

ORIGINAL ARTICLE

17 β -estradiol reduces Ca_v1.2 channel abundance and attenuates Ca²⁺-dependent contractions in coronary arteries

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Abstract

One mechanism by which the female sex may protect against elevated coronary vascular tone is inhibition of Ca²⁺ entry into arterial smooth muscle cells (ASMCs). In vitro findings confirm that high estrogen concentrations directly inhibit voltage-dependent Ca_v1.2 channels in coronary ASMCs. For this study, we hypothesized that the nonacute, in vitro exposure of coronary arteries to a low concentration of 17 β -estradiol (17 β E) reduces the expression of Ca_v1.2 channel proteins in coronary ASMCs. Segments of the right coronary artery obtained from sexually mature female pigs were mounted for isometric tension recording. As expected, our results indicate that high concentrations (≥ 10 μ mol/L) of 17 β E acutely attenuated Ca²⁺-dependent contractions to depolarizing KCl stimuli. Interestingly, culturing coronary arteries for 24 h in a 10,000-fold lower concentration (1 nmol/L) of 17 β E also attenuated KCl-induced contractions and reduced the contractile response to the Ca_v1.2 agonist, FPL64176, by 50%. Western blots revealed that 1 nmol/L 17 β E decreased protein expression of the pore-forming α_{1C} subunit (Ca_v α) of the Ca_v1.2 channel by 35%; this response did not depend on an intact endothelium. The 17 β E-induced loss of Ca_v α protein in coronary arteries was prevented by the estrogen ER α /ER β antagonist, ICI 182,780, whereas the GPER antagonist, G15, did not prevent it. There was no effect of 1 nmol/L 17 β E on Ca_v α transcript expression. We conclude that 17 β E reduces Ca_v1.2 channel abundance in isolated coronary arteries by a posttranscriptional process. This unrecognized effect of estrogen may confer physiological protection against the development of abnormal Ca²⁺-dependent coronary vascular tone.

Abbreviations

17 β E, 17 β -estradiol; Ach, Acetylcholine; ASMCs, arterial smooth muscle cells; Ca_v1.2, L-type Ca²⁺ channels; Ca_v, voltage-gated; Endo, endothelium; ER, estrogen receptor; EtOH, ethanol; FPL64176, 2,5-Dimethyl-4-[2-(phenylmethyl)benzoyl]-1H-pyrrole-3-carboxylic acid methyl ester; GPER, G-protein-coupled estrogen receptor; H₂O₂, hydrogen peroxide; HRP, horseradish peroxidase; ICI 182, 780, 7 α ,17 β -[9-[(4,4,5,5,5-Pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol; RCA, right coronary artery; TBST, Tris-buffered saline containing 0.1% Tween 20; TSG, thapsigargin.

Introduction

The in vivo administration of estrogen to postmenopausal women has a beneficial effect on coronary arterial

function by improving blood flow (Gilligan et al. 1994; Puntawangkoon et al. 2010). The improvement in coronary blood flow during estrogen therapy is typically attributed to activation of Ca²⁺-activated K⁺ channels

and/or inhibition of voltage-gated, L-type Ca²⁺ (Ca_v1.2) channels (Bowles *et al.* 1998a,b). The Ca_v1.2 channels are the major pathway for voltage-dependent Ca²⁺ influx in coronary arterial smooth muscle cells (ASMCs), and as primary mediators of arterial contraction, any imbalance in their activity or expression that favors Ca²⁺ influx may contribute to elevated vascular tone. For example, the expression of Ca_v1.2 channels is increased in coronary arteries susceptible to vasospasm (Kuga *et al.* 2000), and Ca_v1.2 channels are more abundant in small mesenteric and skeletal muscle arteries of genetically hypertensive rats (Pratt *et al.* 2002). Accordingly, the latter arteries exhibit accentuated Ca²⁺-dependent vascular tone, which is normalized by pharmacological blockers of Ca_v1.2 channels.

Using porcine coronary arteries, our laboratory (Tummala and Hill 2007; Hill *et al.* 2010) and others (Han *et al.* 1995; Teoh *et al.* 1999) demonstrated that the primary female estrogenic hormone, 17β-estradiol (17βE), acutely inhibits contractions elicited by depolarizing KCl solutions, suggesting that 17βE reduces Ca²⁺ influx mediated by voltage-sensitive Ca_v1.2 channels. Similarly, Sudhir *et al.* (1997) demonstrated that vasospasms of porcine coronary arteries are alleviated by the intracoronary infusion of 17βE, implying an inhibitory action of pharmacological concentrations of estrogen on coronary reactivity. Finally, Ullrich *et al.* (2007) directly confirmed that 17βE (10 μmol/L) inhibits whole-cell Ca²⁺ current recorded from Ca_v1.2 channel-transfected HEK-293 cells. In all of these studies, high concentrations (≥1 μmol/L) of 17βE were required to reduce Ca²⁺ influx or mediate acute relaxation responses. In agreement with these studies (Han *et al.* 1995; Teoh *et al.* 1999; Tummala and Hill 2007; Ullrich *et al.* 2007; Hill *et al.* 2010), we confirmed that the acute relaxation responses to pharmacological concentrations of 17βE are nongenomic and relate to direct inhibition of Ca_v1.2 channel function (Hill *et al.* 2010). However, a recurring concern of these *in vitro* studies is that the vasodilator effect of high pharmacological 17βE concentrations applied for short exposure times (≤60 min) cannot account for the *in vivo* effect of estrogen on vascular tone, which apparently results from chronic modulation of vascular contractile mechanisms by a much lower concentration of the circulating hormone. It is likely that 17βE's mechanism(s) of action on vascular tone is related to the duration of exposure (*i.e.*, acute vs. chronic) and needs to be further investigated. For example, the estrogen receptor mediated intracellular signaling events (*i.e.*, kinase phosphorylation) in vascular smooth muscle cells are enhanced with prolonged E2 exposure in culture (Ding *et al.* 2009).

