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Metabolite ligands of estrogen receptor-β reduce primate coronary hyperreactivity

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[1]*Dimera, Incorporated, and* [3]*Oregon Health and Science University, Portland, Oregon;* [2]*University of Southern California, Los Angeles, California;* [4]*University of Alabama Birmingham, Birmingham, Alabama; and* [5]*University of Illinois Urbana-Champaign, Urbana, Illinois*

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Mishra, Rajesh G., Frank Z. Stanczyk, Kenneth A. Burry, Suzanne Oparil, Benita S. Katzenellenbogen, Michele L. Nealen, John A. Katzenellenbogen, and R. Kent Hermsmeyer. Metabolite ligands of estrogen receptor-β reduce primate coronary hyperreactivity. *Am J Physiol Heart Circ Physiol* 290: H295–H303, 2006. First published September 30, 2005; doi:10.1152/ajpheart.00468.2005.—Previous reports showed that 17β-estradiol implants attenuate in vivo coronary hyperreactivity (CH), characterized by long-duration vasoconstrictions (in coronary angiographic experiments), in menopausal rhesus monkeys. Prolonged Ca^{2+} contraction signals that correspond with CH in coronary vascular muscle cells (VMC) to the same dual-constrictor stimulus, serotonin + the thromboxane analog U-46619, in estrogen-deprived VMC were suppressed by >72 h in 17β-estradiol. The purpose of this study was to test whether an endogenous estrogen metabolite with estrogen receptor-β (ER-β) binding activity, estriol (E_3), suppresses in vivo and in vitro CH. E_3 treatment in vivo for 4 wk significantly attenuated the angiographically evaluated vasoconstrictor response to intracoronary serotonin + U-46619 challenge. In vitro treatment of rhesus coronary VMC for >72 h with nanomolar E_3 attenuated late Ca^{2+} signals. This reduction of late Ca^{2+} signals also appeared after >72 h of treatment with subnanomolar 5α-androstane-3β,17β-diol (3β-Adiol), an endogenous dihydrotestosterone metabolite with ER-β binding activity. R,R-tetrahydrochrysene, a selective ER-β antagonist, significantly blocked the E_3- and 3β-Adiol-mediated attenuation of late Ca^{2+} signal increases. ER-β and thromboxane-prostanoid receptor (TPR) were coexpressed in coronary arteries and aorta. In vivo E_3 treatment attenuated aortic TPR expression. Furthermore, in vitro treatment with E_3 or 3β-Adiol downregulated TPR expression in VMC, which was blocked for both agonists by pretreatment with R,R-tetrahydrochrysene. E_3- and 3β-Adiol-mediated reduction in persistent Ca^{2+} signals is associated with ER-β-mediated attenuation of TPR expression and may partly explain estrogen benefits in coronary vascular muscle.

menopause; calcium; thromboxane-prostanoid receptor; angiography

CARDIOVASCULAR PHYSIOLOGY is profoundly regulated by multiple endocrine signals via nuclear and cell surface receptors, and age-related hormonal decline may increase the risk of cardiovascular disease (1). Current controversy regarding cardiovascular effects of hormone therapy (35, 38) emphasizes the need for research to enhance our understanding of steroid hormone actions on structural and functional changes in the blood vessel wall (7). Growing evidence suggests a role for estrogen receptors (ER) in regulation of vascular healing and proliferation following injury as well as ER-mediated regulation of endothelial-dependent vasodilator reactivity responses (23). However, published data on ER-mediated effects on coronary vasoconstrictor reactivity responses are sparse.

Despite a large body of evidence on the biological actions of 17β-estradiol (E_2) (33), there is a paucity of information on the biological actions of metabolites of E_2, such as the endogenous ER ligand estriol (E_3), which can be present at significant concentrations at the tissue level (5, 11, 19). E_3 is abundantly produced during late-stage pregnancy and, thus, is present in conjugated equine estrogen preparations, which are known to relieve menopausal symptoms (43), has antiatherosclerotic actions, improves endothelial and bone function (15), and ameliorates symptoms in autoimmune demyelinating disorders such as multiple sclerosis (21). Optimal homeostatic regulation of vascular tone and response to injury and inflammation may require a balance of multiple estrogenic metabolites in the vascular wall. For example, accruing evidence supports the concept of regulation of vascular proliferation by an array of steroid metabolites (4, 5). However, there has been little or no investigation of effects of metabolite ER ligands on the regulation of vascular reactivity.

Our previous research showed that E_2 and progesterone protect surgically menopausal Rhesus Macaque monkeys (RM) from in vivo coronary hyperreactivity (CH) and attenuate persistent Ca^{2+} signals (27, 28). In this study, experiments were designed to investigate the effects of E_3 on regulation of in vivo CH. Furthermore, we tested effects of E_3 and 5α-androstane-3β,17β-diol (3β-Adiol), a dihydrotestosterone (DHT) metabolite with ER-β agonist activity (45), on Ca^{2+} signals in coronary vascular muscle cells (VMC) and, for comparison, examined the effects of the reported pharmacologically selective ER-β ligands diarylpropionitrile (DPN) (40) and genistein (36) on Ca^{2+} signals in coronary VMC. In addition, using immunocytochemistry and Western blotting, we examined the association of regulation of thromboxane-prostanoid receptor (TPR) expression with VMC reactivity.

METHODS

Transdermal E_3 cream application. Six adult female RM (*Macaca mulatta*, 14.7 ± 1.8 yr old, 6.4 ± 1.2 kg body wt) were ovariectomized (Ovx) 1 mo before initiation of daily transdermal application of 1 ml of the 0.3% (g/g) E_3 skin cream for 28 days. The midscapular area was shaved in preparation for application of 1 ml of the 0.3% (g/g) E_3 skin cream (3 mg of E_3 topically applied in each dose). The

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anticipated transdermal systemic absorption (7%) was ~0.2 mg. Trained personnel used a squeeze-box cage for application of the drug through a window in the cage especially designed to allow optimal application with minimal animal discomfort. The cream covered an ~6-cm^2 area on the shaved subscapular area on the back of the trained RM without need for anesthetic or tranquilizer. This subscapular area, which is inaccessible for licking and minimally noticed by the RM, was used for subdermal or transdermal dosing for optimum steroid hormone delivery as reported previously (17).

