Diosgenin Modulates Vascular Smooth Muscle Cell Function by Regulating Cell Viability, Migration, and Calcium Homeostasis

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Received July 20, 2010; accepted December 20, 2010

ABSTRACT

In this study, we compared the potencies of diosgenin, a plant-derived sapogenin structurally similar to estrogen and progesterone, on vascular smooth muscle functions ranging from contraction and migration to apoptosis. The effects of diosgenin on vascular smooth muscle cell viability and migration were measured using a primary mouse aortic smooth muscle cell culture. The effects of diosgenin on smooth muscle cell contraction and calcium signaling were investigated in the isolated mouse aorta using wire myography and confocal microscopy, respectively. Here, we report that in cultured cells diosgenin (≥25 μM) induces apoptosis as measured by the number of annexin V-positive cells and caspase-3 cleavage, while decreasing cell viability as indicated by protein kinase B/Akt phosphorylation. In addition, diosgenin blocks smooth muscle cell migration in a transwell Boyden chamber in response to serum treatment and response to injury in a cell culture system. Diosgenin (≥25 μM) also significantly blocks receptor-mediated calcium signals and smooth muscle contraction in the isolated aorta. There is no difference in the inhibitory effects of diosgenin on vascular smooth muscle contraction between the endothelium-intact and endothelium-denuded aortic segments, indicating that they are caused by altered smooth muscle activity. Our findings suggest that over the concentration range of 10 to 15 μM diosgenin may provide overall beneficial effects on diseased vascular smooth muscle cells by blocking migration and contraction without any significant cytopathic effects, implying a potential therapeutic value for diosgenin in vascular disorders.

Introduction

Vascular smooth muscle cells (SMCs) are key structural and functional components of vessel walls controlling and modulating peripheral resistance and regional blood flow. During the progression and development of occlusive vascular diseases such as atherosclerosis and restenosis SMCs undergo phenotypic changes from a stable quiescent contractile state to a more invasive, synthetic, and proliferative state (Okamoto et al., 1992; Ross, 1999). Thickening and hardening of blood vessels is a complex and multifactorial event associated mainly with changes in lipid homeostasis and deposition, medial SMC proliferation, and/or migration in response to secreted growth factors and inflammatory cytokines, alterations in calcium homeostasis and smooth muscle contraction, and cell apoptosis (Doran et al., 2008; Sprague and Khalil, 2009). Therefore, targeting these smooth muscle aberrations may prove beneficial in the treatment of vascular disease.

Diosgenin (3β-hydroxy-5-spirostene), a plant-derived sapogenin structurally similar to estrogen and progesterone, is the precursor for the industrial large-scale production of progesterone and norethisterone (Marker et al., 1940; Au et al., 2004; Dias et al., 2007). Diosgenin can be extracted from a variety of plants such as wild yam root (Dioscorea villosa), fenugreek (Trigonella foenum graecum), and soybean (Glycine max (soybean)).

ABBREVIATIONS: SMC, smooth muscle cell; Nif, nifedipine; PE, phenylephrine; SR, sarcoplasmic reticulum; GFP, green fluorescent protein; PSS, physiological salt solution; DMSO, dimethyl sulfoxide; EtOH, ethanol; PBS, phosphate-buffered saline; PI, propidium iodide; VGCC, voltage-gated calcium channels; AM, acetoxyethyl ester.

This work was supported by the Canadian Institutes of Health Research (Grant 20R92112). M.E. was supported by fellowships from the Michael Smith Foundation for Health Research and the Heart and Stroke Foundation of Canada/Astrazeneca Canada. H.T.S. was supported by a studentship from the Michael Smith Foundation for Health Research.

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

doi:10.1124/jpet.110.172684.

The online version of this article (available at http://jpet.aspetjournals.org) contains supplemental material.
tic SMCs were isolated as described previously (Srinivasan et al., 1990; Gupta et al., 2001; McAnuff et al., 2005), and gastrointestinal complaints (Pandian et al., 2002; Kaviarasan et al., 2006). Studies have also suggested a lower incidence for coronary artery diseases and disorders related to estrogen deficiencies in humans who have a high consumption of diet rich in phytoestrogens (e.g., genistein, daidzein, and digoestrogen) (Adlercreutz et al., 1992, 1993; Hertog et al., 1995; Figitree et al., 2000; Au et al., 2004).

In previous years, the effects of diosgenin on cellular growth and viability have been tested in different types of cells, but resulted in controversial findings. In human myeloid KBM-5 cells and the 1547 osteosarcoma cell line, diosgenin induced apoptosis and cell cycle arrest (Moalic et al., 2001; Léger et al., 2004; Yen et al., 2005; Shishodia and Aggarwal, 2006), whereas in MC3T3-E1 mouse clonal osteogenic cells it increased cell proliferation and angiogenic activity through the up-regulation of vascular endothelial growth factor production (Yen et al., 2005). Thus, it seems that the effects of diosgenin on cellular homeostasis and function depend on the cell type as well as the dose.

Unfortunately, our understanding of how diosgenin exerts its protective effects in the vasculature is very limited. One study reported that diosgenin could cause endothelium-independent coronary artery relaxation in precontracted porcine left anterior descending coronary artery via activation of iberiotoxin-sensitive Ca\(^{2+}\)-activated K\(^+\) channels (Au et al., 2004). Later, it was suggested that diosgenin-induced relaxation of precontracted rat superior mesenteric artery is mediated by endothelium-dependent mechanisms that involve nitric oxide and cyclooxygenase derivatives (Dias et al., 2007). However, the direct effects of diosgenin on vascular smooth muscle function such as proliferation, migration, contraction, and viability have yet to be determined.