Several studies have explored whether different *in vivo* estrogen levels modulate Ca_v1.2 channel function in ASMCs. Using an ovariectomized rat model, Crews and

Khalil (Crews and Khalil 1999) found less Ca²⁺ influx through Ca_v1.2 channels in aortae isolated from ovary-intact compared to ovariectomized females, implying that higher levels of circulating plasma estrogen may reduce the number of functional Ca_v1.2 channels in ASMCs. Likewise, Bowles (2001) found that coronary arteries of male pigs exhibit a higher expression level of the pore-forming α_{1C} subunit (Ca_vα) of the Ca_v1.2 channel compared to female animals, and also show more voltage-dependent Ca²⁺ current in patch-clamped coronary ASMCs. Recently, Tharp *et al.* (2014) elaborated on this study and demonstrated that coronary ASMCs from ovariectomized female pigs have increased Ca_v1.2 channel current, but not enhanced Ca_vα protein expression, compared to ovary-intact female pigs. The authors hypothesized that endogenous female hormones inhibit Ca_v1.2 channel activity in coronary ASMCs by upregulating the Ca_vβ1 subunit. In this regard, the Ca_vα pore proteins coassemble in 1:1 stoichiometry with smaller β and α_{2δ} subunits to form functional Ca_v1.2 channels in ASMCs; different β subunits modulate expression and properties of the multiprotein channel (Sonkusare *et al.* 2006; Kharrade *et al.* 2013; Cox and Fromme 2015). Based on these *in vivo* studies, the objective of this study was to use an *in vitro* vessel culture approach to define the effect of 17βE on Ca_v1.2 channel expression and function. 17β-Estradiol is the main estrogenic hormone in premenopausal women, and is presumed to be one source of vascular protection (Khalil 2013). We used coronary arteries from sexually mature female pigs as a model because of their close similarity to the human coronary vasculature, and similar development of coronary arterial dysfunctions and disease (Jokinen *et al.* 1985; Tummala and Hill 2007; Sturek 2011).

Materials and Methods

Animals

As previously described (Tummala and Hill 2007), hearts from female Yorkshire pigs (3–4 years of age) were obtained from a local packing plant staffed with a United States Department of Agriculture veterinarian. The distal end of the right coronary artery (RCA) was immediately dissected from the heart in a low Ca²⁺ Krebs solution (in mmol/L: 138 NaCl, 5 KCl, 0.2 CaCl₂, 1 MgCl₂, 10 HEPES, 10 glucose, pH 7.4).

Vessel culture

For tension-recording studies, distal ends of the RCA were carefully sectioned into ring segments (4 mm in length) before placing each ring in the appropriate culture

conditions. The RCA designated for real-time PCR and Western blot analysis were sectioned into longitudinal strips (3 cm in length) prior to vessel culture. To determine the nonacute effect of 17 β -estradiol (17 β E; Sigma Chemical Co., St. Louis, MO) on Ca_v1.2 channel-mediated coronary reactivity and Ca_v α expression, arterial segments were incubated at 37°C in a 5% CO₂ incubator for 24 h in RPMI 1640 phenol-free culture media (Sigma). Arteries either were incubated in: (1) control media, (2) media supplemented with 17 β E (1 pmol/L to 10 μ mol/L), or (3) media supplemented with an equal volume of ethanol solvent (EtOH; 0.1%). In order to implicate specific estrogen receptors (ER), the media in some studies was supplemented either with the ER α /ER β antagonist, ICI 182,780 (Tocris Bioscience; Ellisville, MO) or the G-protein-coupled estrogen receptor (GPER) antagonist, G15 (Tocris Bioscience, Bristol, UK). The latter antagonist reportedly lacks affinity for ER α and ER β at concentrations up to 10 μ mol/L (Dennis et al. 2009; Yu et al. 2011). To evaluate the role of endothelium on the 17 β E effects, the endothelium was removed in a subset of studies by rubbing the luminal vessel surface with a wooden toothpick before vessel culture; its removal was later verified using scanning electron microscopy.

Vascular reactivity

Isometric tension recording was performed using freshly isolated arterial rings or rings cultured for 24 h under one of the three conditions described above. Rings were suspended in organ baths (World Precision Instruments, Sarasota, FL) containing an oxygenated (95% O₂: 5% CO₂) modified Krebs solution (in mmol/L: 138 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, 10 glucose, pH 7.4). All rings were initially stretched to their optimal length (Tummala and Hill 2007). To evaluate 17 β E-induced relaxation, the freshly isolated rings were precontracted with a depolarizing solution containing high (60 mmol/L) KCl (in mmol/L: 83 NaCl, 60 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, 10 glucose, pH 7.4) followed by generation of a concentration–response relationship with 17 β E (0.1–100 μ mol/L) or its EtOH solvent. As reported and discussed earlier (Hill et al. 2010), each 17 β E concentration was equilibrated with the ring for 60 min (to achieve a steady-state relaxation response) before the next sequential concentration was added.

The acute effect of 17 β E on depolarization-induced contractions also was evaluated using freshly isolated rings of RCA. An initial 80 mmol/L KCl-mediated contraction was induced before incubating each ring in a different 17 β E concentration (1 pmol/L to 100 μ mol/L) or its ethanol solvent (0.1%) for 60 min. This was followed by the generation of a KCl concentration–response

relationship (15–80 mmol/L) in all rings. The composition of the KCl solution was a similar to the 60 mmol/L KCl solution described above except for the equimolar substitution of NaCl for KCl.

The concentration–response relationship with KCl was evaluated in rings cultured for 24 h in EtOH, or in 1 pmol/L, 1 nmol/L, and 10 μ mol/L 17 β E. In contrast to the freshly used rings above, cultured rings were not exposed to 80 mmol/L KCl before generating the KCl concentration–response relationship; instead an “untreated group” was cultured without 17 β E or EtOH. During the generation of the KCl concentration–response relationship, rings were allowed to incubate with each KCl solution for only 10 min before exchanging it for the sequential KCl concentration to minimize depolarization-induced Ca_v1.2 channel upregulation (Pesic et al. 2004).

In a subset of studies, the endothelium was denuded from arterial rings using a wooden toothpick prior to vessel culture. After a 24 h culture, the rings were suspended in organ baths and endothelial function was verified by the ability of bradykinin (0.1 μ mol/L; Sigma) to relax a 35 mmol/L KCl depolarizing contraction. In studies using the Ca_v1.2 channel agonist, FPL64176, rings were exposed to acetylcholine (10 μ mol/L; Sigma) and thapsigargin (10 μ mol/L; Sigma) to release and eliminate the reuptake of Ca²⁺ back into the sarcoplasmic reticulum (SR), and thereby eliminate a potential effect of FPL64176 on SR Ca²⁺ release (Wasserstrom et al. 2002). A steady-state contraction was generated to FPL64176 (3 μ mol/L; Tocris Bioscience) followed by application of the Ca_v1.2 channel antagonist, nifedipine (10 μ mol/L; Sigma). Only arterial rings that contracted in response to FPL64176 and relaxed back to baseline in response to nifedipine were used for analysis.

