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Post-translational Regulation of Endothelial Nitric Oxide Synthase (eNOS) by Estrogens in the Rat Vagina

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Abstract

Introduction—Estrogens control vaginal blood flow during female sexual arousal mostly through nitric oxide (NO). Although vascular effects of estrogens are attributed to an increase in endothelial NO production, the mechanisms of endothelial NO synthase (eNOS) regulation by estrogens in the vagina are largely unknown.

Aims—Our hypothesis was that estrogens regulate eNOS post-translationally in the vagina, providing a mechanism to affect NO bioavailability without changes in eNOS protein expression.

Methods—We measured eNOS phosphorylation and eNOS interaction with caveolin-1 and heat shock protein 90 (HSP90) in the distal and proximal vagina of female rats at diestrus, 7 days after ovariectomy and 2 days after replacement of ovariectomized rats with estradiol-17 β (15 μ g).

Main Outcome Measures—Molecular mechanisms of eNOS regulation by estrogen in the rat vagina.

Results—We localized phospho-eNOS (Ser-1177) immunohistochemically to the endothelium lining blood vessels and vaginal sinusoids. Estrogen withdrawal decreased phosphorylation of eNOS on its positive regulatory site (Ser-1177) and increased eNOS binding to its negative regulator caveolin-1 (without affecting eNOS/HSP90 interaction), and they were both normalized by estradiol replacement. Protein expressions of phosphorylated Akt (protein kinase B) and extracellular signal-regulated protein kinase 1/2 (ERK1/2) were not affected by estrogen status, suggesting that the effect of estrogens on eNOS (Ser-1177) phosphorylation was not mediated by activated AKT or ERK1/2. eNOS phosphorylation on its negative regulatory site (Ser-114) was increased in the vagina by estrogen withdrawal and normalized by estradiol replacement, implying that the maintenance of low phosphorylation of eNOS on this site by estradiol may limit eNOS interaction with caveolin-1 and preserve the enzyme's activity. Total eNOS, inducible NOS, caveolin-1, and HSP90 protein expressions were not affected by ovariectomy or estradiol replacement in the distal or proximal vagina.

Conclusions—These results define novel estrogen signaling mechanisms in the vagina which involve eNOS phosphorylation and eNOS-caveolin-1 interaction.

Keywords

Phosphorylation; Caveolin-1; Heat Shock Protein 90

Introduction

Genital arousal, an early physiologic event in the female sexual response, is a complex neurovascular process modulated by neurotransmitters, sex steroid hormones, and growth factors. It is comprised of central and peripheral components. The peripheral component is characterized by an increase in genital blood flow coordinated with clitoral and vaginal smooth muscle relaxation, engorgement of the clitoris and vaginal wall, vaginal lubrication and lengthening [1]. These hemodynamic events are primarily mediated by the nitric oxide (NO)/cyclic guanosine monophosphate pathway [2]. Estrogens are critical in maintaining vaginal blood flow and vaginal lubrication during the sexual response [3]. In rats and rabbits, ovariectomy decreased vaginal blood flow and vaginal lubrication, which were normalized by estradiol replacement [4-6].

Vascular effects of estrogens have been attributed mostly to an increase in endothelial NO production. Endothelial NO synthase (eNOS) has been localized in the rabbit, rat, and human vagina [7-9], but the role of eNOS and the mechanisms that regulate eNOS and endothelial NO bioavailability in the female genital tract are largely unknown. Some investigators have reported that in the rat vagina, both eNOS and neuronal NO synthase (nNOS) expression declined substantially with ovariectomy and increased with estrogen replacement, suggesting that estrogens play a critical role in regulating vaginal NOS expression [8,10]. In contrast, others have reported that in the rabbit vagina, ovariectomy increased, while supraphysiological concentrations of estrogen decreased total NOS activity and eNOS and nNOS protein expression [7,11-13]. While these studies imply that regulation of NOS activity and eNOS expression may be species-specific, they cannot explain the discrepancies between changes in vaginal blood flow and eNOS protein expression in response to estrogens.

