Role of Neuronal Nitric-Oxide Synthase in Estrogen-Induced Relaxation in Rat Resistance Arteries

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ABSTRACT

Estrogen has antihypertensive and vasorelaxing properties, partly via activation of endothelial nitric-oxide synthase (eNOS). Recently, neuronal nitric-oxide synthase (nNOS) has been detected in vascular cells, although the significance of this is unclear. Estrogen was found to stimulate nNOS in certain cultured cells. We hypothesized that estrogen regulates vascular tone partly via endothelium-derived nNOS. Human umbilical vein endothelial cells were used to test whether acute (5 min) stimulation with 17β-estradiol (E2) at 1 or 10 nM affected nNOS activity. Small mesenteric arteries from Sprague-Dawley rats were examined for relaxation to E2 (0.001–10 μM) in the absence or presence of selective nNOS inhibitors [N-propyl-L-arginine (L-NPA); 2 μM] or pan-NOS inhibitors [Nω-nitro-L-arginine methyl ester (L-NAME); 100 μM] using a wire myograph. Immunostaining was used to visualize nNOS in rat mesenteric artery cross-sections. Western blotting measured total and phospho-nNOS in endothelial cell lysates and thoracic aorta homogenates. E2 rapidly increased (p < 0.001) activating phosphorylation of nNOS and nitric oxide (NO) production (as measured by 4-amino-5-methylamino-2,7-difluorofluorescein fluorescence) in endothelial cells. Likewise, E2 caused dose-dependent relaxation of arteries from female rats, which was blunted by both L-NPA and L-NAME (p < 0.001). In contrast, E2 response was modest in male animals and unaffected by NOS inhibition. It is noteworthy that there was a greater baseline presence of phospho-nNOS in male relative to female aortas. Although eNOS is believed to be the main source of NO in the vascular endothelium, we confirmed nNOS expression in endothelial cells. Endothelial nNOS mediated E2 relaxation in isolated arteries from female animals. Altogether, these data suggest vascular nNOS as a novel mechanism in E2 signaling.

Introduction

The female sex hormone estrogen is involved in the regulation of many organ systems in both women and men. Estrogen regulation of key cardiometabolic parameters such as blood pressure is thought to contribute to sex-related differences in cardiovascular function in health and disease (Orshal and Khalil, 2004). However, the molecular mechanisms and pathways of estrogen signaling in the vascular system are not sufficiently understood to allow for optimal clinical applications.

Estrogen, acting directly on vascular cells (i.e., endothelium and smooth muscle), has a vasorelaxant role (Qiao et al., 2008). One of the best-described mechanisms of estrogen-induced vascular relaxation is activation of endothelial nitric-oxide synthase (eNOS), the enzyme that catalyzes nitric oxide (NO) generation in the vascular endothelium.
Estrogen via its plasma membrane-associated receptors on endothelial cells acutely stimulates eNOS activity through the classic calcium/calmodulin-dependent mechanism involving phosphatidylinositol 3-kinase/Akt-mediated phosphorylation at the Ser1177 (Haynes et al., 2000; Florian et al., 2004). The resulting eNOS activity, however, is more complex, because other kinase pathways, such as mitogen-activated protein kinase and Src, can be recruited to modulate eNOS phosphorylation (Chambless and Shaull, 2002; Haynes et al., 2003). This acute local regulation of eNOS function would have an immediate effect on vascular tone.

It is noteworthy that a few years ago another nitric-oxide synthase isoform [neuronal nitric-oxide synthase (nNOS)] was found in endothelial cells in culture (Bachetti et al., 2004). Since then, other studies have identified nNOS in the vascular system in both experimental animals and humans (Seddon et al., 2008; Daneshtalab and Smeda, 2010). Although the functional significance of nNOS in vascular cells is poorly understood, a few studies suggested its role in mediating vascular relaxation in isolated arteries (Capettini et al., 2008; Han et al., 2009). Whereas nNOS and eNOS share some common characteristics (e.g., constitutive expression, calcium-dependent activation, and NO generation), they also possess unique properties and may have distinct roles in vascular function (Huang, 1999; Melikian et al., 2009; Seddon et al., 2009). Similar to eNOS, a change in phosphorylation status determines nNOS enzymatic activity (Zhou and Zhu, 2009); however, little is known about activating (at Ser1417) versus inactivating (at Ser847) regulatory phospho-sites in endothelial nNOS. Collectively, studies available to date suggest that nNOS may be involved in the regulation of vascular function.

