane receptor. Western blots showed that the effects of DHEA on Akt signaling were not mediated by the stimulation of steroid receptors because neither ER (ICI-182,780, fluvestran) nor AR (flutamide) inhibitors12 blocked the effects of DHEA (Figure 1B). Moreover, a cell-impermeable BSA-conjugated form of DHEA decreased P-Akt and P–GSK-3β, suggesting the involvement of a plasma membrane receptor. The existence of a DHEA plasma membrane receptor has recently been suggested in endothelial cells11,24 and neurons, but its presence and function in human VSMCs remain unknown. We speculated that DHEA action might involve a GPCR, particularly because there is some evidence that such receptors can affect downstream Akt
Using GPCR inhibitors (GDP-β-S and pertussis toxin), we demonstrated that the effects of BSA-DHEA on the Akt signaling pathway were inhibited, suggesting that the DHEA plasma membrane receptor in hCASMCs belongs to the GPCR family.

**DHEA-Dependent Activation of GSK-3β Inhibits Hexokinase/VDAC Interaction, Depolarizing ΔΨm and Promoting Cytochrome C Release and Apoptosis**

For DHEA to reverse established vascular remodeling, it should be able to induce apoptosis, ideally sparing quiescent cells. In PDGF-treated hCASMCs, DHEA induced apoptosis measured by terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL), as well as increased caspase 3 and 9 activities (Figure 2A). The activation of caspase 9 and the fact that in the DHEA-treated cells there is leakage of cytochrome c out of the mitochondria into the cytosol (Figure I of the online-only Data Supplement) suggested activation of mitochondria-dependent apoptosis.

The proapoptotic effects of DHEA were once again blocked by a GSK-3β inhibitor (SB216763) and mimicked by another PI3K/Akt inhibitor (LY294002) (Figure 2A). Because the mitochondrial transition pore is voltage dependent, the mitochondrial membrane potential (ΔΨm) is an index of the threshold for mitochondria-dependent apoptosis. As expected, PDGF-treated hCASMCs had increased ΔΨm (indicating a state of apoptosis resistance) compared with quiescent cells (Figure 2B). This mitochondrial hyperpolarization was reversed by DHEA (Figure 2B). Like DHEA, LY294002 (an Akt inhibitor and thus GSK-3β activator) inhibited the increase in ΔΨm in PDGF-treated hCASMCs, whereas PDGF-stimulated hCASMCs without DHEA showed significant colocalization between hexokinase II (green) and mitochondria (red), giving a yellow staining pattern in the merged photographs. PDGF-stimulated hCASMCs treated for 48 hours with a cell-permeable competitive peptide (100 μmol/L) inhibiting the interaction between hexokinase II and VDAC depolarized ΔΨm (n=100 cells from 5 experiments; P<0.05), and addition of DIDS returned ΔΨm to the pretreated hCASMC levels. DIDS blocked the effects of DHEA on ΔΨm, confirming that DHEA promotesVDAC opening.

**Figure 2.** DHEA-dependent activation of GSK3β inhibits hexokinase/VDAC interaction, depolarizing ΔΨm and promoting apoptosis. A, In PDGF-stimulated hCASMCs, DHEA promotes apoptosis (TUNEL; n=150 per group; *P<0.001) and caspase 3 and 9 activation, all of which were inhibited by the GSK3β inhibitor SB216763. B, DHEA-induced mitochondrial depolarization is mediated by the inhibition of Akt signaling. PDGF-stimulated hCASMCs have hyperpolarized ΔΨm vs control cells (TMRM; n=100 per group; P<0.001). Like LY294002, DHEA depolarizes ΔΨm (n=100 per group; P<0.05). Moreover, the GSK3β inhibitor SB216763 (10 μmol/L) or infection with constitutively active Akt adenovirus blocked the effects of DHEA. C, PDGF-stimulated hCASMCs with DHEA showed diffuse cytoplasmic staining of hexokinase II (no colocalization between hexokinase II [green] and mitochondria [red]), whereas PDGF-stimulated hCASMCs without DHEA showed significant colocalization between hexokinase II (green) and mitochondria (red), giving a yellow staining pattern in the merged photographs. PDGF-stimulated hCASMCs treated for 48 hours with a cell-permeable competitive peptide (100 μmol/L) inhibiting the interaction between hexokinase II and VDAC depolarized ΔΨm (n=-100 cells from 5 experiments; P<0.05), and addition of DIDS returned ΔΨm to the pretreated hCASMC levels. DIDS blocked the effects of DHEA on ΔΨm, confirming that DHEA promotesVDAC opening.

