0026-895X/05/6804-959–965$20.00
MOLECULAR PHARMACOLOGY
Vol. 68, No. 4
14662/3051778
*Mol Pharmacol* 68:959–965, 2005
*Printed in U.S.A.*


# Estrogen Increases Mitochondrial Efficiency and Reduces Oxidative Stress in Cerebral Blood Vessels

Chris Stirone, Sue P. Duckles, Diana N. Krause, and Vincent Procaccio

*Department of Pharmacology, College of Medicine, University of California, Irvine, Irvine, California (C.S., S.P.D., D.N.K.); Department of Pediatrics, College of Medicine, University of California, Irvine, Irvine, California (V.P.); Center for Molecular and Mitochondrial Medicine and Genetics, University of California, Irvine, Irvine, California (V.P.)*

Received May 10, 2005; accepted June 28, 2005

**ABSTRACT**

We report here that estrogen ($E_2$) modulates mitochondrial function in the vasculature. Mitochondrial dysfunction is implicated in the etiology of vascular disease; thus, vasoprotection by estrogen may involve hormonal effects on the mitochondria. To test this hypothesis, mitochondria were isolated from cerebral blood vessels obtained from ovariectomized female rats, with or without $E_2$ replacement. Estrogen receptor-$\alpha$ (ER-$\alpha$) was detected in mitochondria by immunoblot and confocal imaging of intact vessels. $E_2$ treatment in vivo increased the levels of specific proteins in cerebrovascular mitochondria, such as ER-$\alpha$, cytochrome *c*, subunit IV of complex IV, and manganese superoxide dismutase, all encoded in the nuclear genome, and subunit I of complex IV, encoded in the mitochondrial genome. Levels of glutathione peroxidase-1 and catalase, however, were not affected. Functional assays of mitochondrial citrate synthase and complex IV, key rate-limiting steps in energy production, showed that $E_2$ treatment increased enzyme activity. In contrast, mitochondrial production of hydrogen peroxide was decreased in vessels from $E_2$-treated animals. In vitro incubation of cerebral vessels with 10 nM 17$\beta$-estradiol for 18 h also elevated levels of mitochondrial cytochrome *c*. This effect was blocked by the estrogen receptor antagonist fulvestrant (ICI-182,780, Faslodex) but was unaffected by inhibitors of nitric-oxide synthase or phosphoinositide-3-kinase. Nuclear respiratory factor-1 protein, a primary regulator of nuclear gene-encoded mitochondrial genes, was significantly increased by long-term estrogen treatment in vivo. In summary, these novel findings suggest that vascular protection by $E_2$ is mediated, in part, by modulation of mitochondrial function, resulting in greater energy-producing capacity and decreased reactive oxygen species production.

Women have a lower risk of cardiovascular disease and stroke and a higher life expectancy compared with men (Sudlow and Warlow, 1997; McCullough and Hurn, 2003; Mensah et al., 2005). Estrogen ($E_2$) is believed to play a protective role, because the incidence of vascular disease increases significantly in women after menopause (Turgeon et al., 2004; Mensah et al., 2005). Indeed, animal studies demonstrate that $E_2$ has a multitude of protective effects on vascular function and experimental stroke (McCullough and Hurn, 2003; Orshal and Khalil, 2004; Turgeon et al., 2004). The mechanisms are not fully understood but include regulation of nuclear gene expression and signal transduction pathways.

This study was supported by National Institutes of Health grant R01-HL50775.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.105.014662.

Recent work strongly supports mitochondrial dysfunction and reactive oxygen species (ROS) production as critical mechanisms in the etiology of vascular disease, including hypertension and atherosclerosis (Ballinger et al., 2000; Lesnefsky et al., 2001; Ramachandran et al., 2002; Touyz and Schiffrin, 2004; Madamanchi et al., 2005; Wisloff et al., 2005). Mitochondria play key roles in cellular energy production, free-radical formation, and apoptosis. However little is known regarding the effects of $E_2$ on mitochondrial function in general, and almost nothing is known regarding mitochondria in the vasculature. In 1996, the mitochondrial DNA (mtDNA) was found to contain estrogen-response elements (Demonacos et al., 1996), but the presence of mitochondrial 17$\beta$-estradiol binding sites, and specifically estrogen receptors, has only recently been demonstrated in several nonvascular tissues and cultured cancer cells (Monje and Boland, 2001; Chen et al., 2004a,b; Yang et al., 2004). Effects of $E_2$ on mitochondrial function and protein expression may explain

**ABBREVIATIONS:** $E_2$, estrogen; ROS, reactive oxygen species; OVX, ovariectomized; OE, estrogen-treated and ovariectomized; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride; ER, estrogen receptor; ICI-182,780, fulvestrant; mtDNA, mitochondrial DNA; MnSOD, manganese superoxide dismutase; GPX-1, glutathione peroxidase-1; NRF-1, nuclear respiratory factor-1; PI, phosphoinositide; NOS, nitric-oxide synthase; L-NAME, $N^G$-nitro-L-arginine-methyl ester; COX IV, complex IV.

differences between male and female mitochondria (Borras et al., 2003; Felty and Roy, 2005). With respect to the vasculature, only one study has shown a mitochondrial effect of $E_2$, that is, an increase in mitochondrial manganese superoxide dismutase (MnSOD) in vascular smooth muscle cells (Strehlow et al., 2003).

Given the tremendous importance of mitochondria to basic cellular functions as well as the critical role of mitochondrial dysfunction in the development of vascular disease, a compelling question is whether vasoprotection by $E_2$ involves effects on the mitochondria. In particular, we hypothesized that $E_2$ modulates mitochondrial function in the vascular bed important for stroke, the cerebral circulation. Our previous work has established that cerebral blood vessels are a target tissue for $E_2$ (Geary et al., 1998; McNeill et al., 2002; Stirone et al., 2003, 2005; Ospina et al., 2004). The present study, to our knowledge, is the first to provide evidence that $E_2$ alters mitochondrial energy production capacity and ROS generation in intact vascular tissue, both in vivo and in vitro. These results implicate mitochondrial regulation as a novel protective mechanism for $E_2$.

