Nongenomic, endothelium-independent effects of estrogen on human coronary smooth muscle are mediated by type I (neuronal) NOS and PI3-kinase-Akt signaling

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Submitted 8 December 2006; accepted in final form 6 March 2007

Han G, Ma H, Chintala R, Miyake K, Fulton DJ, Barman SA, White RE. Nongenomic, endothelium-independent effects of estrogen on human coronary smooth muscle are mediated by type I (neuronal) NOS and PI3-kinase-Akt signaling. Am J Physiol Heart Circ Physiol 293: H314–H321, 2007. First published March 9, 2007; doi:10.1152/ajpheart.01342.2006.—Sex steroids exert profound and controversial effects on cardiovascular function. For example, estrogens have been reported to either ameliorate or exacerbate coronary heart disease. Although estrogen dilates coronary arteries from a variety of species, the molecular basis for this acute, nongenomic effect is unclear. Moreover, we know very little of how estrogen affects human coronary artery smooth muscle cells (HCASMC). The purpose of this study was to elucidate nongenomic estrogen signal transduction in HCASMC. We have used tissue (arterial tension studies), cellular (single-channel patch clamp, fluorescence), and molecular (protein expression) techniques to now identify novel targets of estrogen action in HCASMC: type I (neuronal) nitric oxide synthase (nNOS) and phosphatidylinositol 3-kinase (PI3-kinase)-Akt. 17β-Estradiol (E2) increased NO-stimulated fluorescence in HCASMC, and cell-attached patch-clamp experiments revealed that stimulation of nNOS leads to increased activity of calcium-activated potassium (BKCa) channels in these cells. Furthermore, overexpression of nNOS protein in HCASMC greatly enhanced BKCa channel activity. Immunoblot studies demonstrated that E2 enhances Akt phosphorylation in HCASMC and that wortmannin, an inhibitor of PI3-kinase, attenuated E2-stimulated channel activity, NO production, Akt phosphorylation, and estrogen-stimulated coronary relaxation. These studies implicate the PI3-kinase/Akt signaling axis as an estrogen transduction component in vascular smooth muscle cells. We conclude, therefore, that estrogen opens BKCa channels in HCASMC by stimulating nNOS via a transduction sequence involving PI3-kinase and Akt. These findings provide a molecular mechanism that can explain the clinical observation that estrogen enhances coronary blood flow in patients with diseased or damaged coronary arteries.

CORONARY ARTERY DISEASE (CAD) is the most common cause of death in the Western world and accounts for one-third of all female deaths in the United States (11). Nonetheless, premenopausal women suffer far less CAD than men or postmenopausal women, giving rise to the idea that estrogens somehow protect against CAD. Although earlier studies fostered the idea that estrogen therapy promotes cardiovascular health, the Women’s Health Initiative clinical trial indicates that combination hormone replacement therapy may actually increase the risk of CAD (14). Therefore, there is much confusion concerning the physiological and/or therapeutic effects of estrogen, a hormone with the potential to become one of the most powerful cardiovascular agents available (12).

Acute effects of estrogens on vascular resistance and blood flow continue to be a controversial, if not contradictory, field of study. Clinical studies have demonstrated that acutely administered (15–60 min) estrogen induces vasodilation in both male and female patients. For example, sublingual estrogen delays the onset of exercise-induced myocardial ischemia in women (35), and intravenous estrogen attenuates abnormal coronary vasomotor responses in men (3) or postmenopausal women (33) with established coronary heart disease. In contrast to these vasodilatory studies, we recently identified (40) a molecular mechanism mediating an acute contractile effect of estrogen on coronary arteries in vitro—a finding that may potentially explain the pathological effects of postmenopausal estrogen hormone replacement therapy observed in the Women’s Health Initiative trial (36). Obviously, the cellular/molecular basis of estrogen signaling in the vasculature is very complicated, but understanding these mechanisms is an essential first step in predicting both the physiological effects and potential therapeutic use of estrogens in the cardiovascular system. Our recent molecular and functional studies (19) demonstrated that stimulation of estrogen receptor (ER)-α initiates the estrogen response cascade in human coronary artery smooth muscle cells (HCASMC), as others have described in cardiomyocytes (31); however, downstream estrogen signaling events in HCASMC are poorly understood.

