Diosgenin modulates vascular smooth muscle cell function by regulating cell viability, migration, and calcium homeostasis

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Abbreviations: CPA, Cyclopiazonic Acid; IC_{50}, half maximal inhibitory concentration; Nif, Nifedipine; PE, Phenylephrine; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; SMC, smooth muscle cell; SR, Sarcoplasmic Reticulum; UTP, Uridine 5’-triphosphate

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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 (≥25μmol.L⁻¹) 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 trans-well Boyden chamber in response to serum treatment, and in response to injury in a cell culture system. Diosgenin (≥25μmol.L⁻¹) also significantly blocks receptor-mediated calcium signals and smooth muscle contraction in the isolated aorta. There is no difference between the inhibitory effects of diosgenin on vascular smooth muscle contraction between the endothelium-intact and endothelium-denuded aortic segments, indicating that they are due to altered smooth muscle activity. Our findings suggest that over the concentration range of 10-15μmol.L⁻¹ 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 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 multi-factorial event mainly associated with changes in lipid homeostasis and deposition, medial SMCs 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 (3 beta-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 greacum), and soy bean (Glycine max). Extracts from these plants and a few others have been traditionally used to treat hypercholesterolemia (Valette et al., 1984; Sauvaire et al., 1991), diabetes (Sharma et al., 1990; Gupta et al., 2001; McAnuff et al., 2005), and gastrointestinal complaints (Pandian et al., 2002; Kaviarasan et al., 2006). Recent 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, & diosgenin) (Adlercreutz et al., 1992; Figtree 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 1547 osteosarcoma cell line, diosgenin induced apoptosis and cell cycle arrest (Moalic et al., 2001; Leger et al., 2004; Yen et al., 2005; Shishodia and Aggarwal, 2006), while in MC3T3-E1 mouse clonal osteogenic cells it increased cell proliferation and angiogenic activity through the up-regulation of the vascular endothelial growth factor (VEGF) production (Yen et al., 2005). Thus, it appears 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 recent study reported that diosgenin could cause endothelium-independent coronary artery relaxation in pre-contracted 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 pre-contracted rat superior mesenteric artery is mediated by endothelium-dependent mechanisms that involve nitric oxide (NO) and cyclooxygenase (COX) derivatives (Dias et al., 2007). However, the direct effects of diosgenin on vascular smooth muscle function such as proliferation, migration, contraction, and viability are 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 non-occlusive vascular disease. We have also investigated the effects of
diosgenin on agonist-induced calcium release in vascular SMCs, and on receptor-
mediated vasoconstriction and force development in mouse thoracic aorta.
Methods

Experimental animal & tissue preparation

All animal experiments and procedures were conducted in accordance with the guidelines of the Animal Ethics Board in the University of British Columbia. Male C57BL/6 mice were obtained from Jackson Laboratories and housed in the institutional animal facility (University of British Columbia, Child and Family Research Institute) under standard animal room conditions (12 hr light–12 hr dark, at 25°C). Seven month-old male mice were anesthetized with a mixture of ketamine hydrochloride (80mg.kg\(^{-1}\)) and xylazine hydrochloride (12mg.kg\(^{-1}\)) intraperitoneally. The thoracic aorta was isolated, gently cleaned of connective tissue and blood, and cut into 2 mm segments (5-6 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). Briefly, aortic segments were isolated, cleaned of excess advential tissue, and digested using collagenase II (0.5 mg/ml). Isolated cells were pelleted, resuspended, and grown in Dulbecco's modified Eagle's medium. Sub-confluent 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 G (100 μg.mL\(^{-1}\)) and streptomycin (100 μg.mL\(^{-1}\)) (Invitrogen, CA, USA) 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 alpha actin (see figure S1, supplementary data) 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 HA epitope and control 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, AB), were described previously (Esfandiarei et al., 2007). Smooth muscle cells were infected with adenoviral constructs at a multiplicity of infection of 100. Following overnight incubation at 37°C, cells were replenished with fresh medium. Fluorescence and brightfield microscopy were used to assess transfection efficiency and cellular morphology at 48 h post transfection. Western blot analysis was performed to confirm the over-expression of phosphorylated Akt-Ser in SMC culture compared to control (non-transfected) and AD-GFP-transfected cells to assure the efficiency of the transfection protocol.