In this study, we sought to assess the potential therapeutic value of diosgenin in vascular disease by investigating the role of diosgenin in regulating vascular SMC viability, migration, and apoptosis, all of which contribute to the initiation and progression of occlusive and nonocclusive vascular disease. We also investigated the effects of diosgenin on agonist-induced calcium release in vascular SMCs and receptor-mediated vasoconstriction and force development in mouse thoracic aorta.

**Materials and Methods**

**Experimental Animal and Tissue Preparation.** All animal experiments and procedures were conducted in accordance with the guidelines of the Animal Ethics Board at the University of British Columbia. Male C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in the institutional animal facility (University of British Columbia, Child and Family Research Institute) under standard animal room conditions (12-h light/dark cycle at 25°C). Seven-month-old male mice were anesthetized with a mixture of ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (12 mg/kg) intraperitoneally. The thoracic aorta was isolated, gently cleaned of connective tissue and blood, and cut into 2-mm segments (five or six aortic rings). Experiments were performed using endothelium-intact aortic rings unless otherwise indicated.

**Cell Culture and Transient Transfection.** Primary mouse aortic SMCs were isolated as described previously (Srinivasan et al., 2009). In brief, aortic segments were isolated, cleaned of excess adventitial tissue, and digested using collagenase II (0.5 mg/ml). Isolated cells were pelleted, resuspended, and grown in Dulbecco’s modified Eagle’s medium. Subconfluent primary mouse aortic SMCs were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated newborn calf serum (Invitrogen, Burlington, ON, Canada) at 37°C in a humidified incubator in 5% CO\(_2\). Penicillin (100 μg/ml) and streptomycin (100 μg/ml) (Invitrogen) were added to all culture media. These cells maintain the phenotype and characteristics of vascular smooth muscle cells up to passage 15 as confirmed by positive staining for smooth muscle cell α-actin (see Supplemental Fig. S1) and preservation of contractility (data not shown). To assure the consistency of results, passage 8–13 of SMCs was used for all experiments.

Adenoviral constructs encoding the constitutively active (Ad-Ca-Akt) and wild-type (Ad-Wt-Akt) forms of murine Akt tagged with the hemagglutinin epitope and control green fluorescent protein (GFP) (Ad-GFP), kindly provided by Dr. Kenneth Walsh (Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston, MA) and Dr. Jason Dyck (University of Alberta, Edmonton, Canada), were described previously (Esfandiarei et al., 2007). Smooth muscle cells were infected with adenoviral constructs at a multiplicity of infection of 100. After overnight incubation at 37°C, cells were replated with fresh medium. Fluorescence and bright-field microscopy were used to assess transfection efficiency and cellular morphology at 48 h after transfection. Western blot analysis was performed to confirm the overexpression of phosphorylated Akt-Ser in SMC culture compared with control (nontransfected) and Ad-GFP-transfected cells to assure the efficiency of the transfection protocol.

**Reagents and Antibodies.** HEPES-PSS containing 140 mM NaCl, 10 mM glucose, 5 mM KCl, 5 mM HEPES, 1.5 mM CaCl\(_2\), and 1 mM MgCl\(_2\), pH 7.4 was used for all calcium measurements and confocal microscopy. High-K\(^+\) PSS (60 mM extracellular K\(^+\)) was identical in composition to normal PSS with the exception of 85 mM NaCl and 60 mM KCl. Zero-Ca\(^{2+}\) PSS was prepared in the same way as normal PSS, but CaCl\(_2\) was replaced with 1 mM EGTA. UTP, cyclopiazonic acid, nifedipine (NiF), R(−)-phénylphérique hydrochloride, and diosgenin were obtained from Sigma-Aldrich (Oakville, ON, Canada). Stock solutions of nifedipine and diosgenin were prepared in dimethyl sulfoxide (DMSO) and ethanol (EtOH), respectively. For all experiments with diosgenin and/or nifedipine, vehicle-treated (0 μM) groups were incubated with 1 μl of ethanol and/or DMSO, respectively (the maximum volume of solvents used with the highest concentration of drugs). Further dilutions of reagents were made in zero-Ca\(^{2+}\) PSS buffer. Fluo-4AM was purchased from Invitrogen. All drugs and molecular target nomenclature conform to the British Journal of Pharmacology’s Guide to Receptors and Channels (Alexander et al., 2008). All primary antibodies used in this study were purchased from Cell Signaling Technology (Danvers, MA). Secondary antibodies conjugated with horseradish peroxidase or Alexa Fluor 488 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