Angiography data from 6 Ovx historical control RM (untreated Ovx; 12.4 ± 0.8 yr old, 6.1 ± 0.5 kg body wt), performed in the same laboratory, with use of the same protocol, and by the same investigators, were also included in the study analysis for comparison of the in vivo protective effects of E_3 with CH. These controls were considered appropriate, because no specific effect of the vehicle in a placebo cream was anticipated on the basis of previous demonstration of no treatment effect of placebo with the identical formulation (without the active pharmaceutical ingredient) applied following the same primate center protocol with the same special window cages (17). Experimental protocols were approved by the Oregon Regional Primate Research Center Animal Care and Use Committee.

Provocation of constriction to test for CH. RM were subjected to the coronary artery combined vasoconstrictor stimulus in a catheterization laboratory protocol described previously (16, 17, 26, 28, 29, 31). Briefly, the stepwise injection protocol tests the ability of slow (1 ml over 30 s) intracoronary infusion of vasoactive endogenous substances, 100 μM serotonin (S) and 1 μM U-46619 (U, a thromboxane analog), to initiate the prolonged (>5 min), severe (to <33% of control diameter) vasoconstriction that defines CH producing ischemia and, if unrelieved, myocardial infarction. Acetylcholine-induced vasodilation of 5–8% was found in all E_3-treated RM, indicating normal endothelial dilator function. An observer blinded to the treatment groups measured coronary artery diameters (Φ) from serial angiograms to define the points of minimum Φ after each injection (16, 17, 26, 28, 29, 31). The corresponding anatomic point on the control image was the 100% reference Φ. Average minimum Φ of major epicardial coronary arteries were digitally analyzed to determine minimum Φ. A reduction in Φ to <33% of control for >5 min was classified as CH, whether or not focal vasospasm was observed (16, 17, 26, 28, 29, 31).

Fluorescence polarization ER-β coactivator assay. Fluorescence polarization ER-β coactivator assay (35) was performed according to the manufacturers' published protocol (PanVera Discovery Screening, Madison, WI; Invitrogen, Carlsbad CA) to test ER-β agonist/antagonist activity of 3β-Adiol, DPN, genistein, E_3, or R,R-tetrahydrochrysene (R,R-THC) studied individually using the ER-β coactivator assay along with reference agonists and antagonists. In the ER-β coactivator assay, recombinant human ER-β and a fluorescent ER ligand are used to determine the ER-β agonist/antagonist activity of test compounds. The indicator D22 is a peptide containing an LXXLL motif and flanking sequences that resemble known nuclear receptor coactivators. Agonist-bound ER-β promotes D22 binding, resulting in a larger fraction of bound D22 and measurable increases in polarization value, whereas antagonist-bound ER-β represses D22 binding, yielding a larger fraction of unbound D22 and a measurably lower polarization value. Polarization of fluorescence signals was measured in 96-well microplates using a TECAN Ultra instrument (Research Triangle Park, NC). The concentration of ligand that resulted in half-maximum increase (agonist) or decrease (antagonist) in polarization by sigmoidal B spline curve fit to a dose-response curve (Origin) was taken as the EC_{50} or IC_{50} for the ER-β-D22 interaction.

Vascular muscle culture and VMC reactivity. Coronary VMC used in cell culture live cell fluorescent, immunocytochemistry, and Western blot studies were obtained from a separate group of 12 Ovx RM through the tissue distribution program at the Oregon National Primate Research Center. Coronary VMC culture protocols were performed as described previously (27–29). VMC Ca^{2+} responses to 15 s of S + U pulse stimulation were determined as described previously (17, 27–29) after >72 h of in vitro exposure to ER-β ligand (or placebo). When selective ER-β antagonist treatment (e.g., R,R-THC) was also performed, the antagonist was added to VMC culture plates 3 h before subsequent treatment with the agonist (and continued during the entire agonist treatment). Reactivity effects of chronic (72 h) in vitro treatment with E_3, 3β-Adiol, genistein, and DPN, alone or in the presence of R,R-THC, were examined by VMC Ca^{2+} signal amplitude over 30 min. The VMC Ca^{2+} was determined as percent change in fluo 3 fluorescence from baseline in response to 15 s of stimulation with 10 μM S + 100 nM U. The statistically determined end point was the late (30 min) rise in Ca^{2+} fluo 3 fluorescence, expressed as percent change (27, 28). VMC cultures from three or more separate RM were intrinsic to each resulting data point.

Immunocytochemistry. Immunocytochemistry was performed by an adaptation of the indirect immunofluorescence method of Yu et al. (49), as reported previously (17, 29). Ovx RM coronary arteries were dissected, fixed, and prepared as serial sections for receptor localization. Coronary and aorta cross sections were prepared using a Leica cryomicrotome. ER-β labeling was performed with a mouse monoclonal anti-human ER-β antibody (CFK-E12) (3). TPR labeling was performed with a custom-prepared polyclonal chicken antibody targeted against the ligand binding domain of TPR (Aves Labs, Tigard, OR) based on the reported amino acid sequence for this domain (CFL TLG AES GD) (17, 44) or with a rabbit polyclonal antibody (PH4; courtesy of Dr. P. V. Halushka, Medical University of South Carolina) (28). Controls were included for all immunocytochemical studies (neutralizing antigen peptide or omission of primary antibody) to ascertain the specificity of each antibody. At least four coverslips from each treatment and control group were examined, and images were recorded using a Zeiss Axiovert meta confocal microscope (courtesy of Dr. John Welsh, Neurological Sciences Institute, Oregon Health and Science University) or a Nikon confocal microscope with a Radiance 2100 system (courtesy of Bio-Rad and Dr. Robert Summers, Salk Institute, San Diego, CA). Imaging was performed in nonconfocal studies with a C-Apochromat ×40/1.2 NA water-immersion objective on an Axiovert 200M microscope using a Hamamatsu electron bombardment charge coupled device camera and Compix Simple PCI software. Captured analog images were converted to digital images and digitally stored on an Intel Pentium or AMD Athlon computer hard disk and a DVD disk to allow security, redundant backups, and offline analysis of differences in expression of receptors as measured by intensities under matched conditions.

Western blots. Western blots were performed as described previously (17). Primary cultures of VMC grown to confluence in 100 × 20 mm Falcon tissue culture dishes were treated with agonists for 72 h. If selective ER-β antagonist treatment (e.g., R,R-THC) was performed, the antagonist was added to the cell culture plates for 3 h before addition of the agonist, and antagonist treatment continued during the agonist treatment. Parallel time-matched untreated VMC served as controls.