**Reagents & antibodies**

HEPES-PSS containing (in mmol·L⁻¹) NaCl 140, glucose 10, KCl 5, HEPES 5, CaCl₂ 1.5 and MgCl₂ 1 (pH 7.4) was used for all calcium measurements and confocal microscopy. High-K⁺ PSS (60 mmol·L⁻¹ extracellular K⁺) was identical in composition to normal PSS with the exception of (in mmol·L⁻¹) NaCl 85 and KCl 60. Zero-Ca²⁺ PSS was prepared in the same way as normal PSS, but CaCl₂ was replaced with 1 mmol·L⁻¹ EGTA. Uridine 5'-triphosphate (UTP), cyclopiazonic acid (CPA), nifedipine (Nif), R-(−)-phenylephrine hydrochloride (PE), 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 μmol.L⁻¹) groups were incubated with 1μL of EtOH and/or DMSO, respectively (the maximum volume of solvents used with the highest concentration of drugs). Further dilutions of reagents were made in zero-Ca²⁺ PSS buffer. Fluo-4AM was purchased from Invitrogen (Burlington, ON, Canada). 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 (Beverly, MA, USA). Secondary antibodies conjugated with horseradish peroxidase or Alexa Fluor® 488 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

**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 minutes in lysis buffer containing (mmol.L⁻¹) 50 pyrophosphate, 50 NaF, 50 NaCl, 5 EDTA, 5 EGTA, 10 HEPES (pH 7.4), 1 phenylmethylsulfonyl fluoride, 100μmol.L⁻¹ Na₃VO₄, 0.1% Triton X-100, and 10μg.ml⁻¹ leupeptin. Cell lysates were collected by scraping and protein concentration was determined using Bradford assay. Extracted protein (40-80 μg) was fractionated by electrophoresis in 7% to 9% sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose membranes, and blocked with PBS containing 0.1% Tween-20 and 5% non-fat dry milk for 1 hour. Afterward, the membrane was incubated with specific primary antibody overnight at 4°C, followed by secondary antibody for one hour at room temperature. Immunoblots were visualized with an enhanced chemiluminescence
detection system according to the protocol of the manufacturer (Pierce Biotechnology, IL, USA). Densitometry analysis was performed by using the National Institutes of Health 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**

Sub-confluent SMCs were plated in a 24-well culture plate. After 24 hours, cells were incubated overnight in the serum-free condition to arrest cell growth and proliferation and to assure a synchronized growth following the addition of serum. The following day cells were treated with increasing concentrations of diosgenin or vehicle control (EtOH) for 24 hours in the presence of 10% serum. The CellTiter 96® AQueous Non-Radioactive Cell viability Assay (MTS) was used to measure cell viability according to the manufacturer’s protocol (Promega, WI, USA). Cell viability was presented as percentage compared to the control group (arbitrarily considered as 100 percent of viability).

**Measuring cell death by Cellomics**

To determine the effect of diosgenin on SMCs death, cell apoptosis and necrosis were measured using the ArrayScan HCS system (Cellomics). In this system, numbers of annexin-V-positive cells (apoptotic cells) and Propidium iodide (PI)-positive cells (necrotic cells) were determined. Briefly, SMCs (5 X 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 hours. As the positive control for apoptosis, one group of cells was treated with the apoptosis-inducing drug staurosporine (200nmol.L^-1) for 24 hours. To measure the rate of cell death due to necrosis or apoptosis, cells were then subjected to propidium iodide (1μg.ml⁻¹) or
phycoerythrin-conjugated annexin V (PE-annexin V) according to the manufacturer’s protocol (BD Biosciences, ON, Canada). To measure cell necrosis, around 30 minutes before the ending point of incubation, Hoechst 33342 (1 μg.mL⁻¹) and propidium iodide (1 μg.mL⁻¹) were added to all wells. In order to measure apoptosis, cells were once washed with annexin V binding buffer and then incubated with Hoechst 33342 (1 μg.ml⁻¹) and PE-annexin V (5 μl/well) for 10 minutes in the presence of annexin V binding buffer. Following the proper incubation period, cells were scanned live using the ArrayScan HCS (high content screening) system (Thermo Fisher Scientific, MA, USA). 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 PE-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 staurosporine-treated group (the positive control) is arbitrarily set to 100 percent.

