Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 502 (2004) 123–133

www.elsevier.com/locate/ejphar

Activation of iberiotoxin-sensitive, Ca^{2+}-activated K^+ channels of porcine isolated left anterior descending coronary artery by diosgenin

Alice Lai Shan Au[a], Ching Chi Kwok[a], Allen Ting Chun Lee[a], Yiu Wa Kwan[a,*], Macey Mei Sze Lee[b], Rong-Zhen Zhang[c], Sai Ming Ngai[d], Simon Ming Yuen Lee[e], Guo-Wei He[c], Kwok Pui Fung[b,e]

[a]*Room 409B, Basic Medical Sciences Building, Department of Pharmacology, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong SAR, PR China*

[b]*Department of Biochemistry, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong SAR, PR China*

[c]*Department of Surgery, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong SAR, PR China*

[d]*Department of Biology, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong SAR, PR China*

[e]*Institute of Chinese Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong SAR, PR China*

Received 3 June 2004; received in revised form 11 August 2004; accepted 19 August 2004
Available online 13 September 2004

Abstract

The objective of this study was to determine the vasodilating effect of 3β-hydroxy-5-spirostene (diosgenin), a phytoestrogen found in wild yams, using porcine resistance left anterior descending coronary artery. In 5-hydroxytryptamine (3 μM) pre-contracted preparation, diosgenin caused a concentration-dependent (0.01 to 1 μM), endothelium-independent relaxation, with a maximum relaxation of ~72% at 1 μM. No apparent effect was observed with 17β-oestradiol and progesterone with concentrations ≤0.3 μM, and a relaxation of ~15% and ~23% caused by 17β-oestradiol (1 μM) and progesterone (1 μM), respectively. Diosgenin-elicited relaxation was not altered by 7α,17β-[9[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol (ICI 182,780), mifepristone, (+)-bicuculline, *cis*-*N*-(2-phenylcyclopentyl)azacyclotridec-1-en-2-amine (MDL 12330A), glibenclamide and scavengers of reactive oxygen species. The iberiotoxin-sensitive, Ca^{2+}-activated K^+ (BK_{Ca}) current of single vascular myocytes recorded, using patch-clamp techniques, was markedly enhanced by diosgenin, 17β-oestradiol and progesterone. Application of (9*S*, 10*R*, 12*R*)-2,3,9,10,11,12-hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-*fg*:3′,2′,1′-*kl*]pyrrolo[3,4-*i*][1,6]benzodiazocine-10-carboxylic acid methyl ester (KT 5823, 300 nM) eradicated the enhancement of BK_{Ca} amplitude. Diosgenin, 17β-oestradiol and progesterone did not affect whereas phloretin, biochanin A and zearalanone (1 μM each) significantly suppressed $[Ca^{2+}]_o$-induced contraction. In oestrogen competition essay using human breast cancer cell (MCF-7 cells), diosgenin (0.001 nM to 10 μM) did not interact with oestrogen receptor-α, and no displacement of [^{3}H]17β-oestradiol was observed. In oestrogen receptor α- and β-fluorescence polarization competitor assay, diosgenin (100 μM) demonstrated a greater competition with the β-isoform of oestrogen receptor. These results suggest that diosgenin caused an acute, endothelium-independent coronary artery relaxation via protein kinase G signalling cascade and an activation of BK_{Ca} channel of arterial smooth muscle cells. The oestrogen receptor (α and β-isoforms) and progesterone receptor are probably not involved.

Keywords: Diosgenin; Porcine coronary artery; Oestrogen receptor; Iberiotoxin-sensitive; Ca^{2+}-activated K^+ (BK_{Ca}) channel

1. Introduction

It is well known that women are less susceptible than men to cardiovascular diseases until they reached menopause. Cessation/reduction of the production of ovarian hormone oestrogen as women enter the menopause is accompanied by an increased incidence of coronary vascular diseases (Stampfer et al., 1991) and the development of osteoporosis. In order to solve this problem, hormone replacement therapy (oestrogen plus progestin) is recommended for women who have developed menopausal-

* Corresponding author. Tel.: +852 2609 6884; fax: +852 2603 5139.
E-mail address: b136701@mailserv.cuhk.edu.hk (Y.W. Kwan).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.08.045

related discomforts. However, the cardioprotection conferred by hormone replacement therapy has been questioned recently (Baber et al., 2003; Wassertheil-Smoller et al., 2003). Women who take long-term oestrogen therapy appear to have ~30% greater chance of developing breast cancer (Cummings, 1991) and uterus cancer. In lieu of these side effects, there is an urgent need in searching for an alternative agent that possesses the beneficial property of oestrogen (e.g. coronary artery dilatation).

Phytoestrogens are nonsteroidal compounds present in a variety of products, and consumption of diet (e.g. soybean) rich in phytoestrogens (e.g. genistein and daidzein) has been suggested providing protection against certain oestrogen-dependent diseases. Diosgenin (3β-hydroxy-5-spirostene) is a plant-derived sapogenin that has been suggested structurally "fairly similar" to progesterone (Fig. 1). Diosgenin is the precursor for the industrial large-scale synthesis of different hormones including progesterone (Marker et al., 1940) and norethisterone. It is therefore believed that diosgenin can be converted into progesterone and other sex steroids in vivo. However, the commercial synthesis of progesterone and norethisterone from diosgenin involved very complex, multiple steps. It is unlikely that the metabolic conversion of diosgenin into various sex steroids occurred in vivo. In the market, there are over 50 formulations/creams that contained extract of wild yam *Dioscorea villosa*. Diosgenin present in the cream may confer oestrogen-like beneficial effects when applied topically. However, the scientific evidence is scare, and the long-term beneficial effect of diosgenin on relieving post-menopausal symptoms is questionable (Komesaroff et al., 2001).

It has been reported that diosgenin retarded the progression of osteoporosis (Higdon et al., 2001), and possessed hypocholesterolemic (Juarez-Oropeza et al., 1987) and anti-inflammatory (Yamada et al., 1997) effects in rat. In addition, diosgenin stimulated the growth of