Immunoblots

Cultured strips of RCA were homogenized and membrane lysate prepared (Liu et al. 2001). Equivalent amounts of membrane proteins were run on a 4–15% polyacrylamide gradient gel. After electrophoresis, the proteins were electroblotted to a PVDF membrane. The membrane was subsequently blocked overnight (at 4°C) with 5% non-fat dry milk dissolved in a Tris-buffered saline containing 0.1% Tween 20 (TBST). After decanting off the blocking solution, the membrane was incubated in a 1:100 dilution of rabbit polyclonal anti- α _{1C} (Alomone Labs, Jerusalem, Israel) for 60 min followed by three washes (10 min each) using TBST. The membrane was then incubated with horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (1:1000; Santa Cruz Biotech, Santa Cruz, CA) for 60 min and subsequently washed (3x) with TBST. Chemiluminescence permitted detection of the HRP-labeled

antibody using an AlphaImager Documentation and Analysis System (Alpha Innotech Corp., San Leandro, CA). Likewise, band densitometry was analyzed using AlphaImager software. Smooth muscle β -actin (1:1000; Abcam, Cambridge, MA) was used as a loading control in Western blots.

Real-time PCR

The relative abundance of Ca_v α transcript in arterial strips cultured for 24 h in EtOH or 17 β E (1 nmol/L) was determined by real-time PCR (Tobin *et al.* 2009). Total RNA was isolated using the RNeasy[®] protect mini-kit (Qiagen, Germantown, MD). The resulting RNA was subjected to DNase treatment using DNA-free[™] kit (Ambion, Austin, TX) to remove any contaminating DNA. Five hundred ng of the RNA isolate was reverse transcribed (iScript cDNA synthesis kit; Biorad, Hercules, CA) to generate cDNA. The cDNA was generated at 25°C for 5 min, followed by 42°C for 30 min and 85°C for 5 min. The cDNA sequences were then amplified by RT-PCR (iQ[™] SYBR[®] Green Supermix Kit; Biorad, Hercules, CA) using Ca_v α and β -actin specific primers, and an iCycler iQ[®] Multi-color Real Time PCR Detection System (Biorad, Hercules, CA). The forward and reverse primers used to amplify cDNA were reported earlier (Hirenallur *et al.* 2008): Ca_v α - 5'-ccgccactaccaagatcaac-3' (forward) and 5'-gcatctcgggctcctctc-3' (reverse); β -actin - 5'-accactggcattgtcatggactct-3' (forward) and 5'-tcttcgatgaggtagtcggtcaggt-3' (reverse). After initial denaturation at 95°C for 3 min, the following temperature-cycling profile was used for amplification (40 cycles): 95°C for 10 sec denaturing and 62°C for 1 min for annealing and extension. β -actin was used as an internal standard. Each amplification reaction contained 5 ng of the cDNA product. Control reactions contained all components except the cDNA template. The relative abundance of Ca_v α transcript in EtOH and 17 β E-treated arteries was quantified by the $\Delta\Delta$ Ct method, and reported as percent of EtOH expression (Livak and Schmittgen 2001).

Scanning electron microscopy

As reported by us earlier (Hill and Muldrew 2014), scanning electron microscopy was used to verify the integrity of the endothelium. Arterial strips were pinned lumen side up on a wax surface in individual glass jars, then fixed with 2% glutaraldehyde (Ted Pella, Inc., Redding, CA) for 60 min, and then postfixed with 4% osmium tetroxide (Ted Pella, Inc.) for an additional 60 min. The specimens were dehydrated using ascending ratios of acetone/ethanol (1:1, 2:1, 4:1) followed by descending ratios of acetone/hexamethyldisilazane (4:1, 2:1, 1:1). Next, the

samples were sputter-coated with gold particles and the luminal surface observed using a PSEM II 2000 scanning electron microscope (FEI, Hillsboro, OR).

Statistics

All data are expressed as the mean \pm SE for the number (n) of animals within each group. When comparing two groups, significance was determined using an unpaired t -test. The KCl concentration–response curves were analyzed using two-way repeated measures ANOVA with a Holm–Sidak post hoc analysis. Significant effect of multiple 17 β E concentrations was evaluated using one-way ANOVA followed by Bonferroni's post hoc analysis. Potential endothelial effects were analyzed using a two-way ANOVA (without replication) followed by Tukey HSD post hoc analysis. The EC₅₀ values (-log of the effective concentration required to generate a 50% maximal response) were calculated and analyzed using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA). Statistical analysis was conducted using JMP 8 Statistical Discovery (SAS Institute Inc., USA) using a level of critical significance of $\alpha = 0.05$; therefore, significance was defined as $P < 0.05$.

Results

Vascular reactivity responses

High concentrations of 17 β E were necessary to relax depolarizing KCl-induced contractions (Fig. 1), which fully rely on voltage-dependent Ca²⁺ influx through Ca_v1.2 channels. The progressive relaxation mediated by 17 β E (0.1–100 μ mol/L) significantly attenuated contraction by $17.7 \pm 2.4\%$ and $61.3 \pm 5.4\%$ at concentrations of 10 μ mol/L and 100 μ mol/L 17 β E, respectively ($n = 7$). EtOH served as a solvent and time control for the relaxation experiment. Likewise, when arterial rings were incubated with 17 β E (1 pM to 100 μ mol/L) for 60 min prior to generation of KCl concentration–response curves, only 17 β E concentrations of 10 μ mol/L and 100 μ mol/L significantly attenuated KCl contractions (Fig. 2; $n = 5$). A 17 β E concentration of 1 nmol/L did not acutely relax arteries precontracted by KCl (data not shown) and preincubation for 60 min in 1 nmol/L 17 β E did not suppress KCl concentration–response curves as shown in Figure 2.