**Cell migration assay**

Cell migration was measured using QCM™ Transwell Colorimetric Cell Migration Assay according to the manufacturer’s protocol (Chemicon International, CA, USA). Briefly, 1x10⁵ serum-starved SMCs (to induce quiescence and to synchronize cell proliferation) were loaded onto the upper well of the chamber in the presence or absence of agonist (diosgenin) and vehicle control (EtOH) while lower wells were filled with serum-containing culture medium with various concentrations of the agonist (diosgenin). Following 12 hours incubation, non-migrating cells on the upper side of membranes were removed by wiping and rinsing, and migrated cells on the lower side of membranes were
counted using colorimetric assay. Data are represented as percentage in cell migration where migratory rates for control groups are arbitrarily set to 100 percent.

**Wound healing assay**

Sub-confluent SMCs were grown on glass coverslips. Following overnight serum starvation (to induce quiescence and to synchronize cell proliferation), cell cultures were scratched with a sterile pipette tip to form a wound, washed with pre-warmed sterile PBS, and incubated with medium (containing 10% serum). Cells were then treated with the vehicle (EtOH) or increasing concentrations of diosgenin (10, 25, & 50μmol.L⁻¹) or left untreated (control). At 24 hours post-injury cells were fixed and subjected to imaging using Nikon inverted microscope (X400 magnification) and Spot 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: % of wound closure = \([(L_{0h} - L_{24h})/L_{0h}] \times 100\%\), where \(L_{0h}\) is the distance between two edges of the wound measured at 0 hours post-injury (immediately after scratching), and \(L_{24h}\) is the distance at 24 hours post-injury. Data are represented as percentage of wound closure where the wound size in control (non-treated) group at 24 hours post-injury is arbitrarily set to 100 percent.

**Cytoplasmic measurement of calcium**

In order to measure cytoplasmic calcium in the isolated aorta, 2mm segments of endothelium-intact aortic rings were gently inverted and then loaded with Fluo-4AM (5μmol.L⁻¹ with 5μmol.L⁻¹ Pluronic F-127) for 2 hour at 37°C. Later, loaded aortic rings were isometrically mounted, followed by 15 minute washout time in HEPES-buffered physiological saline solution. For *in vitro* measurement of calcium signals, sub-confluent
SMCs were grown on Matrigel-coated (BD Sciences, ON, Canada) 35-mm glass-bottom culture dishes (MatTek Co., MA, USA) 48 hours prior to each experiment. Cells were then loaded with Fluo-4AM for 1 hour at 37°C, followed by a 15-20 minute wash in HEPES-buffered physiological saline solution.

To investigate the effects of diosgenin on calcium release, cultured SMCs or isolated aortic segments were pre-treated with diosgenin or vehicle (EtOH) for 30 minutes and then stimulated with various pharmacological agents. Images were acquired on an upright Olympus BX50WI microscope with a 60X water-dipping objective (NA 0.9) and equipped with an Ultraview Confocal imaging system (Perkin-Elmer). 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). The representative fluorescence traces shown reflect the averaged fluorescence signals from 6-10 SMCs in each region of interest (ROI). The measured changes in Fluo-4 fluorescence level are proportional to the relative changes in [Ca^{2+}]_{cyto}. The confocal images were analyzed off-line with the Ultraview 4.0 Software (Perkin-Elmer). 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 (A/S Danish Myotechnology, AA, Denmark) for measuring generated force. Krebs solution containing (in mmol·L^{-1}) NaCl 130, KCl 4, MgSO_{4} 1.2, NaHCO_{3} 4, CaCl_{2} 1.5, HEPES 10, KH_{2}PO_{4} 1.18, glucose 6, and EDTA 0.03 (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% O₂–5% CO₂ in Krebs solution. Optimal tension (5mN) was preliminarily determined as described previously (Chung et al., 2008; Syyong et al., 2009). Aortic segments were stretched to the optimal tension for 1 hour and then stimulated twice with 60mmol.L⁻¹ KCl to ensure tissue viability and normal contractile response. To study the effects of diosgenin on contraction, aortic segments were incubated with increasing concentrations of diosgenin (2.5, 10, and 25μmol.L⁻¹) or vehicle (ethanol) for 30 minutes before stimulation with phenylephrine (PE) or 60mmol.L⁻¹ KCl.