**Western Blot Analysis.** Cells either untreated or treated with various experimental reagents were washed twice with ice-cold PBS and kept on ice for 15 min in lysis buffer containing 50 mM pyrophosphate, 50 mM NaF, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 10 mM HEPES, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 100 μM Na\(_3\)VO\(_4\), 0.1% Triton X-100, and 10 μg/ml leupeptin. Cell lysates were collected by scraping, and protein concentration was determined using Bradford (1976) assay. Extracted protein (40–80 μg) was fractionated by electrophoresis in 7 to 9% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and blocked with PBS containing 0.1% Tween 20 and 5% nonfat dry milk for 1 h. Afterward, the membrane was incubated with specific primary antibody overnight at 4°C, followed by secondary antibody for 1 h at room temperature. Immunoblots were visualized with an enhanced chemiluminescence detection system according to the protocol of the
Fig. 1. Apoptotic effects of diosgenin on vascular SMC. A, diosgenin (≥25 µM) increased the number of both annexin V-positive cells (indicator of apoptosis) and PI-positive cells (indicator of necrosis) in a concentration-dependent manner with the EC₅₀ values of 33 and 43 µM, respectively (n = 3 experiments; mean ± S.D.; *, #, P < 0.05). B, diosgenin increases caspase-3 cleavage (an indicator of final stage of apoptosis) in cultured SMCs in a concentration-dependent manner. Total actin protein assures equal protein loading. Immunoblot represents one experiment of three independent experiments. The bar graph presents the average band intensity measurement (n = 3; mean ± S.D.; *, #, P < 0.05). C, at the concentration of 25 µM diosgenin causes cytopathic effects and apoptosis in the SMC culture. Apoptotic bodies are marked by black arrows (original magnification, ×200). Control groups represent nontreated cells, and the 0 µM group represents vehicle (EtOH)-treated cells.
manufacturer (Pierce Biotechnology, Rockford, IL). Densitometry analysis was performed by using the NIH ImageJ software (version 1.27z). Density values for proteins were normalized to the level for control groups (arbitrarily set to 1.0-fold).

**Cell Viability Assay.** Subconfluent SMCs were plated in a 24-well culture plate. After 24 h, cells were incubated overnight in serum-free to arrest cell growth and proliferation and assure synchronized growth after the addition of serum. The next day, cells were treated with increasing concentrations of diosgenin or vehicle control (EtOH) for 24 h in the presence of 10% serum. The CellTiter 96 AQueous Nonradioactive Cell Viability Assay (MTS) was used to measure cell viability according to the manufacturer’s protocol (Promega, Madison, WI). Cell viability was presented as percentage compared with the control group (arbitrarily considered as 100% of viability).

**Measuring Cell Death by Cellomics.** To determine the effect of diosgenin on SMC death, cell apoptosis and necrosis were measured using the ArrayScan HCS system (Cellomics; Thermo Fisher Scientific, Waltham, MA). In this system, numbers of annexin-V-positive cells (apoptotic cells) and propidium iodide (PI)-positive cells (necrotic cells) were determined. In brief, SMCs ($5 \times 10^5$) were seeded into a 96-well culture plate and incubated overnight. Cells were then treated with increasing concentrations of diosgenin or vehicle control (EtOH) for 24 h. As the positive control for apoptosis, one group of cells was treated with the apoptosis-inducing drug staurosporine (200 nM) for 24 h. To measure the rate of cell death caused by necrosis or apoptosis, cells were then subjected to PI (1 μg/ml) or phenylephrine (PE)-conjugated annexin V (5 μl/well) for 10 min in the presence of annexin V binding buffer. After the proper incubation period, cells were scanned live using the ArrayScan HCS (high content screening) system (Thermo Fisher Scientific). Ten focus fields in each well were scanned and analyzed, and the percentage of apoptotic or necrotic cells was calculated based on the proportion of phycoerythrin-annexin V- or propidium iodide-positive cells over the total cell population (Hoechst 33342-positive cells). Data are presented as percentage of cell death where the level of cell death in the staurosporine-treated group (the positive control) was arbitrarily set to 100%.

![Graph A](image1.png)

**Fig. 2.** Effects of diosgenin on vascular SMC viability and Akt phosphorylation. A, diosgenin (25 μM) decreased serum-induced cell proliferation (an index of cell viability) in a concentration-dependent manner with an IC$_{50}$ value of 25.6 μM. Data present three independent experiments ($n = 3$ experiments; mean ± S.D.; *, $P < 0.05$). B, diosgenin decreased Akt phosphorylation (an indicator of cell viability) with no effect on total Akt expression in cultured SMCs. Immunoblot represents one experiment of three independent experiments. The bar graph presents the average band intensity measurement ($n = 3$; mean ± S.D.; *, $P < 0.05$). Control groups represent nontreated cells, and the 0 μM group represents vehicle (EtOH)-treated cells.
Cell Migration Assay. Cell migration was measured using QCM Transwell Colorimetric Cell Migration Assay according to the manufacturer's protocol (Millipore Bioscience Research Reagents, Temecula, CA). In brief, 10^5 serum-starved SMCs (to induce quiescence and synchronize cell proliferation) were loaded onto the upper well of the chamber in the presence or absence of agonist (diosgenin) and vehicle control (EtOH), whereas lower wells were filled with serum-containing culture medium with various concentrations of the agonist (diosgenin). After 24 h incubation, cells were fixed and subjected to imaging using a Nikon (Tokyo, Japan) inverted microscope (×400 magnification) and Spot (Sterling Heights, MI) digital camera. The wound size was measured using Image Pro Plus imaging software. The migration of smooth muscle cells to the site of injury was measured as percentage of wound closure using the following formula: percentage of wound closure = [(L_{0h} - L_{24h})/L_{0h}] × 100%, where L_{0h} is the distance between two edges of the wound measured at 0 h after injury (immediately after scratching), and L_{24h} is the distance at 24 h after injury. Data are represented as percentage of wound closure where the wound size in control (nontreated) group at 24 h after injury was arbitrarily set to 100%.