## Materials and Methods

**In Vivo Treatments.** Animal procedures were approved by the University of California Irvine Institutional Animal Care and Use Committee. Fischer-344 female rats (3 months old; Charles River Laboratories, Inc., Wilmington, MA) were used, and they were ovariectomized (OVX) or ovariectomized and treated continually with a subcutaneous 17$\beta$-estradiol implant (OE) (Geary et al., 1998; Stirone et al., 2003; Ospina et al., 2004). After 4 weeks of treatment, animals were anesthetized by $CO_2$ and killed by decapitation. We have demonstrated previously that estrogen levels in OE animals are within the physiological range and are significantly higher than in OVX animals (Geary et al., 1998; McNeill et al., 2002). Serum 17$\beta$-estradiol levels were 13.58 $\pm$ 2.8 pg/ml for OVX and 77.98 $\pm$ 8.6 pg/ml for OE ($P \leq 0.05$). The physiological relevance of the OE treatment was further validated by expected effects on body and uterine weight. Body weights were 185 $\pm$ 2 g for OVX and 163 $\pm$ 1 g for OE ($P \leq 0.05$). Uterine weights were 35 $\pm$ 2 mg for OVX and 126 $\pm$ 5 mg for OE ($P \leq 0.05$).

**Cerebral Vessel and Mitochondrial Isolation.** Blood vessels were isolated from whole brain homogenates by centrifugation through 16% dextran and collection on a 50-$\mu$m mesh, as described previously (McNeill et al., 2002; Stirone et al., 2003, 2005). This preparation contains a mixture of arteries, arterioles, capillaries, veins, and venules. Blood vessel mitochondria were isolated using a mitochondrial isolation kit from Sigma (MITO-ISO1; Sigma Chemical, St. Louis, MO) according to the manufacturer's protocol, with additional centrifugations at low speed to improve the purity of the mitochondrial fraction. The nuclear marker histone H1 could not be detected in the isolated mitochondrial fractions by immunoblot analysis, indicating an absence of nuclear contamination.

**Immunoblot Analysis.** Whole vessel and mitochondrial lysates were prepared as described previously (Stirone et al., 2005). Equal amounts of protein were loaded in each lane of an 8 or 16% Tris-glycine gel and separated by SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to nitrocellulose membranes, incubated in blocking buffer, and treated with primary antibodies: cytochrome *c*, HC-20 (ER-$\alpha$), H-150 (ER-$\beta$), and histone H1 (Santa Cruz Biochemicals, Santa Cruz, CA); $\alpha$-actin and catalase (Sigma); MnSOD and glutathione peroxidase-1 (GPX-1) (Esposito et al., 1999); complex IV (COX IV) subunits I and IV and porin (Molecular Probes, Eugene, OR); and nuclear respiratory factor-1 (NRF-1; a gift from the Scarpulla laboratory). Appropriate secondary antibodies were used, and the bands were visualized using enhanced chemiluminescence reagent and Hyperfilm (GE Healthcare, Little Chalfont, Buckinghamshire, UK). UN-SCAN-IT software (Silk Scientific Inc., Orem, UT) was used for densitometric analysis of immunoreactive bands. As appropriate, mitochondrial porin or $\alpha$-actin protein levels were determined for each blot to verify equal protein loading.

**Confocal Microscopy.** Pial vessels were prepared as described previously (Ospina et al., 2004; Stirone et al., 2005). Primary antibodies were directed against ER-$\alpha$ HC-20 (Santa Cruz Boichemicals) and COX IV subunit I; secondary antibodies were tagged with fluorescent markers Oregon Green 488 or Texas Red (Molecular Probes). Images were obtained using a Bio-Rad model 1024 laser scanning confocal microscope (Bio-Rad, Hercules, CA).

**In Vitro Experiments.** Freshly isolated cerebral blood vessels from OVX female rats were pre-equilibrated at 37°C, as described previously (McNeill et al., 2002). In all in vitro experiments, vessels were incubated in either 10 nM 17$\beta$-estradiol (encapsulated in 2-hydroxy-propyl-$\beta$-cyclodextrin; Sigma Chemical) or an equivalent concentration of 2-hydroxy-propyl-$\beta$-cyclodextrin alone (vehicle control). In some experiments, the estrogen receptor antagonist ICI-182,780 (1 $\mu$M; Tocris Cookson Inc., Ellisville, MI), PI-3 kinase inhibitor LY294002 (10 $\mu$M; Calbiochem, San Diego, CA), or the endothelial NOS inhibitor $N^G$-nitro-L-arginine-methyl ester (L-NAME) (100 $\mu$M; Sigma Chemical) was included for a 30-min pre-equilibration period and maintained during 17$\beta$-estradiol or vehicle treatment. Vessels were maintained at 37°C in 95% $O_2$/5% $CO_2$ for 6 to 18 h with drug(s), followed by mitochondrial isolation or whole-vessel lysis.

**Enzymatic Measurements and $H_2O_2$ Assays.** Enzyme assays, cytochrome oxidase, and citrate synthase were performed as described previously (Trounce et al., 1996) using mitochondria isolated from brain blood vessels. Cerebrovascular mitochondrial $H_2O_2$ production was measured using an Amplex Red Hydrogen Peroxide assay kit (Molecular Probes), according to the manufacturer's protocol. All enzymatic and $H_2O_2$ measurements were done in triplicate with at least two independent sets of samples.

**Statistical Analyses.** All data values are given as mean $\pm$ S.E.M. Statistical differences were determined by Student's *t* test or, where appropriate, one-way analysis of variance with repeated measures followed by Dunnett's multiple comparison tests. In all cases, statistical significance was set at $P \leq 0.05$.

## Results

**Estrogen Receptor-$\alpha$ in Cerebrovascular Mitochondria.** Mitochondrial lysates from cerebral vessels of OVX and OE rats were probed for ER-$\alpha$ and ER-$\beta$ by immunoblot analysis. A single band at 66 kDa corresponding to ER-$\alpha$ was present in the mitochondrial fractions (Fig. 1A), in contrast to our previous work showing multiple immunoreactive bands for ER-$\alpha$ in lysates of intact cerebral vessels (Stirone et al., 2003). Prior in vivo $E_2$ treatment resulted in significantly higher levels of mitochondrial ER-$\alpha$ (Fig. 1A). ER-$\beta$, however, could not be detected in the mitochondrial lysates (data not shown).