For some experiments and in order to block voltage gated calcium channels, SMC cultures or aorta rings were pre-treated with 1μmol.L⁻¹ Nif or vehicle (DMSO) for ten minutes prior to PE or 60mmol.L⁻¹ KCl stimulation. The concentration-response curve of PE-induced contraction was prepared and the negative logarithm (pD₂) of the concentration of PE giving half-maximum response (EC₅₀) was assessed by linear interpolation on the semi-logarithm concentration-response curve [pD₂=-log (EC₅₀)].

To determine whether diosgenin can modulate smooth muscle contraction, endothelium-intact aortic rings were pre-treated with increasing concentrations of diosgenin (or the vehicle) for 30 before stimulation with PE. For some experiments, the endothelial layer was removed by gently rubbing the intimal layer of aorta with the wire prior to mounting the segments in the myograph chamber. Aortic segments were then treated with 25μmol.L⁻¹ of diosgenin for 30 minutes prior to PE treatment. This procedure abolished aortic relaxation in response to acetylcholine (data not shown).

To calculate the percentage of inhibition of contraction by diosgenin, the force created by 10μmol.L⁻¹ PE in the presence of μmol.L⁻¹ diosgenin (vehicle only, EtOH) was arbitrary
set as 100% of contraction for control group. Percentage of contraction for diosgenin-treated groups was calculated in comparison to the vehicle control group. The percentage of inhibition was calculated as:

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\% \text{ of inhibition} = \% \text{ contraction for vehicle-treated control group} - \% \text{ contraction for diosgenin-treated group}
\]

**Statistical analysis**

Statistical analysis and preparation of concentration-response curves were performed using GraphPad Prism 4.0 software (San Diego, CA, USA). Data was analyzed using One-way analysis of variance (ANOVA). We also used “repeated measures ANOVA” where those different doses were treated as repeated measures (within factor) and different replicates were treated as 'between' factor. We expected "within" to be 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 ± standard deviation (SD) where a value of \( P < 0.05 \) was considered statistically significant. The exact number of repetition (n) for each experiment is specified in the “results” section. 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, while the groups marked as “0μmol.L\(^{-1}\)” 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 (≥25μmol.L⁻¹) 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 25μmol.L⁻¹, diosgenin caused apoptosis in 17% of SMC population. As the concentration of diosgenin increased to 50μmol.L⁻¹, apoptosis was detected in 60% of SMC population (Fig. 1A, n = 3 experiments of 3 replicates, *P < 0.05). Western-blot analysis also confirmed the cleavage of caspase-3 protein in diosgenin-treated SMCs in a concentration-dependent manner (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 SMCs viability and Akt phosphorylation

Diosgenin (≥25μmol.L⁻¹) decreased SMCs viability in a concentration-dependent manner (Fig 2A, n = 3 experiments of 3 replicates, *P < 0.05). At the concentration of 25μmol.L⁻¹, diosgenin caused a significant decrease (40%) in SMC viability. It is well established that Akt (protein kinase B, PKB) 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-Ser 473) following diosgenin treatment. Our results showed that diosgenin decreased Akt
phosphorylation in SMCs without affecting 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 prior to diosgenin treatment. To assure the efficiency of transfection, Akt phosphorylation was measured in transfected SMCs. As shown in figure 3A, phosphorylation of Akt-Ser was significantly increased in SMCs transfected with the constitutively active form of Akt as compared to cells transfected with the adenoviral vector expressing the GFP protein (Fig. 3A, n=3 experiments, *P<0.05). Over-expression 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 cytopathic 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 3 replicates, *P<0.05).