Recent studies indicated that the protein expression of eNOS may not necessarily reflect the functional state of the enzyme. Changes in endothelial NO production may arise from post-translational modification of eNOS without changes in the enzyme's protein expression [14]. Post-translational effects of estradiol on eNOS are mediated by the traditional receptors alpha and beta, or truncated splice variant of receptor alpha, which associate with plasma membrane caveolae [15-17]. The post-translational effects of estradiol on eNOS are tissue specific and involve multiple signal transduction events. In a number of endothelial cells and in the cerebral vessels, estradiol activates eNOS and induces NO release by phosphorylation of eNOS on Ser-1177 (human eNOS sequence, equivalent to bovine Ser-1179) via phosphatidylinositol 3-kinase (PI3-kinase)/AKT signaling pathway [18-22]. In pulmonary and uterine artery, and umbilical vein endothelial cells, eNOS activation by phosphorylation on Ser-1177 is achieved via mitogen-activated protein kinase (MAPK) extracellular signal-regulated protein kinase 1/2 (ERK1/2) [21,23,24]. eNOS phosphorylated on Ser-1177 is the main activated form of the enzyme responsible for NO production [22]. In addition, estradiol may post-translationally activate eNOS by increasing its binding to its positive regulator heat shock protein (HSP) 90 [25-27] and by inhibiting its binding to its negative regulator caveolin-1 [15].

While these multiple modes of post-translational regulation of eNOS by estradiol have been demonstrated mostly in vitro in non-genital endothelial cells, it remains unclear whether these mechanisms have physiologic implications in female genital structures. The ongoing controversy surrounding hormone replacement therapy with respect to stroke and cardiovascular disease [28,29], and the understudied issues of female sexual dysfunction demonstrate that not only are the effects of hormone replacement therapy in women poorly understood, but also that the molecular mechanisms of sex steroid hormone action in normal physiological processes are still unclear. A better understanding of vascular effects of estrogens is warranted.

Aims

Our hypothesis in the present study was that estrogens regulate eNOS post-translationally in the vagina, providing a mechanism to affect endothelial NO bioavailability without changes in eNOS protein content. We specifically evaluated eNOS phosphorylation at its positive (Ser-1177) and negative (Ser-114) regulatory sites, and eNOS interaction with its major regulatory proteins caveolin-1 and HSP90 in the distal and proximal rat vagina.

Materials and Methods

Antibodies

The following antibodies were used: polyclonal rabbit antibodies against phospho (P)-eNOS (Ser-1177 and Ser-114), P-AKT (Ser-473), ERK1/2 (P-ERK1/2; Thr-202 and Tyr-204), AKT, ERK1/2 (Cell Signaling Technology, Beverly, MA, USA); polyclonal rabbit anti-caveolin-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA); monoclonal mouse anti- β -actin (Sigma Chemical, St. Louis, MO, USA); monoclonal mouse antibodies against HSP90, eNOS, and polyclonal rabbit antibody against inducible NOS (iNOS; BD Transduction Laboratories, San Diego, CA, USA).

Animal Model

Female Fischer 344 rats (3-month-old) were purchased from the National Institute of Aging, Bethesda, MD. All animal procedures were conducted in accordance with the ethical standards of the Johns Hopkins University School of Medicine Guidelines for the Care and Use of Laboratory Animals. The phase of the estrous cycle was determined by wet mount smears of vaginal secretions based on the morphological characteristics of the vaginal cells [30]. Bilateral ovariectomy or sham surgery was performed in diestrus to minimize hormone-related variations. Seven days after the surgery, rats were injected s.c. with 15 mg of estradiol-17 β (Sigma, St. Louis, MO, USA) dissolved in vegetable oil, or vehicle [4]. After 48 hours, the entire vagina from vaginal opening to the cervix was removed and divided into two equal parts, upper (proximal) and lower (distal), which were immediately frozen for molecular and biochemical analysis. The proximal vagina is thought to serve as a reservoir for spermatozoa and to maintain their viability, and the distal vagina appears to play a major role in sexual function. Tissue collected from sham-operated animals in diestrus served as control. Uterine horns were removed at necropsy and weighed, and uterine horn mass was used as a bioassay for estradiol concentrations [31].