Wound Healing Assay. Subconfluent SMCs were grown on glass coverslips. After overnight serum starvation (to induce quiescence and synchronize cell proliferation), cell cultures were scratched with a sterile pipette tip to form a wound, washed with prewarmed sterile PBS, and incubated with medium (containing 10% serum). Cells were then treated with the vehicle (EtOH) or increasing concentrations of diosgenin (10, 25, and 50 µM) or left untreated (control). At 24 h after injury, cells were fixed and subjected to imaging using a Nikon confocal microscope (Nikon (Tokyo, Japan) inverted microscope (×400 magnification) and Spot (Sterling Heights, MI) digital camera. The wound size was measured using Image Pro Plus imaging software. The migration of smooth muscle cells to the site of injury was measured as percentage of wound closure using the following formula: percentage of wound closure = [(L_{0h} - L_{24h})/L_{0h}] × 100%, where L_{0h} is the distance between two edges of the wound measured at 0 h after injury (immediately after scratching), and L_{24h} is the distance at 24 h after injury. Data are represented as percentage of wound closure where the wound size in control (nontreated) group at 24 h after injury was arbitrarily set to 100%.

Cytoplastic Measurement of Calcium. To measure cytoplasmic calcium in the isolated aorta, 2-mm segments of endothelium-intact aortic rings were gently inverted and then loaded with Fluo-4AM (5 µM with 5 µM Pluronic F-127) for 2 h at 37°C. Later, loaded aortic rings were isometrically mounted, followed by 15-min washout in HEPES-buffered physiological saline solution. For in vitro measurement of calcium signals, subconfluent SMCs were grown on Matrigel-coated (BD Sciences, Ontario, Canada) 35-mm glass-bottom culture dishes (MatTek, Ashland, MA) 48 h before each experiment. Cells were then loaded with Fluo-4AM for 1 h at 37°C, followed by 24 h of serum starvation. Cells were loaded with Fluo-4AM for 1 h at 37°C, followed by 24 h of serum starvation. Cells were then loaded with Fluo-4AM for 1 h at 37°C, followed by 24 h of serum starvation.

**Effects of Diosgenin on Vascular Smooth Muscle**

Fig. 3. Reversal of diosgenin-induced apoptosis by overexpression of a constitutively active form of Akt. A, SMCs were transfected with the adenoviral vector expressing the constitutively active form of Akt for 48 h. As shown, transfected SMCs express a high level of Akt phosphorylation (2.5-fold increase) as measured by Western blot analysis. β-Actin protein expression confirmed equal protein expression. The blot represents one of three independent experiments. The bar graph is the average band intensity of three independent experiments (n = 3; mean ± S.D.; *, P < 0.05). Control group represents nontransfected SMCs. B, overexpression of an active form of Akt rescues SMCs from the apoptotic effects of diosgenin. Diosgenin-induced caspase-3 cleavage was blocked by overexpression of an activated form of Akt. Control group represents nontransfected SMCs. C, in the presence of serum (a potent activator of Akt), both wild-type and active forms of Akt rescue SMCs from the cytopathic effects of diosgenin. In the absence of serum only the active form of Akt improve cell viability in diosgenin-treated SMCs. The bar graph represents three independent experiments (n = 3; mean ± S.D. (Left, *, **, P < 0.05). Right, *, P = 0.2086; **, P < 0.05). Groups marked as 0 µM represent vehicle (EtOH)-treated cells.
by a 15- to 20-min wash in HEPES-buffered physiological saline solution.

To investigate the effects of diosgenin on calcium release, cultured SMCs or isolated aortic segments were pretreated with diosgenin or vehicle (EtOH) for 30 min and then stimulated with various pharmacological agents. Images were acquired on an upright Olympus (Tokyo, Japan) BX50WI microscope with a 60× water-dipping objective (numerical aperture, 0.9) and equipped with an Ultraview Confocal imaging system (PerkinElmer Life and Analytical Sciences, Waltham, MA). All parameters (laser intensity, gain, etc.) were maintained constant during the experiment. The tissue was illuminated using an argon-krypton laser (488 nm), and a high-gain photomultiplier tube collected the emission (505–550 nm). Representative fluorescence traces reflect the averaged fluorescence signals from 6 to 10 SMCs in each region of interest. The measured changes in Fluo-4 fluorescence level are proportional to the relative changes in [Ca^{2+}]_i. The confocal images were analyzed off-line with Ultraview 4.0 Software (PerkinElmer Life and Analytical Sciences). Fluorescence traces were extracted from the movies to exclude nuclear regions, and traces were normalized to initial fluorescence values.

**Measurement of Isometric Force.** Aortic segments were mounted isometrically in a small vessel wire myograph (Danish Myotechnology AS, Aarhus, Denmark) for measuring generated force. Krebs’ solution containing 130 mM NaCl, 4 mM KCl, 1.2 mM MgSO_4, 4 mM NaHCO_3, 1.5 mM CaCl_2, 10 mM HEPES, 1.18 mM KH_2PO_4, 6 mM glucose, and 0.03 mM EDTA, pH 7.4, was used for all isometric contraction studies. The chambers and bath solutions were kept at 37°C and bubbled continuously with 95% O_2-5% CO_2 in water-dipping objective (numerical aperture, 0.9) and equipped with an Ultraview Confocal imaging system (PerkinElmer Life and Analytical Sciences, Waltham, MA). All parameters (laser intensity, gain, etc.) were maintained constant during the experiment. The tissue was illuminated using an argon-krypton laser (488 nm), and a high-gain photomultiplier tube collected the emission (505–550 nm). Representative fluorescence traces reflect the averaged fluorescence signals from 6 to 10 SMCs in each region of interest. The measured changes in Fluo-4 fluorescence level are proportional to the relative changes in [Ca^{2+}]_i. The confocal images were analyzed off-line with Ultraview 4.0 Software (PerkinElmer Life and Analytical Sciences). Fluorescence traces were extracted from the movies to exclude nuclear regions, and traces were normalized to initial fluorescence values.