Effects of diosgenin on vascular SMCs migration

Diosgenin (≥10μmol.L⁻¹) 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 3 replicates, *P < 0.05). The low concentration of diosgenin (10μmol.L⁻¹) caused a considerable decrease (40%) in SMC migration, while at a higher concentration (50μmol.L⁻¹), diosgenin nearly completely
blocked cell migration. It is of importance that at a low concentration (10μmol.L−1), 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 10μmol.L−1 PE (with the threshold effective concentration of 0.1 μmol.L−1), a concentration that induces 90% of maximal force development (Fig. 5A, n = 5 mice). Diosgenin inhibited the contractile response to PE (10μmol.L−1) both in the presence and absence of extracellular calcium in a concentration-dependent manner with the IC50 values of 25μmol.L−1 (pD2 = -4.5) and 10μmol.L−1 (pD2 = -5.0), respectively (Fig. 5B, n = 5 mice). Interestingly, in the absence of diosgenin, removal of extracellular calcium one minute prior to 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 hours 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; Leger 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 Ca2+ 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\(\mu\)mol.L\(^{-1}\) 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\(\mu\)mol.L\(^{-1}\) 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 pre-treatment of aorta with diosgenin for 30 minutes prior to the stimulation with 60mmol.L\(^{-1}\) KCl had also no effect 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 prior to PE treatment. Diosgenin treatment (25\(\mu\)mol.L\(^{-1}\)) in both groups resulted in 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\(\mu\)mol.L\(^{-1}\) diosgenin. As shown in figure 8A, diosgenin caused a significant decrease (50%) in the PE-induced calcium transient in aortic rings (n = 11 mice, mean ± SD, *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 prior to stimulation of aortic rings with PE, resulted in a decrease in basal [Ca^{2+}]_i, shortening of the PE-induced calcium transient and abolition of the plateau phase (Fig. 8B, n = 9 mice, mean ± SD, *P<0.05). In the absence of extracellular calcium diosgenin completely blocked the PE-induced SR calcium release transient in parallel with blockade of force development (as compared to 50% inhibition in the presence of extracellular calcium in figure 8A).

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 figure 9A, diosgenin caused a significant inhibition of the UTP-induced calcium transient in cultured SMCs (n=5 experiments, mean ± SD, *P < 0.05). It is noteworthy that Nif has no effects on UTP-induced calcium transients in cultured SMCs (Fig. 9B, n=5 experiments, mean ± SD, *P < 0.05). Furthermore, to investigate the effects of diosgenin on UTP-induced force development, isolated aortic rings were pretreated with 25\(\mu\)mol.L\(^{-1}\) diosgenin for 30 minutes prior to UTP application. Diosgenin treatment reduced UTP-induced force development by 50% (figure 10, n=5 mice, mean ± SD, *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\(\mu\)mol.L\(^{-1}\) CPA, a sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor. In control groups, re-perfusion of cell cultures with HEPES buffer containing 1.5mmol. L\(^{-1}\) (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 abolished indicating an inhibitory effect on store-operated calcium channels in aortic SMCs (Fig. 11, n = 6 experiments, *$P<0.05$).
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. Over-expression 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), over-expression of both wild-type and activated forms of Akt were able to improve cell viability following diosgenin treatment. This is due to immediate phosphorylation of the exogenous Akt (over-expressed 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 due to inhibition of Akt signaling and inducing apoptosis in various in vitro culture systems (Moalic et al., 2001; Leger et al., 2004; Shishodia and Aggarwal, 2006). Our findings have shown that at the concentration higher than 10μmol.L⁻¹, 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 25-50μmol.L⁻¹. Fortunately the potency for apoptotic and necrotic effects is low in mouse aortic SMCs,
such that at the concentration of 10μmol.L⁻¹, diosgenin induces 40-50% 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. Similarly, in the presence of extracellular calcium, blocking the L-type calcium channels with nifedipine caused almost 50% decline in PE-induced force, highlighting that in mouse aorta only half of PE-induced contraction is due to calcium entry through L-type calcium channels, and the remaining half is probably dependent 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 due to its effects on calcium release from the intracellular stores and possibly calcium influx through store-operated calcium channels.

Dias et al 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 (Dias et al., 2007). However, in our isolated mouse aorta, the effects of diosgenin on aortic contraction did not seem to be dependent on the endothelial layer, since 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 vs. rat mesenteric artery) or variations in diosgenin concentrations used (25μmol.L⁻¹ vs. 1mmol.L⁻¹).

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 (1mmol.L⁻¹) induced a rapid but transient increase in [Ca²⁺]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 is dependent 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 60mmol.L⁻¹ 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 SERCA 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. 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.

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 vasoconstriction (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 blockade 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 10μmol.L⁻¹ of 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 SMC population suggesting that the use of diosgenin at the concentration range of 10-15μmol.L⁻¹ 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

Designed the experiments: Mitra Esfandiarei

Performed the experiments: Mitra Esfandiarei & Julia T.N. Lam

Helped with cell and tissue culture & confocal microscopy: Sahar A. Yazdi

Performed apoptosis assay by Cellomics: Amina Kariminia & Kaiji Hu

Helped with myograph: Harley T. Syyong & Jorge N. Dorado

Analyzed data: Mitra Esfandiarei & Boris Kuzeljevic

Wrote and revised the manuscript: Mitra Esfandiarei & Cornelis van Breemen
Reference


through estrogen receptor-related phosphatidylinositol 3-kinase/Akt and p38 mitogen-activated protein kinase pathways in osteoblasts. *Molecular Pharmacology* 68:1061-1073.
Footnotes