**Additional Figure:**

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To further elucidate the mechanism by which GSK-3β mediates the effects of DHEA on ΔΨm, we followed a recently described cancer paradigm, ie, Akt-dependent translocation of hexokinase to mitochondrial membranes, where it inhibits VDAC and thus increases ΔΨm and resistance to apoptosis. In an impressively similar manner, in PDGF-treated hCASMCs, hexokinase (shown in green) colocalizes with mitochondria (stained red by MitoTracker Red), giving a yellow pattern; this is prevented by either DHEA or LY294002 (Figure 2C). To show that hexokinase translocation increases ΔΨm via VDAC inhibition in proliferating hCASMCs, we used a cell-permeable competing peptide (RQIKIWFQNRRMKWKKMIASHLLAYFFTELN-amide) that binds VDAC, prohibiting its phosphorylation by hexokinase (mimicking activated GSK-3β). As expected, the increase in ΔΨm was prevented by this peptide mimicking DHEA (Figure 2C). The direct VDAC inhibitor DIDS (which inhibits VDAC by a different mechanism) was still able to increase ΔΨm, indicating that in the DHEA- and peptide-treated cells, the VDAC remained open and functional, thus supporting the notion that the effects of DHEA on VDAC were functional and involved the inhibition of the hexokinase translocation. As previously described, a similar cell-permeable peptide used as control (antennapedia) did not affect ΔΨm (data not shown).

**DHEA-Dependent Activation of GSK-3β Inhibits NFATc1 Activation, Promoting Kv1.5 Expression and Thus Decreasing [Ca2+]i**

In both pulmonary hypertension and cancer, the maintenance of a proproliferative and antiapoptotic phenotype was due to the activation of the transcription factor NFAT. NFAT activation leads to the downregulation of important K+ channels like Kv1.5, which is known to play a critical role in the maintenance of both VSMC membrane potential and apoptosis. We and others have described the ability of DHEA to open both Kv and BKCa channels, decreasing [Ca2+]i acutely. However, to sustain these effects, a mechanism reversing the downregulation of the K+ channels is necessary. In addition to the effects on the VDAC, another pathway by which GSK-3β can affect proliferation and apoptosis is through the inhibition of NFAT. As expected, DHEA reversed NFATc1 activation (ie, translocation to the nucleus) in PDGF-stimulated hCASMCs (Figure 3A). NFATc1, the isoform mostly studied in carotid arteries, was blocked by DHEA-induced Kv1.5 upregulation, demonstrating that the effects of DHEA are mediated by Akt inhibition. Despite increasing the BKCa current, DHEA did not affect BKCa expression, suggesting that NFAT does not control BKCa expression in hCASMCs.