In contrast, prolonged preexposure of similar arteries to 1 nmol/L 17 β E attenuated depolarization-induced contractions (Fig. 3). Coronary arterial rings cultured for 24 h prior to tension recording in RPMI media containing 1 nmol/L 17 β E exhibited an $\sim 25\%$ attenuation of the concentration–response curves to KCl (Fig. 3). This

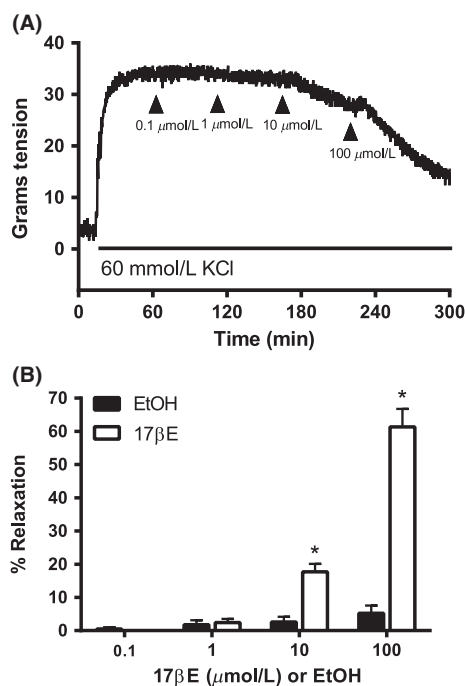


Figure 1. Pharmacological concentrations of 17 β -estradiol (17 β E) are required to relax coronary arterial rings precontracted by 60 mmol/L KCl. (A) Typical recording shows concentration-dependent relaxation by 17 β E of a KCl-contracted arterial ring. Exposure to KCl is indicated by the horizontal line. (B) Average values (mean \pm SE) for 17 β E-induced relaxation of KCl precontracted rings. Equal volumes of EtOH served as solvent control. * $P < 0.05$ using an unpaired t -test, EtOH versus 17 β E groups ($n = 7$ pigs).

inhibitory effect was accentuated in arterial rings preincubated in 10 μ mol/L 17 β E for 24 h, whereas 1 pM 17 β E had no significant effect on the KCl contraction. The acute (1 h) versus longer term (24 h) effects of 1 nmol/L 17 β E on KCl contraction are compared in Table 1.

17 β E for 24 h results in loss of $Ca_v\alpha$ protein

Corroborating our isometric tension-recording findings of reduced Ca^{2+} -dependent contractions after prolonged exposure to 1 nmol/L 17 β E, we also found that a 24 h exposure to 1 nmol/L 17 β E decreased the expression of the pore-forming α_{1C} subunit of the $Ca_v1.2$ channel, $Ca_v\alpha$, by $35 \pm 0.9\%$ (Fig. 4A and B). The Western blots shown in Figure 4 depict immunoreactive doublet bands corresponding to the short (~ 200 kD) and long (~ 240 kD) forms of the $Ca_v\alpha$ subunit. The EtOH solvent (0.1%) and 1 pmol/L 17 β E did not significantly affect $Ca_v\alpha$ protein expression. Immunodensity of the internal standard, β -actin, was similar between treatment groups.

Real-time PCR was used to determine if 1 nmol/L 17 β E reduced $Ca_v\alpha$ subunit abundance by transcriptional

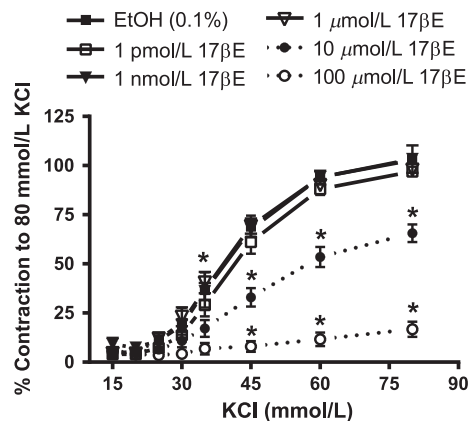


Figure 2. A short-term (60 min) preexposure to 17 β E necessitates the use of high, pharmacological 17 β E concentrations to inhibit depolarization-induced contractions to KCl. Rings were incubated for 60 min in 1 pmol/L, 1 nmol/L, 1 μ mol/L, 10 μ mol/L, or 100 μ mol/L 17 β E, or its EtOH solvent, before generating cumulative concentration–response relationships to KCl. The KCl-induced tension development is expressed as percent (%) of the maximal contractile response to 80 mmol/L KCl before incubating rings in 17 β E or EtOH. The data are shown as the mean \pm SE and analysis was conducted using two-way repeated measures ANOVA (Holm–Sidak post hoc). * $P < 0.05$, 10 μ mol/L or 100 μ mol/L 17 β E versus EtOH at the same KCl concentration ($n = 5$ pigs).

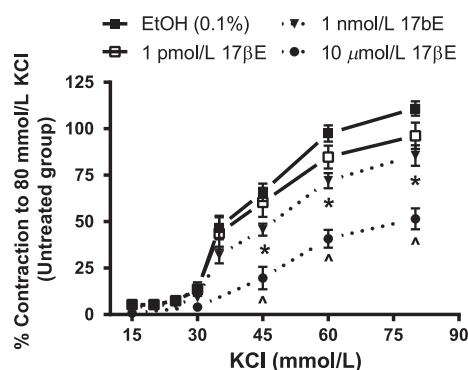


Figure 3. Incubation of coronary arterial rings in a low concentration (1 nmol/L) of 17 β E for 24 h attenuates the concentration–response curve to KCl. Cumulative concentration–response relationships to KCl were generated after rings were cultured for 24 h in 1 pM, 1 nmol/L, or 10 μ mol/L 17 β E or ethanol solvent (EtOH), or left untreated (no addition of 17 β E or EtOH). The KCl contractions are expressed as percent (%) of the maximal contractile response to 80 mmol/L KCl of the untreated group ($n = 5$ pigs). The data are shown as the mean \pm SE and analysis was conducted using two-way repeated measures ANOVA (Holm–Sidak post hoc). * $P < 0.05$, EtOH versus 1 nmol/L 17 β E at the same KCl concentration. Likewise, ^denotes $P < 0.05$, EtOH versus 10 μ mol/L 17 β E at the same KCl concentration.

regulation. Initially, we standardized the real-time PCR assay before comparing $Ca_v\alpha$ subunit mRNA levels. First, the presence of a single peak in the melt curve analysis

Table 1. Comparison between effects of acute (1 h) and prolonged (24 h) exposure to 17 β -estradiol (17 β E) on EC₅₀ values and maximal tension (T_{max}) of KCl concentration–response relationships.