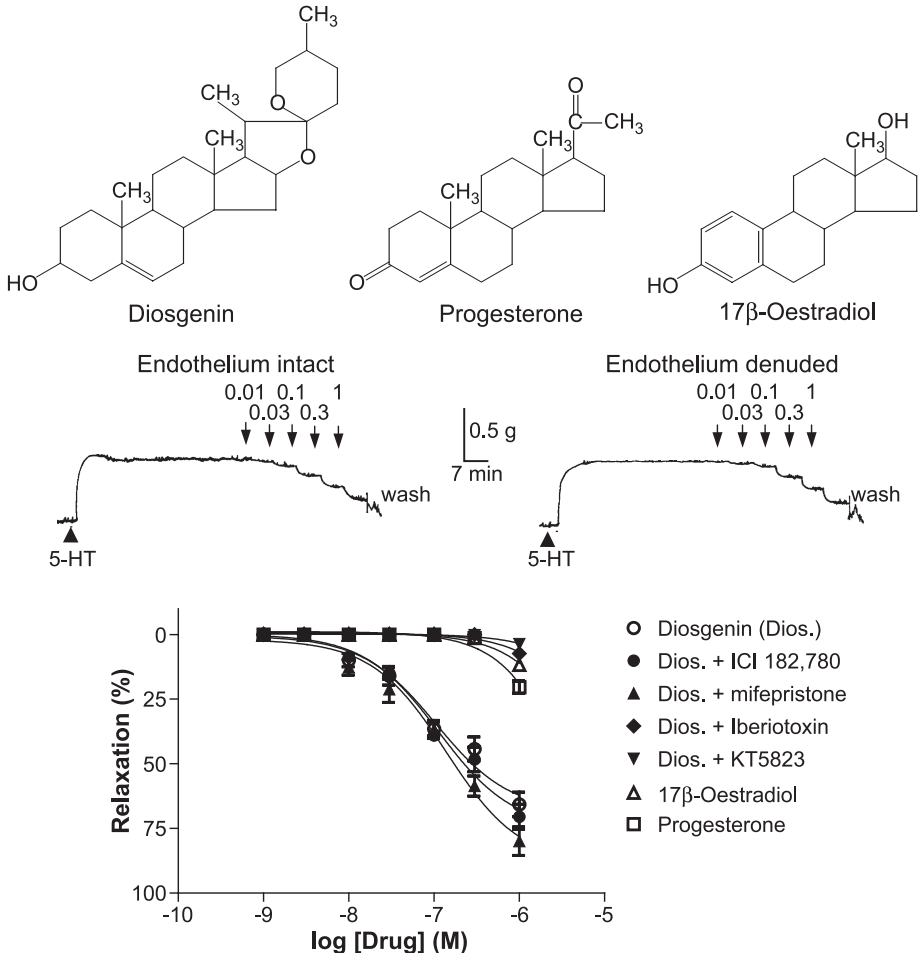

Fig. 1. Chemical structures of diosgenin, progesterone, and 17β-oestradiol (top panel). Representative recordings of the relaxation effects of diosgenin on 5-hydroxytryptamine (5-HT) (5-HT: 3 μM, endothelium intact; 5-HT: 0.9 μM, endothelium denuded) pre-contracted porcine left anterior descending coronary artery (middle panel). Calibration bars: 0.5 g and 7 min. Summary of the relaxation effect of diosgenin (○, control), and in the presence of ICI 182,780 (10 μM, ●), mifepristone (10 μM, ▲), KT 5823 (300 nM, ▼), and iberiotoxin (100 nM, ◆) on 5-hydroxytryptamine pre-contracted porcine left anterior descending coronary artery (endothelium intact). Relaxation effects of progesterone (□) and 17β-oestradiol (△) are included for comparison (bottom panel). Results are expressed as mean±S.E.M., n=5–6.

mammary gland of ovariectomized mouse (Rao and Kale, 1992) as well as inducing apoptosis and cell cycle arrest in osteosarcoma cells (Moalic et al., 2001; Corbiere et al., 2003). In isolated frog hearts (Gomita et al., 1982) and cultured myocardial cells of ICR mouse embryo (Namda et al., 1989), diosgenin glycosides altered myocardial activities probably through the modulation of $[Ca^{2+}]_o$ flux across the plasma membrane. However, the involvement of oestrogen (α- and β-isoforms)/progesterone receptors is not known, as the effect of oestrogen receptor-α antagonist 7α,17β-[9[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol (ICI 182,780) (Wakeling et al., 1991) and progesterone receptor antagonist mifepristone (Schreiber et al., 1983) on diosgenin-induced responses has not been evaluated.

In the literature, there is a wealth of information demonstrated that an acute administration of 17β-oestradiol caused a non-genomic, endothelium-independent vascular relaxation (Jiang et al., 1991) through inhibition of Ca^{2+} influx through L-type Ca^{2+} (I_{CaL}) channel as well as activation of high-conductance, Ca^{2+}-activated K^+ (BK_{Ca}) channels (White et al., 1995). In contrast, the cardiovascular effect of diosgenin and the underlying mechanism involved are not known. Hence, we have examined the coronary artery relaxation effects of diosgenin and the participation of I_{CaL} and BK_{Ca} channels. For comparison, coronary artery relaxation effects of 17β-oestradiol, progesterone and other phytoestrogenic compounds such as phloretin, biochanin A and zearalanone (Li and Li, 1998) were determined.

2. Methods

2.1. Preparation of porcine coronary artery

Fresh hearts were obtained from pigs (either sex, weight ~20 kg, total number of animals used: 186) that were slaughtered in the morning of the experiment at a local slaughterhouse. The heart was immediately immersed in an ice-cold physiological salt solution before transported by train to the laboratory in 30 min. Segment of the left anterior descending coronary artery (tertiary branch, O.D. ~500–800 μm) was dissected within an hour after the animal was slaughtered. Fat and connective tissue were carefully removed under the dissecting microscope. Care was taken not to touch the lumen of the coronary artery during dissection. Three to four arterial rings (each 1 mm in length) were obtained from each heart, and only one ring was used for each drug treatment.

2.2. Isometric tension measurement

2.2.1. Relaxation effect of phytoestrogens on pre-contracted coronary artery

The left anterior descending coronary arterial ring was mounted in a 5-ml organ-bath containing Krebs' solution (gassed with 16% O_2–5% CO_2 balanced with N_2 (pO_2 ~110 mm Hg) (MacLean et al., 1996), pH 7.4, 37±1 °C) of the following composition (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11 and $CaCl_2$ 1.8. In some preparations, endothelium was carefully removed by gentle rubbing the intima of the blood vessel with a stainless steel wire (50 μm), and confirmed by the failure of acetylcholine (10 μM)-induced relaxation. Increasing concentrations of individual phytoestrogen were administered at half-log increments at the plateau of the previous response. The response at each concentration of drug added was measured using the MacLab Chart v 3.6 program (AD Instruments, Australia). Preparations were equilibrated under the resting tension of 10±1 mN in the bath solution for 90 min. Resting tension was readjusted, if necessary, before commencing the experiments. Relaxation in response to relaxant was expressed as % of 5-hydroxytrptamine (3 μM)-induced tone, and 100% relaxation was considered when active tone returned to baseline level. In the preliminary study (n=5), 3 μM 5-hydroxytryptamine yielded a sustained contraction which is ~85% of 5-hydroxytryptamine-induced maximum contraction (observed at 10 μM) of the left anterior descending coronary artery. Manoeuvres such as administration of N^G-nitro-L-arginine methyl ester (L-NAME), iberiotoxin, (9*S*, 10*R*, 12*R*)-2,3,9,10,11,12-Hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-*fg*:3′,2′,1′-*kl*]pyrrolo[3,4-*i*][1,6]benzodiazocine-10-carboxylic acid methyl ester (KT5823) and endothelium denudation resulted in an enhanced 5-hydroxytryptamine (3 μM)-induced tone (~34% increase), concentration of 5-hydoxytryptamine was therefore adjusted accordingly (0.6–1 μM) in order to have a similar magnitude of active tone.

2.2.2. Effect on $[Ca^{2+}]_o$ concentration–response of coronary artery under resting tension

Coronary arterial rings (endothelium-denuded) were incubated in $[Ca^{2+}]_o$-free physiological salt solution containing ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA, 0.5 mM) for 10 min. The $[Ca^{2+}]_o$ concentration-dependent contraction curve was then performed in high $[K^+]_o$ (50 mM) depolarising solution (control, 2.5 mM $[Ca^{2+}]_o$-induced contraction was considered as 100% maximum contraction). In the preliminary study performed in normal $[Ca^{2+}]_o$-containing solution, cumulative addition of $[K^+]_o$ elicited arterial contraction and the maximum response occurred at 50 mM $[K^+]_o$ (n=6). Rings were then washed with Krebs' solution for 30 min and incubated in $[Ca^{2+}]_o$-free physiological salt solution (with 0.5 mM EGTA) for a further 30 min. Subsequently, individual agent of a particular concentration was administered 30 min before the construction of $[Ca^{2+}]_o$ concentration-dependent contraction curve was repeated (treated) and compared. Only one concentration of individual agent was tested in each tissue.

2.3. Enzymatic dissociation of coronary artery smooth muscle cells

Stripe of left anterior descending coronary artery (1×1 mm) was placed in an Eppendorff tube (1.5 ml) containing a low $[Ca^{2+}]_o$ dissociation medium of the following composition (in mM): NaCl 110, KCl 5, NaH_2PO_4 0.5, KH_2PO_4 0.5, $NaHCO_3$ 10, HEPES 10, Phenol Red 0.015, taurine 10, creatine 5, Na pyruvate 5, glucose 11, $CaCl_2$ 0.16, $MgCl_2$ 2, L-ascorbic acid 0.3 and ethylenediaminetetraacetic acid (EDTA) 0.5 (pH 7.0 with NaOH). The arterial stripe was incubated for 45–60 min at 37 °C of the above solution (vol. 1 ml) with the supplement of 6 mg dithiothreitol (Sigma-Aldrich Chemicals, USA), 2.0 mg papain (Fluka, Switzerland), 7.5 mg collagenase (Type 2, Worthington Biochemicals, USA) and 2 mg bovine serum albumin (essential fatty acid-free) (Sigma-Aldrich Chemicals). After incubation, the digested tissue was transferred to enzyme-free dissociation medium, and gently triturated with a fire-polished pasture pipette to dislodge smooth muscle cells. Electrophysiological measurements were carried out only on cells that exhibited morphological features of vascular smooth muscle cells (an elongated, spindle shape, ~100 μm in length) when observed under the microscope (Au et al., 2003). Moreover, parallel observations were made using aliquots of cells (relaxed) and demonstrated a reversible contractile response to high $[K^+]_o$ (50 mM).

