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

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Abstract

Estrogen has anti-hypertensive and vasorelaxing properties, partly via activation of endothelial nitric oxide synthase (eNOS). Recently, neuronal NOS (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 17beta-estradiol (E2) at 1 or 10 nmol/l affected nNOS activity. Small mesenteric arteries from Sprague Dawley rats were examined for relaxation to E2 (0.001-10 μmol/l) in the absence or presence of selective nNOS inhibitor (L-NPA, 2 μmol/l) or pan-NOS inhibitor (L-NAME, 100 μmol/l) using 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 NO production (as DAF-FM 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. Interestingly, 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 signalling.
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 signalling in the vascular system are not sufficiently understood to allow for optimal clinical applications.

Estrogen, acting directly on vascular cells (ie, 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 catalyzing nitric oxide (NO) generation in the vascular endothelium. Estrogen via its plasma membrane-associated receptors on endothelial cells acutely stimulates eNOS activity through the classical calcium/calmodulin dependent mechanism involving the PI3kinase/Akt-mediated phosphorylation at Ser-1177 (Haynes et al., 2000; Florian et al., 2004). The resulting eNOS activity however is more complex, as other kinase pathways – such as, MAPK and Src – can be recruited to modulate eNOS phosphorylation (Chambliss and Shaul, 2002; Haynes et al., 2003). This acute local regulation of eNOS function would have an immediate effect on vascular tone.

Interestingly, a few years ago, another NOS isoform – neuronal NOS (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 Ser 1417) versus inactivating (at Ser 847) regulatory phosphosites in endothelial nNOS. Collectively, studies available to date suggest that nNOS may be involved in regulation of vascular function.

Estradiol was recently shown to activate nNOS via rapid Akt-dependent phosphorylation at the stimulatory site, Ser 1417, 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 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.
Methods

Endothelial cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords obtained from term deliveries at the Royal Alexadra Hospital (Edmonton, Canada). Informed consent was obtained from all women prior to 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 HUVEC as a well characterized widely used model to study human vascular endothelium. Important for the present study, HUVEC constitutively express both eNOS and nNOS, as well as classical estrogen receptors, ERalpha and ERbeta (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 previously described (Chakrabarti et al., 2010). Briefly, the umbilical vein was flushed with PBS 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% FBS as well as L-Glutamine (Gibko/Invitrogen), Penicillin-Streptomycin (Life Technologies) and 1% ECGS. All experiments were conducted on second passage HUVEC. On the day of experiment, confluent monolayers of second passage HUVEC were quiesced in a Q-medium (phenol-free M199 medium with 1% FBS) for 4 hrs prior to treatments. For examining acute effects of estrogen on nNOS activity in the endothelium, we stimulated cells with E2 at 1 and 10 nmol/l 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 acute effects of E2 on nNOS activity are mediated through the ERs, the cells were pre-treated with the classical ER blocker.
ICI 182780 (10 μmol/l) for 1 hr prior to 5 min stimulation with E2 at 10 and 100 nmol/l. At the end of the specified treatment period, the HUVEC 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 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, 1μg/ml) were detected by a Fluor-S-Max multiimager and quantified by densitometry using Quantity One software (Bio-Rad). Data were normalized by re-probing the phospho-nNOS membranes with an antibody against total nNOS (mouse monoclonal antibody from BD Biosciences, 1μg/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 μmol/l of DAF-FM in Q medium at room temperature for 30 min. To examine the effect of E2 on NO production, HUVEC were pre-treated with 10 nmol/l E2 for 30 min prior to application of DAF-FM. To assess the contribution of nNOS in E2-stimulated NO output, HUVEC were pre-treated with L-NPA, the selective nNOS inhibitor (2 μmol/l) for 30 min prior to 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. For each data point, images from 3 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 (MFI/cell) was calculated. NO generation was determined as fold increase in MFI/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 and the United States National Institutes of Health guidelines for the Care and Use of Laboratory Animals.