Exposure time	[17 β E] or EtOH Solvent	<i>n</i>	EC ₅₀ (mmol/L) ¹	T _{max} ²
1 h	EtOH (0.1%)	5	39.89 ± 1.01	102.81 ± 1.86
	1 pmol/L 17 β E	5	41.41 ± 1.01	96.96 ± 1.18
	1 nmol/L 17 β E	5	40.87 ± 1.02	103.33 ± 7.01
24 h	EtOH (0.1%)	5	41.01 ± 1.07	110.87 ± 3.83
	1 pM 17 β E	5	39.67 ± 1.07	96.17 ± 7.15
	1 nmol/L 17 β E	5	44.06 ± 1.08	85.59 ± 5.50 ³

¹KCl concentration required to reach one-half maximal response to 80 mmol/L KCl.

²Maximal contraction to 80 mmol/L KCl. Data are expressed as percent of maximal contractile response to the initial addition of 80 mmol/L KCl.

³*P* < 0.05, EtOH versus 1 nmol/L 17 β E.

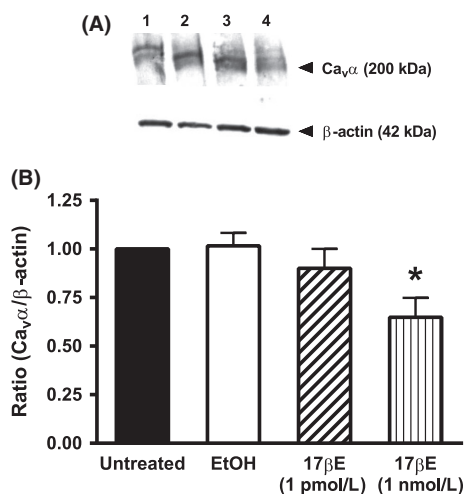


Figure 4. Exposure to 17 β E for 24 h decreases the expression of the α_{1C} pore-forming subunit (Ca_v α) of the Ca_v1.2 channel in coronary arterial strips. (A) Diffuse immunoreactive band corresponding to the Ca_v α protein; the short (~200 kD) and long (~240 kD) forms of Ca_v α are revealed as a doublet in some blots. β -Actin (42 kDa) was used as an internal standard. Protein lysates were obtained from cultured arteries exposed for 24 h to the following conditions: lane 1 = untreated; lane 2 = EtOH (0.1%); lane 3 = 1 pmol/L 17 β E; lane 4 = 1 nmol/L 17 β E. B, The densitometric ratio corresponding to Ca_v α in arterial lysates exposed for 24 h to EtOH or 1 nmol/L 17 β E compared to untreated arteries. Results indicate that 1 nmol/L 17 β E for 24 h decreases Ca_v α protein abundance. Data represent mean \pm S.E. (*n* = 5 pigs) and analyzed using one-way ANOVA followed by Bonferroni's post hoc analysis. There was no difference in β -actin expression between groups.

inferred a single amplification product corresponding to Ca_v α amplified product. Second, we verified that the real-time PCR assay was adequately sensitive to detect 50% change in the expression of amplification products

corresponding to Ca_v α mRNA. Standard amplification curves generated for Ca_v α using twofold serial dilutions of cDNA from 12.5 ng to 1.56 ng per amplification reaction resulted in a one-cycle (1.0 \pm 0.1) increase in threshold cycle as predicted, thereby demonstrating sensitive detection of changes in transcript level in this range. After assay verification, the expression of Ca_v α transcript was compared between arteries cultured in EtOH solvent or 1 nmol/L 17 β E for 24 h (Fig. 5A and B). Representative real-time PCR curves indicated an average cycle threshold of 26.33 \pm 0.52 for the detection of Ca_v α in EtOH-treated arteries (*n* = 6) compared to 26.36 \pm 0.59 in 17 β E-treated arteries (*n* = 6), implying similar abundance of the Ca_v α subunit transcript as estimated by the $\Delta\Delta$ Ct method (Fig. 5C). In the same arterial preparations, as expected, similar cycle thresholds of 16.26 \pm 0.35 (EtOH, *n* = 6) and 16.52 \pm 0.39 (17 β E, *n* = 6) also were calculated for the internal standard β -actin.

17 β E-induced inhibition of KCl contractions and Ca_v α expression is not endothelium dependent

The effect of 24 h exposure to 1 nmol/L 17 β E on Ca_v1.2 channel function also was evaluated by comparing the contractile response to the Ca_v1.2 channel agonist, FPL64176 (3 μ mol/L), between coronary rings exposed to the EtOH solvent or 17 β E. Studies were performed in endothelium intact (+Endo) and denuded (-Endo) arterial rings to evaluate if the inhibitory effect of 1 nmol/L 17 β E on Ca_v1.2 channel-dependent contraction was mediated by endothelium-derived factors. In addition to its intended role as a Ca_v1.2 channel agonist, FPL64176 reportedly can induce Ca²⁺ release from the sarcoplasmic reticulum (SR) by opening ryanodine receptors (Wasserstrom *et al.* 2002; Vaithianathan *et al.* 2010). Therefore, as shown in Figure 6A, arteries were simultaneously exposed to 10 μ mol/L acetylcholine (ACh) and 10 μ mol/L thapsigargin (TSG) to induce SR Ca²⁺ release and prevent its reuptake by inhibiting Ca²⁺-ATPase, respectively. Arteries incubated in 1 nmol/L 17 β E elicited a contractile response to FPL64176 that was 47 \pm 0.56% and 60 \pm 0.33% less (*n* = 6) compared to control arteries (EtOH) in +Endo and -Endo rings, respectively (Fig. 6B). Overall, the presence or absence of the endothelium had no effect on FPL64176-induced tension development (*P* = 0.26).

As shown by anti-Ca_v α Western blots (Fig. 7), coronary arterial strips cultured in 1 nmol/L 17 β E for 24 h exhibited a significant decrease in expression of the pore-forming Ca_v α subunit of the Ca_v1.2 channel in +Endo and -Endo arterial strips. The +Endo and -Endo arteries exposed to 17 β E exhibited a densitometric ratio that was