Drugs, antibodies, and reagents. E_3 [0.3% (g/g)] transdermal cream made to Good Laboratory Practice specifications was provided by Dimera. E_3 and 3β-Adiol were purchased from Steraloids (Newport, RI), DPN and genistein from Tocris Chemicals (Ellisville, MO), fluo 3 from Molecular Probes (Eugene, OR), and secondary antibodies from Jackson Immunolabs (Westgrove, PA). R,R-THC was provided by Dr. John Katzenellenbogen and ER-β mouse monoclonal antibody by Dr. Benita Katzenellenbogen. TPR chicken polyclonal antibody was custom-made for Dimera by Aves Labs. Hexabrix was provided by Tyco-Mallinckrodt (St. Louis, MO). Buffers and solutions used in coronary catheterization studies and in vitro live cell fluorescence experiments have been described elsewhere (26–31). Unless otherwise specified, all other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Statistical analysis. Results were compared by independent *t*-tests and by ANOVA using Origin software, with $P < 0.05$ taken as the

level of statistical significance. For angiographic data analysis, comparisons were made between minimal Φ in control and E_3-treated RM. For live cell fluorescence experiments, comparisons were made between Ca^{2+} signals in Ovx RM coronary control (untreated) VMC and VMC treated with the ER-β agonists E_3, 3β-Adiol, DPN, or genistein, alone or in the presence of the selective ER-β antagonist R,R-THC.

RESULTS

E_3 reduces in vivo CH. Angiography data showed that E_3 treatment effectively reduced S + U-provoked persistent vasoconstriction compared with controls (Fig. 1*A*, E_3-treated Ovx RM vs. untreated Ovx RM). The hyperreactivity in one untreated Ovx RM manifested in the form of a vasospasm (arrow in Fig. 1*A*), i.e., a severe segmental vasoconstriction to 10% of control Φ that persisted for >5 min. As a group, untreated Ovx RM exhibited severe vasoconstriction (<33% for 5–15 min, Φ = 25 ± 3.66% of control; Fig. 1*B*), meeting our criterion for CH. In sharp contrast, E_3 treatment resulted in reduced and only transient vasoconstriction in every case. The minimum (constricted) epicardial coronary artery Φ exceeded 50% of the prestimulus Φ for the E_3 group (Φ = 63.87 ± 3.5% of control; Fig. 1*B*). All six E_3-treated RM showed no CH (by the criterion of <33% of control Φ for >5 min) and completed the entire multiple-challenge protocol without developing severe, persistent constriction.

ER-β agonist activity of 3β-Adiol, genistein, DPN, and E_3. Fluorescence polarization analysis performed in a simultaneous series with ICI-187780, tamoxifen, and 4-hydroxytamoxifen as positive controls demonstrated that, according to changes in rotation of polarized light as effected by ligand-to-receptor binding, 3β-Adiol, E_3, DPN, and genistein act as ER-β agonists, whereas R,R-THC acts as an ER-β antagonist (Fig. 2). Specific EC_{50} values (nM) for the agonists were as follows: 10 for E_2, 17 for E_3, 23 for 3β-Adiol, 15 for DPN, and 20 for genistein. IC_{50} values (nM) for antagonists were as follows: 47 for ICI-182780 (faslodex), 97 for tamoxifen, 79 for 4-hydroxytamoxifen, and 38 for R,R-THC.

E_3 and 3β-Adiol attenuate late intracellular Ca^{2+} signals. The remarkably potent effect of E_3 on reducing the duration and extent of provoked vasoconstriction in primate coronary angiographic studies, the documented affinity of 3β-Adiol for ER-β in rat prostate (45), and independent confirmation of ER-β agonist activity of E_3 and 3β-Adiol in the above-mentioned ER-β coactivator fluorescence polarization assay prompted us to explore VMC effects of these endogenous metabolite ER ligands (in comparison with the more familiar pharmacological ER-β-selective probe genistein) on fluorescent intracellular Ca^{2+}. The physiological range of circulating 3β-Adiol concentration is 100–800 pM in healthy women and men (12). We therefore tested effects of near-physiological 3β-Adiol concentrations.

Treatment with 1 nM E_3 or 0.3 nM in vitro for 72 h significantly reduced persistent (>30 min after the stimulus) Ca^{2+} signals (Fig. 3*A*). VMC stimulation after 0.3–3 nM 3β-Adiol resulted in significantly decreased late Ca^{2+} signals compared with controls. However, 0.03 nM 3β-Adiol was ineffective in preventing late Ca^{2+} signal increases (Fig. 3*B*) and, thus, defined the foot of the 3β-Adiol dose-response curve. The low-physiological-range 72-h treatment with 0.3 nM 3β-Adiol was as effective as 1 nM 3β-Adiol in decreasing