To further validate the presence of ER-$\alpha$ in cerebrovascular mitochondria, we used immunohistochemistry and confocal microscopy. Antibodies against ER-$\alpha$ (green fluorescence) and a mitochondrial marker, subunit I of COX IV (red fluorescence), were used to label these proteins in rat cerebral arteries dissected off the surface of the brain. Figure 1B was taken at a focal plane through the smooth muscle layer, identified by nuclei (4,6-diamidino-2-phenylindole–stained) oriented perpendicular to the direction of blood flow. A merged image of the fluorescence in this focal plane from the two antibody labels reveals colocalization (yellow) of ER-$\alpha$ and the mitochondrial protein COX IV subunit I. In Fig. 1, C

and D, one smooth muscle cell has been enlarged to enhance the detail. Figure 1C shows only the red fluorescence for COX IV subunit I, which is localized in the cell periphery and perinuclear region but is absent from the nucleus. Figure 1D shows the addition of the ER-$\alpha$ fluorescence (green), which is found alone in the nucleus, but is visualized as yellow where it colocalizes with subunit I of COX IV. Colocalization is most striking at one end of the cell (denoted by the arrow) and in the thin perinuclear region.

**Estrogen Increases Cytochrome *c* in Cerebrovascular Mitochondria.** Cytochrome *c* is critically involved in energy production, apoptosis, and ROS production in mitochondria. To determine whether in vivo $E_2$ treatment altered mitochondrial levels of cytochrome *c* protein, mitochondria were isolated from cerebral blood vessels from OVX and OE animals. Western blot analysis revealed that estrogen significantly increased the amount of cytochrome *c* protein in mitochondrial fractions relative to OVX controls (Fig. 2). In contrast, cytochrome *c* was barely detectable in cytosolic fractions prepared from blood vessels of either group.

To validate that this in vivo effect was a direct effect of $E_2$ on the vessel and to determine the time course by which this effect occurred, we isolated cerebral vessels from OVX animals and treated them with 17$\beta$-estradiol (10 nM) in vitro. Figure 3A shows a representative Western blot of cytochrome *c* measured in mitochondrial fractions of cerebral vessels exposed to $E_2$ in vitro for various time periods (6–18 h). Cytochrome *c* protein is significantly elevated after 18 h of $E_2$ exposure.

To determine whether the effect of $E_2$ on mitochondrial cytochrome *c* expression was receptor-mediated, we treated vessels in vitro with the estrogen receptor antagonist ICI-182,780 in the absence and presence of $E_2$. ICI-182,780 fully inhibited the ability of $E_2$ to increase mitochondrial cytochrome *c* (Fig. 3B). We demonstrated previously that cerebrovascular estrogen receptors stimulate PI-3 kinase/Akt signaling and increase endothelial nitric oxide production (McNeill et al., 2002; Stirone et al., 2005). Because both PI-3 kinase activation and nitric oxide have been implicated in modulating nuclear-encoded mitochondrial gene expression, we tested the effects of the PI-3 kinase inhibitor LY294002 and the NOS inhibitor L-NAME (Fig. 3B). Neither inhibitor, however, affected the ability of $E_2$ to increase levels of mitochondrial cytochrome *c*.

**Estrogen Increases Complex IV Protein Expression in Cerebrovascular Mitochondria.** Given the presence of ER-$\alpha$ in cerebrovascular mitochondria and the prior demonstration that $E_2$ increased mitochondrial DNA-encoded COX IV transcripts in MCF-7 cells (Chen et al., 2004a), we sought to determine whether $E_2$ treatment altered protein levels of subunits of COX IV. Western blot analysis of mitochondrial fractions from OVX and OE cerebral vessels revealed that in vivo $E_2$ treatment significantly increased both mtDNA-encoded subunit I (Fig. 4A) and nuclear-encoded subunit IV COX IV (Fig. 4B) relative to OVX levels.

**Estrogen Increases the Activity of Mitochondrial COX IV and Citrate Synthase.** COX IV activity is rate-limiting for electron transport and thus for energy production (Herzig et al., 2000). In the citric acid cycle, the rate-limiting step is the enzymatic condensation reaction of acetyl CoA and oxaloacetate by citrate synthase. Thus, increases in the activities of these enzymes would strongly support an increased capacity for energy production. Given that $E_2$ has significant effects on the mitochondrial levels of two COX IV subunits, we sought to determine whether these effects correlated with a functional change in enzyme activity. Indeed, measure-