Figure 3. DHEA-dependent activation of GSK3β inhibits NFAT activation, promoting Kv1.5 expression and thus decreasing [Ca2+]i. A, Like LY294002, DHEA decreases NFATc1 activation in PDGF-treated hCASMCs (n=75 per group; *P<0.001). B, PDGF-stimulated hCASMCs had smaller K+ current density vs control cells. DHEA treatment (for 48 hours) reversed these effects by increasing both 4-AP- (5 mmol/L) and IbTx- (100 nmol/L) sensitive currents (n=8; *P<0.05). As expected by increasing the K+ current, DHEA decreases [Ca2+]i in proliferative hCASMCs; n=100 per group; *P<0.05. C, In PDGF-stimulated hCASMCs, NFATc1 activation is associated with a downregulation of Kv1.5, whereas both quiescent and DHEA-treated cells showed no NFATc1 activation and maintained Kv1.5 expression (n=50 cells per experiments; 5 experiments; *P<0.05). Constitutively active myr-Akt blocked DHEA-induced Kv1.5 upregulation, demonstrating that the effects of DHEA are mediated by Akt inhibition. Despite increasing the BKCa current, DHEA did not affect BKCa expression, suggesting that NFAT does not control BKCa expression in hCASMCs.
regulates its own expression. Because VSMCs are excitable cells, their K⁺ channels are active at resting state and involved in contraction and proliferation mechanisms. Patch-clamping experiments show that normal hCASMC K⁺ current (IK⁺) is composed of a mixture of both Kv (4-aminopyridine-sensitive [4-AP]) and BKCa (iberiotoxin-sensitive [IbTx]) channels. PDGF decreases both 4-AP– and IbTx-sensitive current, increasing [Ca²⁺]i (Figure 3B) and NFATc1 activation. DHEA increased both Kv and BKCa currents (Figure 3B). Although PDGF did not significantly decrease BKCa protein expression, it significantly decreased Kv1.5, which once again was reversed by DHEA or by the PI3K/Akt blocker LY294002 (Figure 3C). Infection with constitutively active myr-Akt1 adenovirus prevented these effects of DHEA (Figure 3C). These data suggest that the effects of DHEA on Kv1.5 expression are mediated by NFATc1, whereas the ability of DHEA to increase BKCa current without increasing BKCa protein suggested that DHEA activates existing BKCa channels, perhaps by the redox mechanism that we had previously described in pulmonary artery VSMCs.5

DHEA Prevents Vascular Remodeling In Vitro

We studied normal human SVs (obtained at the time of bypass surgery in 8 patients) exposed to PDGF in tissue culture for 5 days, a described model of vascular remodeling.33 In this model, the primary effects of drugs in proliferation of VSMCs in situ can be studied independently of the effects of flow or circulating factors. DHEA prevented PDGF-stimulated SV proliferative remodeling (ie, VSMC proliferation and increased vessel wall thickness). Through an ER/AR-independent mechanism, DHEA decreased Akt activity and increased GSK-3β activity (Figure 4A). The increase in GSK-3β is compatible with the mitochondrial depolarization caused by DHEA in the vessel wall (Figure 4B) and is in agreement with the hCASMC data. Both the mitochondrial depolarization and upregulation of Kv1.5 are associated with a proapoptotic state, which was confirmed by the ability of DHEA to activate apoptosis in the vessel wall as measured by both TUNEL (n=50 per patient; 5 patients; P<0.001) and caspase 3 activity (measured in pooled SVs from 3 patients). The increased apoptosis and decreased proliferation (proliferating cell nuclear antigen; n=50 per patient; 5 patients; P<0.001) explain the reversal of PDGF-induced vascular remodeling, as shown by the decrease in vessel wall thickness (n=8 patients; P<0.05).
apoptosis in the vessel wall (Figure 4B). The increased apoptosis and decreased proliferation explain the reversal of PDGF-induced vascular remodeling, as shown by the decrease in vessel wall thickness (Figure 4B). Once again, DHEA had no effects on healthy SVs (Figure V of the online-only Data Supplement).

**DHEA Inhibits Injury-Induced Vascular Remodeling In Vivo**

Sprague-Dawley rats were subjected to carotid artery balloon injury (versus the sham procedure), and they were randomized after 1 week into vehicle- (water plus ethanol/H110210.1%) and DHEA-treated groups (3 mg/kg) for 1 additional week. The vehicle-treated group showed increased carotid Doppler velocity, decreased carotid artery internal diameter after denudation injury (bottom; n=7; P<0.05). As expected, wall thickness measured on hematoxylin and eosin–stained slides (5 slides per rat; 5 rats) expressed as percent of sham was significantly decreased by DHEA. *P<0.05. B, DHEA inhibits NFAT activation (n=20 cells per rat; 5 rats; P<0.05), thereby reversing Kv1.5 downregulation (n=3 rats; P<0.05) but, as in cultured cells, did not affect BKCa expression (n=3 rats; P=0.05). C, DHEA decreases proliferation (percent proliferating cell nuclear antigen [PCNA]; n=100 cells; 5 rats) and increases apoptosis within the carotid artery wall (percent TUNEL; n=100) and caspase 3 and 9 activities in pooled carotid arteries from 3 rats (P<0.05). D, These effects are associated with a decrease (P<0.05) in Akt activation.