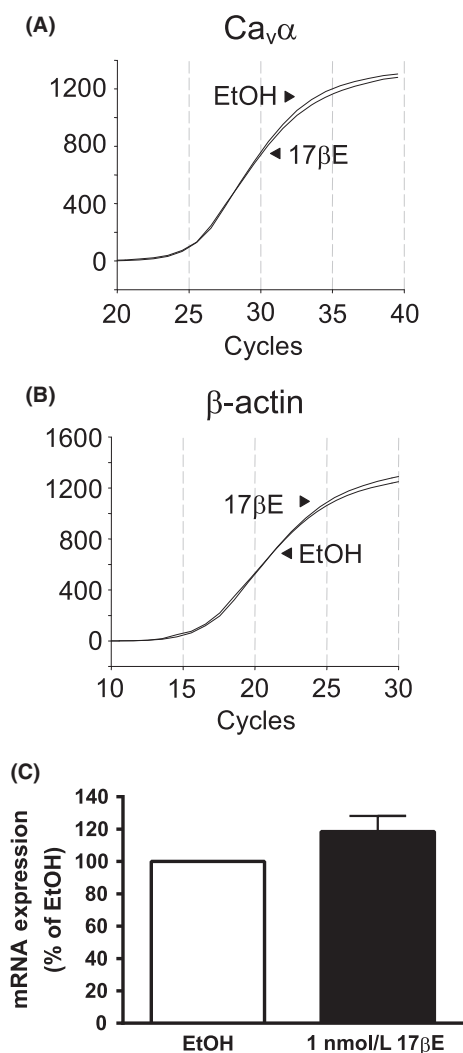


Figure 5. Exposure of arterial segments to 1 nmol/L 17βE for 24 h failed to alter expression of Ca_vα transcript. (A and B) Real-time PCR amplifications of Ca_vα (A) and internal standard β-actin (B) are similar between arteries cultured for 24 h in RPMI media containing 1 nmol/L 17βE or its solvent (EtOH). Relative fluorescence units. (B) Abundance of Ca_vα transcript is not significantly different between 17βE and EtOH-treated arteries as analyzed by the $\Delta\Delta C_t$ method. Data are expressed as percent (%) transcript expression in EtOH ($n = 6$ pigs).

62 ± 9% and 74 ± 14% of the control +Endo arteries treated with EtOH solvent, respectively. Western blot data are expressed as a ratio of Ca_vα expression in +Endo arteries exposed to EtOH. In a control set of experiments, the EtOH solvent had no effect on Ca_vα subunit expression between +Endo and -Endo arteries ($n = 6$, data not shown); the densitometric ratio (Ca_vα/β-actin) was 1.01 ± 0.09 and 0.93 ± 0.17 for +Endo and -Endo arteries, respectively.

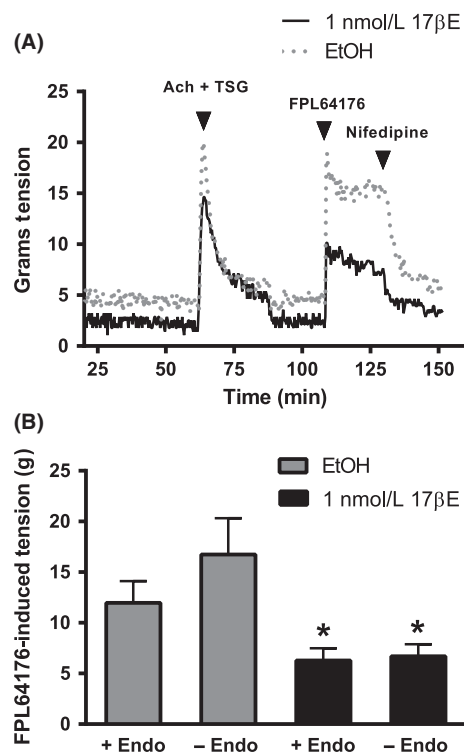


Figure 6. Coronary arterial segments cultured for 24 h in 1 nmol/L 17βE show attenuated contractions to Ca_v1.2 channel activation by 3 μmol/L FPL64176. Endothelium intact (+Endo) and denuded (-Endo) arteries were cultured in 1 nmol/L 17βE or its solvent (EtOH). A, Experimental protocol used to evaluate the contractile response to the Ca_v1.2 channel agonist, FPL64176 (3 μmol/L). To prevent a potentially confounding influence of FPL-induced Ca²⁺ release from sarcoplasmic reticulum (SR), rings initially were exposed to acetylcholine (Ach, 10 μmol/L) and thapsigargin (TSG, 10 μmol/L) before evaluating the contractile response to FPL64176. Complete relaxation of FPL64176-induced contractions by 10 μmol/L nifedipine confirmed dependence on Ca_v1.2 channels. (B) Composite data from studies depicted in panel A. Data are expressed as mean ± S.E. and analyzed using a two-way ANOVA (without replication) followed by Tukey HSD post hoc analysis. * $P < 0.05$, EtOH ± Endo versus 17βE ± Endo; ($n = 7$ pigs).

17βE-induced loss of Ca_vα protein requires ERα/ERβ

Previously in Figs. 4 and 7, we demonstrated a significant reduction in the Ca_vα subunit protein by 1 nmol/L 17βE. Figure 8A shows that the addition of the estrogen receptor (ERα/ERβ) antagonist, ICI 182,780 (10 μmol/L), to the RPMI media significantly prevented the decrease in Ca_vα protein expression induced by 24 h exposure to 1 nmol/L 17βE ($n = 7$). To investigate whether the G-protein-coupled estrogen receptor (GPER) also may mediate the 17βE-induced reduction in Ca_v1.2 channel

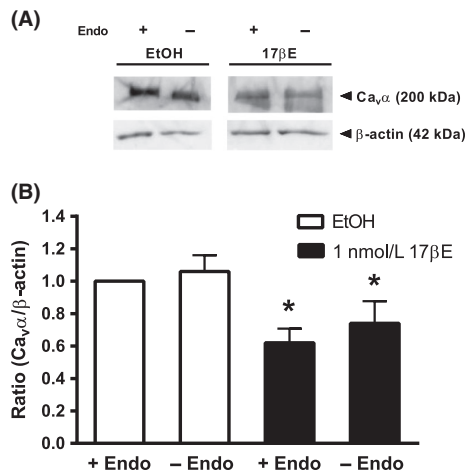


Figure 7. 17βE decreases expression of the Ca_vα pore-forming subunit independently of endothelium. Upper panel shows a representative anti-Ca_vα immunoblot from arteries with intact (+Endo) or denuded (-Endo) endothelium, which were cultured for 24 h in RPMI media containing 1 nmol/L 17βE or ethanol (EtOH). 17βE decreased the densitometric ratio in intact and denuded arteries. β-actin served as an internal standard. The images are from different parts of the same gel. The Ca_vα and β-actin groupings were imaged separately. Band intensity between groups was normalized to β-actin and expressed as a densitometric ratio of the EtOH+Endo group. Data represent mean ± SE (*n* = 7 pigs). **P* < 0.05, EtOH ± Endo versus 17βE ± Endo using a two-way ANOVA (without replication) followed by Tukey HSD post hoc analysis.

expression, arterial strips were incubated for 24 h in 1 nmol/L 17βE in the presence of 10 μmol/L G15, a selective GPER antagonist. This intervention failed to prevent the 17βE-induced decrease in Ca_vα protein expression (Fig. 8B, *n* = 4).

