

Estrogen receptor activation of PI-3 kinase, Akt and nitric oxide signaling in cerebral blood vessels: rapid and chronic effects*

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Running title: Estrogen activates PI-3K/Akt/eNOS in cerebral blood vessels

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Number of text pages: 21

Number of tables: 0

Number of figures: 8

Number of references: 44

Number of words in Abstract: 261

Number of words in Introduction: 721

Number of words in Discussion: 1884

Non-standard abbreviations: NO (nitric oxide); eNOS (endothelial nitric oxide synthase); PI-3 (phosphoinositide-3); OVX (ovariectomized animal); OE (estrogen-treated ovariectomized animal), ER (estrogen receptor)

ABSTRACT

Estrogen receptor regulation of nitric oxide (NO) production by vascular endothelium may involve rapid, membrane-initiated signaling pathways in addition to classic genomic mechanisms. Here we demonstrate using intact cerebral blood vessels that 17 β -estradiol acutely activates endothelial NO synthase (eNOS) via a phosphoinositide-3 (PI-3) kinase-dependent pathway. The effect is mediated by estrogen receptors (ER), consistent with co-localization of ER α and caveolin-1 immunoreactivity at the plasma membrane of endothelial cells lining cerebral arteries. Treatment with 17 β -estradiol (10 nM) for 30 min increased NO production, as measured by total nitrite assay, in cerebral vessels isolated from ovariectomized rats. This effect was significantly decreased by membrane cholesterol depletion with β -methyl-cyclodextrin, the ER antagonist ICI 182,780, and two inhibitors of PI-3 kinase: wortmannin and LY294002. In parallel with NO production, acute 17 β -estradiol treatment increased phosphorylation of both eNOS (p-eNOS) and Akt (p-Akt). PI-3 kinase inhibitors also blocked the latter effects; together these data are consistent with ER activation of the PI-3 kinase-p-Akt-p-eNOS pathway. ER α protein (66 and 50 kDa) co-immunoprecipitated with eNOS as well as with the p85 α regulatory subunit of PI-3 kinase, further implicating ER α in kinase activation of eNOS. Little is known regarding effects of estrogen on cellular kinase pathways *in vivo*; therefore we compared cerebral blood vessels isolated from ovariectomized rats that were either untreated or given estrogen replacement for 4 weeks. Chronic estrogen exposure increased levels of cerebrovascular p-Akt and p-eNOS, as well as basal NO production. Thus, in addition to the rapid activation of PI3 kinase, p-Akt and p-eNOS, estrogen signaling via non-transcriptional, kinase mechanisms has long-term consequences for vascular function.

Upregulation of endothelial nitric oxide (NO) production plays an important role in vasoprotective effects of estrogen (Nilsson, et al., 2001). The cerebral vasculature is a significant target tissue for this hormone, and chronic *in vivo* exposure to estrogen increases NO-mediated vasodilation in rodent cerebral arteries (Geary et al., 1998; 2001). This effect can be explained, in part, by an increase in the expression of endothelial NO synthase (eNOS) in cerebral blood vessels (Stirone, et al., 2003a; McNeill et al., 1999; 2002). Estrogen receptor α (ER α) is present in the endothelium of cerebral arteries (Stirone et al., 2003b), and studies with estrogen receptor antagonists (McNeill et al., 2002) and ER α knock out mice (Geary et al., 2001) suggest that estrogen acts on ER α to modulate levels of cerebrovascular eNOS protein. Chronic estrogen treatment also increases the level of eNOS mRNA in cerebral vessels (Stirone et al., 2003a). Together, these effects are consistent with the classic genomic mechanism of estrogen receptor action that involves transcription of target genes in the nucleus.

However, there is a growing consensus that estrogen receptors also mediate non-genomic effects by activating cell signaling pathways outside the nucleus (Haynes et al., 2002; Ho and Liao, 2002). Much of the current knowledge is based on studies using a variety of transfected and tissue-derived cells in culture, including endothelial cells (Ho and Liao, 2002; Chambliss and Shaul, 2002; Haynes et al., 2000; 2002). In particular, estrogen was found to stimulate NO production within minutes of treatment (Hisamoto et al., 2001; Haynes et al., 2000). Since multiple signal transduction pathways converge to regulate eNOS activity by phosphorylation (Shaul, 2002; Dimmeler et al., 1999; Fulton et al., 1999; Gallis et al., 1999), it was proposed that estrogen activates one or more of these kinase cascades. A number of cell culture studies suggest estrogen rapidly increases NO release by stimulating the phosphoinositide-3 (PI-3)

kinase - Akt kinase (protein kinase B) pathway (Haynes et al., 2002; Hisamoto et al., 2001; Simoncini et al., 2000; 2003). Activation of Src kinase has been suggested to play a role in mediating effects of estrogen on this pathway (Haynes et al., 2003). In contrast, in ovine uterine endothelial cells, estrogen rapidly affects NO production without stimulating Akt phosphorylation (Chen et al., 2004). Other studies with cultured endothelial cells demonstrate that estrogen activation of MAP kinase cascades, in particular ERK 1/2 (extracellular signal-regulated protein kinase) , modulates eNOS activity (Chen et al., 1999, 2004). At this point, it is not known if culture conditions or cell origins, e.g., species and/or vascular bed, contribute to discrepancies in the apparent mechanism of rapid estrogen-stimulated increases in NO production.

It is hypothesized that receptors associated with the plasma membrane are responsible for rapid signaling in response to estrogen (Ho and Liao, 2002; Chambliss, et al., 2000; Haynes et al., 2002; Levin, 2002; Li, et al., 2003). The exact nature of these receptors is still controversial, but data suggest that the traditional nuclear receptors, ER α (Chen et al., 1999; Chambliss et al., 2000) and ER β (Chambliss et al., 2002; Levin, 2002), or truncated splice variants of ER α (Figtree et al., 2003; Li et al., 2003) may associate with plasma membranes, and in particular, with endothelial caveolae (Kim et al., 1999; Chambliss et al., 2000; 2002). Recently, 17 β -estradiol was shown to activate eNOS in caveolae fractions isolated from immortalized ovine pulmonary artery endothelial cells (Chambliss et al., 2000), suggesting that all of the necessary signaling components for rapid estrogen action are associated with membrane caveolae.

The recent data on estrogen membrane receptors and rapid signal transduction are compelling; however, little is known about the presence and significance of these phenomena in

intact vascular tissue. In contrast to cells grown in culture, endothelium under normal physiological conditions is relatively quiescent and subjected to a variety of mechanical and chemical stimuli that differ among vascular beds. Thus the goal of the current study was to determine if 17β -estradiol activates rapid NO release from native blood vessels in an important vascular target, the cerebral circulation. The presence of membrane receptors and the role of kinase pathways also were addressed using intact vessels. Moreover, since vascular consequences of non-genomic mechanisms have not been explored *in vivo*, we determined the long-term impact of chronic exposure to estrogen at physiologically relevant levels on Akt and eNOS activation in rat cerebral blood vessels.

METHODS

In vivo treatments. All animal procedures were approved by the U.C. Irvine Institutional Animal Care and Use Committee. Three groups of Fischer 344 female rats (3 months old, Harlan-Sprague Dawley) were used: unoperated (INT. F), ovariectomized (OVX), or ovariectomized and treated with 17 β -estradiol (OE) as described previously (Geary, et al., 1998; Stirone, et al., 2003b). Rats were anesthetized with 46 mg/kg ketamine and 4.6 mg/kg xylazine (i.p.) for all surgical procedures. For OE animals, hormone treatment was started at the time of ovariectomy with the use of 17 β -estradiol-filled Silastic tubing (1.57 mm inner diameter x 3.18 mm outer diameter; 10 mm in length) implanted subcutaneously (dorsally at the neck). Implants were left in place for 4 weeks; animals were then anesthetized by CO₂ and killed by decapitation. We have previously demonstrated using this procedure that serum estrogen levels in OE animals are within the physiological range (Geary, et al., 1998; McNeill, et al., 2002). Brains were removed and the blood vessels isolated. Body weights were 183 \pm 1 g for OVX and 164 \pm 1 g for OE ($P \leq 0.05$). Uterine weights were 34 \pm 2 mg for OVX and 125 \pm 4 mg for OE ($P \leq 0.05$).

Cerebral vessel isolation. Blood vessels were isolated from whole brain as described previously (McNeill et al., 1999, Stirone, et al., 2003b). Briefly, four brains from each animal group were pooled, gently homogenized in phosphate buffered saline (PBS) and centrifuged (720 x g, 5 min, 4°C). The pellet was resuspended in PBS and layered over 16% dextran (MW=35-45 kDa, Sigma) followed by centrifugation at 4500 x g for 20 min at 4°C. The blood vessel pellet was resuspended in cold PBS and washed over a 50 μ m nylon mesh. This preparation contains both pial and intraparenchymal vessels, that when examined using light microscopy, are a mixture of

arteries, arterioles, capillaries, veins, and venules. Vessels were either used immediately for functional experiments or homogenized in ice-cold lysis buffer and stored at -80°C for later use.