**Discussion**

Here, we show that the clinically available generic drug DHEA might be a novel therapy for the reversal or prevention of injury-induced vascular remodeling. Despite the progress in our understanding of signaling in the injured vascular wall, there are currently no systemically given drugs that can reverse or inhibit remodeling. Recently, encouraging data have been obtained by the use of expensive drug-coated stents at the time of angioplasty, aiming to decrease cell proliferation locally. The ability of DHEA to reverse remodeling in the injured vessels after oral intake without affecting

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**Figure 5.** DHEA inhibits vascular remodeling in vivo. A, DHEA (3 mg/kg) started 1 week after surgery for 1 week improves carotid perfusion (top; Doppler velocity; n=7; P<0.05) and decreases carotid artery internal diameter after denudation injury (bottom; n=7; P<0.05). As expected, wall thickness measured on hematoxylin and eosin–stained slides (5 slides per rat; 5 rats) expressed as percent of sham was significantly decreased by DHEA. *P<0.05. B, DHEA inhibits NFAT activation (n=20 cells per rat; 5 rats; P<0.05), thereby reversing Kv1.5 downregulation (n=3 rats; P<0.05) but, as in cultured cells, did not affect BKCa expression (n=3 rats; P=0.05). C, DHEA decreases proliferation (percent proliferating cell nuclear antigen [PCNA]; n=100 cells; 5 rats) and increases apoptosis within the carotid artery wall (percent TUNEL; n=100) and caspase 3 and 9 activities in pooled carotid arteries from 3 rats (P<0.05). D, These effects are associated with a decrease (P<0.05) in Akt activation.
normal vessels and cells (Figures III and V of the online-only Data Supplement) is very attractive clinically.

More importantly, we show that DHEA can achieve its beneficial effects in vascular remodeling not only by decreasing cell proliferation but also by selectively inducing apoptosis in proliferative cells. We describe a comprehensive mechanism for these effects (Figure 6), showing that DHEA inhibits the activation of the Akt axis, which has been shown to occur in systemic vascular injury. We used several pharmacological agents and adenovirus-mediated Akt overexpression and showed in hCASMCs, human vascular organ cultures, and standard in vivo rat models that DHEA inhibits Akt, thus activating GSK-3\beta. The activation of GSK-3\beta has 2 important effects: By inhibiting the translocation of hexokinase in mitochondria, it promotes mitochondrial depolarization, which decreases the threshold of mitochondria-induced apoptosis, and by promoting the inhibition of the calcium and calcineurin-sensitive NFATc1, it reverses the downregulation of Kv channel expression. This in turn has 2 effects: decreased intracellular calcium levels, limiting proliferation and NFATc1 activation, and inhibited [K\textsuperscript{+}]-induced suppression of caspases. The ability of DHEA to break the described positive feedback loop between [Ca\textsuperscript{2+}], NFATc1 activation, and Kv1.5 expression suggests that the effects of DHEA might be sustained and effective in reversing vascular remodeling.

It is possible that alternative or additional mechanisms might explain our results, at least in part. Namely, DHEA could inhibit G6PDH directly\textsuperscript{34} or indirectly\textsuperscript{35}; this is important because Leopold et al\textsuperscript{36} showed that G6PDH inhibition blocks the Akt axis, at least in bovine endothelial cells. G6PDH is the first enzyme of the pentose-phosphate pathway, a major producer of NADPH (an important regulator of the cellular redox status) and ribulose 5-P (the precursor of ribose 5-P, ie, a critical building block of nucleic acids). Thus, it is not surprising that G6PDH is critical for proliferating cells. Although early work suggested a direct cytosolic inhibition of G6PDH by DHEA,\textsuperscript{34} a later study\textsuperscript{35} showed that aldosterone, a steroid structurally similar to DHEA, blocks G6PDH through a protein kinase A/cAMP responsive element binding–dependent mechanism, resulting in endothelial dysfunction. Other studies in cancer have failed to show any effects of DHEA on G6PDH.\textsuperscript{37,38} We believe that our proposed mechanism is independent of these potential DHEA effects. First, in our human VSMCs, the DHEA-induced inhibition of Akt was prevented in the presence of GPCR inhibitors and mimicked by cell-impermeable BSA-DHEA, suggesting complete dependence on membrane signaling, not cytosolic action. Second, we previously demonstrated in VSMCs that DHEA-mediated effects on plasma membrane K\textsuperscript{+} channels and [Ca\textsuperscript{2+}], were specifically protein kinase A independent.\textsuperscript{6} Nevertheless, our study cannot definitively exclude potential effects on G6PDH in VSMCs. On the other hand, at least in hepatocytes,\textsuperscript{39} G6PDH inhibition could occur downstream of Akt inhibition. Potential cell type–specific effects of DHEA need to be considered. The beneficial effects in VSMCs that we describe might be in conflict with the adverse effects on endothelial cells; however, the former might be relevant to “vascular injury syndrome,” and the latter might be relevant to the endothelial dysfunction in heart failure syndromes. In addition, large-scale epidemiological studies had never demonstrated detrimental vascular effects, at least of the endogenous hormone.\textsuperscript{4}