Estrogen is a coronary vasodilator, and it is often assumed that estrogen-induced relaxation of coronary artery smooth muscle (CASM) is primarily indirect, i.e., mediated by endothelium-derived vasodilatory substances; however, estrogen can also induce endothelium-independent relaxation of human coronary arteries (8, 29), and we demonstrated (41) that estrogen opens K+ channels in isolated HCASMC, possibly by stimulating production of nitric oxide (NO). This direct effect of estrogen on HCASMC may be an important mechanism for helping to counteract pathological coronary vasoconstriction in arteries with a damaged or diseased intima, as evidenced by clinical studies demonstrating that acute (15–30 min) administration of estrogen to postmenopausal women with CAD.
relieves myocardial ischemia (1, 34) and increases coronary blood flow (33). At present, however, we know very little of how estrogen directly relaxes human CASM, especially in arteries with a dysfunctional endothelium. It is unknown how estrogen might stimulate vascular smooth muscle (VSM) to generate NO. We recently identified (40) the type I or neuronal nitric oxide synthase (nNOS) as a potential target of estrogen action in porcine CASM, but there is virtually no evidence that could explain how estrogen might activate nNOS within the arterial wall. To address these important gaps in our knowledge, we have investigated the effect of estrogen on HCASMC and now report evidence from molecular and functional studies indicating multiple components of a novel estrogen signal transduction cascade in human VSM. Our hypothesis is that estrogen stimulates production of NO via activation of nNOS in HCASMC by a mechanism involving the phosphatidylinositol 3-kinase (PI3-kinase)-Akt (protein kinase B) signaling system.

MATERIALS AND METHODS

Cell culture. HCASMC were purchased from Clonetics/Cambrex and were grown in phenol red-free smooth muscle growth medium with 5% FBS as described previously (41). Steroid hormones and growth factors were removed from FBS by charcoal stripping. Only short-term cultures (passages 3–5) were used in the present studies. Because VSM cells may dedifferentiate and develop a secretory phenotype with long-term culture, we used only low-passaged cells (passages 3–5). We observed immunoreactivity of the contractile protein α-actin and responsiveness to the cGMP-dependent protein kinase throughout this period, as these are indicative of protein expression and functional phenotype as exhibited by primary VSM cells.

Patch-clamp studies. For cell-attached patch studies the recording chamber contained the following solution (mM): 140 KCl, 10 MgCl₂, 0.1 CaCl₂, 10 HEPES, and 30 glucose (pH 7.4; 22–25°C). Activity of single potassium channels was recorded (with pClAMP 9, Axon Instruments) in cell-attached patches by filling the patch pipette (2–5 MΩ) with Ringer solution (mM): 110 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂ and 10 HEPES. Voltage across the patch was controlled by clamping the cell at 0 mV with the high-concentration extracellular potassium solution. Currents were filtered at 1 kHz and digitized at 10 kHz. Average channel activity (expressed as number of channels × single-channel open probability, Nₚₒ) in patches with multiple calcium-activated potassium (BKCa) channels was determined as described previously (38). Nₚₒ calculations were based on 10–15 s of continuous recording during periods of stable channel activity. Although channel activity was observed at a variety of membrane potentials, most single-channel data were analyzed at a potential of +40 mV, where BKCa channel openings are easily distinguished from other channel species to permit more accurate statistical analysis (18).