Estradiol has been shown to activate nNOS via rapid Akt-dependent phosphorylation at the stimulatory site, Ser1417, in isolated hypothalamic neurons (Gingerich and Krukoff, 2008) and human coronary artery smooth muscle cells (Han et al., 2007) with implications still remaining to be understood. The aim of our study was to test whether the activity of nNOS in the vascular endothelium is regulated by estradiol. We hypothesized that estradiol mediates its vasorelaxing effects, in part, via endothelial nNOS activation. We further examined differences in this pathway between sexes.

Materials and Methods

Endothelial Cell Culture. Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords obtained from term deliveries at the Royal Alexandra Hospital (Edmonton, Canada). Informed consent was obtained from all women before inclusion into this study. The protocol was approved by the University of Alberta Ethics Committee, and the investigation conformed to the Declaration of Helsinki. We have previously used HUVECs as a well characterized, widely used model to study human vascular endothelium. Important for the present study, HUVECs constitutively express both eNOS and nNOS, as well as classic estrogen receptors, ERα and ERβ (however, GPR30, another ER more recently shown in vascular cells, was below the level of detection in our HUVEC model). HUVEC isolation and culture were as described previously (Chakrabarti et al., 2010). In brief, the umbilical vein was flushed with phosphate-buffered saline to remove blood clots, followed by HUVEC isolation using a type 1 collagenase-containing buffer. The cells were grown in a humidified atmosphere at 37°C with 5% CO2/95% air in M199 medium with phenol red supplemented by 20% fetal bovine serum as well as L-glutamine (Invitrogen, Carlsbad, CA), penicillin-streptomycin (Invitrogen), and 1% endothelial cell growth supplement. All experiments were conducted on second-passage HUVECs. On the day of experiment, confluent monolayers of second-passage HUVECs were quiesced in a Q medium (phenol-free M199 medium with 1% fetal bovine serum) for 4 h before treatments. For examining the acute effects of estrogen on nNOS activity in the endothelium, we stimulated cells with E2 at 1 and 10 nM for 5 min. This is a physiological concentration range for endogenous estrogens that has been shown before to induce eNOS activity within the chosen time frame, 5 min (Chen et al., 1999). To determine whether the acute effects of E2 on nNOS activity are mediated through the ERs, the cells were pretreated with the classic ER blocker 7α,17β-9-[4,4,5,5,5-pentafluoropentyl]-sulfanyl]nonyl[1,3,5,10-tetraene-3,17-diol (ICI 182780) (10 µM) for 1 h before 5-min stimulation with E2 at 10 and 100 nM. At the end of the specified treatment period, the HUVECs were lysed in boiling hot Laemmli’s buffer containing 0.2% Triton X-100 to prepare samples for Western blotting.

Western Blotting for Total and Phosphorylated nNOS. Western blotting was performed on the HUVEC lysates, which were prepared from an equal number of cells in the same volume of the lysis buffer, followed by loading equal volumes of these lysates per well. The protein bands for phospho1417-nNOS and phospho847-nNOS (rabbit polyclonal antibodies from Abcam Inc., Cambridge, MA; 1 µg/ml) were detected by a Fluor-S-Max multi-imager and quantified by densitometry using Quantity One software (Bio-Rad Laboratories, Hercules, CA). Data were normalized by reprobing the phospho-nNOS membranes with an antibody against total nNOS (mouse monoclonal antibody from BD Biosciences, San Jose, CA; 1 µg/ml). Samples generated from a particular umbilical cord were run on the same gel. Cell lysates from untreated cells were loaded on every gel, and all data were expressed as fold change over the corresponding untreated control.