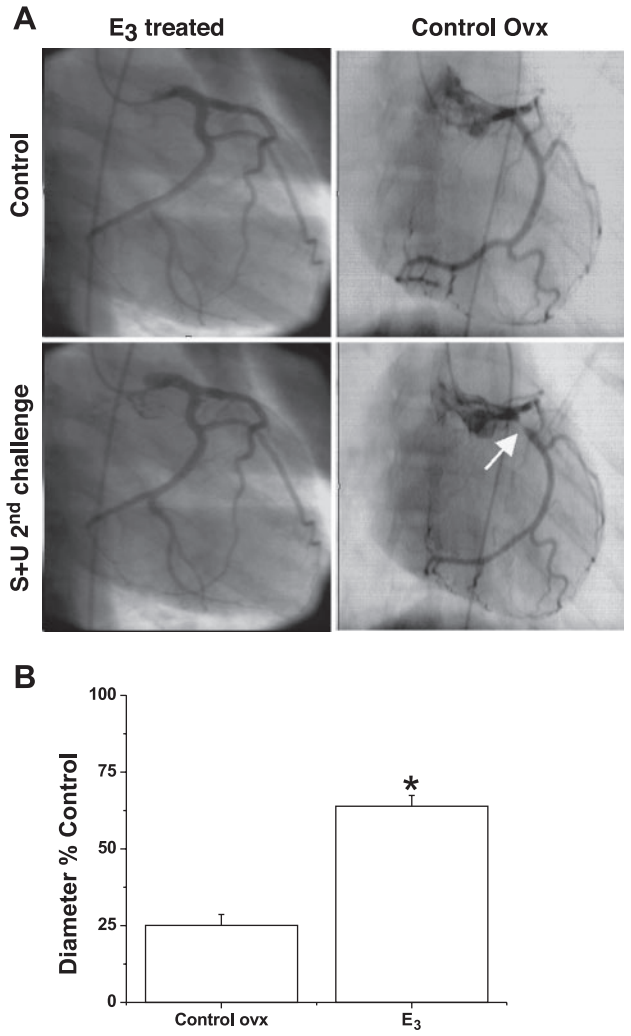

Fig. 1. Effect of estriol (E_3) on rhesus monkey (RM) coronary hyperreactivity (CH) in vivo. *A*: representative angiograms from ovariectomized (Ovx) untreated RM [5 min after 30-s stimulus with serotonin (S) + U-46619 (U)] and E_3-treated RM [3 min after 30-s stimulus with 100 μM S + 3 μM U given following a series of 3 injections of 100 μM S + 1 μM U each and, thus, a cumulative dose of 2,450 nmol of U, the nonmetabolizable thromboxane mimic] showing minimum diameters (Φ) in each respective monkey. Vasoconstriction (arrow) at 5 min in Ovx untreated, but not E_3-treated, RM persisted for >5 min. *B*: angiographically measured minimum Φ as percentage of control (recorded before vasoconstrictor challenge) with intracoronary injections over 30 s of provocative stimulus (S + U challenge). Φ values in Ovx (25 ± 3.66% control) met criterion of reduction to <33% of control Φ for >5 min and are indicative of hyperreactivity vasoconstrictions that occurred in all 6 Ovx monkeys. Φ values in E_3-treated monkeys (63.87 ± 3.55% of control) demonstrate protection against hyperreactivity as suggested by exaggerated, prolonged vasoconstrictions. *Significantly different from Ovx ($P < 0.05$.)

the late Ca^{2+} increase, which suggests a sharp transition between the lowest and an intermediate concentration. The Ca^{2+}-suppressing effect of 0.3 nM 3β-Adiol was blocked by pretreatment with the specific ER-β antagonist R,R-THC at 30 μM (Fig. 3*C*).

Statistical comparisons at 30 min showed that the ER-β ligand genistein did not significantly reduce the late increase in Ca^{2+} compared with untreated control VMC, whereas the recently discovered ER-β agonists 3β-Adiol and DPN, similar to E_3, significantly attenuated late Ca^{2+} increases (Fig. 3*D*).

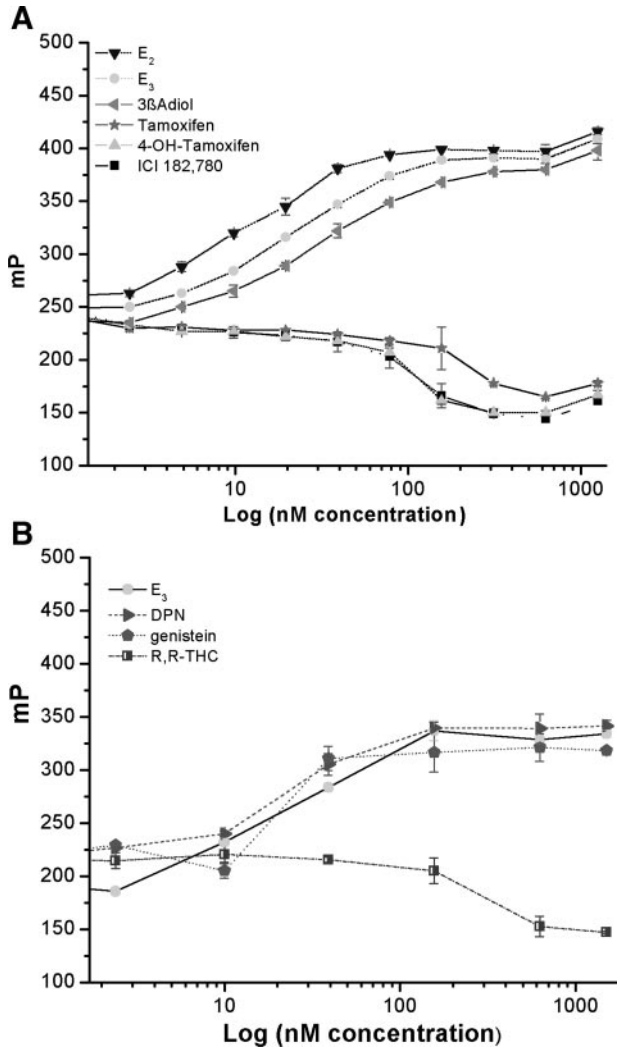

Fig. 2. Estrogen receptor (ER)-β agonist and antagonist binding by fluorescence polarization. *A*: fluorescence polarization ER-β coactivator assays show agonist binding affinity for 17β-estradiol (E_2), E_3, and 5α-androstane-3β,17β-diol (3β-Adiol) as agonists compared with antagonist activity of ICI-182780 (ICI), tamoxifen, and 4-hydroxytamoxifen (4-OH-tamoxifen). Agonists cause a positive and antagonists a negative rotational shift, with 50% of maximum change indicating affinity. EC_{50} and IC_{50} values (nM) from curve fits were as follows: 10 for E_2, 17 for E_3, 23 for 3β-Adiol, 47 for ICI-182780, 79 for 4-OH-tamoxifen, and 97 for tamoxifen. *B*: fluorescence polarization ER-β coactivator assays plotted separately for clarity demonstrate agonist binding affinity for E_3, diarylpropionitrile (DPN), and genistein vs. antagonist activity of R,R-tetrahydrochrysene (R,R-THC). EC_{50} and IC_{50} values (nM) from curve fits were as follows: 19 for E_3, 26 for DPN, 29 for genistein, and 301 for R,R-THC. *x*-Axis, logarithm of ligand concentrations from 0.038 to 1,250 nM in *A* and from 0.15 to 1,500 nM in *B*. Values are means ± SE in millipolarization units (mP).

There were no significant differences among E_3, 3β-Adiol, and DPN in ability to significantly attenuate the late Ca^{2+} signals. DPN (1 nM) and genistein (10 nM) concentrations were based on previously reported ER-β agonist EC_{50} values (25).

In addition, 3β-Adiol not only significantly reduced the late Ca^{2+} increase but also reduced early Ca^{2+} signals (Fig. 3*C*). Because such reductions in intracellular Ca^{2+} could hypothetically occur as direct (nongenomic) actions of 3β-Adiol on VMC, we also tested short-term incubations (5–60 min) with 3β-Adiol. There was no significant reduction in VMC Ca^{2+} signals with any of the short-term (5–60 min) treatments with 3β-Adiol (data not shown).

R,R-THC blocked E_3, 3β-Adiol, and DPN reduction of late Ca^{2+} signals. Pretreatment with a selective ER-β antagonist, R,R-THC (30 μM), significantly blocked the effects of E_3, 3β-Adiol, and DPN in reducing the persistent elevations in Ca^{2+} (Fig. 3*D*). Incubation of VMC with 30 μM R,R-THC alone for 1–6 h had no significant effect on late Ca^{2+} signals. The R,R-THC concentration was chosen on the basis of the molar ratios for selective ER-β blockage by R,R-THC in transactivation assays (24, 41).