Tension studies of endothelium-denuded coronary arteries. Fresh porcine hearts were obtained from a local abattoir, and the left anterior descending coronary artery was dissected and placed into ice-cold Krebs-Henseleit buffer solution of the following composition (mM): 122 NaCl, 4.7 KCl, 15.5 NaHCO₃, 1.2 KH₂PO₄, 1.2 MgCl₂, 1.8 CaCl₂, 11.5 glucose, pH 7.2. Arteries were kept on ice during transport to the laboratory. Vessels were placed under a dissecting microscope, and excess fat and connective tissue were removed in ice-cold buffer solution. Two to four 5-mm rings were obtained from each artery and prepared for isometric contractile force recordings as described previously (38). The endothelium was removed physically by rubbing the intimal surface and tested by observing the absence of acetylcholine-induced relaxation. Rings were mounted on two triangular tissue supports, with one support fixed to a stationary glass rod and the other attached to a force-displacement transducer. Isometric contractions or relaxations were recorded on a PC computer with PowerLab software (ADInstruments). The tissue bathing solution was the modified Krebs-Henseleit buffer described above. The solution was oxygenated continuously (95% O₂-5% CO₂) and maintained at 37°C. Coronary ring preparations were equilibrated for 90 min under an optimal resting tension of 2.0 g, and fresh bath solution was added to the tissue chamber every 30 min to prevent accumulation of metabolic end products. After the initial equilibration, preparations were exposed to maximally effective concentrations of a contractile agonist, e.g., PGF₂α, to ensure stabilization of the muscles. In some experiments wortmannin (100 nM) was allowed to equilibrate with the arteries for at least 30 min before measurement of a complete estrogen concentration-response relationship (1–1,000 nM). Steroid vehicle was usually 50–75% ethanol, and this stock was diluted to a concentration of no more than 0.1% in the vessel chamber.

Western blotting. Expression of nNOS, endothelial NO (eNOS), or inducible NO (iNOS) (BD Transduction Labs) was detected with specific antibodies for the respective isoforms (1:1,000, BD Transduction Labs) as described previously (40). Protein concentrations were determined by Bio-Rad DC protein assay. ADP-Sepharose beads were used to affinity extract NO proteins from lysates (2). Purified positive control proteins (nNOS and iNOS; pituitary extracts) were purchased from Transduction Laboratories. Proteins were separated on SDS-polyacrylamide gels with a Mini Protein II (Bio-Rad) gel kit according to the manufacturer’s instructions. Proteins were then transferred to Hybond enhanced chemiluminescence (ECL) membrane (Amersham Pharmacia BioTech) with a Mini-Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) at 100 V for 1 h. Blots were blocked with 5% nonfat milk overnight at 4°C. The membrane was then rinsed with Tris-buffered saline (TBS)-Tween (TBST) three times for 15 min and two times for 5 min. Blots were then probed with primary antibodies (nNOS, eNOS, or iNOS, 1:1,000; BD Transduction Labs) in TBST containing 1% nonfat milk protein for 1 h. After washing, the membrane was then incubated with anti-rabbit IgG conjugated to horseradish peroxidase and visualized with an ECL system (Amersham). In other experiments, phosphorylation of Akt was determined by immunoblotting with a specific antibody that recognizes the phosphorylated (Ser473) form of the kinase (1:1,000; BD Pharmigen). Phospho-Akt immunoblots were stripped and immunoblotted for total Akt (1:1,000; Sigma).

NO fluorescence studies. The cell-permeant form of the NO fluorescent indicator 4,5-diaminofluorescein diacetate (DAF-2 DA; Sigma) was used to detect NO production in HCASMC. Cells were loaded with 1 μM DAF-2 DA (diluted from a 5 mM stock solution in DMSO; 45 min) and washed several times with Ringer solution. After incubation, Cells were washed with Krebs solution and placed on the stage of a Zeiss confocal laser microscope. A perfusion chamber was created by the addition of silicon grease to form in- and outflow chambers at each end of coverslips inverted onto spacers glued to a slide. Drugs were then added to the cells, and fluorescence was measured. Cell imaging was conducted on a Zeiss 510 NLO laser scanning microscope operating in the confocal mode with a ×40 0.85-numerical aperture objective. Quantitative analysis of confocal images was under the control of Zeiss PHYSIOLOGY software.

Immunofluorescence studies. HCASMC were grown in monolayers on 22 × 22 mm coverslips in 35-mm-diameter dishes (Fisherbrand). After being washed with TBS buffer (50 mM Tris·HCl pH 7.4, 150 mM NaCl) twice, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS for 10 min. Reactive groups were quenched with 0.1% sodium borohydride in TBS for 5 min and then blocked for 1 h in blocking buffer [10% horse serum, 1% bovine serum albumin (BSA), 0.02% NaN₃ in PBS]. Cells were incubated with primary antibody (Affinity BioReagents) (ER-α: 1:2,000, cat. no. PA1-309, anti-human, rabbit polyclonal to NIH₂-terminal residues 21-32 of human ER-α; ER-β: 1:2,000, cat. no. PA1-311, anti-human, rabbit polyclonal to amino acid residues 55–77.
of ER-β) diluted in 1% BSA-TBS at 4°C overnight. After washing, a 1:1,000 dilution in 1% BSA-TBS of goat anti-rabbit FITC-conjugated secondary antibody was placed on the cells for 30 min. After washing three times, the coverslips were mounted on slides. The cellular distribution of the receptors was photographed with a cooled charge-coupled device camera attached to a Zeiss microscope. Receptor expression was detected by using the Deconvolution System on a Zeiss microscope. Control immunofluorescent studies were obtained either by omitting the primary antibody or by including a neutralizing peptide (PEP-011 for ER-α; PEP-037 for ER-β).