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Legends for Figures:

Figure 1. Apoptotic effects of diosgenin on vascular SMC

A) Diosgenin (≥25μmol.L⁻¹) increased the number of both the annexin V-positive (indicator of apoptosis) and PI-positive (indicator of necrosis) cells in a concentration-dependent manner with the EC₅₀ values of 33μmol.L⁻¹ and 43μmol.L⁻¹, respectively (n=3 experiments, mean ± SD, *P < 0.05). B) Diogenin 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 ± SD, *P<0.05). C) At the concentration of 25μmol.L⁻¹, diosgenin causes cytopathic effects and apoptosis in the SMC culture. Apoptotic bodies are marked by black arrows (original magnification, X200). Control groups represent non treated cells while the 0μmol.L⁻¹ group represents vehicle (EtOH)-treated cells.

Figure 2. Effects of diosgenin vascular SMC viability and Akt phosphorylation

A) Diosgenin (≥25μmol.L⁻¹) decreased the serum-induced cell proliferation (an index of cell viability) in a concentration-dependent manner with an IC₅₀ value of (≥25.6μmol.L⁻¹). Data presents three independent experiments (n=3 experiments, mean ± SD, *P<0.05). B) Diosgenin decreased Akt phosphorylation (an indicator of cell viability) with no effects 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 ± SD, *P < 0.05). Control groups represent non treated cells while the 0μmol.L⁻¹ group represents vehicle (EtOH)-treated cells.
Figure 3. Reversal of diosgenin-induced apoptosis by over-expression of a constitutively active form of Akt

A) SMCs were transfected with the adenoviral vector expressing the constitutively active form of Akt for 48 hours. As shown, transfected SMCs express a high level of Akt phosphorylation (2.5 fold increase) as measured by Western blot analysis. Beta actin protein expression confirmed equal protein expression. The blot respresents one of three independent experiments. The bar graph is the average band intensity of three independent experiments (n = 3, mean ± SD, *P < 0.05). Control group represents non-transfected SMCs. B) Over expression of an active form of Akt rescues SMCs from the apoptotic effects of diosgenin. Diosgenin-induced caspase-3 cleavage was blocked by over-expression of activated form of Akt. Control group represents non-transfected 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 ± SD, *P<0.05). Groups marked as [0μmol.L⁻¹] represents vehicle (EtOH)-treated cells.

Figure 4. Effects of diosgenin on vascular SMCs migration

A) Diosgenin (≥10μmol.L⁻¹) blocked SMCs migration into the site of injury and wound closure in a concentration-dependent manner with the IC₅₀ value of 15.3μmol.L⁻¹ (n = 6 experiments, mean ± SD, *P<0.05). B) In the Boyden chamber migration assay, diosgenin (≥10μmol.L⁻¹) caused a marked decrease in serum-stimulated migration in a concentration-dependent manner with the IC₅₀ value of 10.3μmol.L⁻¹ (n = 3 experiments, mean ± SD, *P < 0.05). Graphs represent the average of three independent experiments.
Control groups represent non-treated cells while the 0 μmol.L⁻¹ group represents vehicle (EtOH)-treated cells.

**Figure 5. Effects of diosgenin on α-adrenergic receptor-mediated contraction in the isolated mouse aorta**

A) At the concentration of 10 μmol.L⁻¹ PE induces sub-maximal contraction in the aorta (n=5 mice, *P<0.05). B) In the presence of extracellular calcium, diosgenin causes 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).

**Figure 6. Effects of diosgenin on Ca²⁺ influx via L-type calcium channels in the isolated mouse aorta**

A) 1 μmol.L⁻¹ Nif significantly inhibits PE-induced force development in isolated aortic rings as compared with control and DMSO-treated groups (n = 6 mice, mean ± SD, *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 ± SD, *P<0.05).

**Figure 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 μmol.L⁻¹ diosgenin caused 50% decrease in
PE-induced aortic contraction in both endothelium-denuded and endothelium-intact aortic rings (n = 5 mice, mean ± SD, *P<0.05).

