17β-estradiol reduces \( \text{Ca}_{v}1.2 \) channel abundance and attenuates \( \text{Ca}^{2+} \)-dependent contractions in coronary arteries

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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 \( \text{Ca}^{2+} \)-activated \( K^{+} \) channels.

Abstract

One mechanism by which the female sex may protect against elevated coronary vascular tone is inhibition of \( \text{Ca}^{2+} \) entry into arterial smooth muscle cells (ASMCs). In vitro findings confirm that high estrogen concentrations directly inhibit voltage-dependent \( \text{Ca}_{1.2} \) channels in coronary ASMCs. For this study, we hypothesized that the nonacute, in vitro exposure of coronary arteries to a low concentration of 17β-estradiol (17βE) reduces the expression of \( \text{Ca}_{1.2} \) 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 \( \mu \)mol/L) of 17βE acutely attenuated \( \text{Ca}^{2+} \)-dependent contractions to depolarizing KCl stimuli. Interestingly, culturing coronary arteries for 24 h in a 10,000-fold lower concentration (1 nmol/L) of 17βE also attenuated KCl-induced contractions and reduced the contractile response to the \( \text{Ca}_{1.2} \) agonist, FPL64176, by 50%. Western blots revealed that 1 nmol/L 17βE decreased protein expression of the pore-forming \( \text{Ca}_{1.2} \) subunit (\( \text{Ca}_{v2} \)) of the \( \text{Ca}_{1.2} \) channel by 35%; this response did not depend on an intact endothelium. The 17βE-induced loss of \( \text{Ca}_{v2} \) protein in coronary arteries was prevented by the estrogen ERα/ERβ antagonist, ICI 182,780, whereas the GPER antagonist, G15, did not prevent it. There was no effect of 1 nmol/L 17βE on \( \text{Ca}_{v1} \) transcript expression. We conclude that 17βE reduces \( \text{Ca}_{1.2} \) channel abundance in isolated coronary arteries by a posttranscriptional process. This unrecognized effect of estrogen may confer physiological protection against the development of abnormal \( \text{Ca}^{2+} \)-dependent coronary vascular tone.