2.4. Electrophysiological measurement

A drop of cell suspension was placed in a glass-bottom recording chamber (vol. ~0.5 ml), mounted on the stage of an inverted microscope (Nikon Diaphot 200, Japan) equipped with a Hoffman Modulation Contrast condenser, containing a physiological salt solution of the following composition (mM): NaCl 132, KCl 4.8, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) 10, $MgCl_2$ 2, glucose 5 and $CaCl_2$ 1 (pH 7.4 with NaOH). Cells that were relaxed and have a cleared edge observed under the microscope were used in this study. Voltage-clamp and voltage-pulse generation were controlled with an Axopatch 200A patch-clamp amplifier (Axon Instruments, Forster City, CA, USA), and data were acquired and analysed using pCLAMP software (Axon Instruments).

In all experiments, the large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) current amplitude was recorded at room temperature (~22 °C) using the single micropipette "giga-seal" whole-cell, perforated-patch techniques. Families of macroscopic BK_{Ca} currents were generated by stepwise 20 mV depolarising pulses (pulse duration: 500 ms, holding potential=−70 mV) from −70 to 90 mV, stimulated at 0.166 Hz. The external recording solution contained (mM): NaCl 140, KCl 5, $CaCl_2$ 1, $MgCl_2$ 2, HEPES 10, glucose 5 (pH 7.4 with NaOH). The tip of the pipette was dipped (for ~15 s) into internal solution containing (mM): K_2SO_4 60, KCl 30, $MgCl_2$ 5, $CaCl_2$ 5, HEPES 5 and $MgSO_4$ 40 (pH 7.2 with KOH) (Ruehlmann et al., 1998; White et al., 2002). The remainder of the patch pipette was back-filled with the same solution to which amphotericin B (6 mg ml^{-1}) was added. Series resistance was compensated to provide the fastest decay of the capacitative current with no sign of ringing. Pipettes were fabricated from haematocrit glass capillaries (Accu-Fill 90 Micropet, Clay Adams, USA) and pulled on a two-stage vertical pipette puller (PP-83; Narishige Scientific Instruments, Japan). The pipette tip was position near the nuclear region of the coronary artery smooth muscle cell (dimension ~10 μm) using an oil-based hydraulic micro-manipulator (Narishige Scientific Instruments). Current amplitude recorded was filtered at 5 kHz and sampled at 3–10 kHz. Cell membrane capacitance was estimated, as previously described (Matsuda et al., 1990; Au et al., 2003), and it was 19.2±2.3 pF (n=20). Current amplitude, expressed as current density (in pA/pF), was recorded before (control), during (treated) and after (wash) the administration of a particular drug (Fig. 2). Only one concentration of individual drug was tested in each cell. Cells with visible change in leakage currents during the course of study were discarded and excluded from analysis. External solution was delivered, through gravity, and controlled by solenoid valves coupled to a 4-channel valve driver (General Valve, USA). Solution change (~5 ml, which is 10 times the volume of the recording chamber) could be completed in 15–20 s.

2.5. Measurement of the dissociation of [^{3}H]17β-oestradiol receptor complexes

The procedures of oestrogen competition assay have been reported previously (Olea-Serrano et al., 1985). Human breast cancer cell line (MCF-7 cells) was purchased from American Type Culture Collection (USA), and cells were maintained in our laboratory as monolayer culture. Before the commencement of the competition assay, cells were cultured in Roswell Park Memorial Institute (RPMI) tissue culture medium supplement with phenol red (Gibco-RBI, USA). Cells were then plated on 24-well plates with the cell density of 1×10^5. On the next day, the medium was replaced with assay medium (RPMI medium without phenol red) (Sigma-Aldrich Chemicals), with 10% steroid-depleted fetal calf serum and 1% Penicillin–Streptomycin (Gibco-RBI). Cells were incubated in this medium for two days before ligand binding essay experiment was performed. After 4 days, the growth medium was replaced by serum-free medium containing [^{3}H]17β-oestradiol (71 Ci/mmol) (Life Technology, USA) for 60 min (37 °C) with 2.82 nM [^{3}H]17β-oestradiol in the absence or presence of different concentrations of either non-radioactive 17β-oestradiol (served as a positive control) or diosgenin. After removal of the growth medium, labelled cells were washed three times with ice-cold phosphate buffer saline. The incorporated

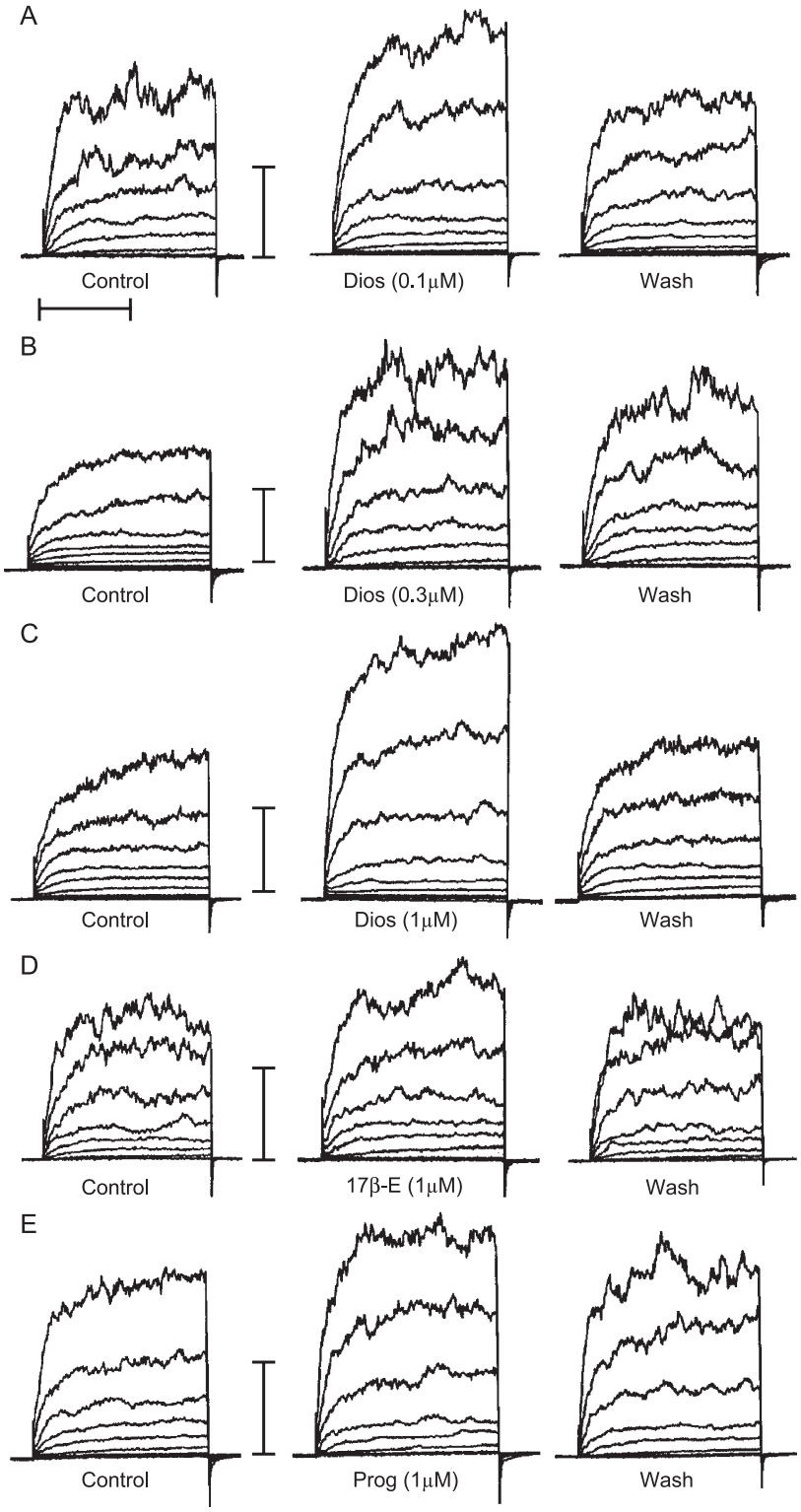

Fig. 2. Effect of diosgenin, 17β-oestradiol (17β-E) and progesterone (Prog) on iberiotoxin-sensitive, Ca^{2+}-activated K^+ channel (BK_{Ca}) current. Inset shows representative steady-state BK_{Ca} recorded in the presence of diosgenin (Dios, 0.1, 0.3, 1 μM), 17β-oestradiol (17β-E, 1 μM) and progesterone (Prog, 1 μM). BK_{Ca} recorded in control and after wash of individual cell are included for comparison. Families of macroscopic BK_{Ca} currents were generated by stepwise 20 mV depolarising pulses (pulse duration: 500 ms, holding potential=−70 mV) from −70 to 90 mV, stimulated at 0.166 Hz. Calibration bars: 1000 pA and 250 ms.