**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 PSS containing in mmol/l: 142 sodium chloride, 4.7 potassium chloride, 1.17 magnesium sulphate, 1.56 calcium chloride, 1.18 potassium phosphate, 10 HEPES, and 5.5 glucose (pH 7.5). Mesenteric arteries with average internal diameter 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), were frozen in liquid nitrogen and stored at -80°C. 10 μm-thick sections were placed on glass slides,
dried overnight and stored at -80°C until experiment. Re-thawed vessel sections were fixed in acetone, incubated with blocking buffer (1% bovine serum albumin in PBS) and immunostained for 2 hr at room temperature with the anti-nNOS mouse monoclonal antibodies (BD Biosciences) in 1:250 dilution. Endothelial layer was visualized by co-immunostaining for von Willebrand’s factor (rabbit polyclonal anti-vWF antibody from Chemicon, 1:400), an endothelial cell specific marker. Incubation with the secondary antibody (Alexa Fluor 546 (green) or 488 (red) from Molecular Probes) was done for 30 min in the dark. Glass coverslips were applied over the vessel sections with Vectashield H-1200 Mounting Kit (which also includes DAPI, a nuclear stain) (Vector Laboratories) and the slides were examined under an Olympus IX81 fluorescence microscope. Images were obtained using SlideBook imaging software and presented in ×100 magnification. A control image, where no primary antibody but the secondary antibody alone were applied, served to detect any non-specific binding. The background autofluorescence was then subtracted from all the images, leaving the control images completely black, and only the true fluorescence from anti-nNOS or anti-vWF binding was visible.

**Ex vivo vessel function assessment**

Arterial rings were mounted in an isometric wire myograph system (Danish Myotechniques, Denmark) with two 40-μm wires threaded through the lumen. Vessels were bathed in 5 ml PSS, gassed with 5% CO$_2$/95% air, and maintained at a temperature of 37°C. Normalization of arteries to an optimal resting tension (set to 0.8 of IC$_{100}$, ie. internal circumference equivalent to a transmural pressure of 100 mmHg) was conducted using LabChart7 software.

After a 30-min equilibration period, vessels were exposed twice to a 10 μmol/l dose of noradrenaline (NA) followed by a single 3 μmol/l 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 EC$_{80}$ of the maximal response to the agonist. To investigate vascular response to E2 (0.001 – 10 $\mu$mol/l) or MCh (0.003 – 3 $\mu$mol/l), the CCRC was conducted following pre-constriction with the EC$_{80}$ of NA. To assess the contribution of nNOS to E2- or MCh- induced vasorelaxation, the selective (N-propyl-L-arginine, L-NPA, 2 $\mu$mol/l) or non-selective (N$\omega$-nitro-L-arginine methyl ester, L-NAME, 100 $\mu$mol/l) NOS inhibitor was applied to vessel bath for 30 min prior to 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 mol/L Tris-HCl buffer (pH 6.8) containing 100 mmol/l KCl, 0.5 mmol/l ZnCl$_2$, 10 mmol/l EDTA, and 1% v/v Protease Inhibitor Cocktail (Sigma). The homogenate was centrifuged at 10,000 rpm for 10 min at 4°C. Protein concentration in the supernatants was determined by 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 beta-
actin (rabbit polyclonal antibody from Abcam, 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, 1/400) were normalized to total nNOS. Bands obtained with anti-ERalpha and anti-ERbeta (rabbit polyclonal antibodies from Santa Cruz, 1/200) were normalized to beta-actin.

Statistical analysis
Data are shown as mean ± SEM. 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 non-linear regression, and EC<sub>50</sub> 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 nmol/l) for 5 min increased nNOS activity. This was evidenced by greater phospho1417-nNOS presence, an activated form of nNOS, in E2-treated cells compared to untreated control HUVEC (Figure 1A). There was no change in the inhibitory phospho847-nNOS expression in the treated relative to untreated cells (Figure 1B). To note, anti-phosphoSer1417 and anti-phosphoSer847 detect multiple bands on the immunoblot, commonly a triple band for p1417-nNOS and a double band for p847-nNOS, the whole area of which is quantified accordingly. E2-induced increase in phospho1417-nNOS was prevented in the cells pre-treated with the ER blocker, ICI 182780 (Figure 1C). Corresponding with the higher nNOS activity, there was an increased NO generation in E2-stimulated HUVEC. This was reflected by a greater intensity of DAF-FM fluorescence (1.4-fold, \( p < 0.01 \)) in the cells treated with E2 (10 nmol/l) compared to control HUVEC (Figure 1D). Moreover, the E2-stimulated NO production was significantly attenuated in the cell pre-incubated 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 co-localized with anti-vWF) and adventitia, with weaker staining in the media (Figure 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 max dose), which was significantly ($p<0.001$) attenuated by pan-NOS inhibition with L-NAME, and also the specific nNOS inhibition with L-NPA (Figure 3A). In the endothelium-denuded arteries, E2 also elicited a relaxation response (19.8±2.3% at max dose), where the nNOS-dependent component was no longer observed (Figure 3B). This suggests that 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 ($p<0.01$) vasorelaxation to exogenous E2 in the arteries isolated from male (6.4±2.9% at max dose) compared to female rats (26.2±3.7% at max dose) (Figure 4A). Neither L-NAME nor L-NPA had any effect on vascular response to E2 in males, suggesting that it is not NOS-dependent (Figure 4B).