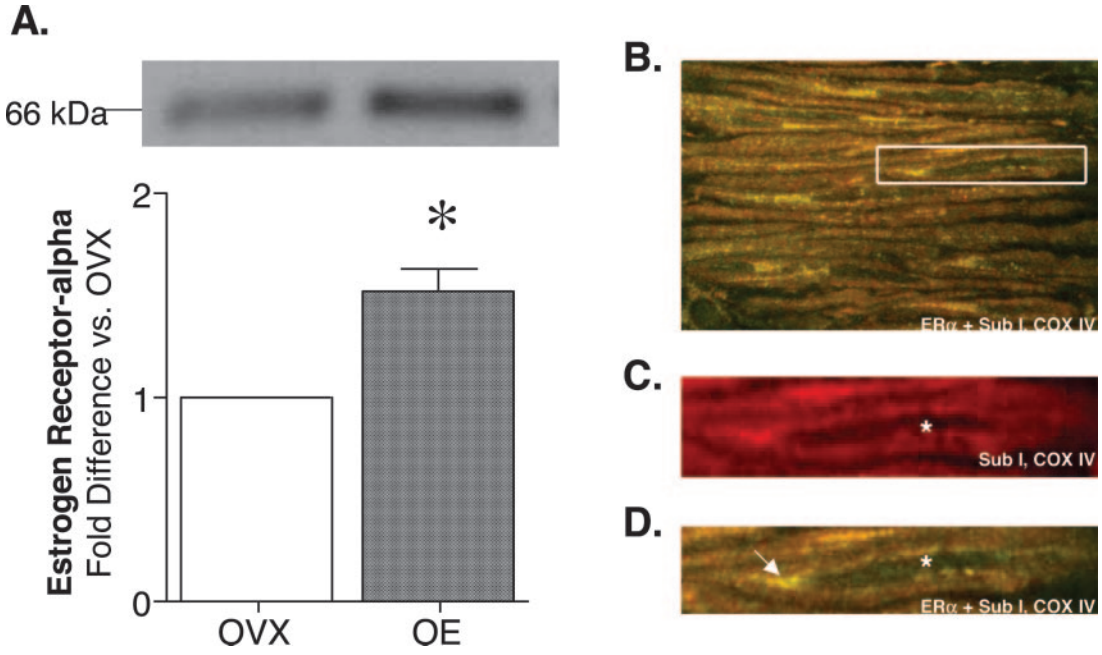


**Fig. 1.** A, effect of $E_2$ on ER-$\alpha$ in cerebral blood vessel mitochondria determined by Western blot. The immunoreactive band shown at 66 kDa was the only band visualized on the Western blot. Mean density values are presented as fold difference compared with OVX ($n = 4$), *, $P \leq 0.05$. B, colocalization of the mitochondrial protein subunit I of COX IV and ER-$\alpha$ by laser scanning confocal microscopy in the smooth muscle of a pial artery from an intact female artery. Dual-staining used an ER-$\alpha$ antibody (green) and an antibody to COX IV subunit I (red). The merged image shows colocalization (yellow). C, enlarged image of a single smooth muscle cell (box in B) shows subunit I of COX IV (red). Asterisk denotes the nucleus, lacking red fluorescence. D, identical cell as in C, revealing colocalization of ER-$\alpha$ with COX IV subunit I (yellow) at one end of the cell and in a thin perinuclear region surrounding the nucleus ($\star$). Only ER-$\alpha$ (green) is present in the nucleus.

ment of COX IV and citrate synthase activities in OE vessel mitochondria relative to OVX controls revealed that $E_2$ treatment in vivo results in a greater than 2-fold increase in both enzyme activities (Fig. 5, A and B).

**Estrogen Increases Nuclear Respiratory Factor-1 Protein Expression in Cerebral Blood Vessels.** NRF-1 is a transcriptional regulator of nuclear-encoded mitochondrial genes, and its induction has been demonstrated to increase protein levels for a wide range of mitochondrial genes (Kelly and Scarpulla, 2004). To determine whether estrogen might be acting through a mechanism involving NRF-1 to modulate mitochondrial protein levels, we performed immunoblot analysis for NRF-1 protein in whole-vessel lysates from cerebral vessels isolated from OVX and OE animals. As shown in Fig. 6, long-term estrogen treatment in vivo significantly increases cerebrovascular NRF-1 protein.

**Effect of Estrogen on Mitochondrial Antioxidant Enzymes and $H_2O_2$ Production in Cerebrovascular Mitochondria.** Our data suggest that $E_2$ can increase the energy capacity of cerebral vessel mitochondria. Increased energy production may also increase ROS production as by-products. Therefore we examined the effect of long-term in vivo $E_2$ treatment on levels of mitochondrial antioxidant enzymes, MnSOD, GPX-1, and catalase. Western blot analysis of mitochondrial fractions isolated from OVX and OE cerebral vessels revealed that $E_2$ significantly increased MnSOD protein but had no effect on levels of GPX-1 or catalase (Fig. 7, A–C).

These data suggest a protective mechanism of $E_2$ through the potential decrease in mitochondrial superoxide levels by MnSOD but also suggest that any increase in energy production caused by $E_2$ stimulation of the electron transport chain could possibly shunt ROS production toward increased hydrogen peroxide. To test this hypothesis, we measured hydrogen peroxide production in mitochondria freshly isolated from OVX and OE cerebral blood vessels (Michelakis et al., 2002). However, as shown in Fig. 7D, succinate-driven mitochondria produce significantly less hydrogen peroxide in $E_2$-treated vessels than in OVX controls.

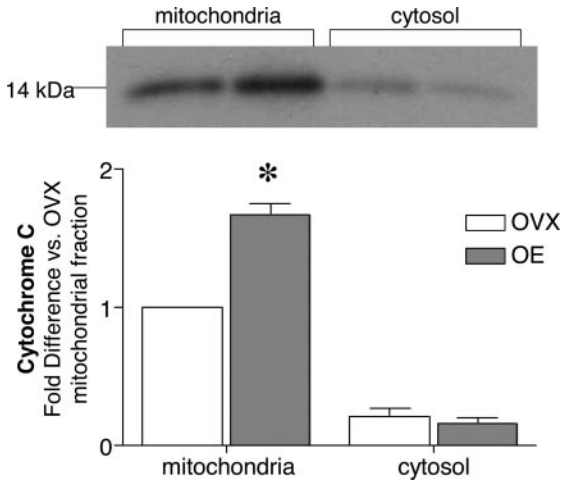


**Fig. 2.** Effect of long-term in vivo $E_2$ treatment on cytochrome *c* in mitochondrial and cytosolic fractions by Western blot analysis. Mean data are represented as fold difference versus OVX mitochondrial fraction ($n = 7$). *, $P \leq 0.05$.

## Discussion

The present study reveals a novel and important protective mechanism exerted by estrogen in the vasculature. We found that estrogen modulates mitochondrial function in cerebral blood vessels, resulting in greater energy production capacity

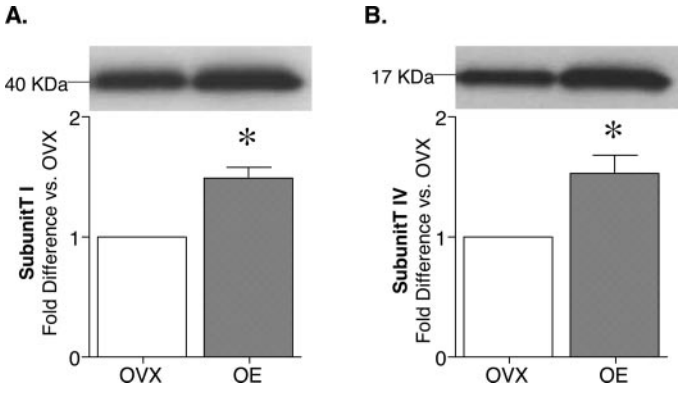


**Fig. 4.** Effect of in vivo $E_2$ exposure on complex IV subunits I and IV from cerebral vessel mitochondria probed by Western blot analysis. Mean values are expressed as fold difference versus OVX ($n = 4$). *, $P \leq 0.05$.