[^{3}H]17β-oestradiol retained in cells (after the administration of different concentrations of unlabelled competitors) was extracted using absolute ethanol (1 ml) (45 min, at room temperature), and transferred the aliquot of ethanol to mini scintillation vials (contained 2 ml of scintillation liquid) for radioactivity counting.

2.6. Oestrogen receptor (α- and β-) competitor assay using fluorescence polarization

In these studies, we have used commercial preparations of full-length recombinant, human oestrogen receptor-α (ERα) and -β (ERβ) (Pan Vera, Madison, WI, USA) and the fluorescein-labeled oestrogen (Fluormone ES2). They were purchased from Pan Vera. An initial oestrogen receptor-fluorescein-labeled oestrogen complex has high polarization value. When the fluorescein-labeled oestrogen complex was titrated with different concentrations of competitors, oestrogen molecules (fluorescein-labeled) are displaced from the oestrogen receptor complex, and a gradual decrease in the fluorescence polarization is observed.

All competitive compounds (17β-oestradiol and diosgenin) were prepared as 10 mM stocks in dimethyl sulphoxide. In triplicate, these compounds were serially diluted in bovine gamma globulin/phosphate buffer (Pan Vera) on a black, flat-bottom, 96-well plate (NUNC, USA). A mixture of either ERα or ERβ and Fluormone ES2 was added to the diluted competitors, and the final concentration of ES2 in all wells was 1 nM. For ERα experiments, the final concentration of the receptor was 15 nM, while in ERβ experiments, the receptor was at a final concentration of 10 nM (Parker et al., 2000). Then, the plate was incubated at room temperature for 2 h. The fluorescence polarization (in milllipolarization, mP) of each well was measured using Polarion Fluorescence Polarization Instrument (TECAN, Research Triangle Park, NC, USA) equipped with a 485-nm excitation filter and 535-nm emission filter, as recommended by the manufacturer.

2.7. Chemicals

All chemicals for preparing physiological salt solution, dissociation medium, external recording solution and internal pipette solution were purchased from Sigma-Aldrich Chemicals. 17β-Oestradiol, progesterone, 3β-hydroxy-5-spirostene (diosgenin), 3-[4-hydroxyphenyl]-1-[2,4,6-trihydroxyphenyl]-1-propanone (phloretin), 5,7-dihydroxy-4′-methoxyisoflavone (biochanin A), 2,4-dihydroxy-6-[10-hydroxy-6-oxo-undecyl]benzoic acid μ-lactone (zearalanone), 5-hydroxytryptamine hydrochloride, indomethacin, sodium nitroprusside dihydrate, glibenclamide, N^G-nitro-L-arginine methylester hydrochloride (L-NAME), 4,5-dihydroxy-1,3-bezene-disulfonic acid disodium (Tiron), superoxide dismutase, catalase and 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) were purchased from Sigma-Aldrich Chemicals. *cis*-*N*-(2-phenylcyclopentyl)azacyclotridec-1-en-2-amine hydrochloride (MDL 12330A) and (9*S*, 10*R*, 12*R*)-2,3,9,10,11,12-hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-*fg*:3′,2′,1′-*kl*]pyrrolo[3,4-*i*][1,6]benzodiazocine-10-carboxylic acid methyl ester (KT 5823) were obtained from Calbiochem–Novabiochem (San Diego, CA, USA). Mifepristone, 7α,17β-[9[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol (ICI 182,780) and (+)-bicuculline were purchased from Tocris Cookson (Bristol, UK). Iberiotoxin was purchased from Alomone Labs (Israel).

2.8. Statistical analysis

All data were expressed as mean±S.E.M. Statistical significance was determined by Student's *t*-test and analysis of variance (ANOVA), where appropriate. In organ-bath experiment, *n*=no. of heart used whereas in patch-clamp electrophysiology study, *n*=no. of single cells used. $P<0.05$ was considered significantly different from controls.

3. Results

3.1. Relaxation effect of different phytoestrogens on porcine isolated left anterior descending coronary artery

Cumulative administration of diosgenin caused a concentration-dependent (0.01 to 1 μM) (*n*=6) relaxation of pre-contracted (by 5-hydroxytryptamine) coronary artery (Fig. 1). The steady-state effect of individual concentration of diosgenin was achieved ~7 min after drug addition (Fig. 1). Maximum relaxation of 72.3±4.1% was achieved at 1 μM (the highest concentration used in this study, as the yield of diosgenin found in *Dioscorea* plants is low) (Quigley, 1978). Removal of endothelium (*n*=5) and inhibition of nitric oxide synthase (using N^G-nitro-L-arginine methyl ester (L-NAME) 20 μM) (*n*=6) caused no apparent effect on diosgenin-elicited relaxation, compared to endothelium-intact controls (Fig. 1). In endothelium-denuded preparations, iberiotoxin (100 nM, a potent large-conductance, Ca^{2+}-activated K^+ channel blocker) (*n*=4) and (9*S*, 10*R*, 12*R*)-2,3,9,10,11,12-hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-*fg*:3′,2′,1′-*kl*]pyrrolo[3,4-*i*][1,6]benzodiazocine-10-carboxylic acid methyl ester (KT 5823, 300 nM, a highly specific protein kinase G inhibitor) (*n*=5) abolished diosgenin-elicited relaxation (Fig. 1). In preparations pre-contracted with high $[K^+]_o$ (35 mM), the magnitude of diosgenin-elicited relaxation observed was drastically attenuated (1 μM diosgenin, relaxation: 13.6±5.2%) (*n*=5, $P<0.01$).

Diosgenin-induced relaxation (in both endothelium-intact and -denuded preparations) was not altered by following drugs pre-treatment. They are: indomethacin (1 μM, a cyclo-oxygenase inhibitor) (*n*=5–6), (+)-bicuculline (1 μM, a $GABA_A$ receptor antagonist) (*n*=5–6), 7α,17β-[9[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol (ICI 182,780) (1 and 10 μM, an oestrogen receptor-α antagonist) (*n*=4–5) (Fig. 1), mifepristone (1 and 10 μM, a progesterone receptor antagonist) (*n*=5–6) (Fig. 1), *cis*-*N*-(2-phenylcyclopentyl)azacyclotridec-1-en-2-amine (MDL 12330A) (1 μM, an adenylate cyclase inhibitor) (*n*=4–5), superoxide dismutase (500 U/ml)

(n=5–7), 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) (500 μM, a cell-permeant nitroxide that acts to catalyse O_2^- dismutation) (n=5–6) (Krishna et al., 1992), catalase (2000 U/ml) (n=5–6), diphenylene iodonium (5 μM, an inhibitor of flavin-containing enzymes such as NAD(P)H oxidases) (n=5–6), 4,5-dihydroxy-1,2-benzene disulfonic acid (Tiron) (10 mM, a superoxide anion scavenger) (n=6–7) and glibenclamide (3 μM, an ATP-sensitive K^+ channel blocker) (n=5–6) (data not shown). None of these drugs altered the resting tension or 5-hydroxytryptamine-induced tone of coronary artery (data not shown).