Discussion

Evidence suggests that circulating estrogen contributes to protection of the female sex against elevated coronary vascular tone by reducing Ca²⁺ entry into ASMCs (Crews et al. 1999; Orshal and Khalil 2004; Tharp et al. 2014). Indeed, high concentrations of estrogen inhibit Ca²⁺ influx and attenuate Ca²⁺-dependent contractions in isolated coronary arteries (Han et al. 1995; Teoh et al. 1999; Hill et al. 2010). However, a confounding finding is that in vitro inhibition of vascular reactivity is only observed at high concentrations of estrogen, which may exceed physiological concentrations by at least 1000-fold (Han et al. 1995; Teoh et al. 1999; Salom et al. 2002; Hill et al. 2010; Reslan et al. 2013). This study addresses this concern with two major findings. First, we observed that a prolonged (24 h) rather than acute exposure to 17βE increases its potency as an inhibitor of Ca²⁺-dependent contractions, such that 1 nmol/L 17βE significantly

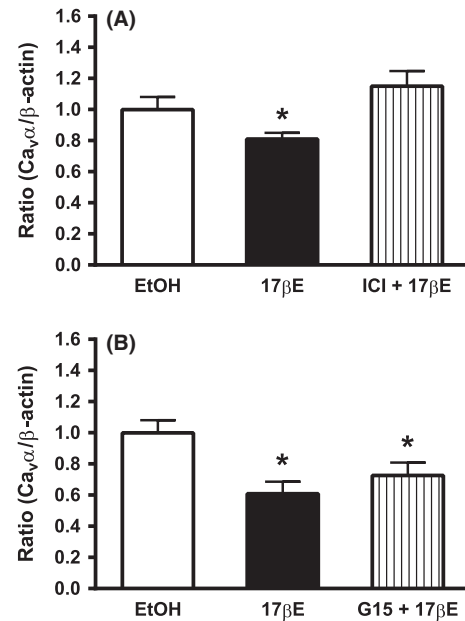


Figure 8. Loss of Ca_vα in cultured arteries exposed to 1 nmol/L 17βE for 24 h is prevented by pharmacological block of ERα/ERβ. (A) The ERα/ERβ antagonist ICI 182,780 (ICI, 10 μmol/L) prevents 17βE-induced downregulation of the Ca_vα subunit. Data represent mean ± SE (*n* = 7 pigs). (B) The G-protein-coupled estrogen receptor (GPER) antagonist, G15 (10 μmol/L) failed to prevent a 17βE-induced loss of Ca_vα. Data represent mean ± SEM. (*n* = 4 pigs) and are expressed as a densitometric ratio. **P* < 0.05 from EtOH using one-way ANOVA followed by Bonferroni's post hoc analysis.

depresses contractions induced by depolarizing KCl and the Ca_v1.2 channel agonist, FPL64176. Second, to our knowledge, we provide the first in vitro findings to directly demonstrate that 17βE results in a posttranscriptional decrease in Ca_v1.2 channel expression. Our results corroborate in vivo sex studies, which have indirectly suggested that the presence of circulating estrogens in female subjects is associated with lower Ca²⁺ channel expression in coronary ASMCs, and thus, less Ca²⁺-dependent tone developed by the coronary arteries (Bowles 2001; Bowles et al. 2004; Tharp et al. 2014). Because Ca_v1.2 channels are the predominant pathway for voltage-dependent Ca²⁺ influx in ASMCs of the coronary circulation (Bowles et al. 1998a), decreasing their expression in the plasma membrane of ASMCs will increase arterial diameter and promote coronary blood flow.

We used 1 nmol/L 17βE to correlate with previous in vitro studies that used 1 nmol/L 17βE as a near “physiological” E2 concentration to evaluate its effect on the vascular mechanics of arteries (Keung et al. 2005, 2011; Cignarella et al. 2009; Lekontseva et al. 2011). Therefore, we did not attempt to use a definite plasma 17βE concentration from cycling sows or premenopausal women

because it is difficult to extrapolate *in vitro* results (which can differ based upon the experimental model) to the *in vivo* condition. In premenopausal women, circulating estrogen is primarily composed of 17 β E, and its concentration approximates 0.2 nmol/L before ovulation; this value rises to 1 nmol/L at ovulation (Marsh et al. 2011), which is comparable to the 1 nmol/L 17 β E concentration used in this study. In mature sows, the plasma estrogen concentration varies from 20 to 840 pmol/L depending on the estrous cycle (Cook et al. 1977; Chatrath et al. 2003). Despite differences in circulating estrogen levels between premenopausal women and our female pig model, there are many advantages to using porcine coronary arteries as a model in this study. The structural similarities of the cardiovascular system between pigs and humans have been well documented (Swindle et al. 2012). In addition, pig coronary arteries similarly express estrogen receptors (ER), ER α and ER β , as well as the G-protein-coupled estrogen receptor, GPER (Traupe et al. 2007; Yu et al. 2011). Overall, circulating plasma estrogens have been well documented to be responsible for differences in coronary vascular reactivity between sexes in swine (Barber and Miller 1997; Jones et al. 1999; Bowles 2001; Heaps and Bowles 2002).

The acute and nongenomic inhibition of Ca_v1.2 channels in porcine coronary arteries by high concentrations of 17 β E has been demonstrated by us and others (Han et al. 1995; Hill et al. 2010). As shown in this study, when coronary arteries are acutely (≤ 60 min) exposed to 17 β E, a 17 β E concentration of ≥ 1 μ mol/L is necessary to inhibit contractile responses to depolarizing KCl solutions. Similarly, our study confirms findings by Teoh et al. (2000) that acutely exposing porcine coronary arteries to a lower 17 β E concentration of 1 nmol/L fails to inhibit Ca²⁺-dependent contractions. It is likely that the apparent lack of *in vitro* sensitivity to 17 β E is partly due to the larger amount of connective tissue and increased diffusion distance of coronary arteries compared to small, resistance arteries. Only after a 24 h exposure to 17 β E, were we able to demonstrate that 1 nmol/L 17 β E attenuates the depolarization-induced contractile response to KCl and inhibits contractions elicited by the Ca_v1.2 channel agonist, FPL64176. Both of these contractile responses are mediated by voltage-dependent Ca²⁺ influx through Ca_v1.2 channels, as verified by their reversal by nifedipine, a selective Ca_v1.2 channel antagonist (Wang et al. 2011; Owen et al. 2013).