Adenovirus infection studies. The gene encoding human nNOS (accession no. U17327) was subcloned into the adenoviral shuttle vector pAdtrack via NotI and XhoI sites. Subsequent clones were verified for integrity via DNA sequencing and protein expression. Replication-deficient adenoviruses expressing either nNOS or green fluorescence protein (GFP), under the control of the cytomegalovirus (CMV) promoter, were generated with the pAdTrack-CMV vector and AdEasy System. Viruses were amplified in HEK293 cells, purified with CsCl, titered with a cytopathic effect assay, and stored in PBS containing 5% sucrose and 2 mM MgCl. HCASMC were transduced with nNOS and control (GFP) adenoviruses at multiplicity of infection of 50 24–72 h later.

Statistical analysis. All data are expressed as means ± SE. Statistical significance between two groups was evaluated by Student’s t-test for paired data. Comparison between multiple groups was made by one-way analysis of variance test. A probability of <0.05 was considered to indicate a significant difference.

RESULTS

Estrogen opens potassium channels in HCASMC via VSM-generated NO. Molecular patch-clamp studies on HCASMC revealed that physiological concentrations of 17β-estradiol (1 nM) stimulated the activity of a prominent, large-conductance potassium channel within 15–20 min (Fig. 1A). This channel dominated membrane electrical activity, exhibited a microscopic conductance of nearly 200 pS in symmetrical potassium gradients [potassium equilibrium potential ($E_K$) = 0 mV], and was stimulated by intracellular calcium (data not shown).

Therefore, we have identified this protein as the BKCa channel, which we have previously characterized in these HCASMC (41). This channel is expressed at high density in primary human coronary and other smooth muscle cells (28). Interestingly, estrogen-stimulated BKCa channel activity was attenuated by $N^G$-monomethyl-$l$-arginine (l-NMMA, 10 μM), an inhibitor of NOS activity, indicating that NO mediates this stimulatory response to estrogen in isolated VSM cells, i.e., without endothelium (Fig. 1B). On average, 1 nM estrogen stimulated channel activity by more than an order of magnitude ($NP_o$: 0.004 ± 0.002 control, 0.035 ± 0.004 estrogen; $n = 3$, $P < 0.001$), whereas l-NMMA completely reversed the effect of estrogen. The degree of selectivity for nNOS (data not shown).

Therefore, subsequent experiments with inhibitory agents used 100 nM estrogen to provide a more rigorous test of an inhibitor’s power to attenuate the stimulatory effect of estrogen.

Neuronal (type I) NOS is a target of estrogen action in HCASMC. Western blot analysis detected expression of type I (n)NOS in lysates of HCASMC (Fig. 2A, inset). In contrast, we were unable to detect significant expression of either the endothelial (eNOS, type III) or inducible (iNOS, type II) isoforms of NOS (data not shown). These immunoblot results identified nNOS as a likely target of estrogen in HCASMC, as we reported previously for porcine coronary arteries (40), and subsequent functional studies verified a role for this specific NOS isoform in the response to estrogen. Patch-clamp recordings of single BKCa channels isolated in cell-attached patches demonstrated that pharmacological inhibition of nNOS activity with $N^G$-propyl-$l$-arginine (l-NPA), which exhibits a high degree of selectivity for nNOS (43), attenuated estrogen-induced channel activity (Fig. 2A). As expected, 100 nM 17β-estradiol significantly increased BKCa channel $NP_o$ from an average of 0.005 ± 0.002 to 0.214 ± 0.067 ($n = 8$, $P < 0.008$). Subsequent inhibition of NOS activity with 100 nM l-NPA reversed this stimulatory effect by 99% ($NP_o$ 0.001 ± 0.001; $n = 7$, $P < 0.006$). As a positive control, BKCa channel activity was restored by the exogenous NO donor 10 μM S-nitroso-N-acetylpenicillamine (SNAP) in the continued presence of l-NPA ($NP_o$ 0.146 ± 0.115; $n = 3$, $P < 0.05$; Fig. 2A),