17β-Oestradiol (≤0.3 μM) failed to cause an apparent effect on active tone of pre-contracted coronary artery, and a relaxation of 15.4±3.3% was achieved at a non-physiological concentration of 1 μM (n=5) (Fig. 1). Endothelial denudation (n=5) and incubation with L-NAME (20 μM) (n=4) resulted in an attenuation of 17β-oestradiol-induced relaxation (8.2±3.1%). In preparations (endothelium-denuded) pre-contracted with high $[K^+]_o$ (35 mM), a minimal relaxation was recorded (3.0±1.1%) (n=5). Application of ICI 182,780 (1 μM) (n=5), iberiotoxin (100 nM) (n=4) eradicated 17β-oestradiol-induced relaxation, whereas mifepristone (1 and 10 μM) (n=5–6), indomethacin (1 μM) (n=5) and MDL 12330A (1 μM) (n=5) failed to alter the relaxation response (in both endothelium-intact and -denuded preparations).

Similar to 17β-oestradiol, progesterone managed to elicit relaxation of pre-contracted coronary artery only with concentrations >0.3 μM, and a relaxation of 23.5±5.4% was recorded at a non-physiological concentration of 1 μM (n=5) (Fig. 1). However, endothelial denudation (n=4) and the presence of L-NAME (20 μM) (n=5) failed to alter progesterone-elicited relaxation (data not shown). In preparations pre-contracted with high $[K^+]_o$ (35 mM), there was a minimal relaxation (endothelium-denuded preparations) recorded (5.1±2.3%) (n=5). Mifepristone (1 μM) (n=6) and iberiotoxin (100 nM) (n=4) abolished progesterone-induced relaxation, whereas ICI 182,780 (1 μM) (n=5), (+)-bicuculline (1 μM) (n=5), indomethacin (1 μM) (n=6) and MDL 12330A (1 μM) (n=5) did not alter progesterone-induced response.

Other phytoestrogenic compounds phloretin, biochanin A and zearalanone failed to elicit relaxation of pre-contracted coronary artery with concentrations ≤0.3 μM. Relaxation response of 12.2±4.1%, 24.3±3.5% and 19.5±3.4% was recorded with phloretin (1 μM) (n=5), biochanin A (1 μM) (n=5) and zearalanone (1 μM) (n=6), respectively. Endothelial denudation, L-NAME (20 μM), iberiotoxin (100 nM) (n=4), mifepristone (1 μM), ICI 182,780 (1 μM), indomethacin (1 μM) and MDL 12330A (1 μM) did not alter the relaxation response (n=5–6 for each agent) (data not shown). A similar magnitude of relaxation caused by phloretin (1 μM), biochanin A (1 μM) and zearalanone (1 μM) was recorded in preparations pre-contracted with high $[K^+]_o$ (35 mM) (phloretin, 10.1±3.3%; biochanin A, 20.8±5.2%; zearalanone, 18.3±4.6%), compared to 5-hydroxytryptamine pre-contracted tissues (n=5–6) (P>0.05).

3.2. Effect on iberiotoxin-sensitive, Ca^{2+}-activated K^+ (BK_{Ca}) channels

The outward current recorded under our conditions was markedly inhibited (~85% inhibition) by iberiotoxin (100 nM) (n=4) (BK_{Ca} amplitude measured at +90 mV, control: 71±5 pA/pF vs. iberiotoxin: 11±4 pA/pF) (P<0.001) indicating that the current recorded in this study is the genuine large-conductance, Ca^{2+}-activated K^+ (BK_{Ca}) channel. Application of diosgenin (0.1, 0.3 and 1 μM) caused a concentration-dependent enhancement of outward BK_{Ca} amplitude (diosgenin: 0.1 μM, 88±4 pA/pF; 0.3 μM, 102±6 pA/pF; 1 μM, 133±9 pA/pF) (n=7–9) (P<0.05 compared to control 71±5 pA/pF) (Fig. 2), and KT 5823 (300 nM) blunted the enhancement (with KT 5823, 78±6 pA/pF, n=5, P>0.05 compared to control).

17β-Oestradiol (1 μM), progesterone (1 μM) and sodium nitroprusside (30 μM, a nitric oxide donor) increased BK_{Ca} amplitude by ~22%, ~18% (Fig. 2) and ~57%, respectively (P<0.05 compared to control). Similar to diosgenin, the enhancement of BK_{Ca} was eradicated by KT 5823 (300 nM) (17β-oestradiol, 78±6 pA/pF; progesterone, 70±4 pA/pF; sodium nitroprusside, 76±5 pA/pF, n=5) (n=4–5, P>0.05 compared to control).

In contrast, phloretin (1 μM) (n=5), biochanin A (1 μM) (n=5) and zearalanone (1 μM) (n=6) failed to modify BK_{Ca} amplitude (phloretin, 75±5 pA/pF; biochanin A, 77±8 pA/pF; zearalanone, 72±5 pA/pF) (P>0.05 compared to control).

3.3. Effect on $[Ca^{2+}]_o$-induced contraction of endothelium-denuded porcine coronary artery

In the preliminary study, the reproducibility of $[Ca^{2+}]_o$-induced coronary artery (endothelium-denuded) contraction was determined. Two concentration–response curves of $[Ca^{2+}]_o$-induced (1 μM to 2.5 mM) contraction were constructed in each preparation (with 45 min in between for rest), and two curves overlapped with each other (n=5) (Au et al., 2003). Diosgenin (1 μM) (n=6) (Fig. 3), 17β-oestradiol (1 μM) (n=5) and progesterone (1 μM) (n=6) did not alter $[Ca^{2+}]_o$-induced contraction. In contrast, phloretin (1 μM) (n=6), biochanin A (1 μM) (n=6) and zearalanone (1 μM) (n=7) markedly suppressed $[Ca^{2+}]_o$-induced contraction (Fig. 3).

3.4. Measurement of the binding of [^{3}H]17β-oestradiol receptor-α, and oestrogen receptor-α and -β competitor fluorescence polarization assay

To evaluate the participation of oestrogen receptor-α, effect of diosgenin on [^{3}H]17β-oestradiol binding was determined using MCF-7 cells (a human breast cancer cell

line used for oestrogen receptor-α binding assay) (Olea-Serrano et al., 1985). Over the concentrations tested (0.001 nM to 10 μM), diosgenin failed to demonstrate a competition with oestrogen, and no apparent displacement of [^{3}H]17β-oestradiol was observed (Fig. 4A). However, the

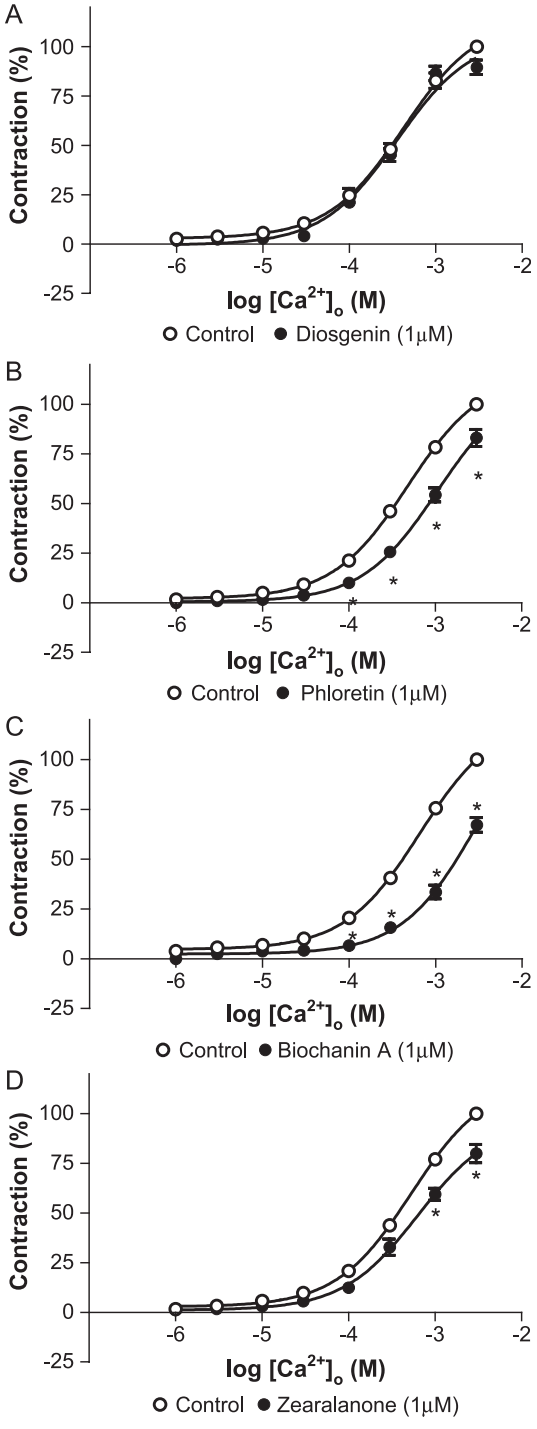

Fig. 3. Effect of diosgenin (A), phloretin (B), biochanin A (C) and zearalanone (D) (each at 1 μM) on $[Ca^{2+}]_o$-induced contraction of porcine isolated left anterior descending coronary artery (endothelium denuded, bathed in a depolarizing (50 mM $[K^+]_o$) physiological salt solution). Results are expressed as mean±S.E.M. * indicated $P<0.05$ compared to control (○), n=5–6.