Nitric-Oxide Detection. Endothelial NO generation was determined by DAF-FM staining (Parker et al., 2003). Confluent HUVEC monolayers were washed once and incubated with 10 µM DAF-FM in Q medium at room temperature for 30 min. To examine the effect of E2 on NO production, HUVECs were pretreated with 10 nM E2 for 30 min before the addition of DAF-FM. To assess the contribution of nNOS in E2-stimulated NO output, HUVECs were pretreated with N-propyl-L-arginine (L-NPA), the selective nNOS inhibitor (2 µM) for 30 min before E2. After incubation with DAF-FM, cells were washed once and examined under a fluorescent microscope. Cell nuclei were visualized with Hoechst 33342 nuclear dye (Invitrogen). For each data point, images from three randomly chosen fields were taken. The total fluorescence intensity and the number of cells in each field were found, and the mean fluorescence intensity per cell was calculated. NO generation was determined as fold increase in mean fluorescence intensity per cell over the untreated control (no E2 or nNOS inhibitor).

Animals. Sprague-Dawley rats (3–4 months old) of both sexes were purchased from Charles River Breeding Laboratories (Quebec, Canada) and housed in the animal facilities of the University of Alberta. The study was approved by the University of Alberta Animal Welfare Committee and followed the Canadian Council on Animal Care guidelines and the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996).

Preparation of Isolated Vessels. At the time of experimentation, rats were euthanized by exsanguination under isoflurane anesthesia. The mesentery was rapidly excised and placed in ice-cold HEPES-buffered physiological saline solution containing 142 mM sodium chloride, 4.7 mM potassium chloride, 1.17 mM magnesium sulfate, 1.56 mM calcium chloride, 1.18 mM potassium phosphate, 10 mM HEPES, and 5.5 mM glucose, pH 7.5. Mesenteric arteries with
average internal diameters of 200 μm were carefully dissected out from surrounding tissue and cut into 2-mm-long segments.

**nNOS Immunofluorescence on Vascular Sections.** Mesenteric artery specimens, embedded in tissue-Tek O.C.T Compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands), were frozen in liquid nitrogen and stored at −80°C. Sections 10 μm thick were placed on glass slides, dried overnight, and stored at −80°C until experiment. Rethawed vessel sections were fixed in acetone, incubated with blocking buffer (1% bovine serum albumin in phosphate-buffered saline), and immunostained for 2 h at room temperature with the anti-nNOS mouse monoclonal antibodies (BD Biosciences) in 1:250 dilution. Endothelial layer was visualized by coimmunostaining for von Willebrand factor (vWF) (rabbit polyclonal anti-vWF antibody from Millipore Bioscience Research Reagents (Temecula, CA); 1:400), an endothelial cell-specific marker. Incubation with the secondary antibody [Alexa Fluor 546 (green) or 488 (red) from Invitrogen] was done for 30 min in the dark. Glass coverslips were applied over the vessel sections with a VECTASHIELD H-1200 Mounting Kit (which also includes DAPI, a nuclear stain) (Vector Laboratories, Burlington, ON, Canada), and the slides were examined under an Olympus (Tokyo, Japan) IX51 fluorescence microscope. Images were obtained using SlideBook imaging software (Olympus) and presented at 100× magnification. A control image, where no primary antibody but the secondary antibody alone was applied, served to detect any nonspecific binding. The background autofluorescence was then subtracted from all of the images, leaving the control images completely black, so only the true fluorescence from anti-nNOS or anti-vWF binding was visible.

**Ex Vivo Vessel Function Assesment.** Arterial rings were mounted in an isometric wire myograph system (Danish Myotechniques, Aarhus, Denmark) with two 40-μm wires threaded through the lumen. Vessels were bathed in 5 ml of physiological saline solution, gassed with 5% CO2/95% air, and maintained at a temperature of 37°C. Normalization of arteries to an optimal resting tension (set to 0.8 of IC₅₀, i.e., internal circumference equivalent to a transmural pressure of 100 mm Hg) was conducted using LabChart7 software (ADInstruments Ltd., Chalgrove, Oxfordshire, UK).