To calculate the percentage of inhibition of contraction by diosgenin, the force created by 10 μM PE in the presence of 0 μM diosgenin (vehicle only, EtOH) was arbitrarily set as 100% of contraction for control group. Percentage of contraction for diosgenin-treated groups was calculated in comparison with the vehicle control group. The percentage of inhibition was calculated as percentage of inhibition = percentage contraction for vehicle-treated control group – percentage contraction for diosgenin-treated group.

**Statistical Analysis.** Statistical analysis and preparation of concentration-response curves were performed using Prism 4.0 software (GraphPad Software, Inc., San Diego, CA). Data were analyzed using one-way analysis of variance. We also used repeated-measures analysis of variance where those different doses were treated as repeated measures (within factor) and different replicates were treated as “between” factor. We expected “within” to be

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**Fig. 4.** Effects of diosgenin on vascular SMC migration. A, diosgenin (> 10 μM) blocked SMC migration into the site of injury and wound closure in a concentration-dependent manner with the IC_{50} value of 15.3 μM (n = 6 experiments; mean ± S.D.; * and **, P < 0.05). B, in the Boyden chamber migration assay diosgenin (10 μM) caused a marked decrease in serum-stimulated migration in a concentration-dependent manner with the IC_{50} value of 10.3 μM (n = 3 experiments; mean ± S.D.; *, P < 0.05). Graphs represent the average of three independent experiments. Control groups represent nontreated cells, and the 0 μM group represents vehicle (EtOH)-treated cells.
significant and both between-group factors (different replicates) not to be significant. Our analyses showed that although there was significant difference between different doses (within groups), there was no difference between replicates (between groups). Therefore, appropriate post hoc tests (Bonferroni or Dunnet’s T3) were used depending on result of Levine’s test for homogeneity of variances (i.e., assumption of equality of variances). Values shown are the mean ± S.D., where a value of $P < 0.05$ was considered statistically significant. The exact number of repetition ($n$) for each experiment is specified under Results. The number for cell cultures, aortic rings, or animals is also noted when different from the number of experiments. For all experiments, the groups marked as “control” represent untreated cells or tissue, whereas the groups marked as “0 µM” represent cells or tissue treated with only vehicle (DMSO or EtOH).

**Results**

**Apoptotic Effects of Diosgenin on Mouse Aortic SMCs.** We investigated the apoptotic and necrotic effects of diosgenin in cultured SMCs. Diosgenin ($≥25$ µM) increased the number of both the annexin V-positive (indicator of apoptosis) and PI-positive (indicator of necrosis) cells in a concentration-dependent manner. At the concentration of 25 µM, diosgenin caused apoptosis in 17% of the SMC population. As the concentration of diosgenin increased to 50 µM, apoptosis was detected in 60% of the SMC population (Fig. 1A; $n = 3$ experiments of three replicates; *, $P < 0.05$). Western blot analysis also confirmed the cleavage of caspase-3 protein in diosgenin-treated SMCs in a concentration-dependent man-

![Graph A](image1.png)

**Fig. 5.** Effects of diosgenin on α-adrenergic receptor-mediated contraction in the isolated mouse aorta. A, at the concentration of 10 µM PE induces submaximal contraction in the aorta ($n = 5$ mice, $P < 0.05$). B, in the presence of extracellular calcium diosgenin causes a maximum of 50% inhibition of the PE-induced contraction in the aorta. Upon removal of extracellular calcium diosgenin completely blocks PE-induced contraction ($n = 5$ mice; $P < 0.05$). C, removal of extracellular calcium also decreases PE-induced force development in mouse aortic rings by 50% ($n = 5$ mice; *, $P < 0.05$).
ner (Fig. 1B; \( n = 3 \) experiments; \( * \, P < 0.05 \)). Morphological study using phase-contrast microscopy also confirmed that the increase in caspase-3 cleavage was associated with the appearance of cytotoxic effects and apoptotic bodies in cultured SMCs (Fig. 1C).

**Effects of Diosgenin on Vascular SMC Viability and Akt Phosphorylation.** Diosgenin (\( \geq 25 \mu M \)) decreased SMC viability in a concentration-dependent manner (Fig. 2A; \( n = 3 \) experiments of three replicates; \( *, P < 0.05 \)). At the concentration of \( 25 \mu M \), diosgenin caused a significant decrease (40%) in SMC viability. It is well established that Akt (protein kinase B) phosphorylation is a key event during cell growth and viability. To understand the cellular mechanism by which diosgenin decreases SMCs viability, we assessed the expression of the phosphorylated form of Akt (phosphor-Akt-Ser473) after diosgenin treatment. Our results showed that diosgenin decreased Akt phosphorylation in SMCs without affecting

![Graph A](image1.png)

![Graph B](image2.png)

**Fig. 6.** Effects of diosgenin on \( \text{Ca}^{2+} \) influx via L-type calcium channels in the isolated mouse aorta. A, Nif (1 \( \mu M \)) significantly inhibits PE-induced force development in isolated aortic rings compared with the control and DMSO-treated groups (\( n = 6 \) mice; mean \( \pm \) S.D.; \( * \, P < 0.05 \)). B, diosgenin does not block aortic contraction in response to high K\(^+\) buffer, indicating that diosgenin has no inhibitory effects on L-type calcium channels in the mouse aorta (\( n = 7 \) mice; mean \( \pm \) S.D.; \( *, P < 0.05 \); \# 0.6515).
the expression of total Akt protein in SMCs (Fig. 2B; n = 3 experiments; *, P < 0.05).