Western Blot Analysis

eNOS was partially purified from the vagina by affinity binding to 2',5'-ADP Sepharose, an nicotinamide adenine dinucleotide phosphate (NADP) structural analog immobilized on Sepharose 4B, which binds and immobilizes enzymes requiring this cofactor. Following tissue homogenization, 50 μ L of packed 2',5'-ADP-Sepharose slurry was added to 0.5–1.5 mg proteins. After 1.5 hours, the beads were washed and bound proteins were eluted in 30 μ L of SDS loading buffer at 100°C for 3 minutes, as described [32]. Partially purified NOS samples or homogenates were resolved on 4–20% Tris gels and transferred to polyvinylidene difluoride membrane. Membranes with partially purified NOS were probed with anti-P-eNOS (Ser-1177 and Ser-114) antibodies at 1:450 dilution (for P-eNOS analyses), anti-caveolin-1 antibody at 1:1,000 dilution (for caveolin-1 bound to eNOS analysis) [33,34], or anti-HSP90 antibody at 1:6,000 dilution (for HSP90 bound to eNOS analysis). After probing for P-eNOS, caveolin-1, or HSP90, these membranes were then stripped and probed with anti-eNOS antibody at 1:1,000 dilution. P-eNOS, caveolin-1, and HSP90 densities were normalized relative to those of eNOS in partially purified samples. For Western blot analysis of P-AKT (at 1:1,000 dilution), P-ERK1/2 (at 1:3,500 dilution), caveolin-1 (at 1:7,000 dilution), total eNOS (at 1:1,000 dilution),

iNOS (at 1:1,000 dilution), and HSP90 (at 1:2,000 dilution), a separate set of homogenates (20–70 µg) was used without purification and standardized to AKT (at 1:1,000 dilution), ERK1/2 (at 1:3,500 dilution), or β -actin (at 1:7,000 dilution). The ratio was determined in terms of arbitrary units and expressed relative to the ratio for vehicle-treated animals in diestrus. To verify that β -actin expression was not affected by any of the treatments, the density of a set of samples was standardized per total proteins (Ponceau S staining). Ratios did not differ from the ones obtained using β -actin for standardization (data not shown).

Immunohistochemistry of P-eNOS (Ser-1177) and eNOS

Distal and proximal vagina parts were embedded in Tissue-Tek (VWR Scientific, Bridgeport, NJ, USA). Transverse sections (6 µm) were cut and mounted on slides, fixed in 4% paraformaldehyde, permeabilized in 0.4% Triton X-100, and quenched in 3% H₂O₂ in PBS for 15 minutes. All slides were subsequently incubated in PBS containing 1% goat serum for 1 hour and then at 4°C overnight in PBS with 0.2% BSA and rabbit anti-eNOS antibody at 1:250 or anti-P-eNOS (Ser-1177) antibody at 1:200 dilutions, as described [35]. Staining was visualized by using an Elite Vectastain kit (Vector Laboratories, Burlingame, CA, USA). Controls without primary antibody were run for each set of slides. Sections were examined and photographed with an Olympus BH-2 photomicroscope.

Statistical Evaluation

Statistical analysis was performed by using oneway ANOVA, followed by Newman–Keuls multiple comparison test or by *t*-test when appropriate. The data are expressed as the mean $\pm$ standard error of the mean. A value of $P < 0.05$ was considered to be significant.

Results

Effect of Estrogens on Uterine Horn Mass

Uterine horn mass was measured as a bioassay for estrogen concentrations. Uterine horn mass was significantly ($P < 0.05$) lower in ovariectomized animals (0.18 ± 0.01 g) compared with sham-operated (0.44 ± 0.03 g) and ovariectomized animals treated with estradiol (0.31 ± 0.02 g). Uterine mass standardized per body weight produced similar results: ovariectomy: 0.0093 ± 0.0007 , which was significantly ($P < 0.05$) lower than in sham-operated (0.023 ± 0.001) and estradiol-treated ovariectomized (0.017 ± 0.0009) animals.

Localization of P-eNOS (Ser-1177) and eNOS in the Vagina

We localized P-eNOS (Ser-1177) and eNOS immunohistochemically in serial sections of rat distal and proximal vagina (Figure 1A). P-eNOS (Ser-1177) and eNOS expressions were localized to the endothelium lining blood vessels and vaginal sinusoids.