After a 30-min equilibration period, vessels were exposed twice to a 10 μM dose of noradrenaline (NA) followed by a single 3 μM dose of methacholine (MCh) to test for smooth muscle and endothelial integrity, respectively. A cumulative concentration-response curve (CCRC) to NA was then performed to determine the EC₅₀ of the maximal response to the agonist. To investigate vascular response to E2 (0.001–10 μM) or MCh (0.003–3 μM), the CCRC was conducted after preconstriction with the EC₅₀ of NA. To assess the contribution of nNOS to E2- or MCh-induced vasorelaxation, the selective (l-NPA, 2 μM) or nonselective (l-NAME; 100 μM) NOS inhibitor was applied to vessel bath for 30 min before preconstriction. To determine the cell-specific source of nNOS, vascular responses were also tested in endothelium-denuded arteries (achieved by threading human hair through the vessel lumen and confirmed by the absence of MCh-stimulated vasorelaxation).

**Vascular Protein Extraction.** Frozen (−80°C) thoracic aortas from male and female rats were thawed on ice and homogenized in 1 mM Tris-HCl buffer, pH 6.8 containing 100 mM KCl, 0.5 mM ZnCl₂, 10 mM EDTA, and 1% (v/v) protease inhibitor cocktail (Sigma, St. Louis, MO). The homogenate was centrifuged at 10,000 rpm for 10 min at 4°C. Protein concentration in the supernatants was determined by the bicinchoninic acid method using bovine serum albumin as a standard. Samples were stored at −80°C until further measurement of NOS proteins by Western blotting.

**Western Blotting for NOS and ER Proteins in Vascular Tissue.** To examine sex-related differences in vascular protein expression, Western blotting was performed on homogenized thoracic aortas isolated from male and female rats. Bands for eNOS and nNOS (mouse monoclonal antibodies from BD Biosciences; 1/250) were normalized to β-actin (rabbit polyclonal antibody from Abcam Inc.; 1/2000) and expressed as fold change male over female samples run on the same gel. Phospho1417-nNOS and phospho847-nNOS (rabbit polyclonal antibodies from Abcam Inc.; 1/400) were normalized to total nNOS. Bands obtained with anti-ERα and anti-ERβ (rabbit polyclonal antibodies from Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1/200) were normalized to β-actin.

**Statistical Analysis.** Data are shown as mean ± S.E.M. One-way analysis of variance (ANOVA) followed by Bonferroni’s test was used for comparisons in cell culture experiments. Two-way repeated-measures ANOVA (RMANOVA) with Bonferroni’s post test was used to compare vascular responses to E2. MCh curves were fitted using nonlinear regression, and EC₅₀ values were compared with one-way ANOVA followed by Bonferroni’s test. NOS and ER expression in the male versus female rat aortas was compared with unpaired t test. A p value < 0.05 was accepted as statistically significant.

**Results**

**Estradiol Acutely Activates nNOS and NO Production in HUVECs.** We found that stimulation of human endothelial cells with physiological doses of E2 (1 or 10 nM) for 5 min increased nNOS activity. This was evidenced by greater phospho1417-nNOS presence, an activated form of nNOS, in E2-treated cells compared with untreated control HUVECs (Fig. 1A). There was no change in inhibitory phospho847-nNOS expression in the treated relative to untreated cells (Fig. 1B). To note, antiphosphoSer1417 and antiphosphoSer847 detect multiple bands on the immunoblot, commonly a triple band for phospho1417-nNOS and a double band for phospho847-nNOS, the whole area of which is quantified accordingly. E2-induced increase in phospho1417-nNOS was prevented in the cells pretreated with the ER blocker ICI 182780 (Fig. 1C). Corresponding with the higher NOS activity, there was an increased NO generation in E2-stimulated HUVECs. This was reflected by a greater intensity of DAF-FM fluorescence (1.4-fold; p < 0.01) in the cells treated with E2 (10 nM) compared with control HUVECs (Fig. 1D). Moreover, E2-stimulated NO production was significantly attenuated in the cells preincubated with the nNOS inhibitor l-NPA.

**nNOS Expression in the Rat Mesenteric Artery.** To confirm the presence of nNOS in whole vessels, we performed immunofluorescence using anti-nNOS antibody on cross-sections of mesenteric arteries from adult female rats. Strong nNOS-specific binding was detected in the endothelium (where it colocalized with anti-vWF) and adventitia, with weaker staining in the media (Fig. 2).