To establish a causal relationship between decreased Akt phosphorylation and increased caspase-3 cleavage and apoptosis in response to diosgenin treatment, SMCs were transfected with the adenoviral vectors carrying either a constitutively active form of Akt (Ad-Ca-Akt) or a wild-type of Akt (Ad-Wt-Akt), or the GFP (Ad-GFP) control construct before diosgenin treatment. To assure the efficiency of transfection, Akt phosphorylation was measured in transfected SMCs. As shown in Fig. 3A, phosphorylation of Akt-Ser was significantly increased in SMCs transfected with the constitutively active form of Akt compared with cells transfected with the adenoviral vector expressing the GFP protein (n = 3 experiments; *, P < 0.05). Overexpression of an active form of Akt blocked diosgenin-induced caspase-3 cleavage (Fig. 3B; n = 3 experiments; *, P < 0.05). In the presence of serum (a potent inducer of Akt phosphorylation), both the wild-type and constitutively active forms of Akt could reverse the cytotoxic effects caused by diosgenin. However, in the absence of serum stimulation, only the active form of Akt could rescue the cells and increase SMC viability (Fig. 3C; n = 3 experiments of three replicates). (Fig. 3C, left, *, **, P < 0.05; right, *, P = 0.2086; **, P < 0.05).

**Effects of Diosgenin on Vascular SMC Migration.** Diosgenin (≥10 μM) blocked SMC migration in a concentration-dependent manner in both wound healing (Fig. 4A; n = 6 experiments; *, P < 0.05) and Transwell Boyden chamber assays (Fig. 4B; n = 3 experiments of three replicates; *, P < 0.05). The low concentration of diosgenin (10 μM) caused a considerable decrease (40%) in SMC migration, whereas at a higher concentration (50 μM) diosgenin nearly completely blocked cell migration. It is of importance that at a low concentration (10 μM) diosgenin provided a significant inhibition in cell migration (Fig. 4B) with no noticeable cytotoxic effects on SMCs (Fig. 1A).

**Effects of Diosgenin on α-Adrenergic Receptor-Mediated Contraction in the Isolated Mouse Aorta.** To induce contraction, aortic rings were stimulated with 10 μM PE (with the threshold effective concentration of 0.1 μM), a concentration that induces 90% of maximal force development (Fig. 5A; n = 5 mice). Diosgenin inhibited the contractile response to PE (10 μM) in both the presence and absence of extracellular calcium in a concentration-dependent manner with IC_{50} values of 25 μM (pD_{2} = 4.5) and 10 μM (pD_{2} = 5.0), respectively (Fig. 5B; n = 5 mice). It is noteworthy that, in the absence of diosgenin, removal of extracellular calcium 1 min before PE application reduced force development by approximately one-half (Fig. 5C; n = 5 mice; *, P < 0.05). It is noteworthy that our time-response experiment with diosgenin has shown that a minimum of 12-h incubation is required to observe the early indications of apoptotic and necrotic responses in cultured SMCs (data not shown). The same observation was reported by other groups using different cell lines (Moalic et al., 2001; Léger et al., 2004; Yen et al., 2005; Shishodia and Aggarwal, 2006). Therefore, we do not expect the involvement of apoptosis in diosgenin-induced inhibition of contraction in the isolated aorta (ex vivo).

**Effects of Diosgenin on Ca^{2+} Influx via L-Type Calcium Channels in the Isolated Mouse Aorta.** We first investigated the contribution of L-type Ca^{2+} channels during PE-induced force development in the isolated mouse aorta. Treatment of aortic rings with 1 μM Nif, a selective inhibitor of L-type Ca^{2+} channels, reduced PE-induced contraction to 69 ± 5% of control and vehicle-treated groups, indicating a partial involvement of L-type Ca^{2+} channels (Fig. 6A; n = 6 mice; *, P < 0.05). However, when the aorta was exclusively activated by voltage-gated calcium channels (VGCC) during high potassium depolarization, the entire contraction was abolished by 1 μM Nif but not by diosgenin (Fig. 6B; n = 7 mice; *, P < 0.05). This observation shows that diosgenin does not block VGCC in the mouse aorta. It is noteworthy that pretreatment of aorta with diosgenin for 30 min before the stimulation with 60 mM KCl also had no effect on force development in aortic rings (data not shown).

To determine whether the diosgenin-induced relaxation had an endothelium-dependent component we treated both endothelium-intact and endothelium-denuded aortic segments with diosgenin before PE treatment. Diosgenin treatment (25 μM) in both groups resulted in a 50% decrease in PE-induced force in the presence of extracellular calcium, indicating an absence of endothelial involvement in our preparation (Fig. 7; n = 5 mice; *, P < 0.05). To minimize tissue trauma during the calcium measurements we thus left the aorta intact and focused the confocal microscope on the medial smooth muscle layer.