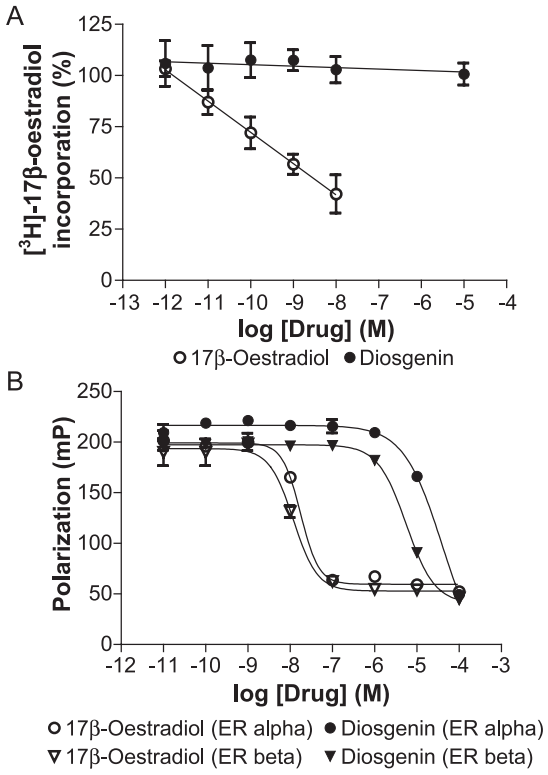

Fig. 4. (A) Effect of diosgenin (●) and 17β-oestradiol (○) on the incorporation of [^{3}H]-17β-oestradiol of human breast cancer cell line (MCF-7 cells). Results are expressed as mean±S.E.M., and experiments were performed in triplicate. (B) The affinity of diosgenin (●, ▼) and 17β-oestradiol (○, ▽) for oestrogen receptor-α (ERα) (circle) and oestrogen receptor-β (ERβ) (inverted triangle) were determined by their ability to displace the fluorescent ligand ER2 from ERα and ERβ using FP-based competition assay. The fluorescence polarization (mP) was plotted against the log concentration of the test compound. Results are expressed as mean±S.E.M., and experiments were performed in triplicate.

non-labelled 17β-oestradiol (0.001 to 10 nM) competed with [^{3}H]17β-oestradiol and a displacement of [^{3}H]17β-oestradiol binding was demonstrated (Fig. 4A).

In oestrogen receptor (α- and β-) competitor assay, diosgenin (0.01 nM to 1 μM) did not cause an apparent change of fluorescence polarization recorded (Fig. 4B). However, a further increase in concentration (10 μM) caused a decrease in polarization of the Fluoromone, with a relatively greater shift in oestrogen receptor-β assay (Fig. 4B). In contrast, 17β-oestradiol elicited a similar magnitude of fluorescence polarization shift in both oestrogen receptor (α- and β-) competitor assays (Fig. 4B).

4. Discussion

In the present study, we provided evidence that diosgenin caused an acute relaxation of pre-contracted (by 5-hydroxytyptamine) porcine isolated left anterior descending coronary artery over nano- and low micromolar (1 μM) concentrations. Our results demonstrate that the functional endothelium/nitric oxide release plays no

apparent role as mechanical removal of endothelium and inhibition of nitric oxide synthase failed to modify diosgenin-induced relaxation.

In arterial preparations pre-contracted with a high $[K^+]_o$ physiological salt solution, diosgenin-mediated coronary artery relaxation was significantly reduced suggesting that opening of K^+ channels is responsible. In rat isolated thoracic aorta, 17β-oestradiol-mediated relaxation is partly due to the opening of ATP-sensitive, K^+ channels IK_{ATP} (Unemoto et al., 2003). However, the failure of glibenclamide (an IK_{ATP} blocker) in modifying diosgenin-elicited vasodilatation argued about the participation of IK_{ATP} opening.

In addition to IK_{ATP}, we have evaluated the participation of iberiotoxin-sensitive, large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels. Interestingly, the outward BK_{Ca} magnitude recorded in vascular myocytes was markedly enhanced by diosgenin in a concentration-dependent manner. Consistent with previous studies (Valverde et al., 1999; Jacob and White, 2000; Liu et al., 2002), 17β-oestradiol (1 μM), progesterone (1 μM) and sodium nitroprusside (30 μM) activated the outward BK_{Ca}. In addition, diosgenin- and sodium nitroprusside-mediated enhancement of BK_{Ca} was abolished by KT 5823 (a protein kinase G inhibitor) (Grider, 1993), but not by MDL 12330A (an adenylate cyclase inhibitor) (Correia-de-Sa and Ribeiro, 1994). Hence, ours results suggest that diosgenin-elicited coronary artery relaxation probably involved activation of protein kinase G whereas the adenylate cyclase pathway is not important.

In addition to K^+ channels opening, inhibition of Ca^{2+} channels is responsible for the relaxation of rat isolated thoracic aorta by 17β-oestradiol and progesterone (Unemoto et al., 2003). In the present study, neither diosgenin, 17β-oestradiol nor progesterone (each at 1 μM) altered $[Ca^{2+}]_o$-induced coronary artery contraction suggesting that an inhibition of the influx of $[Ca^{2+}]_o$ is probably not responsible for the observed relaxation. Other phytoestrogenic compounds phloretin (1 μM), biochanin A (1 μM) and zearalanone (1 μM) elicited an endothelium-independent relaxation, as reported in rabbit isolated coronary artery (Figtree et al., 2000). Consistent with previous study (Figtree et al., 2000), effects of these compounds probably act via an inhibition of influx of $[Ca^{2+}]_o$, as these agents suppressed $[Ca^{2+}]_o$-induced contraction.

Both oestrogen and progesterone receptors existed in coronary artery (Crews and Khalil, 1999), and activation of these receptors resulted in vasodilatation. There are two oestrogen receptor isoforms (α- and β-) and in porcine coronary artery, the α-isoform is present in endothelial cells whereas the β-isoform is found in vascular smooth muscle (Geraldes et al., 2003). Our results demonstrate that neither oestrogen receptor-α nor progesterone receptor is involved because application of ICI 182,780 (a selective oestrogen receptor-α antagonist) (Wakeling et al., 1991) and mifepristone (a progesterone receptor antagonist) (Schreiber et al., 1983) did not alter the relaxation response of diosgenin.

Specificity of individual hormonal receptor antagonist was confirmed as ICI 182,780 and mifepristone abolished 17β-oestradiol- and progesterone-induced vasodilatation, respectively. In addition, the ineffectiveness of mifepristone in modifying diosgenin-elicited relaxation suggests that the conversion of diosgenin into progesterone probably does not occur under our experimental conditions. In addition, diosgenin failed to compete and displace [^{3}H]17β-oestradiol binding in human breast cancer cell (MCF-7 cell, a cell-line commonly used for oestrogen receptor-α binding assay) (Olea-Serrano et al., 1985) strongly suggest that diosgenin-mediated effect does not involve the oestrogen receptor-α, consistent with our receptor antagonist study.

In view of the lack of a selective oestrogen receptor-β antagonist available, the possible involvement of oestrogen receptor-β in mediating diosgenin-elicited response was evaluated using a commercially available kit that is selective for different isoforms (α- and β-) of oestrogen receptor. In oestrogen receptor (both α- and β-isoforms) competition assay, diosgenin (≥10 μM) demonstrates a relatively greater fluorescence polarization shift in β-, than in α-, oestrogen receptor competition assay. Unlike other phytoestrogens (Kuiper et al., 1997; Barkhem et al., 1998), our results suggest that oestrogen receptor-β may not be involved in diosgenin-mediated relaxation.