Assay for NO production. Freshly isolated cerebral blood vessels were pre-equilibrated at 37°C in PBS with 95% O_2 /5% CO_2 for 30 min prior to initiation of the assay. Blood vessels were then incubated for 30 min at 37°C in 100 μL of PBS containing 10 nM 17β -estradiol encapsulated in 2-hydroxy-propyl- β -cyclodextrin; Sigma) or an equivalent concentration of 2-hydroxy-propyl- β -cyclodextrin alone (Sigma; vehicle control). In some cases, vessels were pre-treated with a much higher concentration of β -methyl-cyclodextrin (5 mM; 30 min; Sigma) to deplete membrane cholesterol (Kaiser et al., 2002). In other experiments, the estrogen receptor antagonist ICI-182,780 (10 μM ; Tocris), PI-3 kinase inhibitors wortmannin or LY294002 (10 μM ; Calbiochem), or the src-kinase inhibitor PP2, (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4,*d*]pyrimidine, 10 μM ; Calbiochem) was included during the 30 min pre-equilibration period and maintained during 17β -estradiol treatment. After incubation, samples were centrifuged at 5000 x g for 1 min to pellet vessels, and the supernatant removed for nitrite measurement. Total protein content of the pelleted vessels was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Prior to nitrite measurement, supernatants were centrifuged at 12,000 g for 2 min to remove any remaining particulate matter. Total nitrite levels were measured using a Nitric Oxide Quantitation Kit (Active Motif) as per the manufacturer's protocol. Total nitrite values in each sample were normalized to protein content, and this ratio was expressed as a fold-difference versus vehicle control.

Immunoblot Analysis. Blood vessels were glass homogenized at 4°C in lysis buffer (50 mM β -glycerophosphate, 100 μ M NaVO₃, 2 mM MgCl₂, 1 mM EGTA, 0.5% Triton X-100, 1 mM dl-dithiothreitol, 20 μ M pepstatin, 20 μ M leupeptin, 0.1 U/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 20 min. Samples were then centrifuged for 10 min at 4500 x g at 4°C, the supernatant was collected, and protein concentrations were determined. Lysates were used immediately or stored at -80°C.

In all immunoblot experiments, equal amounts of protein [50 μ g in 1x sodium-dodecyl sulfate (SDS) sample buffer (Invitrogen), boiled for 4 minutes] were loaded in each lane of an 8% Tris-glycine gel (Novex) and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to nitrocellulose membranes (Amersham), incubated in blocking buffer (0.01 M PBS, 0.1% Tween-20, 6.5% nonfat dry milk), and treated with primary antibodies (p-ser-473-Akt, HC-20 (ER- α), p-Ser-1177-eNOS, p85 α , cav-1, Santa Cruz) at dilutions from 1:500 to 1:200. Blots were then incubated in the appropriate secondary antibody (goat-anti-rabbit IgG-HRP, donkey-anti-goat IgG-HRP or goat-anti-mouse IgG-HRP, Santa Cruz) at 1:10,000 dilution, and the bands visualized using enhanced chemiluminescence reagent (ECL, Amersham) and Hyperfilm (Amersham). UN-SCAN-IT software (Silk Scientific) was used for densitometric analysis of immunoreactive bands. In all applicable Western blot experiments, α -actin protein levels were determined to verify equal protein loading of the gel.

Immunoprecipitation. Cerebral vessel lysates from intact females or ovariectomized females with and without 30 min estrogen treatment were centrifuged at 12,000 x g for 10 min to reduce particulate matter. Lysate equivalent to 100 μ g of total protein and 5 μ g of antibody were added

to a total volume of 500 μ L lysis buffer and mixed end-over-end for 1 hr at 4°C. 50 μ L Protein G (50% slurry) was added to the sample and then mixed end-over-end for 1 hr at 4°C. Samples were centrifuged at 12,000 x g for 30 sec, and the pellet was washed four times with 1 ml lysis buffer, with a 30 sec 12,000 x g centrifugation between each wash. The final pellet was suspended in 30 μ L 2x SDS sample buffer and boiled for 3 min, then centrifuged at 12,000 x g for 30 sec to remove the supernatant. The entire 30 μ L supernatant was loaded onto an 8% Tris-glycine polyacrylamide gel and analyzed by SDS-PAGE and immunoblotting as described above. As a negative control, samples of each lysate also were incubated with normal IgG antibodies, followed by the immunoprecipitation protocol and immunoblotting.

Confocal microscopy. Cerebral blood vessels were dissected from the surface of the brain, cut into small segments, fixed in 3% formaldehyde for 30 min and permeabilized using 0.1% Triton X-100 for 5 min. Vessels were then incubated for 30 min in 1% bovine serum albumin (BSA)/PBS, followed by overnight incubation at 4°C in primary antibodies at 1:50 dilution: rabbit anti-ER- α H-184 (Santa Cruz) and mouse anti-caveolin-1 (Zymed) or mouse-anti eNOS (Transduction Laboratories) and rabbit-anti caveolin-1 (Santa Cruz)]. Vessels were then washed for 30 min in PBS and incubated overnight at 4°C with the secondary antibodies at 10 μ g/ml (goat anti-rabbit Oregon Green 488 and goat anti-mouse Texas Red, Molecular Probes), followed by a final wash for 30 min in PBS. Vessels were laid on slides, covered with mounting medium containing DAPI (4',6-diamidino-2-phenylindole), (VectaShield + DAPI, Vector Laboratories) and coverslips applied. Images were obtained using a Bio-Rad model 1024 laser scanning confocal microscope equipped with standard and UV lasers.

Statistics. All data values are given as mean \pm SEM. Statistical differences in immunoblot band densities and total nitrite assays were determined by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison test (GraphPad Prism 2.0 software) and displayed as fold difference relative to OVX or vehicle control. Where appropriate, statistical differences were determined by Student's *t* test. In all cases, statistical significance was set at $P \leq 0.05$.

RESULTS

Estrogen mediates rapid increases in NO production. Cerebral blood vessels isolated from OVX animals were incubated with 10 nM 17 β -estradiol for 30 min at 37°C. NO levels in the media were measured by a total nitrite assay. Estrogen caused a significant increase in cerebrovascular NO production vs. vehicle-treated control (Fig. 1). Preliminary experiments indicated that estrogen increased cerebrovascular NO production within 15 min of exposure; however, due to limitations of the sensitivity of the nitrite assay, the earliest time point at which data could be consistently reproduced was 30 min. For this reason, we used the 30 min time point for further studies. To test whether the estrogen-mediated increase in NO production was mediated by estrogen receptors, the estrogen receptor inhibitor ICI-182,780 was used. ICI-182,780 (10 μ M) fully inhibited estrogen-mediated increases in NO production (Fig. 1). To test whether the effect of estrogen on NO production involved the PI-3 kinase pathway, two inhibitors of PI-3 kinase, wortmannin and LY294002 (10 μ M), were used. As shown in Figure 1, both inhibitors blocked the ability of estrogen to increase NO production.

A recent study suggested that src kinase may play an intermediary role between estrogen receptor activation and PI-3 kinase activation (Haynes et al., 2003). Therefore we tested this hypothesis with the src kinase inhibitor PP2. However, PP2 failed to significantly inhibit estrogen-mediated increases in NO (Fig. 1). To test whether inducible NOS (iNOS) was contributing to NO production, aminoguanidine, an iNOS inhibitor, was added in the presence of estrogen. Aminoguanidine (10 μ M) failed to significantly inhibit estrogen-mediated increases in NO production (aminoguanidine + estrogen treatment resulted in a $49 \pm 5\%$ increase in NO production vs. control, compared to a $54 \pm 7\%$ increase for 30 min estrogen treatment alone,

n=3). Additionally, no iNOS was detected by immunoblot analysis in cerebral vessels before or after incubation.

Acute estrogen exposure increases ser-473 phosphorylated-Akt. To determine whether estrogen activates the PI-3 kinase/Akt pathway, Western blots of lysates from OVX cerebral vessels exposed to 30 min of estrogen treatment (10 nM) were probed for p-ser-473-Akt. Immunoblot analysis demonstrated that 30 min estrogen treatment led to significant increases in p-Akt with no change in total Akt levels (Fig. 2). Furthermore, to demonstrate that the increase in phosphorylation was mediated by estrogen receptors acting through the PI-3 kinase pathway, an estrogen receptor blocker, ICI 182,780, and a PI-3 kinase inhibitor, LY294002, were used. Each inhibitor prevented the effect of estrogen on p-Akt levels (Fig. 2, A and B). Another PI-3 kinase inhibitor, wortmannin, also blocked the estrogen-induced increase in p-Akt levels (91 ± 3% decrease vs. estrogen treatment alone, n=4). Although a physiologically relevant concentration of estrogen was used throughout this study (10 nM), we also verified that concentrations of estrogen from 0.1 nM to 100 μM caused a concentration-dependent increase in p-Akt levels at the 30 min time point (n=3, data not shown).