Abbreviations

17βE, 17β-estradiol; Ach, Acetylcholine; ASMCs, arterial smooth muscle cells; \( \text{Ca}_{1.2} \), L-type \( \text{Ca}^{2+} \) channels; \( \text{Ca}_{v} \), 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; \( \text{H}_{2}\text{O}_{2} \), hydrogen peroxide; HRP, horseradish peroxidase; ICI 182, 780, 7α,17β-[9-[(4,4,5,5-Pentfluoropentyl)sulfinyl]nonyl]estr-1,3,5(10)-triene-3,17-diol; RCA, right coronary artery; TBST, Tris-buffered saline containing 0.1% Tween 20; TSG, thapsigargin.
and/or inhibition of voltage-gated, L-type Ca\(^{2+}\) (Ca\(_{a,1.2}\)) channels (Bowles et al. 1998a,b). The Ca\(_{a,1.2}\) channels are the major pathway for voltage-dependent Ca\(^{2+}\) influx in coronary arterial smooth muscle cells (ASMCs), and as primary mediators of arterial contraction, any imbalance in their activity or expression that favors Ca\(^{2+}\) influx may contribute to elevated vascular tone. For example, the expression of Ca\(_{a,1.2}\) channels is increased in coronary arteries susceptible to vasospasm (Kuga et al. 2000), and Ca\(_{a,1.2}\) 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 Ca\(^{2+}\)-dependent vascular tone, which is normalized by pharmacological blockers of Ca\(_{a,1.2}\) 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, 17\(\beta\)-estradiol (17\(\beta\)E), acutely inhibits contractions elicited by depolarizing KCl solutions, suggesting that 17\(\beta\)E reduces Ca\(^{2+}\) influx mediated by voltage-sensitive Ca\(_{a,1.2}\) channels. Similarly, Sudhir et al. (1997) demonstrated that vasospasms of porcine coronary arteries are alleviated by the intracoronary infusion of 17\(\beta\)E, implying an inhibitory action of pharmacological concentrations of estrogen on coronary reactivity. Finally, Ullrich et al. (2007) directly confirmed that 17\(\beta\)E (10 \(\mu\)mol/L) inhibits whole-cell Ca\(^{2+}\) current recorded from Ca\(_{a,1.2}\) channel-transfected HEK-293 cells. In all of these studies, high concentrations (\(\geq 1 \mu\)mol/L) of 17\(\beta\)E were required to reduce Ca\(^{2+}\) 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 17\(\beta\)E are nongenomic and relate to direct inhibition of Ca\(_{a,1.2}\) channel function (Hill et al. 2010). However, a recurring concern of these in vitro studies is that the vasodilator effect of high pharmacological 17\(\beta\)E concentrations applied for short exposure times (\(\leq 60\) 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 17\(\beta\)E’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\(_{a,1.2}\) channel function in ASMCs. Using an ovariectomized rat model, Crews and Khalil (Crews and Khalil 1999) found less Ca\(^{2+}\) influx through Ca\(_{a,1.2}\) channels in aortae isolated from ovari-intact compared to ovariectomized females, implying that higher levels of circulating plasma estrogen may reduce the number of functional Ca\(_{a,1.2}\) channels in ASMCs. Likewise, Bowles (2001) found that coronary arteries of male pigs exhibit a higher expression level of the pore-forming \(\alpha_{1C}\) subunit (Ca\(_{a,\alpha}\)) of the Ca\(_{a,1.2}\) channel compared to female animals, and also show more voltage-dependent Ca\(^{2+}\) 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\(_{a,1.2}\) channel current, but not enhanced Ca\(_{a,\alpha}\) protein expression, compared to ovari-intact female pigs. The authors hypothesized that endogenous female hormones inhibit Ca\(_{a,1.2}\) channel activity in coronary ASMCs by upregulating the Ca\(_{a,\beta1}\) subunit. In this regard, the Ca\(_{a,\alpha}\) pore proteins coassemble in 1:1 stoichiometry with smaller \(\beta\) and \(\gamma_2\delta\) subunits to form functional Ca\(_{a,1.2}\) channels in ASMCs; different \(\beta\) subunits modulate expression and properties of the multiprotein channel (Sonkusare et al. 2006; Kharade et al. 2013; Cox and Fromme 2015). Based on these in vitro studies, the objective of this study was to use an in vitro vessel culture approach to define the effect of 17\(\beta\)E on Ca\(_{a,1.2}\) channel expression and function. 17\(\beta\)-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 Ca\(^{2+}\) Krebs solution (in mmol/L: 138 NaCl, 5 KCl, 0.2 CaCl\(_2\), 1 MgCl\(_2\), 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 17β-estradiol (17βE; Sigma Chemical Co., St. Louis, MO) on Ca_{1.2} channel-mediated coronary reactivity and Ca_{ax} expression, arterial segments were incubated at 37°C in a 5% CO_{2} incubator for 24 h in RPMI 1640 phenol-free culture media (Sigma). Arteries either were incubated in: (1) control media, (2) media supplemented with 17βE (1 pmol/L to 10 μmol/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 ERα/ERβ 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 ERα and ERβ at concentrations up to 10 μmol/L (Dennis et al. 2009; Yu et al. 2011). To evaluate the role of endothelium on the 17βE 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% O_{2}:5% CO_{2}) modified Krebs solution (in mmol/L: 138 NaCl, 5 KCl, 2 CaCl_{2}, 1 MgCl_{2}, 10 HEPES, 10 glucose, pH 7.4). All rings were initially stretched to their optimal length (Tummala and Hill 2007). To evaluate 17βE-induced relaxation, the freshly isolated rings were preconstricted with a depolarizing solution containing high (60 mmol/L) KCl (in mmol/L: 83 NaCl, 60 KCl, 2 CaCl_{2}, 1 MgCl_{2}, 10 HEPES, 10 glucose, pH 7.4) followed by generation of a concentration–response relationship with 17βE (0.1–100 μmol/L) or its EtOH solvent. As reported and discussed earlier (Hill et al. 2010), each 17βE 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 17βE 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 17βE concentration (1 pmol/L to 100 μmol/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 μmol/L 17βE. 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 17βE 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_{1.2} 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 μmol/L; Sigma) to relax a 35 mmol/L KCl depolarizing contraction. In studies using the Ca_{1.2} channel agonist, FPL64176, rings were exposed to acetylcholine (10 μmol/L; Sigma) and thapsigargin (10 μmol/L; Sigma) to release and eliminate the reuptake of Ca^{2+} back into the sarcoplasmic reticulum (SR), and thereby eliminate a potential effect of FPL64176 on SR Ca^{2+} release (Wasserstrom et al. 2002). A steady-state contraction was generated to FPL64176 (3 μmol/L; Tocris Bioscience) followed by application of the Ca_{1.2} channel antagonist, nifedipine (10 μmol/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 lyate 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_{3C} (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 Cav1.2 transcript in arterial strips cultured for 24 h in EtOH or 17βE (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 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 Cav1.2 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): Cav1.2 - 5’-ccgccactacaagatgagctc-3’ (forward) and 5’-gcatttctc-3’ (reverse); β-actin - 5’-acacggcattgtcatggactct-3’ (forward) and 5’-ttctatagatgcctcggt-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 Cav1.2 transcript in EtOH and 17βE-treated arteries was quantified by the ΔΔCt 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 ± 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 17βE 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 α = 0.05; therefore, significance was defined as P < 0.05.