Effect of Estrogens on P-eNOS (Ser-1177) and Total eNOS Protein Expression in the Vagina

Because eNOS is a key mediator of endothelium-dependent relaxation and estrogen action in the vasculature, we assessed the amount of the active P-eNOS (Ser-1177) in the distal and proximal vagina. Ovariectomy significantly ($P < 0.05$) decreased P-eNOS (Ser-1177) levels in the distal (Figure 1B) and proximal (Figure 1C) vagina compared with those in control animals in diestrus. Estradiol replacement restored ($P < 0.05$) P-eNOS (Ser-1177) levels in the distal and proximal vagina of ovariectomized rats to levels comparable with those of control rats in diestrus. In contrast to P-eNOS (Ser-1177) expression, total eNOS expression was not affected by ovariectomy or estradiol replacement in the distal or proximal vagina (Figure 1D, E). Distal and proximal vagina did not differ in P-eNOS (Ser-1177; 6.98 ± 1.8 arbitrary units vs. 4.15 ± 1.1 arbitrary units, respectively) and eNOS (4.82 ± 1.7 arbitrary units vs. 4.62 ± 1.7 arbitrary units, respectively) expressions.

Effect of Estrogens on P-AKT and P-ERK1/2 Protein Expressions in the Vagina

We next examined whether the stimulatory effect of estradiol on eNOS phosphorylation on Ser-1177 in the vagina was mediated by P-AKT or P-ERK1/2. While the levels of P-AKT were significantly ($P < 0.05$) reduced in the distal and proximal vagina of ovariectomized rats relative to levels in control animals in diestrus, these levels remained reduced after estradiol replacement (Figure 2A, B). The levels of P-ERK1/2 in the distal and proximal vagina were not affected by ovariectomy or estradiol replacement (Figure 2C, D).

Effect of Estrogens on eNOS Bindings to Caveolin-1 and HSP90 in the Vagina

Binding of eNOS to caveolin-1 and HSP90, a negative and positive regulator of eNOS function, respectively, was evaluated in the distal and proximal vaginal samples partially purified for NOSs, thus allowing the detection of eNOS and proteins bound to eNOS using specific antibodies [33,34]. Figure 3A, B shows that the ratio of caveolin-1/eNOS was significantly ($P < 0.05$) increased in the distal and proximal vagina of ovariectomized rats compared with that of control rats in diestrus, implying increased eNOS interaction with caveolin-1, and it was normalized by estradiol replacement. These changes were unrelated to caveolin-1 expression, which was unaffected by ovariectomy or estradiol replacement (Figure 3C, D). eNOS binding to its positive regulator HSP90, as well as the protein expression of HSP90 in the distal and proximal vagina, was unaffected by ovariectomy and estradiol replacement (Figure 4).

Effect of Estrogens on P-eNOS (Ser-114) Protein Expression in the Vagina

In vitro mutagenesis studies recently demonstrated that phosphorylation of eNOS on Ser-114 increases eNOS association with caveolin-1 and inhibits eNOS activity [36]. We evaluated whether estradiol affects this phosphorylation site on eNOS in the vagina as a possible mechanism underlying its effect on eNOS–caveolin-1 interaction. Figure 5 shows that in both the distal and proximal vagina, eNOS phosphorylation on Ser-114 was dramatically ($P < 0.05$) increased by ovariectomy, and it was normalized by estradiol replacement.

Effect of Estrogens on iNOS Protein Expression

We evaluated whether estrogens affect iNOS, a classical indicator of vascular inflammation. Protein expression of iNOS was not affected by ovariectomy or estradiol replacement in the distal or proximal vagina (Figure 6).

Discussion

The present study demonstrates that estrogens post-translationally regulate eNOS in the rat vagina by its phosphorylation on a positive site Ser-1177, by limiting its phosphorylation on a negative site Ser-114, and by limiting its interaction with the negative protein regulator caveolin-1. The latter effect of estrogens appears to occur by preserving low phosphorylation of eNOS on Ser-114. These findings provide a molecular basis for improved eNOS function and vascular function in the vagina by estrogens, which conceivably increases blood flow involved in the perfusion of the vagina in response to sexual stimuli, and ultimately improves vascular homeostasis. These data also suggest a new molecular target for pharmacologic therapies as well as a nonhormonal treatment for female sexual arousal disorder, although more mechanistic studies will be required to demonstrate how the eNOS protein can be regulated or adjusted.

eNOS catalyzes the synthesis of NO, a potent vasodilator and a key mediator of vascular homeostasis. The bioavailability of endothelial NO is largely regulated by mechanisms of post-translational regulation of eNOS, such as phosphorylation, protein interaction, dimer

stabilization, and localization [14]. Our data show that estrogens maintain eNOS phosphorylation on Ser-1177, which is the main activated form of the enzyme. This effect was not due to changes in overall enzyme levels, as eNOS protein expression was unaffected by estrogens. eNOS phosphorylation on Ser-1177 apparently activates the enzyme's catalytic function by reducing the enzyme's calcium requirement and facilitating electron transfer [22].