Acute estrogen exposure increases ser-1177 phosphorylated-eNOS. Western blot analysis was used to determine if estrogen also increased phosphorylation of eNOS at serine-1177. Acute estrogen exposure *in vitro* (30 min) led to significant increases in p-eNOS levels in isolated cerebral blood vessels with no change in total eNOS protein (Fig. 3). To determine if the increase in p-eNOS required PI-3 kinase activation, vessels were pre-treated with LY294002 prior to estrogen exposure. As demonstrated in Fig. 3, LY294002 treatment inhibited the estrogen-

mediated increase in p-eNOS levels. As p-eNOS was difficult to detect by Western blotting of cerebral vessel lysates, a second approach was also used. Total eNOS was first immunoprecipitated from vessels with and without 30 min estrogen exposure. The samples were then analyzed by SDS-PAGE and probed for phosphorylated eNOS. Immunoprecipitation of eNOS from vessels treated with estrogen also demonstrated significant increases in the phosphorylated form of eNOS ($47 \pm 4\%$ increase vs. OVX controls, $n=4$).

ER α co-immunoprecipitates with p85 α and eNOS. To further explore the mechanism by which estrogen acutely mediates increases in NO production, the interaction of ER α with proteins involved in eNOS activation was explored through immunoprecipitation experiments. As shown in Fig. 4A, in vessel lysates from intact female rats immunoprecipitation of ER α reveals an association with the p85 α regulatory subunit of PI-3 kinase as well as eNOS.

Immunoprecipitation of eNOS confirms the association of this protein with at least two forms of ER α , one at 66 and the other at 50 kDa (Stirone et al, 2003b). Similarly, immunoprecipitation of ER α from vessels of OVX females also indicated association of the receptor with eNOS and p85 α (data not shown). Estrogen treatment in vitro (10 nM, 30 min) significantly increased the amount of eNOS protein associated with ER α (Fig. 4B), but under these conditions we did not detect any change in the levels of ER α associated with p85 α (data not shown).

ER- α is co-localized with caveolin-1 in cerebrovascular endothelium. We previously visualized ER α immunoreactivity in the endothelium of cerebral blood vessels (Stirone, et al., 2003b). In the current study, we addressed the possibility that ER α may be localized at the endothelial cell membrane by using immunohistochemistry and confocal microscopy at a higher

magnification (100 X). Antibodies against ER α and a marker for caveolae, caveolin-1, were used to stain rat cerebral arteries dissected off the surface of the brain. Figure 5 shows that both ER α (Fig. 5B) and caveolin-1 (Fig. 5A) are present in endothelial cells. The merged image reveals co-localization of these two proteins at the membrane, which can be seen outlining the individual endothelial cells, oriented in the direction of blood flow (Fig. 5C).

Caveolar disruption by β -methyl-cyclodextrin inhibits acute estrogen-mediated NO

production, but not Akt phosphorylation. To test the functional significance of ER α localized to membrane caveolae of cerebrovascular endothelial cells, isolated cerebral arteries were pre-treated for 30 min with 5 mM β -methyl-cyclodextrin (β -mCD), a concentration and form of cyclodextrin previously shown to significantly deplete cholesterol and disrupt the structure of caveolae from aortic rings within 30 min (Kaiser, et al., 2002). As shown in Fig. 6A, 30 min β -mCD pre-treatment significantly inhibited the effect of estrogen to acutely increase vessel NO production. However this regimen of β -mCD pretreatment failed to inhibit the effect of estrogen on cerebrovascular Akt phosphorylation (Fig. 6B).

Chronic estrogen treatment increases NO production. To determine if chronic treatment with estrogen to maintain physiologically relevant hormone levels also increases NO production in the cerebral circulation, vessels were isolated from OVX and OE animals and assayed for total nitrite levels after 30 min incubation in PBS at 37°C. As shown in Fig. 7, basal NO production was significantly higher in vessels from OE animals compared to OVX controls. However, in contrast to what was found in OVX vessels, addition of estrogen (10 nM) during the 30 min

incubation period did not further increase NO production in vessels from females treated chronically with estrogen (data not shown).

Chronic in vivo estrogen treatment increases p-Akt and p-eNOS levels. In order to determine if estrogen-mediated phosphorylation of Akt and eNOS occur under conditions of prolonged exposure to estrogen *in vivo*, immunoblot analysis of cerebral vessels from OVX animals exposed to estrogen for one month (OE) were compared to vessels from OVX females and intact females. As shown in Fig. 8A, cerebrovascular p-Akt levels were significantly elevated in cerebral vessels from animals chronically exposed to estrogen as well as from intact females as compared to the OVX group. Total Akt protein levels did not change with chronic estrogen treatment.

As we have previously demonstrated (McNeill et al., 1999), total eNOS protein was increased following chronic estrogen exposure. Levels of p-eNOS were also significantly greater in vessels from intact females and OVX animals chronically exposed to estrogen compared to OVX controls (Fig. 8B).

DISCUSSION

The major finding of this study is that estrogen receptors activate a PI-3 kinase cascade in rat cerebral blood vessels that leads to both acute and chronic increases in levels of p-Akt and p-eNOS as well as increases in NO production. This study is an important verification of rapid, non-genomic signaling by estrogen in intact vasculature. Furthermore, it demonstrates that chronic exposure to physiologically relevant hormone levels *in vivo* also stimulates kinase activation of cerebrovascular Akt and eNOS. Confocal imaging of cerebral arteries reveals a significant pool of ER α colocalized with caveolin-1 at the plasma membrane of endothelial cells *in situ*. Furthermore, cerebrovascular ER α co-immunoprecipitates with the p85- α regulatory subunit of PI-3 kinase and eNOS. Both the full-length ER α (66 kDa) and a truncated ER α (about 50 kDa) are found in the immunoprecipitates, suggesting that multiple receptor types might mediate non-genomic signaling by estrogen in native tissue. Disruption of vascular caveolae inhibits estrogen-stimulated NO production; but interestingly, this perturbation did not prevent estrogen-mediated phosphorylation of Akt. Together these data suggest that non-genomic actions of estrogen, mediated by caveolae-associated ER α , contribute to cerebrovascular protective effects of estrogen.

Using intact cerebral vessels *ex vivo*, we demonstrate that physiological concentrations of 17 β -estradiol rapidly increase NO production. A similar effect of estrogen is observed using endothelial cells in culture (Chen, et al., 1999; Chambliss et al., 2002; Haynes, et al., 2000). Estrogen appears to act by triggering eNOS phosphorylation since cerebrovascular levels of p-eNOS, but not total eNOS protein, increase in parallel with NO production. Phosphorylation of eNOS at serine-1117 is known to increase enzyme activity by 10-20 fold (Gallis et al., 1999).

Mutation of eNOS serine-1177 to aspartate, which mimics the negative charge afforded by phosphorylation, leads to constitutively active eNOS even at low levels of Ca^{2+} (Dimmeler et al., 1999). This implies that activation of p-eNOS is less calcium-dependent than the unmodified form of eNOS (Dimmeler et al., 1999). Some cell culture studies, in fact, have shown that estrogen elevates NO production even in the absence of a Ca^{2+} stimulus (Caulin-Glaser et al., 1997; Haynes et al., 2000), which is consistent with an effect of estrogen on eNOS phosphorylation.

We also found that basal levels of p-eNOS are higher in cerebral blood vessels taken from animals chronically exposed to estrogen. Thus estrogen alters the sensitivity of eNOS *in vivo* such that the enzyme is more easily activated by a variety of chemical and mechanical stimuli (Shaul, 2002). In addition, there is more basal NO production in cerebral vessels taken from intact females and estrogen-treated animals as compared with the ovariectomized group. This correlates with our previous finding that chronic estrogen treatment decreases cerebral artery tone via endothelial, NOS-dependent mechanisms (Geary et al., 1998; 2001; Ospina et al., 2004). Interestingly, *ex vivo* stimulation of NO release by 17β -estradiol could only be detected in vessels from ovariectomized animals, perhaps because p-eNOS is already elevated in tissue exposed to estrogen *in vivo*. The *in vivo* data underscore the physiological relevance of estrogen-induced eNOS phosphorylation. Moreover, this signaling mechanism complements genomic actions of estrogen on vascular eNOS. We previously found that estrogen increases eNOS mRNA (Stirone et al., 2003a) and protein (McNeill et al., 1999, Stirone et al., 2003a) in cerebral vessels. Thus, the higher level of basal NO in vessels from estrogen-exposed animals likely reflects both genomic and nongenomic actions of the hormone.