Finally, although we do not know the exact mechanism by which DHEA inhibits the growth factor–induced activation of Akt, we provide novel evidence for human VSMCs that it might be mediated by a plasma membrane GPCR receptor. This is the first time in carotid VSMCs that a GPCR is

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**Figure 6.** DHEA reduces vascular remodeling via inhibition of the Akt/GSK3β/NFAT signaling axis. Originating at an as-yet unidentified plasma membrane GPCR, DHEA inhibits Akt, which in turn increases GSK3β activation. Increased GSK3β activation leads to deactivation of NFAT, which reverses Kv1.5 downregulation, thereby decreasing [Ca\textsuperscript{2+}] and further decreasing NFAT activation and its proproliferative and antiapoptotic effects. Direct effects of DHEA on Kv and BKCa channels might also contribute to reduced [Ca\textsuperscript{2+}]. Additionally, increased GSK3β activity prevents the binding of hexokinase II to VDAC, thereby inducing mitochondrial depolarization and the efflux of cytochrome c into the cytoplasm to induce apoptosis. These effects combined reduce the neointimal hyperplasia seen in vascular remodeling diseases.
proposed to mediate the effects of such a commonly used compound. In fact, there has been emerging evidence that DHEA might have ER- and AR-independent effects in endothelial and cancer cells.40 We confirm these findings in VSMCs and extend them by proposing the involvement of a GPCR.

The fact that in our protocol DHEA was initiated well after the vascular injury and was able to reverse established vascular remodeling increases the clinical relevance of our findings. Oparil et al41 showed that estrogen administration 72 hours before rat carotid injury prevents vascular remodeling, but the molecular targets of that intervention are different from ours because we show that our Akt-dependent mechanism is independent of estrogen receptor signaling. Araneo et al42 showed that DHEA before and 4 hours after injury was able to decrease structural vascular changes in thermal injury models; however, DHEA given 6 hours after the injury had no therapeutic effects. The pleiotropic effects of DHEA and the likely dependence on specific cell types suggest that some of our proposed mechanisms might not be applicable to other tissues or disease states.

The translational potential of our work is strengthened by the fact that we used clinically relevant doses in both our in vitro and in vivo experiments. Recently, Svec and Porter43 and Tummala and Svec44 reviewed several clinical trials of DHEA supplementation and correlated the oral dose with its serum levels. The dose range in most trials was between 50 and 230 nmol/L, whereas sulfate DHEA (the active form of DHEA) varied between 10 and 60 μmol/L.44,45 We have previously published the finding that DHEA 3 to 30 mg · kg⁻¹ · d⁻¹ in the rat (ie, similar to the doses in humans) results in serum levels ≥10⁻⁷ mol/L; thus, our currently used doses in vivo (3 mg · kg⁻¹ · d⁻¹) and in vitro (5 to 100 μmol/L) are similar to the levels achieved in humans in vivo.

We propose that oral ingestion of DHEA simultaneously or after vascular intervention might be a novel therapeutic strategy to prevent restenosis, particularly because DHEA does not appear to affect quiescent cells and therefore spares healthy vessels. In addition, the structural similarity with estrogen molecules suggests the ability to develop slow-release DHEA-coated stents.46 Our findings might explain the inverse relationship between the low levels of endogenous DHEA and the burden of systemic vascular disease3,47 and provide enough mechanistic data to support the conduction of DHEA studies in early-phase human studies.