**nNOS Plays a Role in Acute E2-Induced Relaxation in Rat Mesenteric Arteries.** In adult female rats, E2 elicited dose-dependent vascular relaxation of mesenteric arteries (26.2 ± 3.7% at maximum dose), which was significantly (p < 0.001) attenuated by pan-NOS inhibition with l-NAMe and also specific nNOS inhibition with l-NPA (Fig. 3A). In the endothelium-denuded arteries, E2 also elicited a relaxation response (19.8 ± 2.3% at maximum dose), where the nNOS-dependent component was no longer observed (Fig. 3B). This suggests that the predominantly endothelial source of nNOS contributes to E2 relaxation within the chosen concentration range.

**Vascular Response to E2 in Male Rats Is NOS-Independent.** Given that nNOS activity in the vascular system is regulated by E2, we tested whether there were sex-specific differences in this pathway. We observed reduced
vasorelaxation to exogenous E2 in the arteries isolated from male rats (6.4 ± 2.9% at maximum dose) compared with female rats (26.2 ± 3.7% at maximum dose) (Fig. 4A). Neither L-NAME nor L-NPA had any effect on vascular response to E2 in males, suggesting that it is not NOS-dependent (Fig. 4B).

Sex-Specific Differences in Vascular Protein Expression. There was no statistical difference in the total expression of either ERα or ERβ between female and male thoracic aortas (data not shown). Total eNOS or nNOS content in the thoracic aortas was also not significantly different between sexes (Fig. 5, A and B). However, phosphorylation of nNOS is an important determinant of enzyme activity. It is noteworthy that there was a greater baseline expression of both stimulatory (Ser1417) (1.9 ± 0.2-fold) and inhibitory (Ser847) (1.7 ± 0.3-fold) phosphorylated forms of nNOS in male compared with female vessels (Fig. 5, C and D). Unlike human endothelial phosho-nNOS, phosho-nNOS from the rat aortas demonstrated increased gel electrophoretic mobility. The latter depends on not only size, but also charge of proteins. Thus, although according to its amino acid composition the true molecular weight of phospho-nNOS is higher, it reproducibly runs at a smaller apparent molecular weight. Such a discrepancy between actual and apparent molecular weights is not uncommon in Western blotting applications.

nNOS, Unlike eNOS, Does Not Contribute to MCh-Induced Relaxation. An endothelium-dependent agonist, MCh, leads to vascular smooth muscle relaxation via the rapid release of endothelial vasoactive substances, including NO. We tested whether this MCh-stimulated NO is partially derived from nNOS. As illustrated in Fig. 6A, MCh sensitivity was significantly reduced in the presence of the general NOS inhibitor L-NAME (MCh EC50 = 0.28 ± 0.09 µM versus 0.04 ± 0.01 µM with and without L-NAME), but not the specific nNOS inhibitor L-NPA (MCh EC50 = 0.04 ± 0.01 µM with or without L-NPA). Similar responses to MCh were observed in both female (Fig. 6A) and male (Fig. 6B) mesen-
teric vascular beds. These results support the known role for NOS (inhibitable by L-NAME), likely to be eNOS, in the vasorelaxation elicited by stimulation of muscarinic receptors on the endothelium.

**Discussion**

The key findings of the present study are the following: 1) E2 rapidly increases nNOS activity and nNOS-mediated NO production in endothelial cells; 2) endothelial-derived
nNOS contributes to E2-induced vascular relaxation in female but not male arteries; and 3) there is a greater presence of nNOS in its phosphorylated state in male compared with female aortas.