In a number of endothelial cells, estradiol induces eNOS phosphorylation on Ser-1177 via PI3-kinase/AKT [18-22] and/or MAPK ERK1/2 [21,23,24] signaling pathways. Based on our results, however, P-AKT and P-ERK1/2 do not appear to mediate this effect of estradiol in the vagina. Ovariectomy-induced reduction in the active form of AKT, which is also an anti-apoptotic factor [37], may rather indicate reduced anti-apoptotic capability of the vagina after estrogen withdrawal. Indeed, a considerable level of apoptosis has been observed in the mouse vagina as early as 1 day after ovariectomy [38]. At present, the exact mechanism of estrogen-mediated eNOS (Ser-1177) phosphorylation in the rat vagina is unknown. In addition to activated AKT and ERK1/2, several other protein kinases can phosphorylate eNOS on this site, such as protein kinase A, AMP-activated protein kinase, protein kinase G, calcium/calmodulin-dependent protein kinase II, and protein kinase C. In addition, the phosphorylation of Ser-1177 may also be affected by the state of phosphorylation/dephosphorylation of other residues on eNOS [14].

In endothelial cells eNOS targets to caveolae in plasma membrane where it is tonically inhibited by binding to caveolin-1. Caveolin-1, the resident membrane protein of caveolae, can directly interact with eNOS and inhibit its activity by occupying its calmodulin-binding site [39,40]. In COS-7 cells transfected with estrogen receptor alpha and eNOS, activation of eNOS by estradiol involves decreased eNOS binding to caveolin-1 [15]. In the rat heart, ovariectomy increased the interaction between eNOS and caveolin-1, and decreased NOS activity, which was corrected by estradiol replacement [41]. We now show that estradiol maintains limited interaction between eNOS and caveolin-1 in the rat vagina, which conceivably provides a molecular basis for improved eNOS function. This was not due to changes in caveolin-1 levels, which were unaffected by estrogens. Furthermore, our findings suggest that a plausible mechanism by which estradiol limits eNOS interaction with caveolin-1 in the vagina involves eNOS phosphorylation site Ser-114 (human sequence, equivalent to bovine Ser-116). In cultured bovine aortic endothelial cells and in the mouse aorta mutagenesis studies recently demonstrated that eNOS phosphorylation on Ser-114 negatively modulates eNOS activity indirectly by increasing its association with caveolin-1 [36]. Apparently, estrogens, by maintaining low phosphorylation of eNOS on Ser-114, limit the interaction of eNOS with caveolin-1 and preserve the enzyme's activity in the vagina. This novel mechanism of estrogen action has not been demonstrated before in vitro or in vivo. Whether estrogens regulate eNOS–caveolin-1 interaction by similar mechanisms in other tissues and the physiological significance of this effect as it pertains to multiple disease states associated with endothelial dysfunction and alterations in hormonal milieu, await further investigation.

HSP90 is another crucial regulator of eNOS and positively influences its function. Various stimuli, including vascular endothelial growth factor (VEGF), fluid shear stress, and estradiol, enhance the interaction between eNOS and HSP90 to increase NO production [42]. The mechanism of eNOS activation by HSP90 apparently involves calmodulin-dependent disruption of eNOS binding with caveolin-1, recruitment of eNOS and AKT to adjacent regions on HSP90, reduced dephosphorylation of AKT, and increased ability of AKT to phosphorylate HSP90-bound eNOS [42]. In human umbilical vein [26] and porcine aortic [25] endothelial cells, estradiol stimulates eNOS-HSP90 association, which enhances the enzyme's activity. The effect of estrogens on eNOS interaction with HSP90 in vivo is, however, not known. We now show that estrogens do not affect eNOS/HSP90 interaction in the vagina, suggesting that this interaction does not play a critical role in eNOS regulation by estrogens in the vagina in

vivo. The discrepancy between in vitro and in vivo findings may be related to multiple factors affecting eNOS function in vivo, as well as to differences in vascular beds studied.

iNOS has been localized in the vagina [43], and increased iNOS levels were seen in the aorta [44] and coronary artery [45] of ovariectomized rats, while iNOS down-regulation by estrogen has been suggested to mediate beneficial vascular effects of estrogen [45]. However, iNOS levels in the vagina were unaffected by estrogen levels, implying that in this model, estrogens did not exhibit anti-inflammatory or anti-fibrotic effects.