**Results**

**Vascular reactivity responses**

High concentrations of 17βE were necessary to relax depolarizing KCl-induced contractions (Fig. 1), which fully rely on voltage-dependent Ca2+ influx through Cav1.2 channels. The progressive relaxation mediated by 17βE (0.1–100 μmol/L) significantly attenuated contracture by 17.7 ± 2.4% and 61.3 ± 5.4% at concentrations of 10 μmol/L and 100 μmol/L 17βE, respectively (n = 7). EtOH served as a solvent and time control for the relaxation experiment. Likewise, when arterial rings were incubated with 17βE (1 pM to 100 μmol/L) for 60 min prior to generation of KCl concentration–response curves, only 17βE concentrations of 10 μmol/L and 100 μmol/L significantly attenuated KCl contractions (Fig. 2; n = 5). A 17βE 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 17βE did not suppress KCl concentration–response curves as shown in Figure 2.

In contrast, prolonged preexposure of similar arteries to 1 nmol/L 17βE attenuated depolarization-induced contractions (Fig. 3). Coronary arterial rings cultured for 24 h prior to tension recording in RPMI media containing 1 nmol/L 17βE exhibited an ~25% attenuation of the KCl concentration–response curves to KCl (Fig. 3). This
inhibitory effect was accentuated in arterial rings preincubated in 10 μmol/L 17βE for 24 h, whereas 1 pM 17βE had no significant effect on the KCl contraction. The acute (1 h) versus longer term (24 h) effects of 1 nmol/L 17βE on KCl contraction are compared in Table 1.

17βE for 24 h results in loss of Ca_{α2} protein

Corroborating our isometric tension-recording findings of reduced Ca^{2+}-dependent contractions after prolonged exposure to 1 nmol/L 17βE, we also found that a 24 h exposure to 1 nmol/L 17βE decreased the expression of the pore-forming α_{1C} subunit of the Ca_{α2} channel, Ca_{α2}, by 35 ± 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_{α2} subunit. The EtOH solvent (0.1%) and 1 pmol/L 17βE did not significantly affect Ca_{α2} 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 17βE reduced Ca_{α2} subunit abundance by transcriptional regulation. Initially, we standardized the real-time PCR assay before comparing Ca_{α2} subunit mRNA levels. First, the presence of a single peak in the melt curve analysis (A) Figure 1. Pharmacological concentrations of 17β-estradiol (17βE) are required to relax coronary arterial rings precontracted by 60 mmol/L KCl. (A) Typical recording shows concentration-dependent relaxation by 17βE of a KCl-contracted arterial ring. Exposure to KCl is indicated by the horizontal line. (B) Average values (mean ± SE) for 17βE-induced relaxation of KCl precontracted rings. Equal volumes of EtOH served as solvent control. *P < 0.05 using an unpaired t-test, EtOH versus 17βE groups (n = 7 pigs).
Table 1. Comparison between effects of acute (1 h) and prolonged (24 h) exposure to 17β-estradiol (17βE) on EC50 values and maximal tension (Tmax) of KCN concentration–response relationships.