The localization of nNOS in the vascular endothelium is a recent finding with the full implications yet to be understood. We validated basal expression of nNOS in HUVECs, as well as cross-sections of rat mesenteric arteries, where nNOS staining was strongly present in the endothelium and, to some extent, in the vessel media and adventitia. Historically, nNOS (named NOS I) was the first of NOS isozymes purified in 1990 from rat and porcine cerebellum (Bredt and Snyder, 1990; Mayer et al., 1990), which today is known as a key source of NO in the nervous system. As a vital neurotransmitter system in brain development, defective NO/nNOS function has been implicated in devastating neurodegenerative processes such as dementia (Zhou and Zhu, 2009). Other studies have linked nNOS hyperactivation to neuronal damage after cerebrovascular accident, i.e., stroke (Eliasson et al., 1999; Huang, 1999), suggesting a potential for nNOS inhibitors to ameliorate ischemic brain injury (Nanri et al., 1998). It is likely that understanding the role of vascular nNOS may as well uncover a far-reaching significance in health and/or disease.

To our knowledge, the ability of estradiol to rapidly activate nNOS in the endothelium has not been reported before. We found that E2 applied to endothelial cells in physiological concentrations increased levels of activating phospho1417-nNOS within 5 min, without affecting the levels of inhibitory phospho847-nNOS. Our observation complements two reports suggesting that E2 activates nNOS in neuronal cells (Gingerich and Krukoff, 2008) and VSMCs (Han et al., 2007) via the rapid change in its phosphorylation status. Moreover, the E2-stimulated nNOS activity in HUVECs was ER-dependent and associated with an increased NO production sensitive to nNOS inhibition. Previously, E2-mediated phosphorylation and activation of eNOS in the vascular endothelium was thought to be the major signaling pathway underlying female-specific systemic vasoprotection. It is noteworthy that activating the Ser1417 phospho-site of nNOS is deemed analogous to the established phosphorylation site of Akt at Ser1177 in eNOS (Adak et al., 2001). On the other hand, the Ser847 phospho-site is located within the autoinhibitory loop of nNOS and functions to stabilize the inactive enzyme con-
formation (Rameau et al., 2004). This may resemble the constitutively phosphorylated (inhibitory) Thr495 residue of resting eNOS, which is located within the calmodulin binding domain and stabilizes the inactive enzyme. Changes in Thr495 phosphorylation are generally associated with stimuli that elevate intracellular Ca\(^{2+}\) and increase eNOS activity manyfold (e.g., bradykinin and acetylcholine). However, stimulation with estrogen does not seem to act on Thr495; rather it moderately increases NO production (2- to 4-fold over basal levels) via exclusively Ser1177 phosphorylation (Fleming and Busse, 2003).

It is important to note that many studies on the role of eNOS in vessel function have been conducted using l-NAME, which is a nonselective NOS inhibitor (in fact, there are no selective eNOS inhibitors available to date). Thus, a cautious interpretation of results is warranted because we realize that eNOS is not the only NOS isoform in vascular cells, whereas some of the l-NAME effects may well be attributed to nNOS. Indeed, highly selective nNOS inhibition with 2 \(\mu\)M l-NPA or the use of l-NAME, 100 \(\mu\)M in the vessel bath experiments, supported our hypothesis that E2-induced vascular relaxation was mediated, in large part, by nNOS. Some of the key findings were further validated by using another very selective nNOS inhibitor, N-(1-imino-3-butenyl)-L-ornithine, at 2 \(\mu\)M (data not shown). The fact that both l-NPA and l-NAME had similar inhibitory effects on E2 relaxation suggests that nNOS seems to be a predominant signaling mechanism for E2. Although the possibility for eNOS contribution is not altogether excluded, there might be important differences in the functional role of these two NOS isoforms. For example, unlike eNOS, we and others have shown that nNOS expression extends beyond the endothelium; therefore, endothelium-independent (i.e., VSMC) mechanisms can potentially contribute to vascular regulation. In our experimental protocols with endothelium-denuded arteries, the effect of nNOS inhibition was not evident until the higher doses of E2 (>10 \(\mu\)M) were reached in the vessel bath (data not shown). Although interpretation regarding the physiological role is difficult, this might be an indication of functional nNOS in VSMCs. Likewise, it has been suggested that the VSMC-derived nNOS has a role in the relaxation of isolated porcine coronary arteries (Han et al., 2009). The identified mechanism for relaxation is via E2-initiated phosphatidylinositol 3-kinase-Akt signaling, leading to rapid nNOS activation and NO/cGMP-mediated opening of calcium-activated potassium channels (BK\(_{Ca}\)) on VSMCs. Rosano et al. (1993) propose this endothelium-independent mechanism to explain the clinical observation that E2 is able to enhance coronary blood flow in diseased coronary arteries with dysfunctional endothelium.