Several signal transduction mechanisms have been implicated in rapid effects of estrogen, including activation of the PI-3 kinase/Akt kinase cascade (Haynes et al., 2000; 2002; Ho and Liao, 2002; Simoncini et al., 2003). PI-3 kinase phosphorylates Akt at serine-473; and then p-Akt directly phosphorylates eNOS at serine-1177 (Dimmeler et al., 1999; Fulton et al., 1999; Shiojima and Walsh, 2002; Gallis et al., 1999). As illustrated by a study where constitutively-active Akt was transfected into rat femoral arteries (Luo et al., 2000), Akt regulates production of NO and affects blood vessel diameter. In isolated cerebral vessels, 17 β -estradiol rapidly increases levels of p-Akt, but not total Akt protein; these increases in p-Akt, p-eNOS and NO production are all blocked by two inhibitors of PI-3 kinase, wortmannin and LY294002. These results are consistent with estrogen signaling via the PI-3 kinase/Akt/eNOS pathway, in agreement with some (Haynes et al., 2000; Simoncini et al., 2000), but not all (Haynes et al., 2003; Chen et al., 1999; Chen et al., 2004) studies in cultured endothelium. In particular, we found no evidence in cerebral vessels that estrogen-mediated activation of eNOS involves src kinase (Haynes et al., 2003), and our data contrast with findings from uterine artery endothelial cells that show no effect of estrogen on Akt (Chen et al., 2004). Together, these studies underscore the potential for endothelial differences due to cell environment and/or tissue origin.

Acute effects of 17 β -estradiol on cerebral blood vessels appear to be mediated by estrogen receptors, in particular ER α and/or a splice variant of ER α . Stimulation of NO production and Akt phosphorylation in intact vessels is blocked by the ER antagonist ICI 182 780, in agreement with findings from cultured endothelial cells (Chen et al., 1999, Kim et al., 1999, Stefano et al., 2000, Haynes et al., 2000). ICI 182 780, however, does not distinguish

receptor subtypes; therefore rapid signaling by estrogen could be mediated by the well-defined nuclear receptors ER α and ER β (Ho and Liao, 2002; Nilsson et al., 2001, Chambliss et al., 2000; 2002), their splice variants (Figtree et al., 2003; Pendaries et al., 2002; Li et al., 2003) or other putative membrane receptors (Russell et al, 2000). Involvement of ER α is indicated by co-immunoprecipitation of this receptor subtype with the p85- α regulatory subunit of PI-3 kinase. We have previously shown that ER α is present in both the intimal and medial layers of cerebral vessels (Stirone et al., 2003b); thus, using vessel lysates, we cannot definitively conclude that the ER α - p85- α interaction we have identified occurs in the endothelium. However, our data agree with recent studies of cultured endothelial cells which show that ER α , but not ER β , associates with the p85- α subunit of PI-3 kinase (Simoncini et al., 2000; 2003).

However, we also demonstrate that cerebrovascular ER α co-immunoprecipitates with eNOS, and there is a significant increase in the association of eNOS and ER α after 30 min of estrogen treatment. At this point, we cannot distinguish between a direct protein-protein interaction and co-existence of the two proteins within a tight complex, e.g., caveolae. We previously reported that cerebral vessels express the full-length ER α (66 kDa) as well as a truncated ER α that has a molecular weight of about 50 kDa and lacks the N-terminal region (Stirone et al., 2003b). We detected both the 50 kDa, as well as the 66 kDa, bands in immunoprecipitates with eNOS. In recent cell transfection studies, a splice variant of human ER α (46 kDa), in which the N-terminal is deleted, was found to be more effective than the full-length ER α (66 kDa) at rapidly stimulating NO release from cultured cells (Li et al., 2003; Figtree et al., 2003). The present study suggests that a truncated form of ER α could play a role

in eNOS activation in intact vascular tissue. The full-length ER α , although less effective (Li et al., 2003), may contribute as well.

Because eNOS and signaling molecules such as PI3 kinase are localized together within caveolar regions of the plasma membrane, it has been hypothesized that estrogen receptors act at the membrane to mediate rapid hormone signaling (Chambliss and Shaul, 2002; Levin, 2002). Using confocal microscopy, we demonstrate that ER α immunoreactivity co-localizes with the key caveolae scaffolding protein, caveolin-1, at the plasma membrane of endothelial cells lining intact cerebral arteries. Further studies are required to establish the forms of ER α present at the membrane and the mechanisms underlying this localization. Hydrophobicity profiling of native ER α indicates that this receptor does not contain a domain sufficiently hydrophobic to exist as an integral membrane protein, nor does it contain a consensus glycosylation or acylation site. However, ER α has been shown to bind directly to caveolin-1 (Schlegel et al., 2001). In addition, palmitoylation may be involved in translocating the 46kDa variant of ER α to the caveolar membrane (Li et al., 2003). To date, the evidence for membrane estrogen receptors in endothelial cells has come primarily from cultured cells or immortalized cell lines (Deecher et al., 2003; Chambliss and Shaul, 2002; Chen et al., 2004; Figtree et al., 2003; Li et al., 2003; Simoncini et al., 2003). However, resting cells in culture tend to exhibit primarily intracellular estrogen receptors (Li et al., 2003; Figtree et al., 2003; Simoncini et al., 2003), and transfection techniques are commonly employed to study membrane receptors (Levin, 2002; Li et al., 2003). The present study is one of the first to establish the presence of ER α at the endothelial membrane in native blood vessels; and, in contrast to cultured cells, there appears to be a significant pool of native estrogen receptors localized in or near the plasma membrane.

Using β -methyl-cyclodextrin treatment to disrupt caveolae by sequestration of cholesterol (Kaiser et al., 2002; Parpal et al., 2001), we found that 17β -estradiol activation of NO production in cerebral vessels was prevented. However, acute 17β -estradiol treatment still resulted in robust phosphorylation of Akt at Ser-473. This was unexpected based on reports that activation of Akt, for example by insulin in adipocytes (Parpal et al., 2001), is inhibited by β -cyclodextrin disruption of caveolae under similar conditions. However there are some differences between insulin and estrogen activation of Akt (Sasaki et al., 2003). It is also possible that loss of cholesterol can activate, rather than inhibit, caveolar signaling molecules (Fielding and Fielding, 2003). Alternatively, estrogen stimulates Akt phosphorylation at intracellular sites (Sasaki et al., 2003) that may or may not require caveolar receptors; our pAkt measurements no doubt reflect multiple pools within the blood vessel.

In experimental models, estrogen has a clear protective effect against stroke (Hurn and Brass, 2003). Acute activation of p-eNOS via PI3 kinase/Akt also appears to increase cerebral blood flow and decrease cerebral infarct size (Limbourg et al., 2002); thus this mechanism likely contributes to the effect of estrogen. By increasing cerebrovascular p-eNOS and NO, estrogen can increase vasodilation and inhibit vascular inflammation, leukocyte adhesion, smooth muscle cell proliferation, platelet aggregation and free radical activity (Geary et al., 1998; Shaul, 2002; Ospina et al., 2004).

Implications for estrogen regulation of the vascular PI3-kinaseAkt cascade, however, go beyond stimulation of NO production. Recently it was shown that acute stimulation of PI3 kinase

by estrogen upregulates gene expression in cultured umbilical vein endothelial cells (Pedram et al., 2002). Akt also regulates endothelial cell survival and angiogenesis (Shiojima and Walsh, 2002); a mechanism that may underlie 17β -estradiol inhibition of endothelial apoptosis (Alvarez et al., 1997). Thus activation by estrogen of the PI-3 kinase/Akt pathway can have far-reaching effects on vascular function.

Much discussion has focused on rapid effects of estrogen, but in fact, in the physiological setting, hormone levels do not fluctuate over a period of minutes. Thus the significance of these mechanisms may lie not in how fast estrogen can signal, but in what are the consequences of activating specific signal transduction mechanisms. One of the most interesting findings of the present study is that chronic *in vivo* estrogen treatment increases basal levels of p-Akt and p-eNOS. This suggests that acute, nongenomic effects are maintained under prolonged exposure to physiological levels of estrogen.

ACKNOWLEDGMENTS

The skillful technical assistance of Jonnie Stevens is gratefully acknowledged.

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FOOTNOTES

*Supported by NIH Grant R01 HL 50775 and by a Grant-in-Aid from the American Heart Association, National Center.

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FIGURE LEGENDS

Figure 1. Effect of acute estrogen treatment on NO production. Isolated cerebral blood vessels from OVX females were incubated in PBS at 37°C for 30 min in the presence of 10 nM 17 β -estradiol or vehicle (CTRL). As indicated antagonists for estrogen receptors (ICI-182,780), PI-3 kinase (wortmannin and LY294002), or src kinase (PP2) were added (each at a concentration of 10 μ M) in the presence of 17 β -estradiol. Nitric oxide production was determined by total nitrite assay and expressed as a ratio of total nitrite levels to vessel protein concentration. Values are normalized to vehicle control (n=9 for estradiol (E₂); n=4 for E₂ + ICI; n=4 for E₂ + W; n=4 for E₂ + LY; n=5 for E₂ + PP2); *P \leq 0.05 vs. E₂ alone

Figure 2. Effect of estrogen treatment on levels of ser-473 phosphorylated Akt. Representative Western blots for p-Akt and Akt (A) or p-Akt and α -actin (B) are shown for vessels from OVX females incubated 30 min with vehicle (CTRL), estrogen (E₂, 10 nM), or estrogen plus the antagonists (A) ICI-182,780 (10 μ M) or (B) LY294002 (10 μ M). Mean density values are presented as fold difference compared to control (N=4). *P \leq 0.05 vs. CTRL.