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Disclosures
None.

References

CLINICAL PERSPECTIVE

In response to injury, whether by an instrument during a percutaneous intervention or by inflammation, vascular smooth muscle cells proliferate excessively, leading to lumen obliteration. In coronary interventions, there has been some success in the prevention of restenosis by expensive therapies like drug-eluting stents. Systemic and nontoxic therapies to treat restenosis at the time of vascular interventions (coronary or peripheral vessels) or locally (via a device or stent) to limit vascular remodeling.

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Dehydroepiandrosterone Reverses Systemic Vascular Remodeling Through the Inhibition of the Akt/GSK3-β/NFAT Axis
Sébastien Bonnet, Roxane Paulin, Gopinath Sutendra, Peter Dromparis, Melanie Roy, Kristalee O. Watson, Jayan Nagendran, Alois Haromy, Jason R.B. Dyck and Evangelos D. Michelakis

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Methods:

Reagents: The following reagents and their working concentrations were used: platelet-derived growth factor-BB (PDGF BB), 30 ng/mL (Upstate, Lake Placid, NY); dehydroepiandrosterone (DHEA), 5-500µM was prepare in ethanol as previously described 5(Calbiochem, San Diego, CA) proper control experiments were performed with ethanol and did not shown any effects; bovine serum albumin-conjugated DHEA (BSA-DHEA), 100 µM (Steraloids, Newport, RI); estrogen receptor blocker ICI 182,780, 100nM (Sigma-Aldrich, St. Louis, MO); androgen receptor blocker Flutamide, 100nM (Sigma-Aldrich, St. Louis, MO); GSK3β inhibitor SB216763, 10µM (Sigma-Aldrich, St. Louis, MO); PI3K inhibitor LY294002, 10µM (Calbiochem, San Diego, CA); VDAC inhibitor 4,4′-Diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), 0.5mM (Calbiochem, San Diego, CA); HexokinaseII/VDAC cell-permeable competitive binding peptide, 100µM (Calbiochem, San Diego, CA); guanosine 5′-[β-thio]diphosphate trilithium salt (GDP-β-S), 100µM (Sigma-Aldrich, St. Louis, MO); Pertussis toxin (PTx), 100 ng/mL (List Biological Laboratories, San Jose, CA); 4-aminopyridine (4-AP), 5mM (Sigma-Aldrich, St. Louis, MO); Iberiotoxin (IbTx) 100nM (Sigma-Aldrich, St. Louis, MO).

Confocal Microscopy / immunofluorescence: Apoptag apoptosis detection kit (TUNEL Serologicals, Norcross, GA) and the proliferating cell nuclear antigen (PCNA) antibody (DAKO, CA) were used as previously described 4, 6. Antibodies to cytochrome c (Calbiochem, San Diego, CA), hexokinase II (Santa Cruz Biotechnology, Santa Cruz, CA), NFAT-c1 (Novus Biologicals, Littleton, CO), Kv1.5 (Santa Cruz Biotechnology, Santa Cruz, CA), as well as Mitotracker Red, 500 nM (Molecular Probes, Invitrogen, Burlington, ON), were used as previously described 4, 6. Percent of TUNEL, PCNA or NFAT positive cells were measured in 3 different slides/experiment and minimum of 5 experiments were performed. FITC- and TRITC-conjugated (DAKO Canada, Mississauga, ON) secondary antibodies were used in immunofluorescence. For the VSMC infected with adenovirus, to avoid conflict with the GFP, we used Marina Blue (Molecular Probes, Invitrogen, Burlington, ON) secondary antibody for the cytochrome c staining. Using LSM Zeiss software, GFP was imaged in blue and cytochrome c in green, to keep consistency with the non-infected cells. Mitochondrial membrane potential was studied using tetramethylrhodamine methyl-ester perchlorate (TMRM) as previously described 4, 6. [Ca2+]i was studied using FLUO-3AM (Molecular Probes, Invitrogen, Burlington,
Adenoviral Infection: VSMC were grown on poly-L-lysine (Sigma-Aldrich, St. Louis, MO)-coated glass coverslips, and infected at a MOI of 100 in serum-free DMEM (Gibco, Invitrogen, Burlington, ON) for 6 hr allowing an infection rate of ≈60 to 80%. Media was then exchanged for DMEM with 10% FBS. Cells were treated with PDGF±DHEA 24 hr post-infection for 48 hr. Unfortunately the virus we used does not include a GFP construct to track infection rates. However, we used an identical strain of virus, only carrying a GFP construct to determine optimal MOI and infection efficiency. Infection rate was measured in every experiment by % GFP positive cells via fluorescence. Gene product activity was confirmed by immunoblot using Akt and P-Akt antibodies at dilutions of 1/1000.