**Effects of Diosgenin on α-Adrenergic Receptor-Mediated Calcium Signals in the Isolated Mouse Aorta.** We measured PE-induced calcium signals in the absence and presence of 25 μM diosgenin. As shown in Fig. 8A, diosgenin caused a significant decrease (50%) in the PE-induced calcium transient in aortic rings (n = 11 mice; mean ± S.D.; *, P < 0.05). As shown, the plateau phase of calcium signal (required for maintaining the force) was also affected by diosgenin. Removal of extracellular calcium before stimulation of aortic rings with PE resulted in a decrease in basal [Ca^{2+}], shortening of the PE-induced calcium transient, and abolition of the plateau phase (Fig. 8B; n = 9 mice; mean ± S.D.; *, P < 0.05). In the absence of extracellular calcium diosgenin completely blocked the PE-induced sarcoplasmic reticulum (SR) calcium release transient in parallel with blockade of force development (compared with 50% inhibition in the presence of extracellular calcium in Fig. 8A).

**Fig. 7.** Role of endothelial layer in diosgenin-induced inhibition of aortic contraction. Similar inhibitory effect for diosgenin was observed in the absence and presence of endothelial layer. At the concentration of 25 μM diosgenin caused a 50% decrease in PE-induced aortic contraction in both endothelium-denuded and endothelium-intact aortic rings (n = 5 mice; mean ± S.D.; *, P = 0.9600).
Effects of Diosgenin on Purinergic Receptor-Mediated Calcium Signals in Mouse Aortic SMC Culture. We investigated whether the effect of diosgenin on SR Ca^{2+} release and possibly store-operated Ca^{2+} channels was also present in the cultured cells. As shown in Fig. 9A, diosgenin caused a significant inhibition of the UTP-induced calcium transient in cultured SMCs (n = 5 experiments; mean ± S.D.; * P < 0.05). It is noteworthy that Nif had no effects on UTP-induced calcium transients in cultured SMCs (Fig. 9B; n = 5 experiments; mean ± S.D.; *, P < 0.05). Furthermore,
to investigate the effects of diosgenin on UTP-induced force development, isolated aortic rings were pretreated with 25 μM diosgenin for 30 min before UTP application. Diosgenin treatment reduced UTP-induced force development by 50% (Fig. 10; *P < 0.05). To test for a possible effect of diosgenin on store-operated Ca\(^{2+}\) channels in vitro, extracellular calcium was removed from SMC cultures, and SR calcium content was depleted using 10 μM cyclopiazonic acid, a sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase inhibitor. In control groups, reperfusion of cell cultures with HEPES buffer containing 1.5 mM calcium readmission resulted in a rapid and transient 3-fold increase in [Ca\(^{2+}\)]\(_{cyto}\) followed by a lower plateau phase. In the diosgenin-treated cells the transient increase in [Ca\(^{2+}\)]\(_{cyto}\) was significantly reduced and the plateau was abolished, indicating an inhibitory effect on store-operated calcium channels in aortic SMCs (Fig. 11; *P < 0.05).
Discussion

In the present study, we describe various mechanisms by which diosgenin modulates vascular SMC function and viability in the mouse aorta. In cell culture, diosgenin inhibits aortic SMC proliferation and migration, but also induces apoptosis. The apoptotic effect of diosgenin in aortic SMCs is associated with a significant reduction in Akt phosphorylation and a marked increase in caspase-3 cleavage. Overexpression of an active form of Akt rescued SMCs from the apoptotic effects of diosgenin, establishing a causal relationship between the loss of Akt activity and increased apoptosis in diosgenin-treated SMCs. We also studied the effects of diosgenin on SMC viability and found that in the presence of serum (an activator of Akt) overexpression of both the wild-type and activated forms of Akt were able to improve cell viability after diosgenin treatment. This is caused by immediate phosphorylation of the exogenous Akt (overexpressed wild-type Akt) by serum. However, in a serum-starved condition, only a constitutively active form of Akt was able to improve SMC viability, suggesting that only the active form of Akt could reverse the cytopathic effects caused by diosgenin.

Diosgenin has been reported to inhibit cancer growth caused by inhibiting Akt signaling and inducing apoptosis in various in vitro culture systems (Moalic et al., 2001; Léger et al., 2004; Shishodia and Aggarwal, 2006). Our findings have shown that at the concentration higher than 10 μM diosgenin could cause apoptosis in SMCs. This observation is in agreement with previous studies in tumor cells, in which the apoptotic effects of diosgenin were observed within the concentration range of 25 to 50 μM. Fortunately, the potency for apoptotic and necrotic effects is low in mouse aortic SMCs, such that at the concentration of 10 μM diosgenin induces 40 to 50% inhibition of SMC migration without any significant apoptotic or necrotic effects (Fig. 12).