Figure 3. Effect of acute estrogen treatment on phosphorylated eNOS. Cerebral blood vessels from OVX females were treated for 30 min with vehicle (CTRL), estrogen, or estrogen plus LY294002 and then probed by Western blot analysis for ser-1177 phosphorylated eNOS and total eNOS. Mean density values are presented as fold difference vs. control (N=6). *P \leq 0.05 vs. CTRL.

Figure 4. ER- α co-immunoprecipitates with the p85 α subunit of PI3 kinase and with eNOS.

(A) Lysate of intact female cerebral blood vessels was used. From the top: Immunoprecipitation of ER α reveals co-immunoprecipitation with both p85 α and eNOS as determined by Western blot. Immunoprecipitation of eNOS shows co-immunoprecipitation of ER α . (B) Vessel lysates from OVX females with and without 30 min estrogen exposure were compared. Mean data are shown for immunoprecipitation of ER- α , followed by Western blot for eNOS. N=5.

Figure 5. Co-localization of caveolin-1 and ER- α in the endothelium of a cerebral artery. Laser scanning confocal microscopy was used to image a small segment of a pial artery isolated from intact female rat brain. The artery was dual-stained with an N-terminal ER α antibody (H-184, green) and an anti-caveolin-1 antibody (red). The merged image shows areas of co-localization (orange). Images represent a single focal plane through the endothelial cell layer as identified by orientation of cell nuclei stained with DAPI.

Figure 6. Effect of disruption of caveolae on estrogen-stimulated NO production and Akt phosphorylation. Cerebral vessels from OVX females were pretreated with or without 5 mM β -methyl-cyclodextrin (β -mCD) to remove membrane cholesterol and disrupt caveolae. (A) NO production as measured by total nitrite assay from vessels incubated in vehicle (CTRL) or 10 nM estrogen (E_2) for 30 min (n=4). (B) Western blot for p-Akt and α -actin (loading control) from vessels exposed to 10 nM estrogen or vehicle for 30 min. Protein levels are expressed as a fold difference from vehicle control (n=4).

Figure 7. Effect of chronic *in vivo* estrogen treatment on cerebrovascular NO production. Basal NO production was measured by total nitrite assay in vessels isolated from OVX females and chronic estrogen-treated females (OE). Values were calculated as the ratio of total nitrite production to vessel total protein content, and expressed as a fold difference vs. the OVX control. (N=4).

Figure 8. Effect of chronic *in vivo* estrogen exposure on levels of p-Akt, Akt, eNOS and p-eNOS. Cerebral blood vessels from OE, OVX and intact female (INT.F) rats were probed for p-Akt and Akt (A) and p-eNOS and eNOS (B) immunoreactivity by Western blot analysis. A representative blot is shown for each protein or phosphoprotein. The optical density for each OE and INT.F. band is expressed as fold difference relative to the OVX band on the same blot; and the mean values \pm SEM are plotted. (N=4). *P \leq 0.05 compared to the OVX value for the relevant protein or phosphoprotein.

Fig. 1

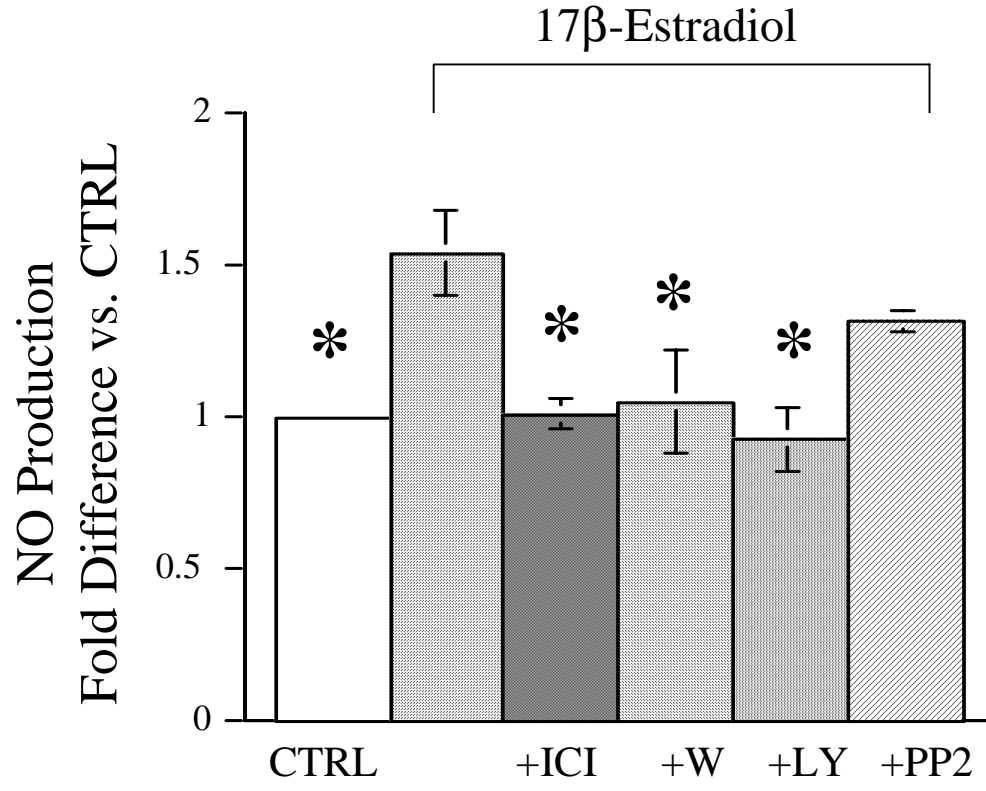


Fig. 2A

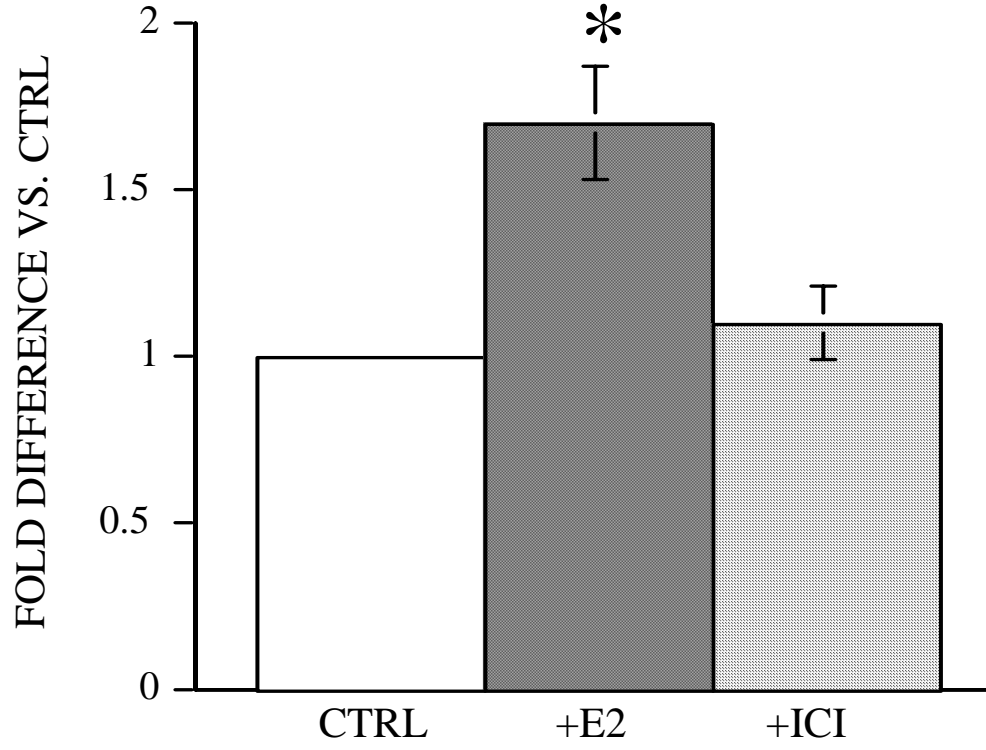
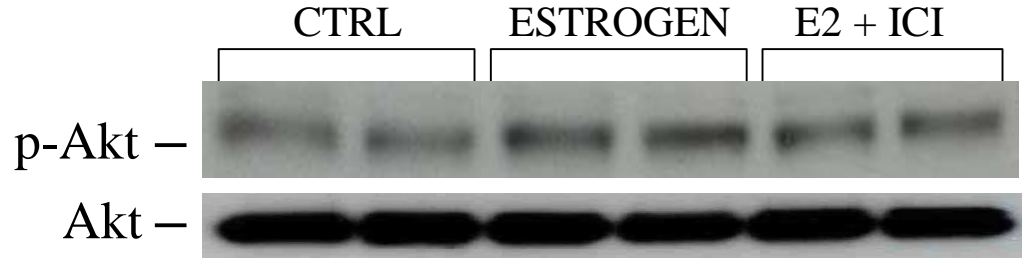