There are several limitations of this study. We have only investigated molecular changes at one time point (7 days after ovariectomy with and without 2 days estrogen replacement). While we did not detect any changes in eNOS protein expression, it is possible that longer time of estrogen withdrawal/replacement is required for estrogens to exhibit transcriptional regulation of eNOS. Also, we did not confirm mediatory role of activated AKT or ERK1/2 on estradiol-induced eNOS phosphorylation on Ser-1177. Conceivably, higher doses of estradiol may be required to activate these kinases. Similarly, we recognize that coimmuno-precipitation experiments would further support eNOS-protein interaction in the vagina. However, these experiments are technically difficult in tissues (performed, to our knowledge, in just a very limited number of studies), in contrast to isolated endothelial cells. It is also recognized that blood flow is the final measure of female sexual arousal. In this study, we did not measure vaginal blood flow, and we acknowledge this limitation.

Despite these limitations, this work establishes basic molecular mechanisms of estrogen effect on eNOS in the vagina, and future studies are needed to correlate eNOS post-translational modification by estrogen with physiological measurements of blood flow in response to a sexual stimulus. Similarly, future studies will determine how these post-translational effects of estrogens affect endothelial function in the vagina, whether they are mediated by plasma membrane estrogen receptors alpha and beta, and whether estrogens affect the RhoA/Rho kinase contractile pathway in the vagina. Furthermore, in addition to its acute vasodilatory actions, eNOS may act as a coactivator of transcription when bound to estrogen receptor alpha in the nucleus [46]. Whether this also may be a mechanism by which eNOS mediates trophic responses to estrogen in the vagina remains to be determined.

Conclusion

Our study shows for the first time that estrogens post-translationally regulate eNOS in the vagina by phosphorylation of the enzyme on a positive (Ser-1177) regulatory site, by limiting its phosphorylation on a negative (Ser-114) regulatory site, and by limiting its interaction with caveolin-1. Estrogen-stimulated eNOS function would conceivably lead to vasodilatation in the vagina in response to a sexual stimulus. This work establishes the role of post-translational mechanisms for estrogen action on eNOS function in the vagina. Future investigation is required to confirm the physiologic and pathophysiologic relevance of eNOS post-translational modification in the vagina.

Statement of Authorship

Category 1

(a) Conception and Design

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Figure 1.

Immunohistochemistry of P-eNOS (Ser-1177) and eNOS, and the effect of ovariectomy and estradiol replacement on P-eNOS (Ser-1177) and eNOS protein expressions in the distal and proximal rat vagina. (A) Representative immunohistochemical localization of P-eNOS (Ser-1177) and eNOS in serial sections of distal and proximal vagina of control (D, diestrus) rats, 7-day ovariectomized (Ovx) rats, and ovariectomized rats treated 2 days with estradiol (OvxE). P-eNOS (Ser-1177) and eNOS are localized to the vascular (white arrows) and sinusoidal (black arrows) endothelium lining blood vessels and vaginal sinusoids, respectively. Magnification 400 $\times$. N = 2–3. (B–E) Western blot analysis of P-eNOS (Ser-1177; B, C) and eNOS (D, E) in the distal (left panels) and proximal (right panels) vagina of control (D), Ovx, and OvxE rats. Upper panels are representative Western blot data of P-eNOS (Ser-1177) and eNOS, and lower panels represent quantitative analysis of P-eNOS (Ser-1177) and eNOS. Phosphorylation of eNOS on Ser-1177 was decreased by ovariectomy compared with control animals in diestrus, and this was restored by estradiol replacement. eNOS protein expression was unaffected by any treatment. Values are expressed as percentage of the value in control (diestrus) group. Each bar represents the standard error of the mean. N = 6–8; *P < 0.05 vs. control-diestrus. eNOS = endothelial nitric oxide synthase; P-eNOS = phospho-eNOS.