Hypersensitivity of vascular smooth muscle to physiological stimuli results in enhanced vasoconstriction in a wide variety of vascular disorders such as diabetic vascular disease, hypertension, pulmonary vasoconstriction, and coronary vasospastic angina pectoris (Brondum et al., 2008; Burger, 2009; Eijking et al., 2009; Morrell et al., 2009). To determine whether diosgenin could affect vascular smooth muscle responses to physiological stimuli, we measured the contractile behavior of the isolated mouse aorta in the absence and presence of diosgenin. In the presence of extracellular calcium, diosgenin caused a maximum of 50% inhibition of contraction in response to PE stimulation. Likewise, in the presence of extracellular calcium, blocking the L-type calcium channels with nifedipine caused an almost 50% decline in PE-induced force, highlighting that in mouse aorta only half of PE-induced contraction is caused by calcium entry through L-type calcium channels, and the remaining half probably depends on calcium release from intracellular stores and/or calcium entry through store operated channels. Consistent with this hypothesis, in the absence of extracellular calcium, diosgenin treatment caused 100% inhibition of contraction in response to PE stimulation. Likewise, in the presence of extracellular calcium, blocking the L-type calcium channels with nifedipine caused an almost 50% decline in PE-induced force, highlighting that in mouse aorta only half of PE-induced contraction is caused by calcium entry through L-type calcium channels, and the remaining half probably depends on calcium release from intracellular stores and/or calcium entry through store operated channels. Consistent with this hypothesis, in the absence of extracellular calcium, diosgenin treatment caused 100% inhibition of contraction in response to PE. The observation that diosgenin did not block L-type channel-mediated calcium influx from the extracellular space in the aorta further confirmed our assumption that the 50% blockade caused by diosgenin was probably caused by its effects on calcium release from the intracellular stores and possibly calcium influx through store-operated calcium channels.

Dias et al. (2007) reported that treatment of rat superior mesenteric arteries with diosgenin resulted in endothelium-dependent vasorelaxation that could be significantly blocked
by the inhibitor of endothelial nitric-oxide synthase. However, in our isolated mouse aorta, the effects of diosgenin on aortic contraction did not seem to depend on the endothelial layer, because removal of the endothelium in mouse aorta did not affect vasodilatation in response to diosgenin. This difference could be related to the origin of the blood vessels (mouse aorta versus rat mesenteric artery) or variations in diosgenin concentrations used (25 µM versus 1 mM).

As expected, diosgenin also decreased the PE-induced calcium transient in the mouse aorta by almost half. Removal of the extracellular calcium in the presence of diosgenin resulted in complete blockade of the PE-induced calcium transient, which corresponds with complete inhibition of force development in the aorta.

UTP, an effective agonist for induction of calcium release in cultured SMCs, exerts its effects via purinergic P2Y receptors that activate the phospholipase C pathway (Horiuchi et al., 2001). In our primary aortic SMC culture, application of UTP (1 mM) induced a rapid but transient increase in [Ca^{2+}]_{cyto}, which was largely blocked by diosgenin.

In these aortic SMC cultures, blocking L-type calcium channels with nifedipine had no effect on UTP-induced calcium transients, indicating that in these cells the agonist-induced calcium transient does not depend on the opening of VGCC, but depends on SR calcium release and possibly calcium influx via the store-operated calcium channels. Consistent with the above, no elevation in calcium concentration was observed in cultured SMCs upon application of 60 mM KCl, suggesting that L-type calcium channels are not expressed or are possibly inactivated in our primary SMC cultures (data not shown). To test the effects of diosgenin on store-operated calcium channels in cultured SMCs, we depleted the SR by sarco/endoplasmic reticulum Ca^{2+}-ATPase inhibition in the absence of extracellular calcium and readmitting calcium in the absence or presence of diosgenin. Our data clearly showed blockade of store-operated calcium channels in mouse SMCs by diosgenin. An interesting aspect of diosgenin is its close chemical relationship to estrogens, which are also known to target a variety of calcium transporters and enzymes (Martin et al., 1978; Miksicek, 1993). Thus for the purpose of widening our understanding of physiological smooth muscle regulation and possibly correcting pathological dysregulation, further investigation of the molecular mechanisms of diosgenin is desirable.

Fig. 11. Effects of diosgenin on store-operated channels in aortic SMCs. In the absence of diosgenin and after SR depletion, reperfusion of cells with HEPES buffer containing 1.5 mM Ca^{2+} resulted in a rapid and transient increase in cytoplasmic calcium that quickly dropped and then was maintained at the basal level. However, in the presence of 25 µM diosgenin the peak response (caused by the opening of store-operated calcium channels) was significantly blocked (n = 6 experiments; mean ± S.D.; *, P < 0.05).
In conclusion the approach taken in this study relies on the notion that during the progression of vascular diseases the arterial walls undergo structural remodeling that is associated with an increase in SMC migration and proliferation and irregular vasoconstruction (Okamoto et al., 1992; Ross, 1999). In occlusive vascular disorders, SMC apoptosis also significantly contributes to disease progression and end-stage plaque rupture, resulting in blockage of the arteries and thus impaired blood flow to the targeted organ (Doran et al., 2008; Sprague and Khalil, 2009). Therefore, a desirable and effective therapeutic approach would control enhanced SMC migration and contraction, while viability of vascular SMC is maintained. In this study, we have shown that diosgenin inhibits both SMC migration and contraction. By performing comprehensive concentration-response experiments we were able to show that treatment of vascular smooth muscle with 10 μM diosgenin could provide 45% inhibition of migration and 25% inhibition of contraction (Fig. 12). At this concentration, diosgenin causes apoptosis in less than 3.5% of the SMC population, suggesting that the use of diosgenin at the concentration range of 10 to 15 μM may be expected to provide overall beneficial effects on diseased vascular SMCs. In short, comparison of the concentration-response curves for these various pharmacological actions of diosgenin indicates a possible separation of “desirable effects” such as inhibition of migration and contraction from the “harmful effects” of apoptosis and loss of viability, making it a possible candidate for the treatment of vascular diseases.

Authorship Contributions

**Participated in research design:** Esfandiarei.

**Conducted experiments:** Esfandiarei, Lam, Yazdi, Kariminia, Syyong, Dorado, and Hu.

**Performed data analysis:** Esfandiarei and Kuzeljevic.

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