Electrophysiology: Whole-cell patch clamping will be performed on cells voltage-clamped at a holding potential of -70 mV. Currents will be evoked by 200-ms test pulses from -70 to +70 mV with 20mV steps, filtered at 1kHz and sampled at 2-4kHz as previously described 4, 6.

Immunoblotting: VSMC were collected and immunoblotting was performed on pooled samples from 4 T-25 dishes (25 µg protein in pooled sample/lane). The films were digitized and quantified using 1D Image Analysis Software (Kodak, Rochester, NY). Expression was normalized to Ponceau-S to correct for loading differences. Antibodies: Kv1.5 (1:500, Sigma, Oakville, Ontario), BKCa (1:500, Sigma, Oakville, Ontario), caspase 9 and 3 (1:1000, Upstate) Akt, P-Akt; GSK3, P-GSK3 (1:1000, Cell signaling USA).

Vascular Doppler and ultrasound techniques: Carotid artery blood flow and wall thickness were measured using 15Mhz probe from a Sonos 5500 ultrasound system. M-mode was used to measure wall thickness, B-mode was used to measure internal diameter and Doppler was used to measure flow velocity.

Supplemental figure 1: A: By depolarizing mitochondria DHEA induces the release of cytochrome c into the cytosol. In PDGF-stimulated human carotid artery VSMC using confocal imaging and triple staining for mitochondria (red), cytochrome c (green), and nuclei (blue) we showed co-localization between mitochondria and cytochrome-c giving a yellow pattern, while in DHEA or LY294002 (another Akt inhibitor) treated VSMC, cytochrome c is released into the cytosol giving a diffuse green pattern (n=25 cells/per experiments, 5 experiments were performed).

Supplemental figure 2: DHEA dose-dependently depolarizes mitochondria of proliferative VSMC.
Supplemental figure 3: DHEA depolarizes mitochondria of proliferative VSMC via activation of a plasma membrane GPCR without affecting quiescent VSMC. Human carotid artery VSMC were incubated with PDGF, the PI3K inhibitor LY294002 (10µM), DHEA (100µM), or BSA-DHEA (100µM), and the G-protein inhibitors GDP-β-S (100µM) or PTx (100 ng/mL) for 48 hr. Mitochondrial membrane potential was determined by tetramethylrhodamine methyl-ester perchlorate (TMRM) (10nM) fluorescence. Mean relative fluorescences±SEM are shown; n=120, *p<0.05.

Supplemental figure 4: Infection efficiency was measured in every experiment by immunofluorescence (Ad-GFP) using GFP confocal microscopy. At the concentration of 100 MOI we obtained an infection rate of approximately 65 to 80%.
Adv-GFP did not affect mitochondrial membrane potential in neither control VSMC nor PDGF-stimulated VSMC. As shown by the arrows TMRM signal is similar between GFP positive and negative cells (p>0.05; n=30).

Supplemental figure 5: As in cultured VSMC, DHEA did not affect mitochondrial membrane potential of human saphenous veins VSMC.
Mitochondrial (red) localization of Cytochrome c (green)

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DOSE RESPONSE

% decrease in TMRM intensity

- PDGF+No DHEA
- PDGF+5μM DHEA
- PDGF+10 μM DHEA
- PDGF+100 μM DHEA
DHEA induces mitochondrial depolarization through a GPCR only in PDGF-stimulated VSMC.
DHEA has no effects on control human saphenous veins

TMRM (F.U)
Infection efficiency was measured by fluorescence (GFP)

Infection with AdvGFP does not change resting ΔΨm in control VSMC