**Figure 8. Effects of diosgenin on α-adrenergic receptor-mediated calcium signals in the isolated mouse aorta**

A) Treatment of the mouse aorta with 25μmol.L⁻¹ of diosgenin resulted in a significant inhibition of PE-induced calcium transient as compared to vehicle (EtOH)-treated aorta (n = 11 mice, mean ± SD, *P<0.05). B) In the absence of extracellular calcium, diosgenin completely blocked PE-induced calcium transient in the mouse aorta (n = 9 mice, mean ± SD, *P<0.05).

**Figure 9. Effects of diosgenin on purinergic receptor-mediated calcium signals in mouse aortic SMC culture**

A) At the concentration of 25μmol.L⁻¹ diosgenin caused a marked decrease in the UTP-induced calcium transient in cultured SMCs as compared to vehicle (EtOH)-treated group (n = 5 experiments, mean ± SD, *P<0.05). B) Nifedipine (1μmol.L⁻¹) did not affect the UTP-induced calcium signal indicating that L-type calcium channel were not activated during UTP stimulation. Bar graphs represents the average of three independent experiments (n = 5 experiments, mean ± SD, *P<0.05).

Figure 10. Effects of diosgenin on UTP-induced force development in the mouse aorta

Pre-treatment of aortic segments with 25μmol.L⁻¹ diosgenin resulted in a 50% decrease in UTP-induced force development in the aorta (n = 5 experiments, mean ± SD, *P<0.05).

**Figure 11. Effects of diosgenin on store-operated channels in aortic SMCs**
In the absence of diosgenin and following SR depletion, re-perfusion of cells with HEPES buffer containing 1.5mmol.L\(^{-1}\) Ca\(^{2+}\) resulted in a rapid and transient increase in cytoplasmic calcium that quickly dropped and then maintained at the basal level. However, in the presence of 25µmol.L\(^{-1}\) diosgenin the peak response (caused by the opening of store-operated calcium channels) was significantly blocked (n = 6 experiments, mean ± SD, *P<0.05).

Figure 12. Family of curves representing various effects of diosgenin on mouse aortic smooth muscle cells

Treatment of vascular smooth muscle with 10µmol.L\(^{-1}\) diosgenin provides 13% inhibition of proliferation, 45% inhibition of migration, 24% inhibition of contraction. Note that no significant apoptotic (less than 3%) effects are apparent in vascular SMCs when treated with 10µmol.L\(^{-1}\) diosgenin. The table summarized the inhibitory effects of diosgenin and the IC\(_{50}\) for all the observed effects.
Figure 1

A) Bar graph showing the % Apoptosis + Necrosis with different concentrations of Diosgenin (µmol.L⁻¹). The graph includes control, 25, and 50 µmol.L⁻¹ treatments. Two bars are indicated with * and # symbols, indicating statistical significance (P < 0.05).

B) Western blot analysis of Cleaved Caspase-3 and β-Actin with control, 0, 5, 10, 25, and 50 µmol.L⁻¹ treatments. The graph shows fold change with control and treated conditions, indicating a significant increase (P < 0.05).

C) Microscopy images showing the effect of Diosgenin (24 hours) on the cell morphology. Control and treated conditions are compared at 0 µmol.L⁻¹, 25 µmol.L⁻¹, and 50 µmol.L⁻¹ with arrows indicating changes in cell structure.
Figure 2

A) % Cell Viability

B) % Cell Viability

*D P < 0.05*
Figure 3

A) 

Control  Ad-Ca-Akt  Ad-GFP  
P-Akt-s  
β-Actin

B) 

Control  Ad-Ca-Akt  Ad-GFP  
0  5  10  25  25  25  Diosgenin (μmol.L⁻¹)  
Cleaved Caspase-3  
β-Actin

C) 

% Cell Viability  
Control  Ad-GFP  Ad-Wt-Akt  Ad-Ca-Akt  
0  25  25  25  25  
(+) 10% Serum

Control  Ad-GFP  Ad-Wt-Akt  Ad-Ca-Akt  
0  25  25  25  25  
(-) 10% Serum  

*  **  P < 0.05

**  P < 0.05

*  P = 0.2086

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Figure 4

A) % Wound Closure

<table>
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<tr>
<th>Diosgenin (μmol.L⁻¹)</th>
<th>0</th>
<th>10</th>
<th>25</th>
<th>50</th>
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<tbody>
<tr>
<td>Control (0 hr)</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>24 hours post-injury</td>
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</table>