It has been reported that 17β-oestradiol-mediated vasodilatation involved the cyclo-oxygenase cascade (Calkin et al., 2002), and diosgenin possess a cyclo-oxygenase-related anti-inflammatory effects in rats (Yamada et al., 1997). In our study, pre-treating the coronary artery with indomethacin (a common cyclo-oxygenase inhibitor) did not modify diosgenin-mediated vasodilatation arguing about the role of cyclo-oxygenase pathway. In recent years, reactive oxygen species have been recognized as a significant determinant of K^+ channels function. In fact, 17β-oestradiol-mediated relaxation in some blood vessels (Barbacanne et al., 1999; Gragasin et al., 2003), and the immunological effects of diosgenin in macrophage cells of Swiss mice (Moreira et al., 2001) involved various reactive oxygen species generated. In this regard, we have evaluated this possibility by examining a range of reactive oxygen species inhibitors/modulators on diosgenin-mediated relaxation. However, our results rule out the participation of reactive oxygen species as none of these agents modified diosgenin-elicited coronary artery relaxation.

In the present study, progesterone (1 μM) produced a mifepristone-sensitive relaxation of porcine coronary artery in an endothelium-independent manner. Similar observations have been reported in human umbilical artery (Ramirez et al., 1998) and rabbit jugular vein (Herkert et al., 2000), but not in coronary artery of anaesthetized pigs (Molinari et al., 2001). It has been shown that progesterone-induced in vitro relaxation of porcine left anterior descending coronary artery involved the (+)-bicuculline-sensitive,

$GABA_A$-like receptors (Jacob and White, 2000), and an activation of BK_{Ca} channels of single vascular myocytes. In our study, progesterone (1 μM) enhanced BK_{Ca}, but the presence of (+)-bicuculline (a selective $GABA_A$ receptor antagonist) (Shirakawa et al., 1989) did not modify progesterone-elicited relaxation. Hence, our results suggest that the (+)-bicuculline-sensitive, $GABA_A$ receptors are not involved. The discrepancy (i.e. the participation of $GABA_A$ receptors) between our results and the observation reported by Jacob and White (2000) is not known. We have used tertiary branch of left anterior descending coronary artery (O.D. ~500–800 μm) whereas in Jacob and White's study (2000), the size/anatomical location of left anterior descending coronary artery was not mentioned.

In summary, we have found that diosgenin caused an acute, endothelium-independent relaxation of porcine left anterior descending coronary artery via activation of protein kinase G and opening of iberiotoxin-sensitive BK_{Ca} channels. Our results suggest that oestrogen receptor (α- and β-isoforms) and progesterone receptors are probably not involved.

4.1. Potential limitations

In this study, we have used pigs that are ~2 months old (~20 kg, either sex) and they are sexually immature. A commercial slaughterhouse kindly provided the animals for our study, and it is therefore very difficult to request for a particular sex (e.g. female) of the animal as well as measuring the hormone level of individual animal. We understand that sex hormones could have a significant impact on the outcomes of our study. However, our results demonstrate that in these sexually immature animals, diosgenin produced coronary artery relaxation with no participation of oestrogen receptor (α- and β-isoforms) and progesterone receptors. Of course, we could not rule out the possible influence of female hormones on the physiological effects of different phytoestrogens. We admitted that further mechanistic studies on sexually mature female pigs are required before this can be confirmed or refuted.

Acknowledgements

The authors would like to thank Mr. W.C. Lai (Manager of Production Department, Ng Fung Slaughterhouse (H.K.)) and staff of the Sheung Shui Slaughterhouse (Hong Kong) for their excellent technical assistance in providing fresh pig's heart for this study. The financial support provided by The Hong Kong/United Kingdom Joint Research Scheme (The British Council) to YW Kwan (as one of the co-investigators) for learning the enzymatic dissociation of viable vascular smooth muscle cells in Prof. AM Gurney's laboratory (December, 1999) (Department of Physiology and Pharmacology, Strathclyde Institute for Biomedical Science, Strathclyde University, Scotland) is appreciated. Assistance provided by Prof. Sylvain Bernès (Centro de Química del IC-UAP, AP 1613, Puebla 72000, México) during the preparation of this manuscript is also acknowledged.

References

Au, A.L.S., Kwan, Y.W., Kwok, C.C., Zhang, R.Z., He, G.W., 2003. Mechanisms responsible for the in vitro relaxation of ligustrazine on porcine left anterior descending coronary artery. Eur. J. Pharmacol. 468, 199–207.

Baber, R.J., O'Hara, J.L., Boyle, F.M., 2003. Hormone replacement therapy: to use or not to use? Med. J. Aust. 178, 630–633.

Barbacanne, M.A., Rami, J., Michel, J.B., Souchard, J.P., Philippe, M., Besombes, J.P., Bayard, F., Arnal, J.F., 1999. Estradiol increases rat aorta endothelium-derived relaxing factor (EDRF) activity without changes in endothelial NO synthase gene expression: possible role of decreased endothelium-derived superoxide anion production. Cardiovasc. Res. 41, 672–681.

Barkhem, T., Carlsson, B., Nilsson, Y., Enmark, E., Gustafsson, J.A., Nilsson, S., 1998. Differential response of estrogen receptor α and estrogen receptor β to partial estrogen agonists/antagonists. Mol. Pharmacol. 54, 105–112.

Calkin, A.C., Sudhir, K., Honisett, S., Williams, M.R., Dawood, T., Komesaroff, P.A., 2002. Rapid potentiation of endothelium-dependent vasodilation by estradiol in postmenopausal women is mediated via cyclooxygenase 2. J. Clin. Endocrinol. Metab. 87, 5072–5075.

Corbiere, C., Liagre, B., Bianchi, A., Bordji, K., Dauca, M., Netter, P., Beneytout, J.L., 2003. Different contribution of apoptosis to the antipoliferative effects of diosgenin and other plant steroids, hecogenin and tigogenin, on human 1547 osteosarcoma cells. Int. J. Oncol. 22, 899–905.

Correia-de-Sa, P., Ribeiro, J.A., 1994. Tonic adenosine A_{2A} receptor activation modulates nicotinic autoreceptor function at the rat neuromuscular junction. Eur. J. Pharmacol. 271, 349–355.

Crews, J.K., Khalil, R.A., 1999. Antagonistic effects of 17β-estradiol, progesterone and testosterone on Ca^{2+} entry mechanisms of coronary vasoconstriction. Arterioscler. Thromb. Vasc. Biol. 19, 1034–1040.

Cummings, S.R., 1991. Evaluating the benefits and risks of postmenopausal hormone therapy. Am. J. Med. 91, 14S–18S.

Figtree, G.A., Griffiths, H., Lu, Y.Q., Webb, C.M., MacLeod, K., Collins, P., 2000. Plant-derived estrogens relax coronary arteries in vitro by a calcium antagonistic mechanism. J. Am. Coll. Cardiol. 35, 1977–1985.

Geraldes, P., Sirois, M.G., Tanguay, J.F., 2003. Specific contribution of estrogen receptors on mitogen-activated protein kinase pathways and vascular cell activation. Circ. Res. 93, 399–405.

Gomita, Y., Moriyama, M., Ichimaru, Y., Uchikado, A., Nohara, T., Kawasaki, T., 1982. Cardiovascular effects of pennogenin tetraglycoside (Tg) extracted from *Paris quadrifolia* Linn. Yakugaku Zasshi 102, 495–498.

Gragasin, F.S., Xu, Y., Arenas, I.A., Kainth, N., Davidge, S.T., 2003. Estrogen reduces angiotensin II-induced nitric oxide synthase and NAD(P)H oxidase expression in endothelial cells. Arterioscler. Thromb. Vasc. Biol. 23, 38–44.

Grider, J.R., 1993. Interplay of VIP and nitric oxide in regulation of the descending relaxation phase of peristalsis. Am. J. Physiol. 264, G334–G340.

Herkert, O., Kuhl, H., Busse, R., Schini-Kerth, V.B., 2000. The progestin levonorgestrel induces endothelium-independent relaxation of rabbit jugular vein via inhibition of calcium entry and protein kinase C: role of cyclic AMP. Br. J. Pharmacol. 130, 1911–1918.