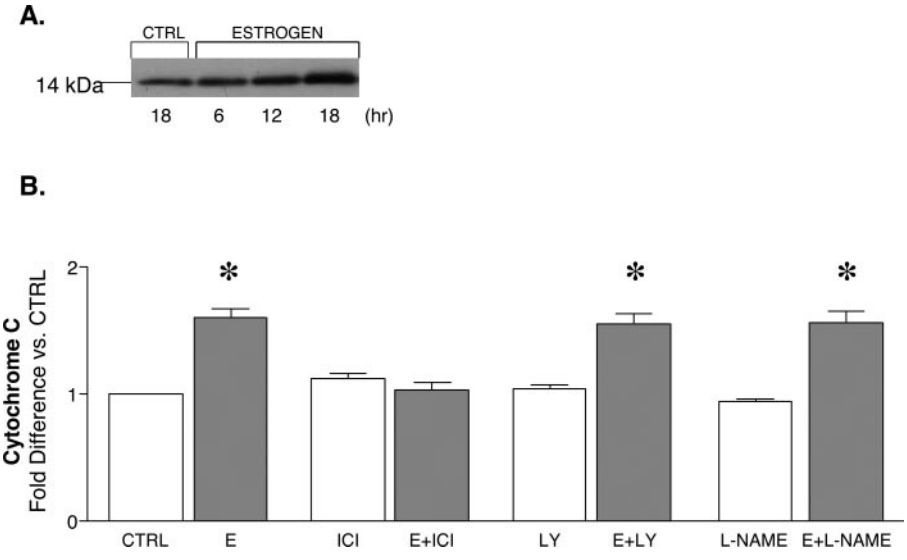


**Fig. 3.** Effect of in vitro $E_2$ on mitochondrial cytochrome *c*. A, Western blot shows time course of in vitro $E_2$ (10 nM) on mitochondrial cytochrome *c*. B, densitometric analysis for mitochondrial cytochrome *c* at 18 h in the absence and presence of different inhibitors ICI-182,780 (ICI), LY294002 (LY), or L-NAME. Mean values are presented as fold difference versus 18-h vehicle control (E alone, $n = 12$; all others, $n = 4$) *, $P \leq 0.05$.

with decreased production of reactive oxygen species. A number of vascular protective mechanisms have been demonstrated for estrogen (McCullough and Hurn, 2003; Orshal and Khalil, 2004; Turgeon et al., 2004). In the cerebral vasculature, we showed previously that estrogen treatment enhances endothelial-dependent dilation and increases the levels and activity of endothelial NOS (Geary et al., 1998; McNeill et al., 2002; Stirone et al., 2005). Estrogen also suppresses the induction of inflammatory markers in cerebral blood vessels (Ospina et al., 2004). Vascular effects of estrogen have been shown to include modulation of nuclear genomic expression and rapid activation of cellular kinase pathways (Orshal and Khalil, 2004; Turgeon et al., 2004; Stirone et al., 2005). We now show that vascular mitochondria are a target for estrogen action. The presence of mitochondrial estrogen receptors and effects on mitochondrial gene expression suggest that estrogen may act directly on the mitochondria. However, estrogen also affects NRF-1, a transcription factor that acts on nuclear genes encoding respiratory subunits such as cytochrome *c* or cytochrome oxidase. Together, this suggests that estrogen coordinates a number of cellular processes to impact mitochondrial function in vascular tissue.

The mtDNA encodes 13 polypeptides of the mitochondrial respiratory chain; the remaining genes reside in the nuclear genome. In addition, mtDNA encodes for two ribosomal RNAs and 22 transfer RNAs required for mitochondrial protein synthesis. Cross-talk between both genomes is required not only for the biogenesis and function of mitochondria, but also for rapid response to changing cellular energy demands and redox status; however, this process remains poorly understood. ER-$\alpha$ is known traditionally as a nuclear receptor, but our demonstration that it is also present in mitochondria suggests the possibility that $E_2$ exerts coordinated effects on both nuclear and mitochondrial gene expression. Several recent studies in nonvascular cultured cells also demonstrated the presence of estrogen receptors in mitochondria and suggest effects of $E_2$ on mitochondrial function and protein expression (Chen et al., 2004b; Yang et al., 2004; Felty and Roy, 2005). Estrogen response elements have been found in the D-loop, in the master regulatory region, and within the structural genes of the mtDNA (Demonacos et al., 1996). A previous study showed that $E_2$ can increase mtDNA transcripts for COX IV subunits I and II in cultured cancer cells (Chen et al., 2004b), which is consistent with the $E_2$-mediated increases in cerebrovascular subunit I protein found in the present study. We reported previously an immunoblot analysis of cerebral vessel lysate that reveals multiple forms of ER-$\alpha$ in the tissue, all of which are increased by the presence of $E_2$ (Stirone et al., 2003). It is interesting to note that in isolated mitochondrial fractions, only the 66-kDa form of ER-$\alpha$ is detected, and its levels are also increased in mitochondria from OE animals. It has been reported that 66-kDa ER-$\alpha$ has superior ability to bind DNA and alter nuclear transcription versus other ER-$\alpha$ subtypes (Li et al., 2003). Thus, it is likely that ER-$\alpha$ binds mtDNA and increases transcription in cerebral vessels, as recently shown in MCF-7 cells using human recombinant 66-kDa ER-$\alpha$ (Chen et al., 2004b).

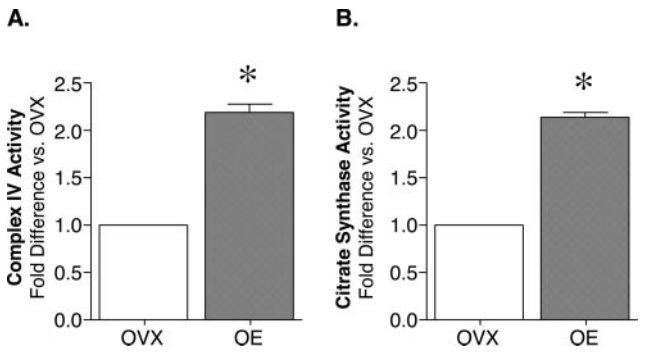


**Fig. 5.** Effect of long-term in vivo $E_2$ exposure on enzyme activities of COX IV (A) and citrate synthase (B) in cerebral vessel mitochondria. Mean data are expressed as fold difference versus OVX ($n = 8$). *, $P \leq 0.05$.