Differences between sexes in vascular function are now well recognized at various levels: from populations and subjects, extending into fundamental molecular pathways (Knot et al., 1999). Indeed, understanding the basis for these differences probably will lead to innovations in the cardiovas-
cular medicine of near future. Relevant to our hypothesis, estrogen-eNOS signalosome constitutes one of the important vasoprotective mechanisms under normal physiological conditions. Males (who are naturally deficient in this mechanism) demonstrate reduced compensatory reserve in NO-mediated vascular function in response to vascular risk factors (Kähönen et al., 1998). Following this logic, numerous laboratory studies proved the concept that stimulation of NO-dependent mechanisms can also benefit male species treated with estrogen (Sobey et al., 2004; Chan et al., 2010). Because a nonspecific NO inhibition was conducted in the above studies, the source of E2-enhanced NO production remains unclear. In the present study, we, too, found that E2 stimulation elicited lesser relaxation in male compared with female isolated vessels. There was also no effect of O2 or nNOS inhibition in male arteries, suggesting that, unlike in females, E2 causes relaxation via other NO-dependent pathways. Because vascular expression of both classic ERs was not significantly different between females and males, potentially other factors (e.g., postreceptor events) might account for the sex differences in vasorelaxation to E2. Although nNOS expression in thoracic aorta was also not different between the sexes, we measured a significantly greater presence of phospho-nNOS in male compared with female vascular tissue. To note, phosphorylated nNOS forms display multiple bands, which is not surprising given variations in the native protein itself. Indeed, vascular nNOS appears in multiple splice variants (Wilcox et al., 1997). As a result, multiple nNOS bands in Western blots have been observed by different groups (Chaudhury et al., 2008; Jin et al., 2009) and may also reflect mixed endothelium- and VSMC-derived pools of nNOS. Speculatively, increased base-line phosphorylation of nNOS in males may indicate the pool of the enzyme that is not amenable to acute regulation/phosphorylation by estrogen, although may contribute to the basal vessel tone. This may explain the observed sex differences in E2-mediated vascular responses.

Finally, our data suggest that nNOS is not involved in the classic endothelium-dependent relaxation stimulated by MCh. In both male and female arteries, L-NAME resulted in a right shift of MCh CCRC, whereas L-NPA did not have a significant effect on MCh dose response. These results are consistent with the previously known role for eNOS (rather than nNOS) in stimulated NO-dependent vasodilation to agonists (such as acetylcholine analogs) or shear stress. Indeed, there is speculation in the literature about whether stimulated versus basal vasomotor tone might be regulated/subserved by different sources of NO, i.e., eNOS versus nNOS, respectively (Seddon et al., 2008, 2009). For example, some investigations showed a poor correlation between stimulated and basal NO-dependent vasodilation within the same vascular bed, where stimulated response tended to be lost in disease settings (termed “endothelial dysfunction”), while there was a relative preservation of the basal tonic NO generation (Deanfield et al., 2007).

In summary, we demonstrated the presence of functional nNOS in vascular cells. Stimulation of the vascular endothelium with physiological concentrations of E2 led to a rapid increase in activating phosphorylation of nNOS and nNOS-dependent NO production, a novel mechanism of estrogen action in this cell type. At the level of whole vessels ex vivo E2 elicited dose-dependent relaxation, largely via nNOS activation. It is noteworthy that this vascular mechanism was found in female but not male rat vessels and was associated with a greater presence of nNOS in a chronically phosphorylated state in males. Further research is needed to delineate the role and regulation of nNOS in vascular health and disease.

It is intriguing that now, two decades after nNOS was first described in neuronal tissue, nNOS inhibitors are being tested through different phases of clinical trials as potential therapeutic agents for neurological conditions. If better understood, vascular nNOS may also yield yet unknown possibilities in vascular pharmacology.

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Authorship Contributions

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