Figure 2.

Effect of ovariectomy and estradiol replacement on P-AKT and P-ERK1/2 protein expressions in the distal and proximal rat vagina. Upper panels are representative Western blot data, and lower panels represent quantitative analysis of P-AKT (A, B) and P-ERK1/2 (C, D) in the distal (left panels) and proximal (right panels) vagina of control (D, diestrus) rats, 7-day ovariectomized (Ovx) rats, and ovariectomized rats treated 2 days with estradiol (OvxE). Ovariectomy decreased P-AKT, which was not corrected by estradiol replacement. P-ERK1/2 protein expression was unaffected by any treatment. Values are expressed as percentage of the value in control (diestrus) group. Each bar represents the standard error of the mean. N = 5–8; * $P < 0.05$ vs. control-diestrus. ERK1/2 = extracellular signal-regulated protein kinase 1/2; P-ERK1/2 = phospho-ERK1/2.

Figure 3.

Effect of ovariectomy and estradiol replacement on caveolin-1 binding to eNOS and caveolin-1 protein expression in the distal and proximal rat vagina. Upper panels are representative Western blot data, and lower panels represent quantitative analysis of the ratio of caveolin-1/eNOS (A, B), and caveolin-1 protein expression (C, D) in the distal (left panels) and proximal (right panels) vagina of control (D, diestrus) rats, 7-day ovariectomized (Ovx) rats, and ovariectomized rats treated 2 days with estradiol (OvxE). Vaginas from ovariectomized rats exhibited increased ratio of caveolin-1/eNOS, indicative of increased binding of caveolin-1 to eNOS, compared with control animals in diestrus, which was restored by estradiol replacement. Caveolin-1 protein expression was unaffected by any treatment. Values are expressed as percentage of the value in control (diestrus) group. Each bar represents the standard error of the mean. $N = 6-8$; $*P < 0.05$ vs. control-diestrus. eNOS = endothelial nitric oxide synthase.

Figure 4.

Effect of ovariectomy and estradiol replacement on HSP90 binding to eNOS and HSP90 protein expression in the distal and proximal rat vagina. Upper panels are representative Western blot data, and lower panels represent quantitative analysis of HSP90/eNOS, indicative of HSP90 binding to eNOS (A, B), and HSP90 protein expression (C, D) in the distal (left panels) and proximal (right panels) vagina of control (D, diestrus) rats, 7-day ovariectomized (Ovx) rats, and ovariectomized rats treated 2 days with estradiol (OvxE). No statistically significant differences were found among treatment groups. Values are expressed as percentage of the value in control (diestrus) group. Each bar represents the standard error of the mean. N = 4–8. HSP90 = heat shock protein 90; eNOS = endothelial nitric oxide synthase.

Figure 5.

Effect of ovariectomy and estradiol replacement on P-eNOS (Ser-114) protein expression in the distal and proximal rat vagina. Upper panels are representative Western blot data, and lower panels represent quantitative analysis of P-eNOS (Ser-114) in the distal (left panel) and proximal (right panel) vagina of control (D, diestrus) rats, 7-day ovariectomized (Ovx) rats, and ovariectomized rats treated 2 days with estradiol (OvxE). Phosphorylation of eNOS on Ser-114 was increased by ovariectomy compared with control animals in diestrus, and this was restored by estradiol replacement. Values are expressed as percentage of the value in control (diestrus) group. Each bar represents the standard error of the mean. $N = 4-5$; $*P < 0.05$ vs. control-diestrus. eNOS = endothelial nitric oxide synthase; P-eNOS = phospho-eNOS.

Figure 6.

Effect of ovariectomy and estradiol replacement on iNOS protein expression in the distal and proximal rat vagina. Upper panels are representative Western blot data, and lower panels represent quantitative analysis of iNOS protein expression in the distal (left panel) and proximal (right panel) vagina of control (D, diestrus) rats, 7-day ovariectomized (Ovx) rats, and ovariectomized rats treated 2 days with estradiol (OvxE). No statistically significant differences were found among treatment groups. Values are expressed as percentage of the value in control (diestrus) group. Each bar represents the standard error of the mean. N = 6–8. iNOS = inducible nitric oxide synthase.