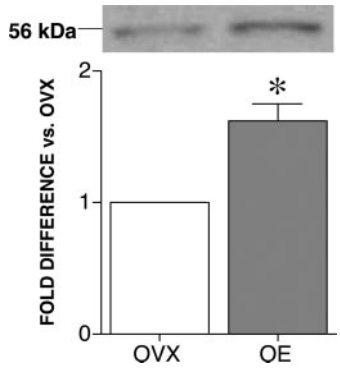


**Fig. 6.** Effect of long-term $E_2$ on cerebrovascular NRF-1 protein. Representative Western blot for NRF-1. Mean data are expressed as fold difference versus OVX ($n = 6$). *, $P \leq 0.05$.

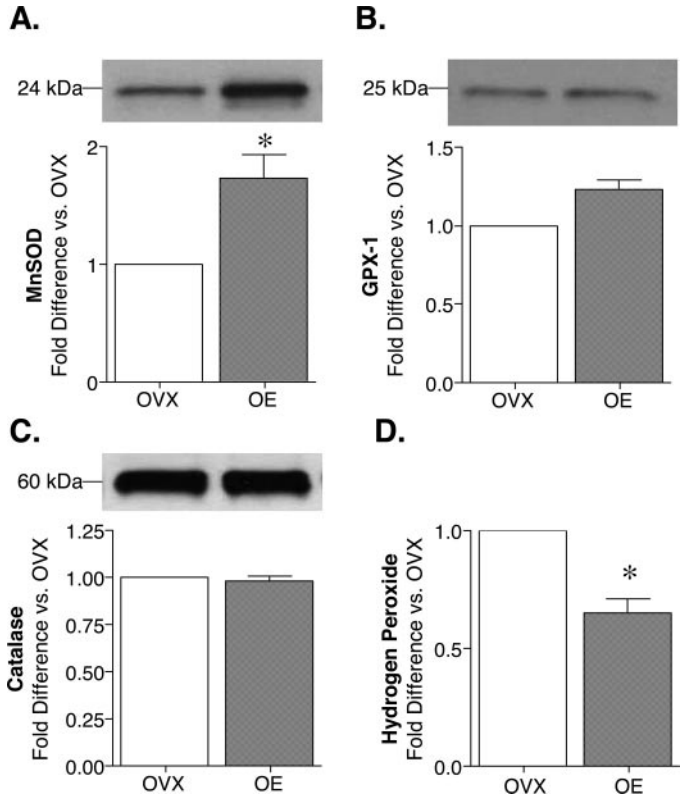


**Fig. 7.** Effect of long-term $E_2$ on mitochondrial ROS-converting proteins and hydrogen peroxide production in cerebral blood vessels. Western blots are shown for MnSOD (A), GPX-1 (B), and catalase (C). D, mitochondrial hydrogen peroxide production. Mean data are expressed as fold difference versus OVX ($n = 4$, MnSOD; $n = 5$, GPX-1; $n = 4$, catalase; and $n = 8$, hydrogen peroxide production). *, $P \leq 0.05$.

Whereas the presence of ER in vascular mitochondria is an important observation, it does not explain estrogen regulation of nuclear-encoded mitochondrial proteins and its overall influence on mitochondrial function. NRF-1 is believed to be a key nuclear transcription regulator responsible for increasing the transcription of nuclear-encoded mitochondrial genes (Kelly and Scarpulla, 2004). Induction of NRF-1 has been demonstrated to increase cytochrome *c* protein and a wide range of other nuclear-encoded mitochondrial proteins (Kelly and Scarpulla, 2004). Although we cannot rule out other mechanisms, the estrogen-mediated increase in cerebrovascular NRF-1 protein in vivo suggests that estrogen acts through NRF-1 to elevate levels of nuclear-encoded mitochondrial proteins. Effects of $E_2$ on the mtDNA may coordinate mitochondrial gene transcription in concert with the transcription of mitochondrial nuclear-encoded genes to regulate oxidative capacity. Together, increases in the levels of both subunits I and IV of complex IV provide a mechanism for the greater COX IV enzyme activity observed in cerebrovascular mitochondria from OE animals. It is important to note that all of these effects were obtained using physiological levels of $E_2$ both in vivo and in vitro.

It is well established that mitochondrial energy production decreases and ROS production increases with age and diseases, including vascular disease (Wallace, 2001; Wisloff et al., 2005). Mitochondrial disorders, which impair bioenergetics and promote ROS production, are common and have been linked to a number of important diseases in humans, including those that result in stroke-like episodes, diabetes, and metabolic and neurological syndromes (Smeitink et al., 2001; Wallace, 2001; Wilson et al., 2004; Lowell and Shulman, 2005). Because mtDNA is in close proximity to ROS produced by electron transport yet has inadequate DNA repair mechanisms, it is prone to oxidative damage (Madamanchi et al., 2005). In the vasculature, the extent of atherosclerosis correlates well with mtDNA damage in both humans and ApoE knockout mice (Ballinger et al., 2002).

Thus, maintenance of energy capacity should provide protection against disease and aging, yet it may occur at the expense of increased ROS production that could negate those benefits. Therefore, we examined mitochondrial protein levels of the three primary antioxidant enzymes, MnSOD, GPX-1, and catalase, involved in scavenging mitochondrial ROS and found that $E_2$ treatment only affected MnSOD. An increase in MnSOD was reported previously for vascular smooth muscle cells, albeit at supraphysiological $E_2$ levels. Increased MnSOD implies that $E_2$ reduces superoxide production in mitochondria (Strehlow et al., 2003); however, without compensatory changes in GPX-1 or catalase, a decrease in superoxide levels could result in increased hydrogen peroxide. To determine whether $E_2$ treatment alters ROS metabolism, we measured hydrogen peroxide produced in OVX and OE vessel mitochondria, driven by succinate. It was surprising that hydrogen peroxide levels were significantly lower in OE vessel mitochondria.