![Fig. 1. Estrogen opens calcium-activated potassium (BKCa) channels in human coronary artery smooth muscle cells (HCASMC) via nitric oxide (NO). A: BKCa channel activity (expressed as number of channels × single-channel open probability, $NP_o$) recorded from the same cell-attached patch before (control) and after 1 nM 17β-estradiol (E2). Inhibition of nitric oxide synthase (NOS) activity with 10 μM $N^G$-monomethyl-$l$-arginine (l-NMMA) reversed the effect of E2. Channel openings (40 mV) are upward deflections from the baseline (closed) state (dashed line). B: summary of experiments demonstrating the effect of estrogen and l-NMMA on cell-attached patches. Each bar represents the average ± SE; $n = 3$. *$P < 0.001$ compared with control (untreated), $\#P < 0.001$ compared with estrogen-stimulated channel activity.](image-url)
channels in HCASMC expressing only GFP was similar to that observed in wild-type cells (NPo ≈ 0). On the other hand, overexpression of nNOS in HCASMC increased baseline BKCa channel activity >50-fold compared with control or GFP-expressing myocytes (n = 7–10, P < 0.05; Fig. 2B). In addition, estrogen stimulated BKCa channel activity to much higher levels in myocytes overexpressing nNOS compared with channel activity in either wild-type or GFP-expressing cells (NPo; wild type 0.132 ± 0.028, GFP 0.041 ± 0.14, GFP + nNOS 0.269 ± 0.074; n = 7–10, P < 0.01; Fig. 2B). These studies strongly suggest that basal activity of nNOS regulates BKCa channel activity in HCASMC, and the ability of estrogen to increase BKCa channel activity in these cells can clearly involve stimulation of nNOS activity.

Estrogen stimulates NO production via PI3-kinase-Akt signaling in HCASMC. In addition to patch-clamp analysis of single molecules, the importance of NO in mediating the response of HCASMC to estrogen was examined in intact cells. These studies yielded results that were completely consistent with our single-channel patch-clamp findings. Estrogen-stimulated NO generation was observed in HCASMC (Fig. 3A). Under control (nonstimulated) conditions we detected a small amount of fluorescence which was stable for more than an hour; however, treatment of HCASMC with 100 nM 17β-estradiol produced an acute, time-dependent increase in fluorescence, indicating stimulation of NO production in these cells. Estrogen increased fluorescence intensity by an average of 12.0 ± 2.4% (n = 4) above control levels at only 10 min, and the signal increased by 54.7 ± 3.3% over control levels after 30-min exposure to estrogen (n = 4, P < 0.001; Fig. 3). In contrast to these studies, the stimulatory effect of estrogen on NO production was greatly attenuated by first treating HCASMC with 100 nM L-NNA (15 min; Fig. 3A, bottom), an inhibitor of PI3-kinase. For example, in wortmannin-treated HCASMC 100 nM 17β-estradiol increased NO-dependent fluorescence by only an average of 10.3 ± 1.4% (n = 4–7; P < 0.001) after a full 30 min of exposure (≥80% inhibition; Fig. 3). These findings suggested that the effect of estrogen on HCASMC involves stimulation of the PI3-kinase-Akt signaling cascade.