Our findings further suggest that the attenuation of Ca²⁺-dependent contractions observed after a 24 h exposure to 1 nmol/L 17 β E is mediated by a posttranscriptional downregulation of the pore-forming Ca_v α subunit of the Ca_v1.2 channel. To our knowledge, no studies have reported an effect of near physiological estrogen levels on

Ca_v α expression in ASMCs, although it is well recognized that Ca_v1.2 channels are dynamically regulated *in vivo* by circulating factors and disease states. For example, an overabundance of arterial Ca_v1.2 channels is a shared feature of pig, rat, and mouse models of hypertension, which also exhibit elevated myogenic tone and arterial reactivity (Molero et al. 2002; Pratt et al. 2002; Pesic et al. 2004; Hirenallur et al. 2008; Kharade et al. 2013). Likewise, Wang et al. (2008) reported that angiotensin II increases Ca_v α subunit expression without changes in Ca_v α mRNA levels in cultured rat mesenteric arteries, implying involvement of posttranscriptional regulation of Ca_v1.2. However, Tharp et al. (2014) reported that ASMCs of female pigs subjected to ovariectomy exhibited a twofold increase in Ca²⁺ current in the absence of altered Ca_v α transcript or protein expression. Overall, these findings infer that the particular experimental model may determine the mechanism by which the abundance of Ca_v1.2 channels is regulated in the plasma membrane (Mazzuca et al. 2015).

Importantly, our results suggest that the loss of Ca_v1.2 channels in coronary arteries in response to 24 h exposure to 1 nmol/L 17 β E is prevented by the estrogen receptor (ER α /ER β) antagonist ICI 182,780, whereas the GPER antagonist G15 has no effect. It remains unlikely that G15 was ineffective as a GPER inhibitor in our studies, because Yu et al. (2011) previously used a similar concentration of G15 (3 μ mol/L) to inhibit the GPER-mediated vasorelaxation induced by its selective agonist, G-1, in porcine coronary arteries. Instead, our findings imply that 17 β E may bind to ER α /ER β to alter posttranscriptional regulation of Ca_v1.2 channels, a process that has not been defined in ASMCs. However, in nonvascular cell types and heterologous expression systems, the posttranscriptional mechanisms that regulate Ca_v1.2 channel expression include ubiquitination and proteosomal degradation of the Ca_v α subunit (Altier et al. 2011; Rougier et al. 2011). Thus, it is possible that ER α /ER β signaling disrupts the fine balance between Ca_v1.2 channel biogenesis and degradation, potentially resulting in a loss of Ca_v1.2 channels at the plasma membrane.

The relaxation response to high, pharmacological concentrations of estrogen relies on direct inhibition of Ca²⁺ influx into coronary ASMCs, and does not require the vascular endothelium (Gilligan et al. 1994; Han et al. 1995; Nakajima et al. 1995; Hill et al. 2010; Yang and Reckelhoff 2011). However, the endothelium has been implicated in the regulation of Ca_v1.2 channel expression in ASMCs (Wang et al. 2008, 2011). Therefore, this study also sought to define the contribution of endothelium to the 17 β E-induced loss of Ca_v1.2 channels and Ca²⁺-dependent contractions. However, we failed to find a role for the endothelium in 17 β E actions. Instead, the

17 β E-mediated decrease in Ca_v1.2 channel numbers appears to be mediated by ER α /ER β expressed by ASMCs, and we suggest the downregulation of Ca_v1.2 channels by ER α /ER β may attenuate the development of abnormal coronary arterial tone. The ER α /ER β -mediated, endothelium-independent dilation and decrease in voltage-gated Ca²⁺ influx has been similarly shown using rat mesenteric microvessels (Mazzuca *et al.* 2015).

Although our porcine coronary artery model offers many experimental advantages, several limitations of this study should be acknowledged. First, as discussed earlier, we did not use a defined “physiological” plasma 17 β E concentration. Although our porcine coronary artery model offers many experimental advantages, we cannot ensure our results directly translate to the human condition. Second, the limitations of *in vitro* vascular studies that remove ASMCs from modulating endogenous influences and place them in artificial vessel culture conditions are appreciated. In earlier studies (Lindqvist *et al.* 1999; Thorne and Paul 2003), and also here, we used serum-free RPMI media to maintain arterial contractility and avoid cell proliferation. Third, we attempted to perform whole-cell patch clamp on ASMCs freshly isolated from our cultured coronary arteries to directly confirm a loss of functional Ca_v1.2 channels in response to 17 β E. However, we were unable to maintain the high resistance (≥ 10 G Ω) seals required for high-quality recordings of Ca²⁺ currents, although we routinely record ion currents from ASMCs freshly isolated from many other arterial preparations (Pesic *et al.* 2004; Hirehallur *et al.* 2008; Thakali *et al.* 2010; Kharade *et al.* 2013). For this reason, our study relied on two different vascular reactivity assays using depolarizing KCl solution and FPL64176, which directly activate Ca_v1.2 channels in ASMCs *in situ* to allow for detection of changes in Ca_v1.2 channel function.

In conclusion, this study provides initial evidence that a nonacute, low concentration of 17 β E for 24 h results in loss of functional Ca_v1.2 channels in porcine coronary arteries, and this event appears to rely on posttranscriptional modifications enacted by 17 β E through ER α /ER β signaling. This finding is significant because most *in vitro* studies have used high pharmacological estrogen concentrations to emphasize the antagonistic effects of estrogen on Ca²⁺ signaling in ASMCs and on arterial contraction. It is possible that this previously unrecognized effect of estrogen contributes to the attenuation of coronary vascular contractions reported by sex studies in females compared to males (Jones *et al.* 1999; Bowles 2001; Tharp *et al.* 2014).

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Disclosure

None declared.

Author Contributions

Research design: Hill, Dalton, Joseph, and Rusch; Conducted experiments: Hill, Dalton, Joseph, and Thakali; Performed data analysis: Hill, Dalton, Joseph, and Rusch; Contributed to the writing of the manuscript: Hill, Dalton, Joseph, Thakali, and Rusch.

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