Two possible explanations for lower $H_2O_2$ levels arise from our observation of an $E_2$-mediated increase in cytochrome *c*. Cytochrome *c* is the only known mitochondrial protein proven to be directly and critically involved in all three major functions of mitochondria: energy production, ROS production, and apoptosis. With respect to energy production, cytochrome *c* transports electrons between complexes III and IV, and increased cytochrome *c* could result in increased efficiency of electron transport between these two complexes. Indeed, it has been reported that serum-induced increases in cytochrome *c* are sufficient to enhance mitochondrial respiration, even in the absence of increased citrate synthase activity or increases in COX IV subunit expression (Herzig et al., 2000). Given that complexes I and III produce the bulk of mitochondrial ROS, it has been shown that increases in cytochrome *c* significantly reduce complex III ROS production (Barros et al., 2003; Chen et al., 2003). This is significant, because mitochondria represent the major source of ROS in the cell.

Our data clearly indicate that $E_2$ increases cytochrome *c* in the mitochondrial fractions but not in the cytosol, in which it is important for apoptosis. In addition to well-established roles in energy production and cell-death pathways, cytochrome *c* has been shown to act as an antioxidant and modulate ROS production (Zhao et al., 2003). Thus, a second possible explanation for decreased levels of $H_2O_2$ after $E_2$ treatment may depend on an alternative pathway in which cytochrome *c* can directly supply electrons to superoxide and especially hydrogen peroxide, converting them to $H_2O$ and $O_2$, thus reducing mitochondrial ROS via the so-called electron-leak pathway (Skulachev, 1998; Xu, 2004; Zhao and Xu, 2004). Furthermore, loss of mitochondrial cytochrome *c* has been associated with increased ROS production at complex I, suggesting that in addition to electron-leak, cytochrome *c* may also improve the efficiency of the entire electron transport process (Kushnareva et al., 2002). Although these effects remain to be experimentally demonstrated in the vasculature, they strongly support the hypothesis of a novel vasoprotective mechanism of $E_2$, possibly mediated through increased mitochondrial cytochrome *c* protein.

Our study provides the first evidence for the presence of mitochondrial estrogen receptors in intact vascular tissue and in an important $E_2$ target tissue, the cerebral circulation. Furthermore, these data suggest that physiological levels of $E_2$ in vivo result in both a significant increase in the capacity for cerebrovascular mitochondria to produce energy and a reduction in mitochondrial ROS production. Cytochrome *c* may play a crucial role in these protective effects. Our data indicate that $E_2$ can act through both mitochondrial and nuclear genomes, involving multiple mechanisms, to enhance cerebral vascular mitochondrial function. Given supporting evidence in the literature that beneficial $E_2$-mediated effects on mitochondrial function occur in other cell types, effects of $E_2$ on mitochondrial function may represent a general phenomenon. Thus the ability of $E_2$ to protect against age-related and disease-related decreases in bioenergetics and ROS production may contribute to the longer life span of females.

**Acknowledgments**

We thank Jonnie Stevens for animal surgeries and expert technical assistance and Antonio Davila for technical expertise.

**References**

Ballinger SW, Patterson C, Knight-Lozano CA, Burow DL, Conklin CA, Hu Z, Reuf J, Horaist C, Lebovitz R, Hunter GC, et al. (2002) Mitochondrial integrity and function in atherogenesis. *Circulation* **106:**544–549.

Ballinger SW, Patterson C, Yan CN, Doan R, Burow DL, Young CG, Yakes FM, Van Houten B, Ballinger CA, Freeman BA, et al. (2000) Hydrogen peroxide- and peroxynitrite-induced mitochondrial DNA damage and dysfunction in vascular endothelial and smooth muscle cells. *Circ Res* **86:**960–966.

Barros MH, Netto LE, and Kowaltowski AJ (2003) $H_2O_2$ generation in *Saccharomy-*