Fig. 2B

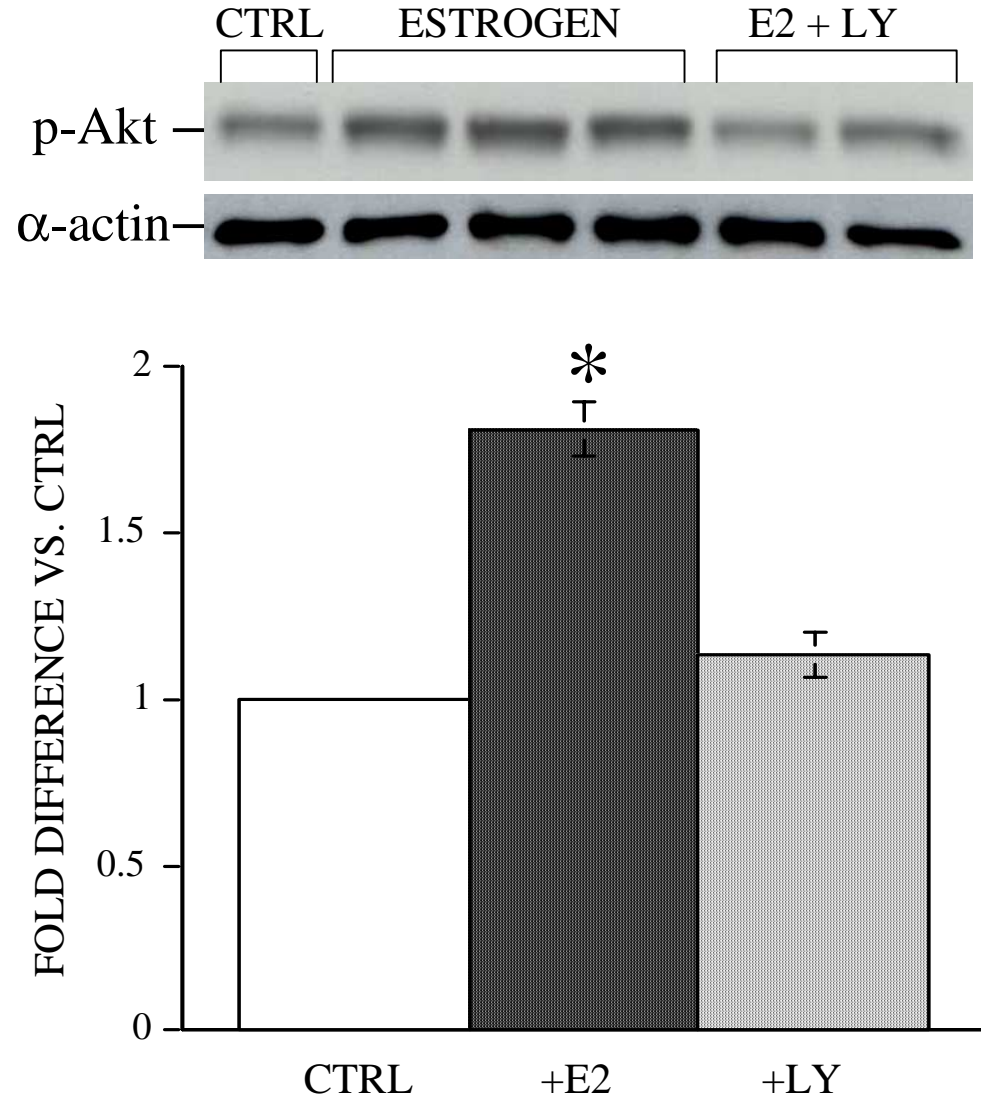


Fig. 3

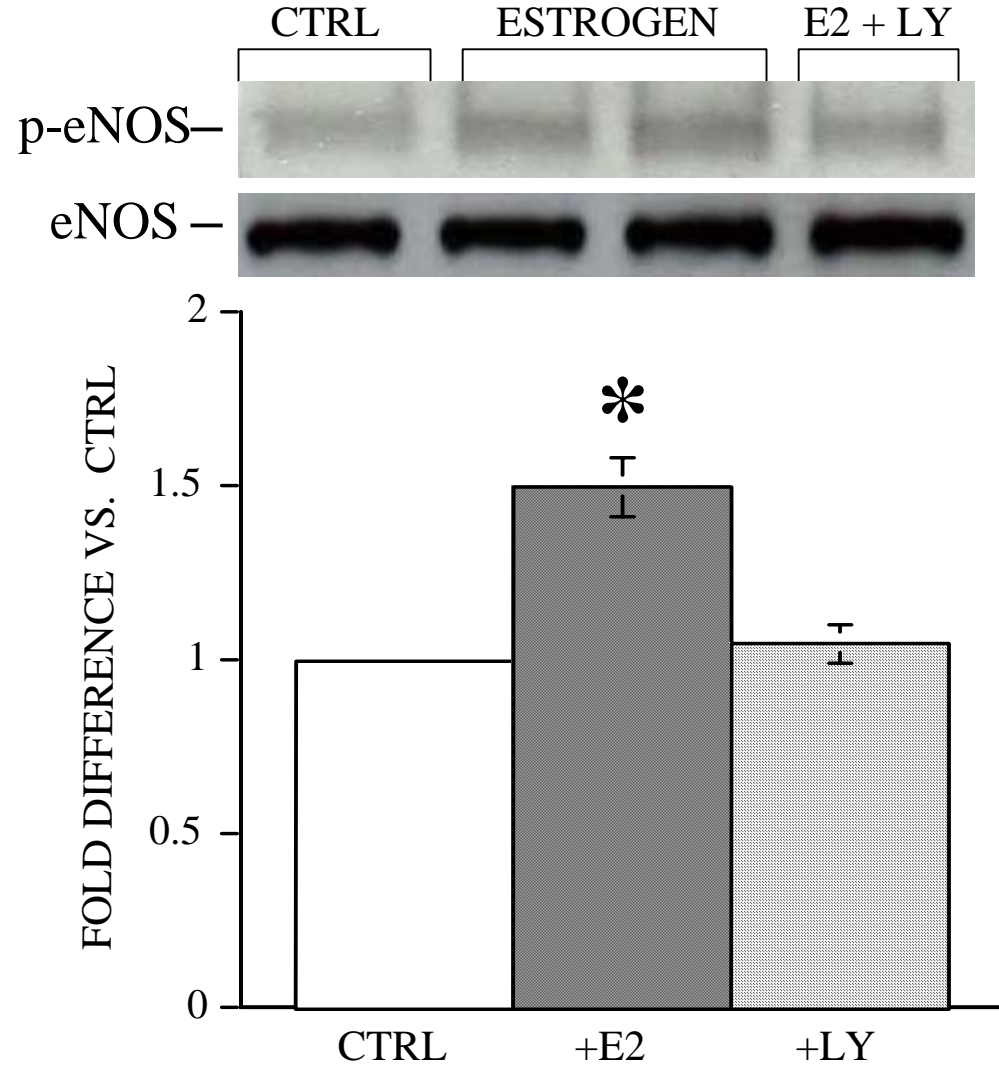


Fig. 4A

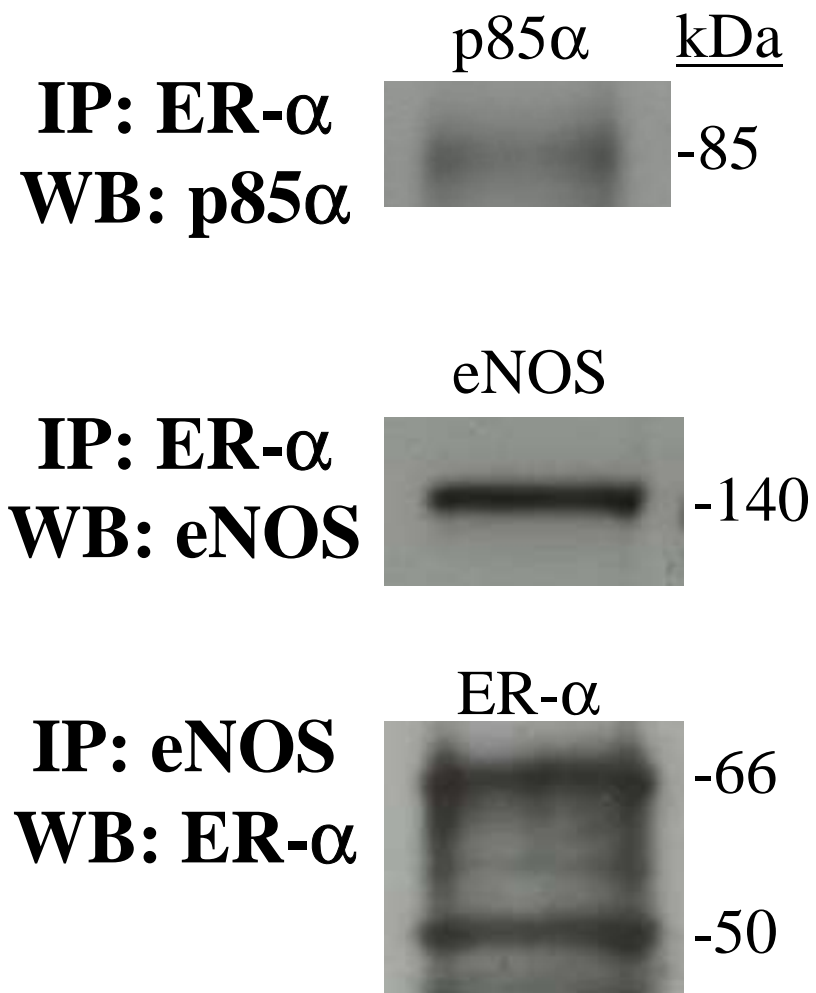


Fig. 4B