| Exposure time | Solvent | [17βE] or EtOH | n | EC50 (mmol/L)1 | Tmax2 |
|---------------|---------|----------------|---|---------------|-------|
| 1 h           | EtOH (0.1%) | 5 | 39.89 ± 1.01 | 102.81 ± 1.86 |
| 1 pmol/L 17βE | 5 | 41.41 ± 1.01 | 96.96 ± 1.18 |
| 1 nmol/L 17βE | 5 | 40.87 ± 1.02 | 103.33 ± 7.01 |
| 24 h          | EtOH (0.1%) | 5 | 41.01 ± 1.07 | 110.87 ± 3.83 |
| 1 pmol/L 17βE | 5 | 39.67 ± 1.07 | 96.17 ± 7.15 |
| 1 nmol/L 17βE | 5 | 44.06 ± 1.08 | 85.59 ± 5.50 |

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.

P < 0.05, EtOH versus 1 nmol/L 17βE.

The effect of 24 h exposure to 1 nmol/L 17βE on Ca1.2 channel function also was evaluated by comparing the contractile response to the Ca1.2 channel agonist, FPL64176 (3 μmol/L), between coronary rings exposed to the EtOH solvent or 17βE. Studies were performed in endothelium intact (+Endo) and denuded (-Endo) arterial rings to evaluate if the inhibitory effect of 1 nmol/L 17βE on Ca1.2 channel-dependent contraction was mediated by endothelium-derived factors. In addition to its intended role as a Ca1.2 channel agonist, FPL64176 reportedly can induce Ca2+ release from the sarcoplasmic reticulum (SR) by opening ryanodine receptors (Wasserstrom et al. 2002; Vaiithianathan et al. 2010). Therefore, as shown in Figure 6A, arteries were simultaneously exposed to 10 μmol/L acetylcholine (Ach) and 10 μmol/L thapsigargin (TSG) to induce SR Ca2+ release and prevent its reuptake by inhibiting Ca2+-ATPase, respectively. Arteries incubated in 1 nmol/L 17βE elicited a contractile response to FPL64176 that was 47 ± 0.56% and 60 ± 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-Ca1.2 Western blots (Fig. 7), coronary arterial strips cultured in 1 nmol/L 17βE for 24 h exhibited a significant decrease in expression of the pore-forming Ca1.2 subunit of the Ca1.2 channel in +Endo and -Endo arterial strips. The +Endo and -Endo arteries exposed to 17βE exhibited a densitometric ratio that was
62 ± 9% and 74 ± 14% of the control +Endo arteries treated with EtOH solvent, respectively. Western blot data are expressed as a ratio of Ca₉α expression in +Endo arteries exposed to EtOH. In a control set of experiments, the EtOH solvent had no effect on Ca₉α subunit expression between +Endo and -Endo arteries (n = 6, data not shown); the densitometric ratio (Ca₉α/β-actin) was 1.01 ± 0.09 and 0.93 ± 0.17 for +Endo and -Endo arteries, respectively.