*ces cerevisiae* respiratory pet mutants: effect of cytochrome c. *Free Radic Biol Med* **35:**179–188.
Borras C, Sastre J, Garcia-Sala D, Lloret A, Pallardo FV, and Vina J (2003) Mitochondria from females exhibit higher antioxidant gene expression and lower oxidative damage than males. *Free Radic Biol Med* **34:**546–552.
Chen JQ, Delannoy M, Cooke C, and Yager JD (2004a) Mitochondrial localization of ERalpha and ERbeta in human MCF7 cells. *Am J Physiol* **286:**E1011–E1022.
Chen JQ, Eshete M, Alworth WL, and Yager JD (2004b) Binding of MCF-7 cell mitochondrial proteins and recombinant human estrogen receptors alpha and beta to human mitochondrial DNA estrogen response elements. *J Cell Biochem* **93:**358–373.
Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, and Lesnefsky EJ (2003) Production of reactive oxygen species by mitochondria: central role of complex III. *J Biol Chem* **278:**36027–36031.
Demonacos CV, Karayanni N, Hatzoglou E, Tsiriyiotis C, Spandidos DA, and Sekeris CE (1996) Mitochondrial genes as sites of primary action of steroid hormones. *Steroids* **61:**226–232.
Esposito LA, Melov S, Panov A, Cottrell BA, and Wallace DC (1999) Mitochondrial disease in mouse results in increased oxidative stress. *Proc Natl Acad Sci USA* **96:**4820–4825.
Felty Q and Roy D (2005) Estrogen, mitochondria and growth of cancer and non-cancer cells. *J Carcinog* **4:**1.
Geary GG, Krause DN, and Duckles SP (1998) Estrogen reduces myogenic tone through a nitric oxide-dependent mechanism in rat cerebral arteries. *Am J Physiol* **275:**H292–H300.
Herzig RP, Scacco S, and Scarpulla RC (2000) Sequential serum-dependent activation of CREB and NRF-1 leads to enhanced mitochondrial respiration through the induction of cytochrome c. *J Biol Chem* **275:**13134–13141.
Kelly DP and Scarpulla RC (2004) Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. *Genes Dev* **18:**357–368.
Kushnareva Y, Murphy AN, and Andreyev A (2002) Complex I-mediated reactive oxygen species generation: modulation by cytochrome c and NAD(P)+ oxidation-reduction state. *Biochem J* **368:**545–553.
Lesnefsky EJ, Moghaddas S, Tandler B, Kerner J, and Hoppel CL (2001) Mitochondrial dysfunction in cardiac disease: ischemia–reperfusion, aging and heart failure. *J Mol Cell Cardiol* **33:**1065–1089.
Li L, Haynes MP, and Bender JR (2003) Plasma membrane localization and function of the estrogen receptor $\alpha$ variant (ER46) in human endothelial cells. *Proc Natl Acad Sci USA* **100:**4807–4812.
Lowell BB and Shulman GI (2005) Mitochondrial dysfunction and type 2 diabetes. *Science (Wash DC)* **307:**384–387.
Madamanchi NR, Hakim ZS, and Runge MS (2005) Oxidative stress in atherogenesis and arterial thrombosis: the disconnect between cellular studies and clinical outcomes. *J Thromb Haemost* **3:**254–267.
McCullough LD and Hurn PD (2003) Estrogen and ischemic neuroprotection: an integrated view. *Trends Endocrinol Metab* **14:**228–235.
McNeill AM, Zhang C, Stanczyk FZ, Duckles SP, and Krause DN (2002) Estrogen increases endothelial nitric oxide synthase via estrogen receptors in rat cerebral blood vessels: effect preserved after concurrent treatment with medroxyprogesterone acetate or progesterone. *Stroke* **33:**1685–1691.
Mensah GA, Mokdad AH, Ford ES, Greenlund KJ, and Croft JB (2005) State of disparities in cardiovascular health in the United States. *Circulation* **111:**1233–1241.
Michelakis ED, Hampl V, Nsair A, Wu X, Harry G, Haromy A, Gurtu R, and Archer SL (2002) Diversity in mitochondrial function explains differences in vascular oxygen sensing. *Circ Res* **90:**1307–1315.
Monje P and Boland R (2001) Subcellular distribution of native estrogen receptor alpha and beta isoforms in rabbit uterus and ovary. *J Cell Biochem* **82:**467–479.
Orshal JM and Khalil RA (2004) Gender, sex hormones and vascular tone. *Am J Physiol* **286:**R233–R249.
Ospina JA, Brevig HN, Krause DN, and Duckles SP (2004) Estrogen suppresses IL-1beta-mediated induction of COX-2 pathway in rat cerebral blood vessels. *Am J Physiol* **286:**H2010–H2019.
Ramachandran A, Levonen AL, Brookes PS, Ceaser E, Shiva S, Barone MC, and Darley-Usmar V (2002) Mitochondria, nitric oxide and cardiovascular dysfunction. *Free Radic Biol Med* **33:**1465–1474.
Skulachev VP (1998) Cytochrome c in the apoptotic and antioxidant cascades. *FEBS Lett* **423:**275–280.
Smeitink J, van den Heuvel L, and DiMauro S (2001) The genetics and pathology of oxidative phosphorylation. *Nat Rev Genet* **2:**342–352.
Stirone C, Boroujerdi A, Duckles SP, and Krause DN (2005) Estrogen receptor activation of phosphoinositide-3 kinase, akt and nitric oxide signaling in cerebral blood vessels: rapid and long-term effects. *Mol Pharmacol* **67:**105–113.
Stirone C, Duckles SP, and Krause DN (2003) Multiple forms of estrogen receptor-alpha in cerebral blood vessels: regulation by estrogen. *Am J Physiol* **284:**E184–E192.
Strehlow K, Rotter S, Wassmann S, Adam O, Grohe C, Laufs K, Bohm M, and Nickenig G (2003) Modulation of antioxidant enzyme expression and function by estrogen. *Circ Res* **93:**170–177.
Sudlow CL and Warlow CP (1997) Comparable studies of the incidence of stroke and its pathological types: results from an international collaboration. International Stroke Incidence Collaboration. *Stroke* **28:**491–499.
Touyz RM and Schiffrin EL (2004) Reactive oxygen species in vascular biology: implications in hypertension. *Histochem Cell Biol* **122:**339–352.
Trounce IA, Kim YL, Jun AS, and Wallace DC (1996) Assessment of mitochondrial oxidative phosphorylation in patient muscle biopsies, lymphoblasts and transmitochondrial cell lines. *Methods Enzymol* **264:**484–509.
Turgeon JL, McDonnell DP, Martin KA, and Wise PM (2004) Hormone therapy: physiological complexity belies therapeutic simplicity. *Science (Wash DC)* **304:**1269–1273.
Wallace DC (2001) A mitochondrial paradigm for degenerative diseases and ageing. *Novartis Found Symp* **235:**247–266.
Wilson FH, Hariri A, Farhi A, Zhao H, Petersen KF, Toka HR, Nelson-Williams C, Raja KM, Kashgarian M, Shulman GI, et al. (2004) A cluster of metabolic defects caused by mutation in a mitochondrial tRNA. *Science (Wash DC)* **306:**1190–1194.
Wisloff U, Najjar SM, Ellingsen O, Haram PM, Swoap S, Al-Share Q, Fernstrom M, Rezaei K, Lee SJ, Koch LG, et al. (2005) Cardiovascular risk factors emerge after artificial selection for low aerobic capacity. *Science (Wash DC)* **307:**418–420.
Xu JX (2004) Radical metabolism is partner to energy metabolism in mitochondria. *Ann NY Acad Sci* **1011:**57–60.
Yang SH, Liu R, Perez EJ, Wen Y, Stevens SM Jr, Valencia T, Brun-Zinkernagel AM, Prokai L, Will Y, Dykens J, et al. (2004) Mitochondrial localization of estrogen receptor $\beta$. *Proc Natl Acad Sci USA* **101:**4130–4135.
Zhao Y, Wang ZB, and Xu JX (2003) Effect of cytochrome c on the generation and elimination of $O2^{*-}$ and $H_2O_2$ in mitochondria. *J Biol Chem* **278:**2356–2360.
Zhao Y and Xu JX (2004) The operation of the alternative electron-leak pathways mediated by cytochrome c in mitochondria. *Biochem Biophys Res Commun* **317:**980–987.

**Address correspondence to:** Dr. Vincent Procaccio, Center for Molecular and Mitochondrial Medicine and Genetics, University of California Irvine, Irvine, CA 92697. E-mail: vproca@uci.edu