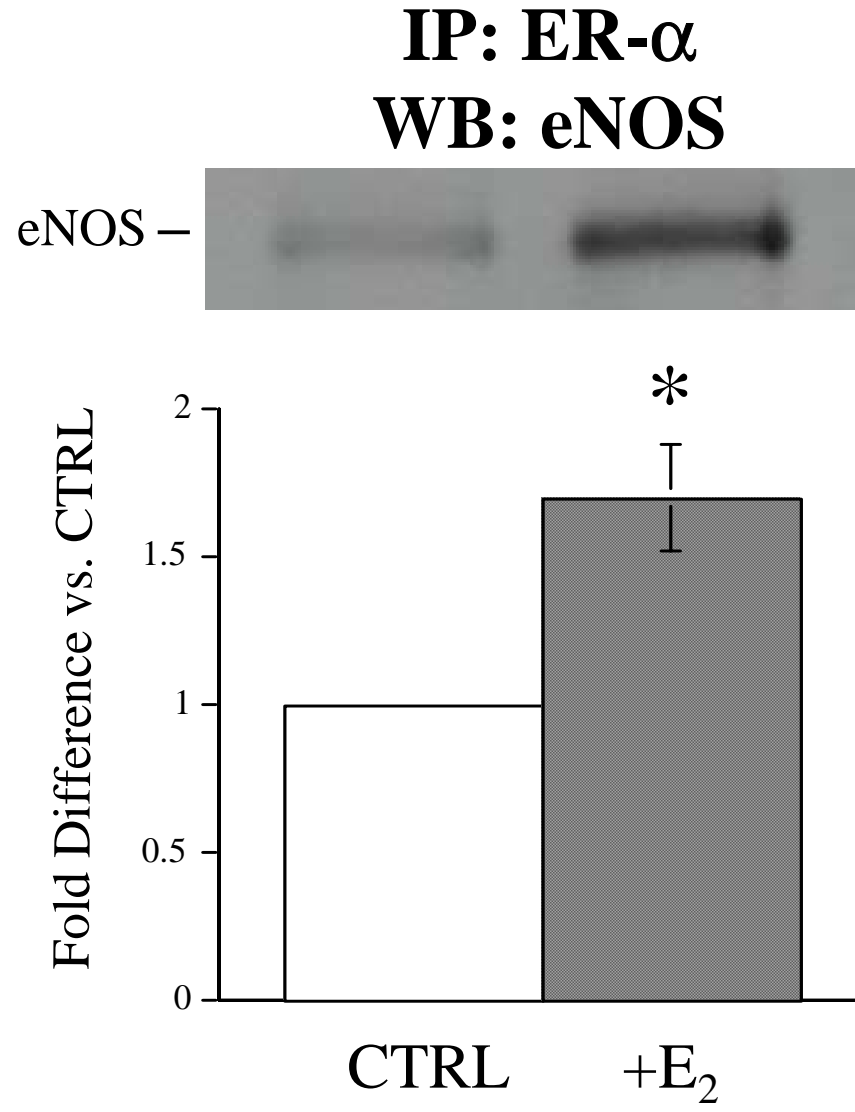


Fig. 5

Caveolin-1

ER- α

Merged

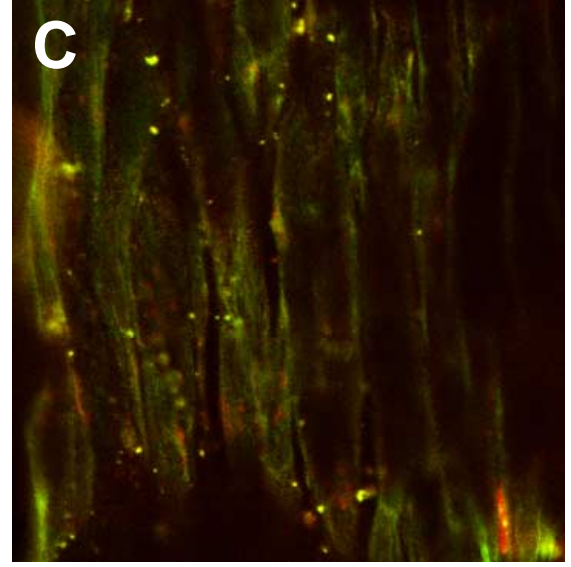
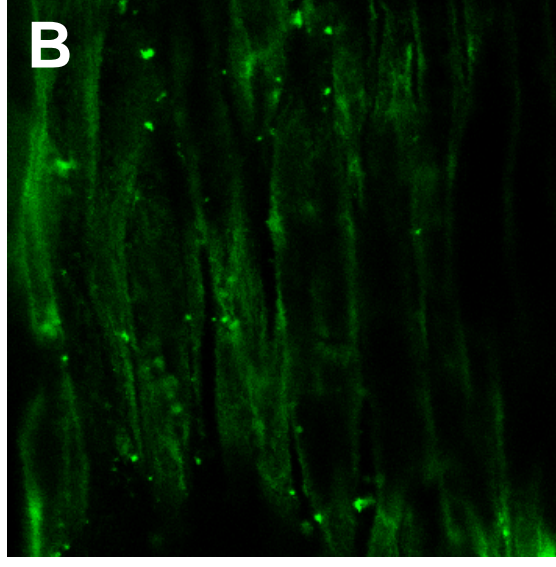
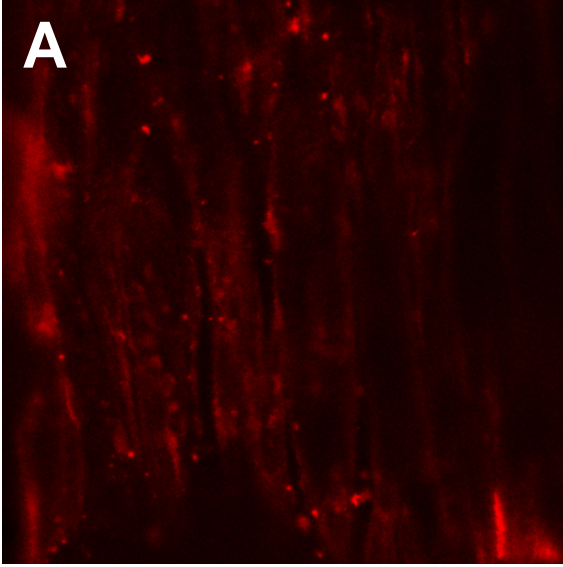