* P < 0.05
** P < 0.05

B) % Migration in Boyden Chamber

<table>
<thead>
<tr>
<th>Diosgenin (μmol.L⁻¹)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>25</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control non-treated</td>
<td>100</td>
<td>80</td>
<td>60</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.05
Figure 5

A) 

B) 

C)
Figure 6

A)  

**Control**  
**DMSO**  
**Nifedipine**

B)  

**60 mmol.L⁻¹ K⁺**  
**25 µmol.L⁻¹ Diosgenin**  
**60 mmol.L⁻¹ K⁺**  
**1 µmol.L⁻¹ Nifedipine**

* P < 0.05  
# P = 0.6515
Figure 7

![Graph showing inhibition of PE-induced contraction](image)

% Inhibition of PE-induced contraction

+ Endothelium

- Endothelium

μmol.L⁻¹ Diosgenin (30min)

* P = 0.9600
**Figure 8**

A) 0 μmol.L⁻¹ Diosgenin

Cytoplasmic Calcium (Fold Change)

<table>
<thead>
<tr>
<th></th>
<th>Base</th>
<th>PE</th>
<th>Base</th>
<th>PE</th>
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<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>25</td>
<td>25</td>
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</table>

μmol.L⁻¹ Diosgenin (30 min)

* P < 0.05

B) 0 μmol.L⁻¹ Diosgenin

Cytoplasmic Calcium (Fold Change)

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>0 Ca²⁺</th>
<th>PE</th>
<th>Baseline</th>
<th>0 Ca²⁺</th>
<th>PE</th>
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<tr>
<td></td>
<td>0.5F/F₀</td>
<td>200s</td>
<td></td>
<td>0.5F/F₀</td>
<td>200s</td>
<td></td>
</tr>
</tbody>
</table>

- Diosgenin + Diosgenin

* P < 0.05
Figure 9

A) UTP-Induced Calcium Release (Fold Change)

- 0 μmol.L⁻¹ Diosgenin
  - 1 mmol.L⁻¹ UTP
- 25 μmol.L⁻¹ Diosgenin
  - 1 mmol.L⁻¹ UTP

* P < 0.05

B) Calcium Release (Fold Change)

- 0 μmol.L⁻¹ Nifedipine
  - 1 mmol.L⁻¹ UTP
- 1 μmol.L⁻¹ Nifedipine
  - 1 mmol.L⁻¹ UTP

* P = 0.9369
Figure 10

* P < 0.05

% Inhibition of Force (of 1 mmol.L⁻¹ UTP)

0 μmol.L⁻¹ Diosgenin

25 μmol.L⁻¹ Diosgenin

1 mmol.L⁻¹ UTP

0 25 μmol.L⁻¹ Diosgenin
Figure 11

- **Cytoplasmic Calcium (Fold Change)**

<table>
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<tr>
<th>Condition</th>
<th>Baseline</th>
<th>Peak</th>
<th>Plateau</th>
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<tbody>
<tr>
<td>- Diosgenin</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>+ Diosgenin</td>
<td></td>
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</tbody>
</table>

- **Legend**

- 0 μmol L⁻¹ Diosgenin
- 25 μmol L⁻¹ Diosgenin

- **Significance**

* P < 0.05
Figure 12

![Graph showing the effects of Diosgenin on apoptosis, inhibition of migration, and inhibition of contraction.](image)

<table>
<thead>
<tr>
<th>Effect (%)</th>
<th>0μM</th>
<th>5μM</th>
<th>10μM</th>
<th>25μM</th>
<th>50μM</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (μmol.L&lt;sup&gt;-1&lt;/sup&gt;)</th>
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</thead>
<tbody>
<tr>
<td>Apoptosis</td>
<td>2.7±0.15</td>
<td>2.80±0.32</td>
<td>3.01±0.39</td>
<td>17.29±1.01</td>
<td>60.65±2.13</td>
<td>32.99</td>
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<tr>
<td>Inhibition of Migration</td>
<td>0.1±0.1</td>
<td>7.3±0.1</td>
<td>45.1±2.02</td>
<td>69.1±2.3</td>
<td>85.4±3.3</td>
<td>10.19</td>
</tr>
<tr>
<td>Inhibition of Contraction</td>
<td>1.5±0.4</td>
<td>6.7±2.8</td>
<td>23.9±4.7</td>
<td>46.7±5.0</td>
<td>48.2±4.9</td>
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