Higdon, K., Scott, A., Tucci, M., Benghuzzi, H., Tsao, A., Puckett, A., Cason, Z., Hughes, J., 2001. The use of estrogen, DHEA, and diosgenin in a sustained delivery setting as a novel treatment approach for

osteoporosis in the ovariectomized adult rat model. Biomed. Sci. Instrum. 37, 281–286.

Jacob, M.K., White, R.E., 2000. Diazepam, γ-aminobutyric acid, and progesterone open K^+ channels in myocytes from coronary arteries. Eur. J. Pharmacol. 403, 209–219.

Jiang, C., Sarrel, P.M., Lindsay, D.C., Poole-Wilson, P.A., Collins, P., 1991. Endothelium-independent relaxation of rabbit coronary artery by 17β-oestradiol in vitro. Br. J. Pharmacol. 104, 1033–1037.

Juarez-Oropeza, M.A., Diaz-Zagoya, J.C., Rabinowitz, J.L., 1987. In vivo and in vitro studies of hypocholesterolemic effects of diosgenin in rats. Int. J. Biochem. 19, 679–683.

Komesaroff, P.A., Black, C.V., Cable, V., Sudhir, K., 2001. Effects of wild yam extract on menopausal symptoms, lipids and sex hormones in healthy menopausal women. Climacteric 4, 144–150.

Krishna, M.C., Grahame, D.A., Samuni, A., Mitchell, J.B., Russo, A., 1992. Oxoammonium cation intermediate in the nitroxide-catalyzed dismutation of superoxide. Proc. Natl. Acad. Sci. U. S. A. 89, 5537–5541.

Kuiper, G.G.J.M., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S., Gustafsson, J.A., 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 138, 863–870.

Li, J.J., Li, S.A., 1998. Natural and anthropogenic environmental oestrogens: the scientific basis for risk assessment. Breast cancer: evidence for xeno-oestrogen involvement in altering its incidence and risk. Pure Appl. Chem. 70, 1713–1723.

Liu, Y., Terata, K., Chai, Q., Li, H., Kleinman, L.H., Gutterman, D.D., 2002. Peroxynitrite inhibits Ca^{2+}-activated K^+ channel activity in smooth muscle of human coronary arterioles. Circ. Res. 91, 1070–1076.

MacLean, M.R., Sweeney, G., Baird, M., McCulloch, K.M., Houslay, M., Morecroft, I., 1996. 5-Hydroxytryptamine receptors mediated vasoconstriction in pulmonary arteries from control and pulmonary hypertensive rats. Br. J. Pharmacol. 119, 917–930.

Marker, R.E., Tsukamoto, T., Turner, D.L., 1940. Sterols. C. Diosgenin. J. Am. Chem. Soc. 62, 2525–2532.

Matsuda, J.J., Volk, K.A., Shibata, E.F., 1990. Calcium currents in isolated rabbit coronary arterial smooth muscle myocytes. J. Physiol. 427, 657–680.

Moalic, S., Liagre, B., Corbiere, C., Bianchi, A., Dauca, M., Bordji, K., Beneytout, J.L., 2001. A plant steroid, diosgenin induced apoptosis, cell cycle arrest and COX activity in osteosarcoma cells. FEBS Lett. 506, 225–230.

Molinari, C., Battaglia, A., Grossini, E., Mary, D.A., Surico, N., Vacca, G., 2001. Effect of progesterone on peripheral blood flow in prepubertal female anesthetized pigs. J. Vasc. Res. 38, 569–577.

Moreira, R.R.D., Carlos, I.Z., Vilegas, W., 2001. Release of intermediate reactive hydrogen peroxide by macrophage cells activated by natural products. Biol. Pharm. Bull. 24, 201–204.

Namda, T., Huang, X.L., Shu, Y.Z., Huang, S.L., Hattori, M., Kakiuchi, N., Wang, Q., Xu, G.J., 1989. Chronotropic effect of the methanolic extracts of the plants of the *Paris* species and steroidal glycosides isolated from *P. vietnamensis* on spontaneously beating of myocardial cells. Planta Med. 55, 501–505.

Olea-Serrano, N., Devleeschouwer, N., Leclercq, G., Heuson, J.C., 1985. Assay for estrogen and progesterone receptors of breast cancer cell lines in monolayer culture. Eur. J. Cancer Clin. Oncol. 21, 965–973.

Parker, G.J., Law, T.L., Lenoch, F.J., Bolger, R.E., 2000. Development of high throughput screening assays using fluorescence polarization: nuclear-ligand-binding and kinase/phosphatase assays. J. Biomol. Screen. 5, 77–88.

Quigley, F.R., 1978. Diosgenin in West Africa Dioscorea plants. Planta Med. 33, 414–415.

Rao, A.R., Kale, R.K., 1992. Diosgenin—a growth stimulator of mammary gland of ovariectomized mouse. Indian J. Exp. Biol. 30, 367–370.

Ramirez, R.J., Gibson, M., Kalenic, J., Einzig, S., Omar, H.A., 1998. In vitro vascular relaxation to progesterone and its metabolites in human umbilical and placental blood vessels. J. Matern.-Fetal Investig. 8, 61–65.

Ruehlmann, D.O., Steinert, J.R., Valverde, M.A., Jacob, R., Mann, G.E., 1998. Environmental estrogenic pollutants induce acute vascular relaxation by inhibiting L-type Ca^{2+} channels in smooth muscle cells. FASEB J. 12, 613–619.

Schreiber, J.R., Hsueh, A.J., Baulieu, E.E., 1983. Binding of the anti-progestin RU-486 to rat ovary steroid receptors. Contraception 28, 77–85.

Shirakawa, J., Hosoda, K., Taniyama, K., Matsumoto, S., Tanaka, C., 1989. Gamma-aminobutyric acid-A receptor-mediated suppression of 5-hydroxytryptamine-induced guinea-pig basilar artery smooth muscle contractility. Blood Vessels 26, 129–136.

Stampfer, M.J., Colditz, G.A., Willet, W.C., Manson, J.E., Rosner, B., Speizer, F.E., Hennekens, C.H., 1991. Postmenopausal estrogen therapy and cardiovascular disease: ten-year follow-up from the Nurse's Health Study. N. Engl. J. Med. 325, 756–762.

Unemoto, T., Honda, H., Kogo, H., 2003. Differences in the mechanisms for relaxation of aorta induced by 17β-estradiol or progesterone between normotensive and hypertensive rats. Eur. J. Pharmacol. 472, 119–126.

Valverde, M.A., Rojas, P., Amigo, J., Cosmelli, D., Orio, P., Bahamonde, M.I., Mann, G.E., Vergara, C., Latorre, R., 1999. Acute activation of Maxi-K channels (*hSlo*) by estradiol binding to the β subunit. Science 285, 1929–1931.

Wakeling, A.E., Dukes, M., Bowler, J., 1991. A potent specific pure antiestrogen with clinical potential. Cancer Res. 51, 3867–3873.

Wassertheil-Smoller, S., Hendrix, S.L., Limacher, M., Heiss, G., Kooperberg, C., Baird, A., Kotchen, T., Curb, J.D., Black, H., Rossouw, J.E., Aragaki, A., Safford, M., Stein, E., Laowattana, S., Mysiw, W.J., WHI Investigators, 2003. Effect of estrogen plus progestin on stroke in postmenopausal women: the Women's Health Initiative: a randomized trial. JAMA 289, 2673–2684.

White, R.E., Darkow, D.J., Lang, J.L.F., 1995. Estrogen relaxes coronary arteries by opening $BK_{(Ca)}$ channels through a cGMP-dependent mechanism. Circ. Res. 77, 936–942.

White, R.E., Han, G., Maunz, M., Dimitropoulou, C., El-Mowafy, A.M., Barlow, R.S., Catravas, J.D., Snead, C., Carrier, G.O., Zhu, S., Yu, X., 2002. Endothelium-independent effect of estrogen on Ca^{2+}-activated K^+ channels in human coronary artery smooth muscle cells. Cardiovasc. Res. 53, 650–661.

Yamada, T., Hoshino, M., Hayakawa, T., Ohhara, H., Yamada, H., Nakazawa, T., Inagaki, T., Iida, M., Ogasawara, T., Uchida, A., Hasegawa, C., Murasaki, G., Miyaji, M., Hirata, A., Takeuchi, T., 1997. Dietary diosgenin attenuates subacute intestinal inflammation associated with indomethacin in rats. Am. J. Physiol. 273, G355–G364.