Fig. 6A

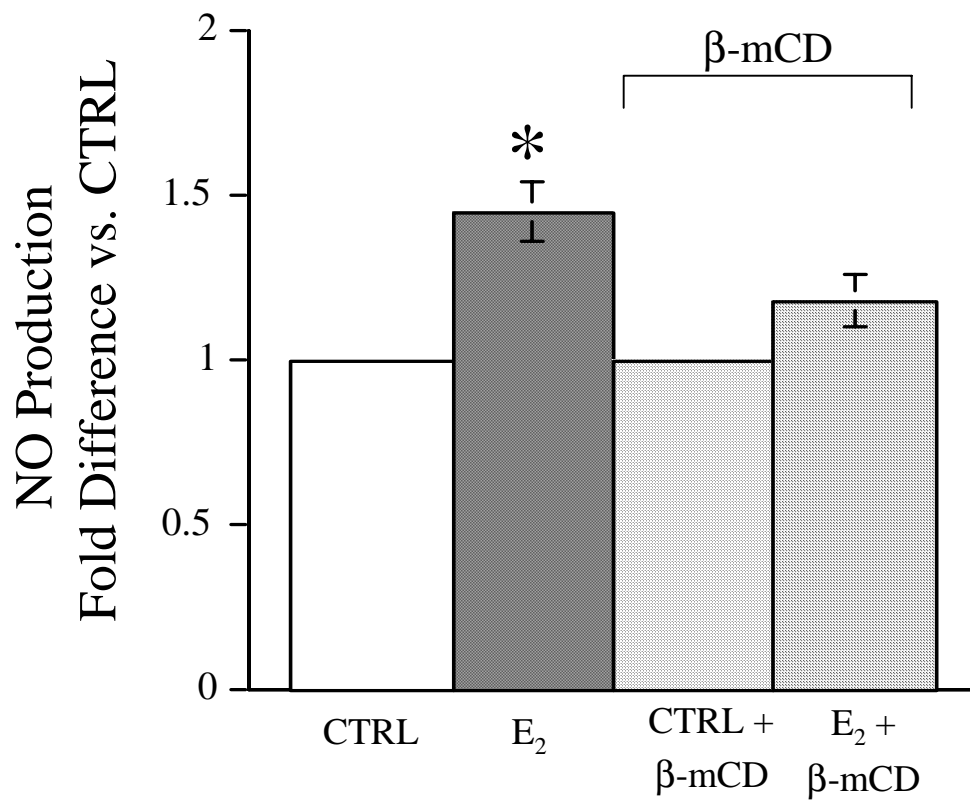


Fig. 6B

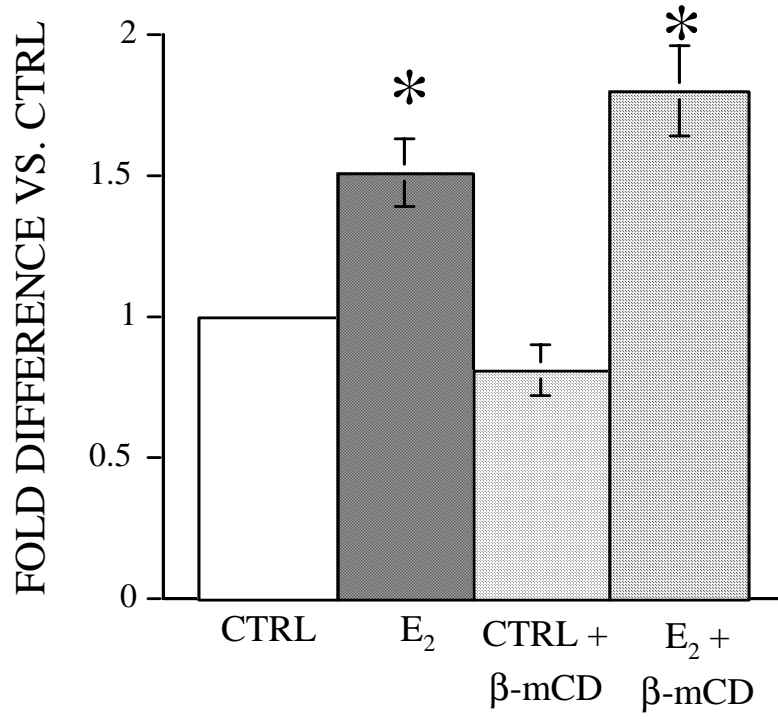
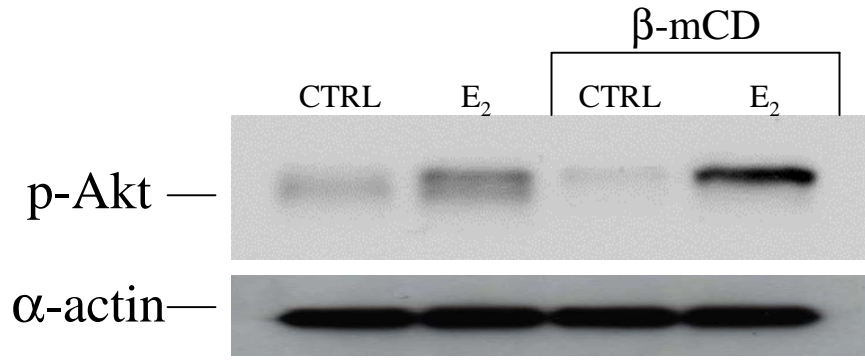


Fig. 7

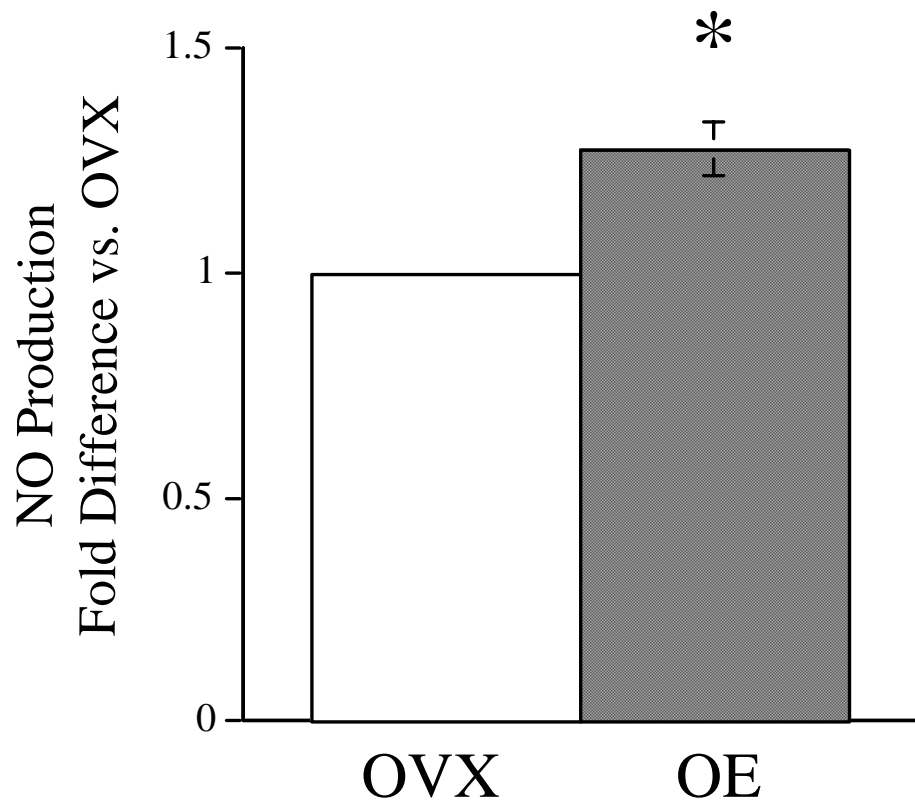


Fig. 8A

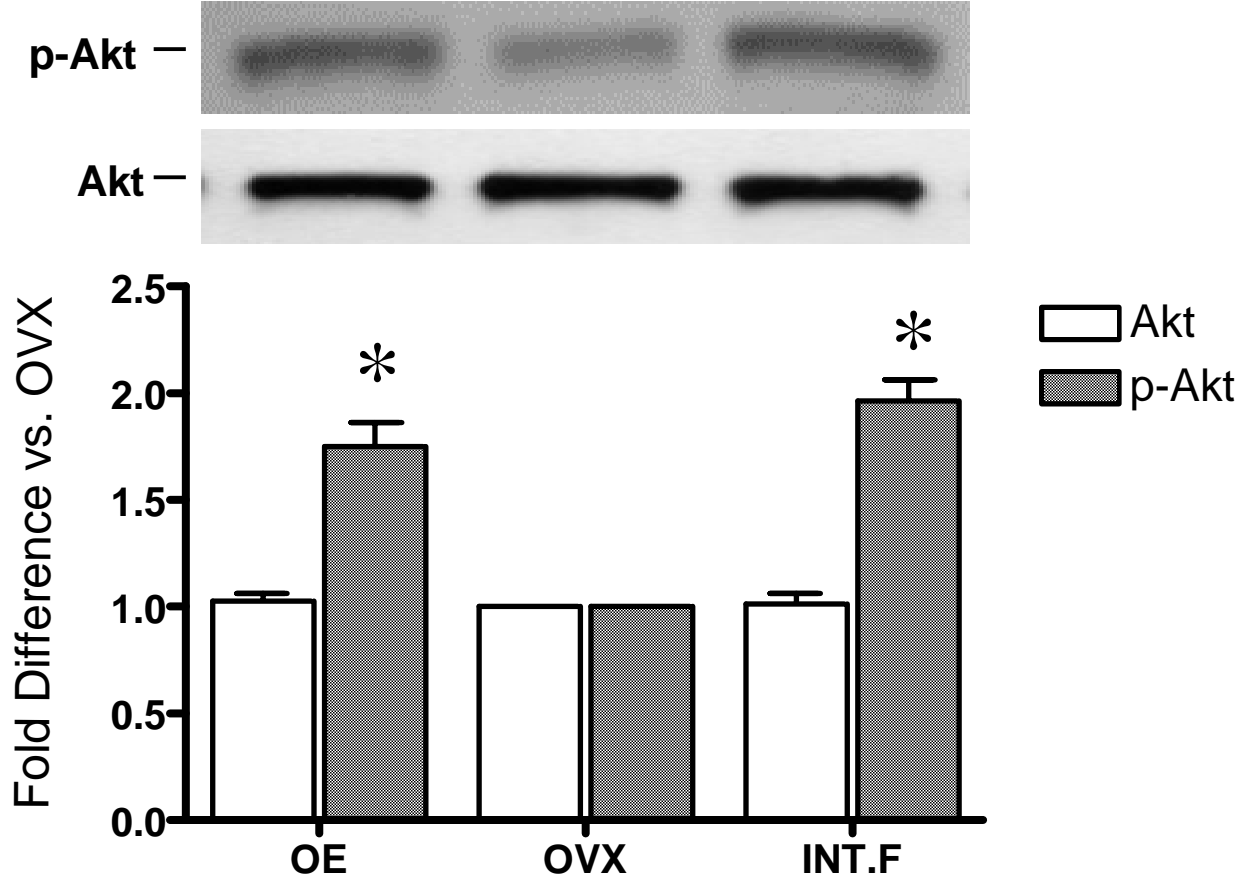


Fig. 8B