ER-β and TPR are expressed in RM coronary arteries and VMC. Double-labeling immunocytochemistry studies employing indirect immunofluorescence showed ER-β and TPR coexpression in primate coronary arteries and aorta (Fig. 4*A*). TPR expression in aorta from E_3-treated RM was dramatically attenuated compared with control RM (Fig. 4*B*). In addition, ER-β and TPR were coexpressed in immunocytochemistry studies of coronary VMC, with a tendency for suppression of TPR in the E_3- or 3β-Adiol-treated groups (Fig. 4*C*). In primary coronary VMC lysates, ER-β was detected as a 52-kDa protein band with use of mouse monoclonal ER-β antibody (Fig. 5*A*).

E_3 and 3β-Adiol attenuated TPR expression in VMC, which is blocked by R,R-THC. Although immunocytochemistry results only qualitatively implied decreased TPR expression by 3β-Adiol and E_3 (Fig. 4*C*), Western blotting demonstrated the quantitative reduction of TPR by 3β-Adiol or E_3 (Fig. 5, *B* and *C*). Studies of the effects of the other ER-β ligands, with or without R,R-THC, on TPR expression showed that in vitro treatment with DPN or genistein also significantly decreased TPR. There were no significant differences among the effects of ER-β agonists in attenuating TPR. Although R,R-THC alone did not significantly change TPR expression (data not shown), R,R-THC pretreatment significantly blocked the reduction of TPR by E_3 or 3β-Adiol (Fig. 5, *B* and *C*). Similarly, R,R-THC significantly blocked the reduction of TPR by genistein or DPN (Fig. 5, *B* and *C*).

DISCUSSION

This study is the first demonstration of a coronary protective effect of low-dose transdermal E_3 in a surgically menopausal primate model, as evidenced by the reduction of in vivo CH. Reduction of CH by E_3 treatment was, in fact, equivalent to reduction of CH in previous studies in which primates were treated for 2–4 wk with E_2 subdermal implants with plasma E_2 levels of 60 pg/ml (26, 27, 31). Although GC-MS demonstrated that serum estrogen metabolites in the E_3-treated RM remained at Ovx levels (data not shown) and, despite a short half-life (due to a single pass through the liver) with rapid glucuronide or sulfate conjugation and subsequent rapid renal excretion, application of transdermal low-dose E_3 for 4 wk significantly reduced CH.

Whereas the literature is replete with evidence (animal and human data) supporting beneficial cardiovascular effects of E_2, few studies have examined E_3 actions on cardiovascular pathophysiology. In a spontaneously hypertensive stroke-prone rat model of moderate renal dysfunction, E_3 treatment significantly reduced cardiac lesions (10). In humans, E_3 augments the beneficial effect of pravastatin in retarding the progression