Immunofluorescence studies indicated expression of both subtypes of ER (ER-α and ER-β) in HCASMC (Fig. 3C). These findings are consistent with our previous molecular functional studies (19) indicating that estrogen stimulation of BKCa channels in HCASMC is mediated primarily via the ER-α subtype. In addition, time course experiments characterizing the effect of both 17α- and 17β-estradiol on NO-stimulated fluorescence in HCASMC indicated that only 17β-estradiol increased NO production in these cells. For example, 5 min of exposure to 100 nM 17α-estradiol did not affect cellular fluorescence significantly (mean change of only 0.46 ± 0.31%, n = 3; Fig. 3D). In contrast, 100 nM 17β-estradiol increased fluorescence by 4.55 ± 0.15% during this same time period (P = 0.001; Fig. 3D). Furthermore, the magnitude of 17β-estradiol-stimulated NO production increased as exposure time was lengthened (Fig. 3D); however, even after a full 30-min exposure to 17α-estradiol there was no significant change in cellular fluorescence. These experiments with 17α-estradiol (a biologically less active steroid) controlled for possible nonspecific effects (e.g., changes in membrane fluidity) and/or vehicle and demonstrated the lack of spontaneous “run-up” of cellular...
fluorescence during the course of an experiment. Furthermore, these experiments substantiate a highly acute effect of 17β-estradiol on ER-NOS coupling in HCASMC.

Patch-clamp studies further supported a role for PI3-kinase in mediating the effect of estrogen in HCASMC. Experiments on cell-attached patches indicated, as expected, that 100 nM 17β-estradiol increased BKCa channel activity substantially (NPo from 0.002 ± 0.003 to 0.230 ± 0.065, n = 5; Fig. 4); however, subsequent addition of 50 nM wortmannin completely reversed (>99%; P < 0.004) the effect of estrogen on BKCa channel activity (NPo 0.001 ± 0.001; n = 5). To control for the possibility that wortmannin was simply blocking the channel (i.e., independent of PI3-kinase inhibition), we demonstrated that wortmannin did not inhibit BKCa channel activ-
Estrogen-induced coronary artery relaxation involves PI3-kinase-Akt signaling cascade. We demonstrated that such acute exposure to estrogen relieves myocardial ischemia (1, 34) and increases coronary blood flow (33) in patients with established CAD and/or intimal dysfunction. Therefore, the present findings provide a molecular mechanism that can at least partially explain how estrogen enhances coronary blood flow via a NO-dependent, endothelium-independent mechanism. We speculate that estrogen would activate nNOS in normal or diseased arteries but that the importance of such VSM-derived NO would be greatly enhanced when the endothelium is dysfunctional (e.g., diabetes, atherosclerosis, aging).

Because NO is highly membrane permeant and cannot accumulate intracellularly, the primary means of controlling NO action is by regulating its synthesis at the level of NOS. Estrogen increases NO in the cardiovascular system, indicating stimulation of vascular NOS activity. For example, arteries from females release more NO than corresponding vessels from males (21), and NO production is enhanced in pregnancy (9), suggesting that gestational declines in systemic vascular resistance and diastolic blood pressure (despite increased cardiac output) could result from estrogen-induced, NO-mediated vasodilation. Nonetheless, our knowledge of how estrogen stimulates NO production in the vasculature is far from complete. It is clear that estrogen enhances eNOS activity via both genomic and nongenomic actions (7, 22); however, these mechanisms cannot explain the numerous reports of estrogen-induced, endothelium-independent relaxation of coronary arteries (8, 29).

**DISCUSSION**

The present studies now provide evidence for a novel target of estrogen action in the human cardiovascular system: type I (n)NOS expressed in HCASMC. Furthermore, we also provide direct evidence that the signal transduction mechanism that couples activation of ER to nNOS activity and coronary artery relaxation involves the PI3-kinase-Akt signaling axis. We observed effects of 17β-estradiol within 5–15 min, suggesting an acute, nongenomic action. Clinical studies have demonstrated that such acute exposure to estrogen relieves myocardial ischemia (1, 34) and increases coronary blood flow (33) in patients with established CAD and/or intimal dysfunction.

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Type I NOS (nNOS) is expressed in ovine uterine (37), bovine carotid (5), rat carotid (4), and mouse coronary (24) arteries and has also been detected in cultured human aortic VSM cells (30). Our immunoblot studies detected only the nNOS isoform in HCASMC, strongly suggesting that this enzyme mediates estrogen-stimulated NO production and BKCa channel activity. In addition, a functional role of nNOS was evidenced by the fact that 1-1P, a compound that enhances 3,000-fold greater selectivity for nNOS over iNOS and 150-fold greater selectivity over eNOS (43), completely reversed the stimulatory effect of estrogen on BKCa channels. Furthermore, we demonstrated that overexpression of nNOS in HCASMC increased estrogen-stimulated and nonstimulated BKCa channel open probability substantially. In light of these parallel biochemical and functional results, we conclude that estrogen stimulates nNOS activity in the hippocampus (17), suggesting that nNOS is an important target of acute estrogen action in the brain and the cardiovascular system and that PI3-kinase-Akt signaling is an essential component that transduces the response of activated ER to nNOS to modulate cellular excitability, as we (D. J. R. Fulton) had reported previously for endothelial cells (22).