**Sex-specific differences in vascular protein expression**

There was no statistical difference in the total expression of either ERalpha or ERbeta 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 (Figure 5A and B). However, phosphorylation of nNOS is an important determinant of the enzyme activity. Interestingly, 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 to female vessels (Figure 5C and D). Unlike human endothelial phospho-nNOS, phospho-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, methacholine (MCh), leads to vascular smooth muscle relaxation via rapid release of endothelial vasoactive substances, including nitric oxide. We tested whether this MCh-stimulated nitric oxide is partially derived from nNOS. As illustrated by the Figure 6A, MCh sensitivity was significantly reduced in the presence of the general NOS inhibitor, L-NAME (MCh EC₅₀=0.28±0.09 μmol/l vs 0.04±0.01 μmol/l with and without L-NAME), but not the specific nNOS inhibitor, L-NPA (MCh EC₅₀=0.04±0.01 μmol/l with or without L-NPA). Similar responses to MCh were observed in both female (Figure 6A) and male (Figure 6B) mesenteric 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 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, (3) There is a greater presence of nNOS in its phosphorylated state in male compared to 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 human endothelial cells (HUVEC), 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 following cerebrovascular accident, ie. 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 5min, without affecting the levels of inhibitory phospho847-nNOS. Our observation complements two recent reports suggesting that E2 activates nNOS in neuronal cells (Gingerich and Krukoff, 2008) and VSMC
(Han et al., 2007) via the rapid change in its phosphorylation status. Moreover, the E2-stimulated nNOS activity in HUVEC 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 signalling pathway underlying female-specific systemic vasoprotection. Of note, activating 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, Ser847 phospho-site is located within the autoinhibitory loop of nNOS and functions to stabilize the inactive enzyme conformation (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 many-fold (e.g., bradykinin and acetylcholine). However stimulation with estrogen does not appear to act on Thr495; rather it moderately increases NO production (2-4-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 were conducted using L-NAME, which is a non-selective NOS inhibitor (in fact, there are no selective eNOS inhibitors available to date). Thus, a cautious interpretation of results is warranted as 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 μmol/l L-NPA or the use of L-NAME, 100 μmol/l 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, L-VNIO at 2 μmol/l (data not shown). The fact that both L-NPA and L-NAME had similar inhibitory
effects on E2 relaxation suggests that nNOS appears to be a predominant signalling 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 (ie, 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 μmol/l) 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 VSMC. Likewise, it has been recently 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 PI3kinase-Akt signalling, leading to rapid nNOS activation and NO/cGMP-mediated opening of calcium-activated potassium channels (BKCa) on VSMC. The authors 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 (Rosano et al., 1993).

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 will likely lead to innovations in the cardiovascular 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 (Kahonen et al., 1998). Following this logic, numerous laboratory studies proved the
concept that stimulation of NO-dependent mechanisms can as well benefit to male species treated with estrogen (Sobey et al., 2004; Chan et al., 2010). Since a non-specific NOS inhibition was conducted in the above studies, the source of E2-enhanced NO production remained unclear. In the present study, we too found that E2 stimulation elicited lesser relaxation in male compared to female isolated vessels. There was also no effect of NOS or nNOS inhibition in male arteries, suggesting that unlike in females, E2 causes relaxation via other NOS-independent pathways. Since vascular expression of both classical ERs was not significantly different between females and males, potentially other factors (e.g., post-receptor 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 significantly greater presence of phospho-nNOS in male compared to 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 appear 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 baseline 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.

Lastly, our data suggest that nNOS is not involved in the classical 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 are speculations in the literature whether stimulated versus basal vasomotor tone might be regulated/subserved by different sources of NO, ie. eNOS vs nNOS, respectively (Seddon et al., 2008; Seddon et al., 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 the 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. Interestingly, 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 as well yield yet unknown possibilities in vascular pharmacology.
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Authorship Contributions

Participated in research design: Lekontseva, Chakrabarti

Conducted experiments: Lekontseva, Chakrabarti, Jiang, Cheung

Contributed new reagents or analytic tools:

Performed data analysis: Lekontseva, Chakrabarti

Wrote or contributed to the writing of the manuscript: Lekontseva, Chakrabarti, Davidge
References


Footnotes

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Part of his work has been previously presented in a form of abstracts:


Figure Legends

Figure 1. Estradiol acutely activates nNOS and increases NO production in HUVECs. Representative immunoblots from lysates of HUVECs stimulated for 5 min with 1 or 10 nmol/l of E2. The HUVEC lysates were probed with antibodies against either the stimulatory (Ser1417, figure 1A) or inhibitory (Ser847, figure 1B) phosphorylation sites of nNOS. The phospho-nNOS bands were normalized to the corresponding total nNOS bands. The results from 6-7 independent experiments were summarized as mean ± SEM. ** and *** indicate $p<0.01$ and $p<0.001$ respectively, compared to the untreated control. One-way ANOVA for the E2 treatment effect $p<0.001$. Figure 1C. HUVECs were stimulated for 5 min with varying concentrations of E2 with or without 1 hr pre-treatment with the classical ER-antagonist ICI 182780 (10 μmol/l). The cell lysates underwent western blotting with antibodies against p1417-nNOS (the activating site phosphorylation) and total nNOS. Representative images from 2 independent experiments are shown. Figure 1D. HUVECs were pre-treated with L-NPA (2 μmol/l) for 30 min prior to a 30-min stimulation with E2 (10 nmol/l). Cells were washed and treated with DAF-FM (10 μmol/l) for NO detection. Data presented as mean ± SEM of 3 independent experiments. ** and *** indicate $p<0.01$ and $p<0.001$ respectively (one-way ANOVA).

Figure 2. nNOS expression in the rat mesenteric artery. Cross-sections of mesenteric arteries from female Sprague Dawley rats were immunostained for nNOS and the endothelium-specific marker, von Willebrand’s factor (vWF). A control image, from which the background autofluorescence was estimated and then subtracted, is shown with the vascular cells nuclei visualized by DAPI stain. A set of representative images from 3 independent experiments are shown.
Figure 3. **nNOS contributes to acute E2-induced relaxation in mesenteric arteries from female rats.** Dose-dependent relaxation to E2 was reduced in the presence of L-NAME (100 μmol/l) or L-NPA (2 μmol/l) in the vessels with intact endothelium (Figure 3A). In endothelium-denuded arteries, NOS inhibition did not have an effect on vascular response to E2 (Figure 3B). Data are shown as mean ± SEM of 5-10 independent experiments. ** and *** indicate $p<0.01$ and $p<0.001$ for L-NAME treated vessel vs control. # and ### indicate $p<0.05$ and $p<0.001$ for L-NPA treated vessel vs control. Two-way RMANOVA for the effect of NOS inhibitor $p<0.001$; for the interaction NOS inhibitor/ E2 concentration $p<0.001$.

Figure 4. **Vascular response to E2 in male rats does not depend on NOS.** Dose-dependent relaxation of mesenteric arteries to E2 was less in male compared to female rats (figure 4A). In males, pre-incubation of vessels with either L-NAME (100 μmol/l) or L-NPA (2 μmol/l) for 30 min had no effect on E2 response (figure 4B). Data are shown as mean ± SEM of 6-10 independent experiments. * and *** indicate $p<0.05$ and $p<0.001$, respectively. Two-way RMANOVA for the effect of sex $p<0.01$; for the interaction sex/ E2 concentration $p<0.05$.

Figure 5. **Greater presence of nNOS phosphorylation in males.** Protein was extracted from rat thoracic aortas. Representative immunoblots are shown. The expression of total eNOS (figure 5A) and nNOS (figure 5B) was normalized to the corresponding actin bands. The two phosphorylated forms of nNOS (figures 5C and D) were normalized to the total nNOS. Male/female fold change was calculated and the results are shown as mean ± SEM of 8 samples/group. * and ** indicate $p<0.05$ and $p<0.01$, respectively ($t$-test).
Figure 6. nNOS, unlike eNOS, does not have a role in MCh-induced relaxation. Either in female (figure 5A) or male (figure 5B) rats nNOS inhibition with L-NPA (2 μmol/l) did not have an effect on MCh-dependent relaxation in mesenteric arteries. In contrast, general NOS inhibitor, L-NAME (100 μmol/l) attenuated responses to MCh. Data are shown as mean ± SEM of 6-10 independent experiments. * and ** indicate \( p<0.05 \) and \( p<0.01 \). One-way ANOVA \( p=0.01 \) for the effect of NOS inhibitors in females; \( p<0.05 \) for the effect of NOS inhibitors in males.
Figure 1

A. Activating Ser 1417 phosphorylation

B. Inactivating Ser 847 phosphorylation

C. Role of estrogen receptors

D. Nitric oxide production
Figure 2

nNOS  vWF  Merged

Secondary Ab control (+ DAPI)
A. Female: L-NAME vs L-NPA

B. Endothelium-denuded
A. Female vs Male

B. Male: L-NAME vs L-NPA
Figure 6

A. Female

![Graph showing relaxation percentage vs. log [MCh], mol/l for female participants with different treatments: Control, L-NAME (100 μmol/l), and L-NPA (2 μmol/l).](image)

B. Male

![Graph showing relaxation percentage vs. log [MCh], mol/l for male participants with different treatments: Control, L-NAME (100 μmol/l), and L-NPA (2 μmol/l).](image)