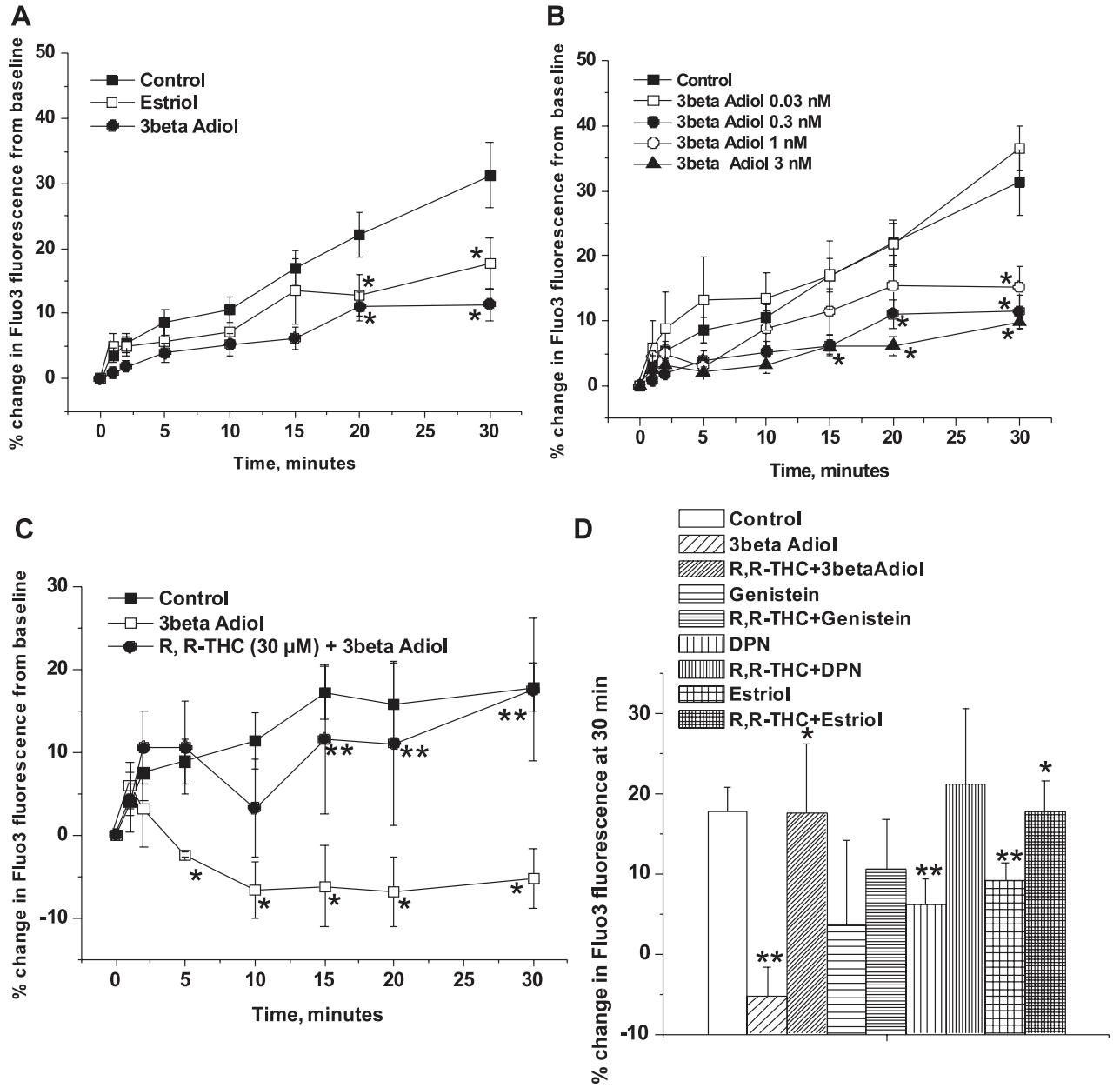

Fig. 3. Effect of ER-β agonist and antagonist on intracellular Ca^{2+} signals. *A*: effects of 72 h of in vitro treatment of RM coronary VMC with 0.3 nM 3β-Adiol ($n = 6$) and 1 nM E_3 ($n = 5$) on fluo 3 fluorescence in 30-min protocol compared with untreated control ($n = 21$). Ca^{2+} signals are shown as percent change in fluo 3 fluorescence from baseline in response to a 15-s stimulation (starting at *time 0*) with 10 μM S + 100 nM U in VMC treated with the steroid receptor modulator in vitro (for >72 h). *Significantly different from control at that time point ($P < 0.05$). *B*: time course of VMC Ca^{2+} signals after >72 h of treatment with 0.03 nM ($n = 5$), 0.3 nM ($n = 6$), 1 nM ($n = 5$), and 3 nM ($n = 5$) 3β-Adiol vs. untreated controls. *Significantly different from control at that time point ($P < 0.05$). *C*: effects of 72 h of treatment with 30 μM R,R-THC + 0.3 nM 3β-Adiol ($n = 7$) vs. 0.3 nM 3β-Adiol alone ($n = 5$) compared with control VMC ($n = 4$). *Significantly different from control ($P < 0.05$). **Significantly different from 3β-Adiol alone ($P < 0.05$). *D*: average of late (30 min) Ca^{2+} signals in control VMC and in VMC in which ER-β was blocked by 3 h of pretreatment with 30 μM R,R-THC vs. 0.3 nM 3-Adiol ($n = 7$), 10 nM genistein ($n = 6$), 1 nM DPN ($n = 5$), or 1 nM E_3 ($n = 5$) compared with VMC exposed only to 0.3 nM ($n = 10$), 1 nM E_3 ($n = 6$), 10 nM genistein ($n = 6$), or 1 nM DPN ($n = 5$). *Significantly different from 3β-Adiol and E_3 alone ($P < 0.05$). **Significantly different from control ($P < 0.05$).

of atherosclerosis in postmenopausal women (48) and improves endothelial function (14). Despite these recent data showing favorable cardiovascular effects of E_3, the exact role of E_3 in cardiovascular physiology remains to be determined. Formation of E_3 from E_2 may allow continued stimulation of ER at the vascular tissue level, thereby sustaining the biological effects of E_2. Furthermore, because it is less potent than E_2, E_3 may allow fine tuning via stimulation of ER of responses at the tissue and cellular level.

In the ER-β coactivator assay, the EC_{50} values for E_3, 3β-Adiol, and DPN were equivalent, suggesting similar ER-β agonist activity, and there were no significant differences among E_3, 3β-Adiol, and DPN in reducing the late intracellular Ca^{2+} signals. E_2 showed the highest ER-β agonist activity. E_2 has previously been shown in these protocols to be potent in reducing intracellular Ca^{2+} signals (27, 30). In vitro pharmacological data showing that E_3 and 3β-Adiol treatment reduced late Ca^{2+} signals (which can be blocked by the selective ER-β