Involvement of the PI3-kinase/Akt signaling axis in the acute response of HCASMC to estrogen was indicated by multiple measures. We observed that estrogen stimulated phosphorylation (i.e., activation) of Akt and that this stimulation was inhibited by either 100 nM ICI-182780 (ER antagonist) or 50 nM wortmannin, which at concentrations <100 nM is a selective inhibitor of PI3-kinase (16). In addition, attenuation of PI3-kinase activity in HCASMC inhibited estrogen-stimulated BKCa channel activity, prevented estrogen-stimulated DAF-2 DA (NO) fluorescence, and inhibited estrogen-induced relaxation of endothelium-denuded porcine coronary arteries. These findings implicate PI3-kinase as a mediator of estrogen action in HCASMC. Therefore, it seems that estrogen signaling via the PI3-kinase-Akt pathway may be an important, even ubiquitous, transduction mechanism mediating nongenomic effects of the steroid. For example, estrogen enhances the transport, motility, and penetrating ability of sperm cells via a rapid second-messenger system coupled to the ER (13). Because sperm cells express nNOS (27) and low concentrations of sodium nitroprusside stimulate sperm motility (23), it is tempting to speculate that the same nongenomic estrogen signaling mechanism we have characterized in HCASMC also functions in sperm cells. In addition, estrogen regulates Akt activity in cardiac cells (6), which express nNOS in sarcoplasmic reticulum (42).

The question arises, however, as to whether such acute, nongenomic effects are relevant in the body, where more chronic exposure to estrogen is the norm. There is some degree of normal fluctuation in plasma estrogen levels, and, interestingly, an inverse correlation exists between the frequency of ischemic episodes and plasma estradiol levels during the menstrual cycle (25). Interestingly, CASM cells exhibit aromatase (i.e., estrogen synthase) immunoreactivity (10), as do VSM cells from other human arteries (20). Therefore, estrogen can be synthesized within the vascular wall independent of plasma levels, and thereby act on VSM cells in an autocrine/paracrine manner. Consequently, expression and regulation of aromatase activity, especially in males, could permit “acute,” localized exposure to estrogen and facilitate nongenomic actions of the steroid by a mechanism completely independent of plasma estradiol. As an example, there is evidence that ethanol promotes aromatization of androgens to estrogen, and it has been demonstrated that serum estradiol levels can increase only minutes after a single dose of ethanol (15); however, questions remain as to whether or not significant aromatization occurs with moderate alcohol ingestion (32). Therefore, the tacit assumption that only long-term, genomic effects of estrogens have a real physiological relevance may need to be reevaluated in light of potentially important acute, localized effects, and this mechanism may be especially important in terms of understanding the effects of estrogen on the human coronary circulation in both healthy and diseased states.

In summary, our findings have identified a novel signaling mechanism of estrogen action in the vasculature: estrogen-stimulation of PI3-kinase-Akt, which in turn enhances NO production via type I (n)NOS. This mechanism is clearly endothelium independent, and therefore may help explain the many reports demonstrating that estrogen relaxes endothelium-denuded arteries in vitro and promotes vasodilation in patients with damaged or dysfunctional endothelium. It is quite possible that this same mechanism underlies estrogen stimulation of NO production in the brain via nNOS, and therefore may also contribute to the reported ability of estrogen to improve cognition and lessen dementia (26).

ACKNOWLEDGMENTS

We thank Nancy Godin for expert technical assistance.

GRANTS

This study was supported by grants from the National Heart, Lung, and Blood Institute [HL-073890 (R. E. White, S. A. Barman, and D. J. R. Fulton), HL-080402 (R. E. White), HL-074279 (D. J. R. Fulton), and HL-68026 (S. A. Barman and R. E. White)] and the American Heart Association [SDG 0430226N (G. Han) and 0555149B (S. A. Barman)].

REFERENCES