Previously in Figs. 4 and 7, we demonstrated a significant reduction in the Ca₉α 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 Ca₉α 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 Ca₉α.2 channel.
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 Cav1.2 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 depresses contractions induced by depolarizing KCl and the Cav1.2 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 Cav1.2 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 et al. 2001; Bowles et al. 2004; Tharp et al. 2014). Because Cav1.2 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 17β/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 17β/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), ERα and ERβ, 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 Caα1.2 channels in porcine coronary arteries by high concentrations of 17β/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 17β/E, a 17β/E concentration of ≥1 μmol/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 17β/E concentration of 1 nmol/L fails to inhibit Ca2+-dependent contractions. It is likely that the apparent lack of in vitro sensitivity to 17β/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 17β/E, were we able to demonstrate that 1 nmol/L 17β/E attenuates the depolarization-induced contractile response to KCl and inhibits contractions elicited by the Caα1.2 channel agonist, FPL64176. Both of these contractile responses are mediated by voltage-dependent Ca2+ influx through Caα1.2 channels, as verified by their reversal by nifedipine, a selective Caα1.2 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 17β/E is mediated by a posttranscriptional downregulation of the pore-forming Caα1.2 subunit of the Caα1.2 channel. To our knowledge, no studies have reported an effect of near physiological estrogen levels on Caα1.2 expression in ASMCs, although it is well recognized that Caα1.2 channels are dynamically regulated in vivo by circulating factors and disease states. For example, an overabundance of arterial Caα1.2 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 Caα1.2 subunit expression without changes in Caα1.2 mRNA levels in cultured rat mesenteric arteries, implying involvement of posttranscriptional regulation of Caα1.2. 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 Caα1.2 transcript or protein expression. Overall, these findings infer that the particular experimental model may determine the mechanism by which the abundance of Caα1.2 channels is regulated in the plasma membrane (Mazzuca et al. 2015).

Importantly, our results suggest that the loss of Caα1.2 channels in coronary arteries in response to 24 h exposure to 1 nmol/L 17β/E is prevented by the estrogen receptor (ERα/ERβ) 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 μmol/L) to inhibit the GPER-mediated vasorelaxation induced by its selective agonist, G-1, in porcine coronary arteries. Instead, our findings imply that 17β/E may bind to ERα/ERβ to alter posttranscriptional regulation of Caα1.2 channels, a process that has not been defined in ASMCs. However, in nonvascular cell types and heterologous expression systems, the posttranscriptional mechanisms that regulate Caα1.2 channel expression include ubiquitination and proteosomal degradation of the Caα1.2 subunit (Altier et al. 2011; Rougier et al. 2011). Thus, it is possible that ERα/ERβ signaling disrupts the fine balance between Caα1.2 channel biogenesis and degradation, potentially resulting in a loss of Caα1.2 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 Caα1.2 channel expression in ASMCs (Wang et al. 2008, 2011). Therefore, this study also sought to define the contribution of endothelium to the 17β/E-induced loss of Caα1.2 channels and Ca2+-dependent contractions. However, we failed to find a role for the endothelium in 17β/E actions. Instead, the
17βE-mediated decrease in Ca_{1.2} channel numbers appears to be mediated by ERα/ERβ expressed by ASMCs, and we suggest the downregulation of Ca_{1.2} channels by ERα/ERβ may attenuate the development of abnormal coronary arterial tone. The ERα/ERβ-mediated, endothelium-independent dilation and decrease in voltage-gated Ca^{2+} influx has been similarly shown using rat mesenteric microvessels (Mazzucca 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 17βE 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_{1.2} channels in response to 17βE. However, we were unable to maintain the high resistance (≥10 GΩ) seals required for high-quality recordings of Ca^{2+} currents, although we routinely record ion currents from ASMCs freshly isolated from many other arterial preparations (Pesci et al. 2004; Hirenallur 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_{1.2} channels in ASMCs in situ to allow for detection of changes in Ca_{1.2} channel function.

In conclusion, this study provides initial evidence that a nonacute, low concentration of 17βE for 24 h results in loss of functional Ca_{1.2} channels in porcine coronary arteries, and this event appears to rely on posttranscriptional modifications enacted by 17βE through ERα/ERβ signaling. This finding is significant because most in vitro studies have used high pharmacological estrogen concentrations to emphasize the antagonistic effects of estrogen on Ca^{2+} 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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