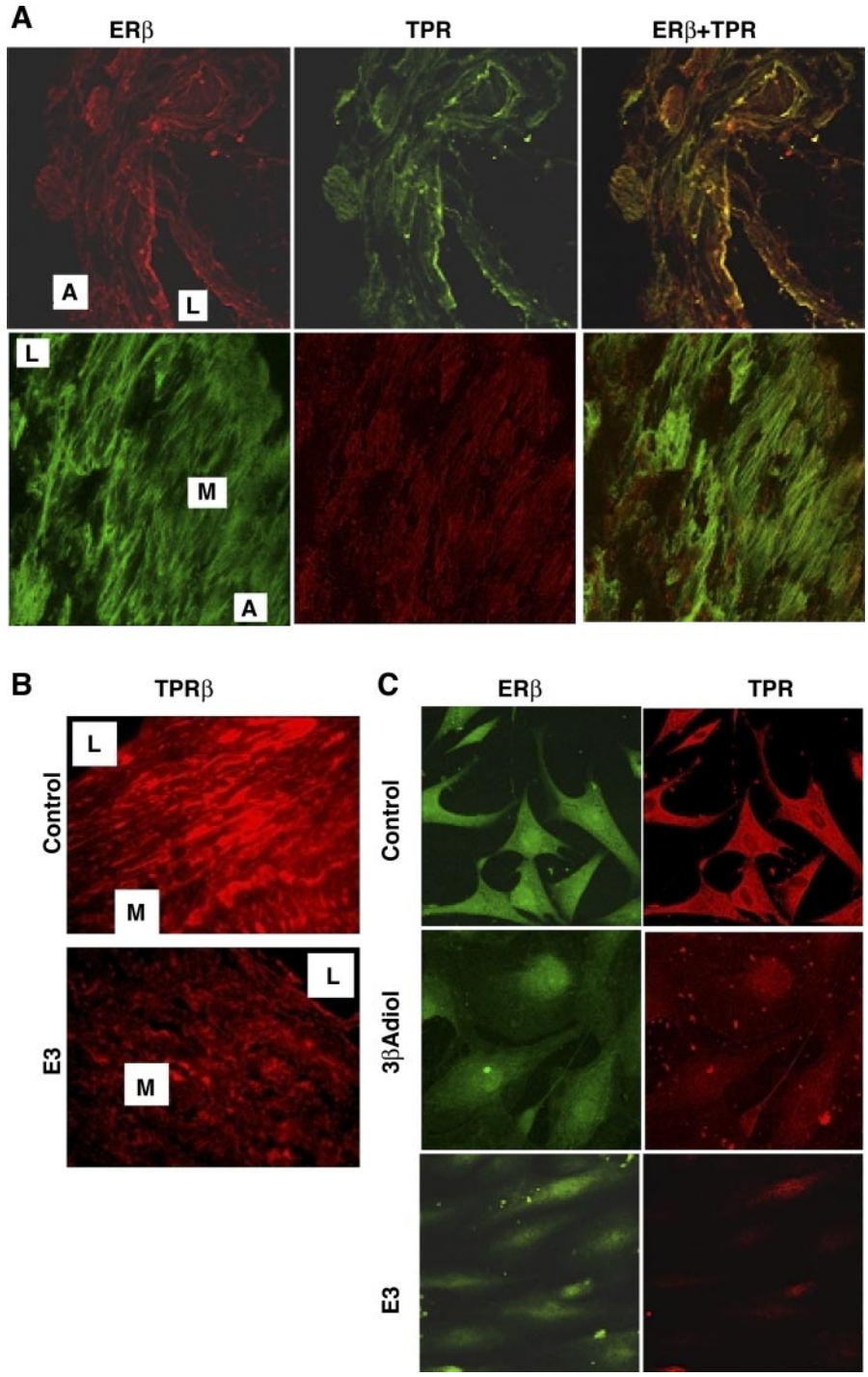

Fig. 4. ER-β agonist-induced suppression of thromboxane-prostanoid receptor (TPR) in primate arteries and coronary VMC. *A*: ER-β and TPR coexpression (double-labeling) in 5-μm cryosections of control (no steroid treatment) Ovx RM coronary arteries (*top*) and aortae (*bottom*). ER-β and TPR are expressed in media (M) and adventitia (A). ER-β and TPR are observed in endothelial cells, VMC, and pluripotent mesenchymal cells (fibroblasts). ER-β receptors were labeled with a mouse monoclonal antibody (CFK-E12) and TPR with a rabbit polyclonal antibody (PH4). Images were recorded with a ×20/0.75 NA water-immersion objective on a Zeiss Axiovert 510 Meta confocal laser scanning microscope. Data are representative of results from 3 cross sections from 5 (control) RM. L, lumen. *B*: TPR in 5-μm-thick cryosections of aorta from control (untreated) and E_3-treated RM under comparable conditions. Note suppression of TPR by E_3. TPR were labeled with a custom-made primary chicken polyclonal TPR antibody (Aves Labs) based on the peptide sequence for the ligand binding domain of thromboxane-prostanoid. Images were recorded with a C-Apochromat ×40/1.2 NA water-immersion objective on a Axiovert 200M microscope using an electron bombardment charge coupled device camera and Compix Simple PCI software. Data are representative of results from cryosections from 3 control and 3 E_3-treated monkeys. *C*: immunocytochemistry study of coronary VMC with ER-β mouse monoclonal antibody to human ER-β (CFK-E12) and TPR primary chicken polyclonal antibody (Aves Labs) under identical conditions. Double-labeled VMC primary cultures treated for 72 h with 0.3 nM 3β-Adiol and 1 nM E_3 and parallel time-matched, untreated Ovx control VMC are shown. The images were recorded with a ×40/1.4 NA oil-immersion objective on a Nikon-Bio-Rad Radiance 2,100 multiphoton system. Data are representative of results from 3 independent experiments.

antagonist R,R-THC) point to a possible contribution of ER-β to the in vitro reduction of late Ca^{2+} signals by these endogenous metabolites.

CH is hypothesized to involve TPR upregulation, which has been documented in ovarian steroid-deficient states (17, 28, 29). Because E_3 and 3β-Adiol independently attenuated the persistent Ca^{2+} signals, we reasoned that ER-β-mediated downregulation of TPR expression and signaling may contribute to the reduction in late Ca^{2+} signals. Attenuation of TPR expression in the aorta by in vivo E_3 treatment was corroborated by evidence demonstrating attenuated TPR expression in VMC after in vitro ER-β agonist treatment. Specificity shown by in vitro R,R-THC-antagonizing effects against E_3 or 3β-Adiol (which, unless they were blocked, would reduce late Ca^{2+} signals and attenuate TPR expression) suggests that ER-β may suppress TPR expression and associated downstream persistent Ca^{2+} signaling. To our knowledge, these data provide the first evidence that downregulation of TPR expression, including an obligatory contribution of ER-β that results in decreased late Ca^{2+} signals in coronary vascular muscle, may significantly alleviate or prevent CH. Suppression of TPR-mediated exaggerated, persistent VMC Ca^{2+} signals

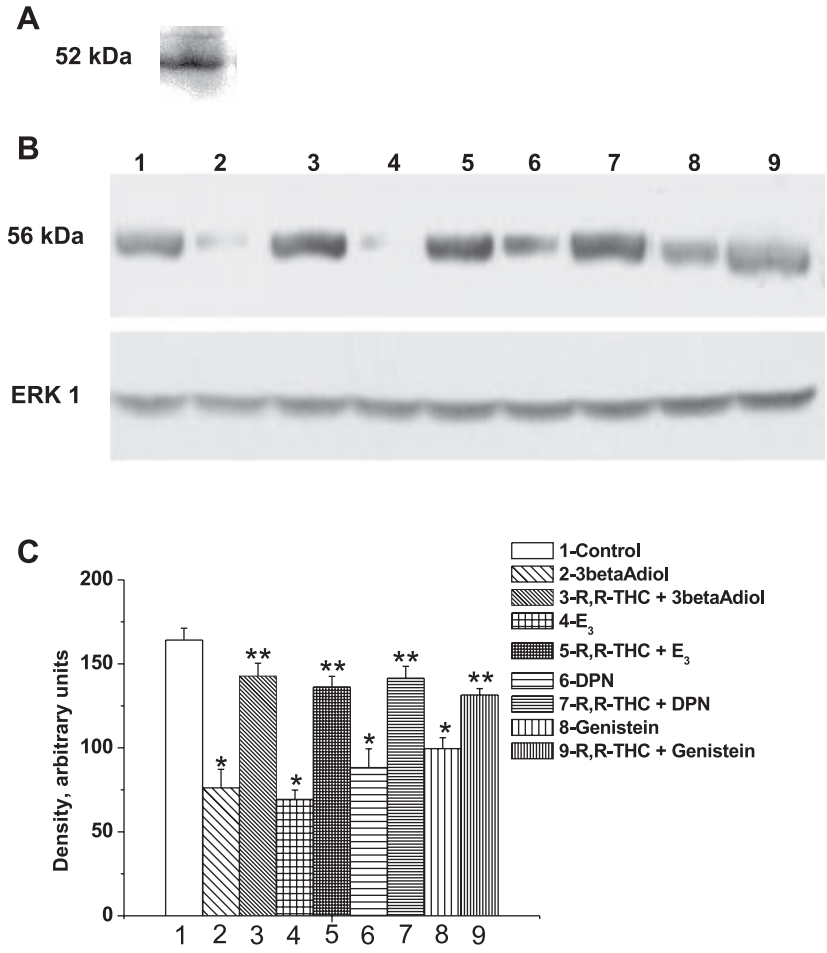

Fig. 5. Western blot of TPR in RM coronary VMC with ER-β ligands. *A*: ER-β was detected as a 52-kDa protein with use of a mouse monoclonal antibody (CFK-E12) (26) and was not different among treatments. This blot was formed from mixed extract. *B*: TPR (56-kDa band) expression with ERK1 as loading reference control. *Lane 1*, control VMC; *lane 2*, 0.3 nM 3β-Adiol; *lane 3*, 3 μM R,R-THC + 0.3 nM 3β-Adiol; *lane 4*, 1 nM E_3; *lane 5*, 3 μM R,R-THC + 1 nM E_3; *lane 6*, 1 nM DPN; *lane 7*, 3 μM R,R-THC + 1 nM DPN; *lane 8*, 10 nM genistein; *lane 9*, 3 μM R,R-THC + 10 nM genistein. *C*: quantitation of TPR in RM coronary VMC in *B* by densitometric analysis of blots using NIH Scion Image analysis software. Data are representative of results from 3 independent experiments. *Significantly different from untreated control. **Significantly different from respective agonist alone ($P < 0.05$).

would therefore appear to offer a cellular mechanism for physiological suppression of CH.

Although pharmacological data from the present study suggest a role for ER-β in reducing the late intracellular Ca^{2+} signals and in attenuating TPR expression in coronary VMC, an acknowledged limitation of this study is that we have not examined the specific contribution of ER-α to regulation of the abnormal late Ca^{2+} signals and TPR expression. Therefore, we cannot rule out a significant contribution of ER-α in suppressing TPR expression and diminishing the late Ca^{2+} signals. Nonetheless, the R,R-THC (a selective ER-β antagonist) evidence suggests that ER-β stimulation is a major contributor. Moreover, because ER-β is the dominant isoform of ER expressed in vascular muscle (18), its role in regulation of vascular reactivity warrants evaluation. Relative contributions of the roles of ER-α and ER-β in CH regulation will depend on clarification in future studies as more selective ER agonists and antagonists become available.

Increased late Ca^{2+} signals, in the presence of pharmacological ER-β blockade with R,R-THC observed in this study, appear to be an important cellular mechanistic extension of the concept of enhanced vasoconstrictor sensitivity reported in ER-β-knockout (KO) mice (50). KO studies showed that ER-α stimulation in the absence of ER-β resulted in hypertension in the ER-β-KO mice, which was hypothesized to be due to increased sensitivity to vasoconstrictors in female and male mice (50). An unexplored question is whether regulation of sensitivity to vasoconstrictors is mediated specifically by ER-α-ER-β heterodimers or, rather, as a balance of dual actions mediated by ER-α and ER-β. Because binding of ligands to a particular ER subtype and the resulting balance of ER-α and ER-β actions in a tissue are clearly species dependent (14), there is a potential pitfall in a global interpretation of primate and mouse steroid receptor data. Because of uncertainties inherent in KO approaches and steroid receptor phenomena that are uniquely primate, predictions of human ER actions should rely on primate data when differences from mouse or other nonprimate studies are reported (14).

We previously demonstrated the presence of ER-α (25) and TPR (17, 28, 29) in RM aorta and coronary arteries. Although the predominant ER expressed in human VMC has been shown to be ER-β (18) and mRNA expression for ER-α and ER-β has been previously demonstrated in primate coronary artery and aorta (37), in this study we demonstrate, for the first time, ER-β protein expression in RM aorta, coronary arteries, and coronary VMC (isolated cells). Expression of two forms of ER in the blood vessel wall requires consideration of the possibility that ER-α and ER-β separately or cooperatively, with synergistic or opposing molecular actions, regulate reactivity (and probably also proliferation) in VMC and endothelial cells. ER-β has been implicated in the VMC antiproliferative effects of E_2 during the repair response to vascular injury in both genders (20). In contrast, E_2-mediated reendothelialization (46) is promoted by ER-α (2). This dichotomy of E_2 effects be-

tween endothelium and vascular muscle would be consistent with the hypothesis that ER-α predominates in the endothelium but ER-β predominates in vascular muscle. Recent data suggest that E_2 contributes to the vascular healing process and that restenosis is prevented by promoting reendothelialization via ER-α activation (9). There may be a concomitant decrease in VMC migration and proliferation via ER-β actions that allows for repair without occlusion (9). ER-β may also mediate gender differences in ischemia-reperfusion injury, inasmuch as ER-β-KO female mice display significantly less functional recovery (and more necrosis) than wild-type female (or male) control mice; thus ER-β may have a greater cardioprotective role in females (8).

Cyclooxygenase-2 (COX-2) is the dominant source of prostaglandins, which mediate pain and inflammation, and also biosynthesis of the cardiovascular protective substance prostacyclin, a short-lived autacoid that is normally continuously synthesized in the blood vessel wall (32). The central role of COX-2 in the beneficial effects of estrogens has been recently recognized, with the implication that COX-2 inhibition might remove beneficial protection, particularly in women (6). In view of recent adverse outcomes with COX-2 inhibitors, the hypothesis that COX-2 inhibition may unbalance estrogen benefits is a concept that should be carefully examined.

The reduction of intracellular Ca^{2+} by 3β-Adiol (an androgen metabolite), which implies beneficial coronary vascular effects, is counterintuitive to reported potentially deleterious effects of androgens on CH (27). However, cardiovascular actions of androgens are conflicting and poorly understood (22, 47). The 3β-Adiol (a direct DHT metabolite) data, showing reduction of persistent Ca^{2+} signals, virtually mirror E_3 in vitro data, which correlate strongly with E_3 in vivo data to implicate reduction of CH as a salutary ER-β agonist effect on coronary artery function. We speculate that such an ER-β-mediated action of androgen metabolites, e.g., 3β-Adiol, may counterbalance potentially adverse coronary vascular actions of the major active androgen DHT, which is only one enzymatic step away. This possibility warrants further exploration.

Clinical outcomes of treatment with estrogens to achieve cardiovascular protection against dysfunction may depend on the state of blood vessels (whether diseased or relatively normal), the duration of a deficiency, and the form of drug delivery (type of estrogen, dose, and route of administration). Therefore, timing of the intervention, dose, and continuous circadian release may be important in optimizing vascular benefits and outcomes (13, 17, 38). Future research to enhance understanding of mechanisms of CH and its complex regulation by steroid receptor signal transduction pathways has the promise of leading to the discovery of better coronary protective strategies focused on transcriptional origins of vascular reactivity.

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DISCLOSURES

R. K. Hermsmeyer owns stock or options in Dimera, Inc., and has patent(s) pending.

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