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Estrogen increases mitochondrial efficiency and reduces oxidative stress in cerebral blood vessels*

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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-α (ER-α) 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, i.e., ER-α, cytochrome c, subunit IV of complex IV, and manganese superoxide dismutase, all encoded in the nuclear genome, as well as 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 E_2 treatment increased enzyme activity. In contrast, mitochondrial production of hydrogen peroxide was decreased in vessels from E_2-treated animals. I*n vitro* incubation of cerebral vessels with 10 nM 17ß-estradiol for 18 hr also elevated levels of mitochondrial cytochrome c. This effect was blocked by the ER antagonist ICI-182,780 [fulvestrant (Faslodex)], but unaffected by inhibitors of nitric oxide synthase or phosphoinositide-3-kinase. Nuclear Respiratory Factor-1 protein, a primary regulator of nuclear gene-encoded mitochondrial proteins, was significantly increased by chronic estrogen treatment *in vivo.* In summary, these novel findings suggest 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 as compared to men (McCullough and Hurn, 2003; Mensah et al., 2005; Sudlow and Warlow, 1997). Estrogen (E_2) is thought to play a protective role, since the incidence of vascular disease increases significantly in women after menopause (Mensah et al., 2005; Turgeon et al., 2004). 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.

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; Madamanchi et al., 2005; Ramachandran et al., 2002; Touyz and Schiffrin, 2004; 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 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 17ß-estradiol binding sites, and specifically estrogen receptors, has only recently been demonstrated in several non-vascular tissues and cultured cancer cells (Chen et al., 2004a; Chen et al., 2004b;; Monje and Boland, 2001; Yang et al., 2004). Effects of E_2 on mitochondrial function and protein expression may explain 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; Ospina et al., 2004; Stirone et al., 2005; Stirone et al., 2003). 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.

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Methods

In vivo treatments. Animal procedures were approved by the U.C. Irvine Institutional Animal Care and Use Committee. Fischer 344 female rats (3 months old, Charles River Laboratories) were used: ovariectomized (OVX), or ovariectomized and treated chronically with a subcutaneous 17β-estradiol implant (OE) (Geary et al., 1998; Ospina et al., 2004; Stirone et al., 2003). After 4 weeks of treatment animals were anesthetized by CO_2 and killed by decapitation. We have previously demonstrated that estrogen levels in OE animals are within the physiological range and significantly higher than OVX (Geary et al., 1998; McNeill et al., 2002). Serum 17β-estradiol levels were 13.58 ± 2.8 pg/ mL for OVX and 77.98 ± 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 ± 2 g for OVX and 163 ± 1 g for OE ($P \leq 0.05$). Uterine weights were 35 ± 2 mg for OVX and 126 ± 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 μm mesh as described previously (McNeill et al., 2002; Stirone et al., 2005; Stirone et al., 2003). 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) as per 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 previously described (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-α), H-150 (ER-β), histone H1 (Santa Cruz); α-actin, catalase (Sigma); MnSOD, glutathione peroxidase-1 (GPX-1) (Esposito et al., 1999); complex IV (COX IV) subunits I and IV, porin (Molecular Probes); Nuclear Respiratory Factor-1 (NRF-1; a gift from the Scarpulla laboratory). Appropriate secondary antibodies were used, and the bands visualized using enhanced chemiluminescence reagent and Hyperfilm (Amersham). UN-SCAN-IT software (Silk Scientific) was used for densitometric analysis of immunoreactive bands. As appropriate, mitochondrial porin or α-actin protein levels were determined for each blot to verify equal protein loading.

Confocal microscopy. Pial vessels were prepared as previously described (Ospina et al., 2004; Stirone et al., 2005). Primary antibodies were directed against ER-α HC-20 (Santa Cruz) 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.

In vitro experiments. Freshly isolated cerebral blood vessels from OVX female rats were pre-equilibrated at 37ºC as described previously (McNeill et al., 2002). In all *in vitro* experiments, vessels were incubated in either 10 nM 17ß-estradiol (encapsulated in 2-hydroxy-propyl-ß-

cyclodextrin; Sigma) or an equivalent concentration of 2-hydroxy-propyl-ß-cyclodextrin alone (vehicle control). In some experiments, the estrogen receptor antagonist ICI-182,780 (1 μM; Tocris), PI-3 kinase inhibitor LY294002 [2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride] (10 μM; Calbiochem), or the eNOS inhibitor, L-NAME [N^G-nitro-L-arginine-methyl ester] (100 μM; Sigma) were included for a 30 min pre-equilibration period and maintained during 17ß-estradiol or vehicle treatment. Vessels were maintained at 37°C in 95% O_2/5% CO_2 for 6-18 hours with drug(s), followed by mitochondrial isolation or whole vessel lysis.

Enzymatic Measurements and H_2O_2 assays. Enzyme assays, cytochrome c 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 2 independent sets of samples.

Statistical analyses. All data values are given as mean ± SEM. Statistical differences were determined by Student's *t* test, or where appropriate, one-way ANOVA with repeated measures followed by Dunnett's multiple comparison tests. In all cases, statistical significance was set at $P \leq 0.05$.

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Results

Estrogen receptor-alpha in cerebrovascular mitochondria

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

To further validate the presence of ER-α in cerebrovascular mitochondria, we used immunohistochemistry and confocal microscopy. Antibodies against ER-α (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 (DAPI-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 co-localization (yellow) of ER-α and the mitochondrial protein, COX IV, subunit I. In Figure 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 absent from the nucleus. Figure 1D shows the addition of the ER-α fluorescence (green), which is found alone in the nucleus, but is visualized as yellow where it co-localizes with subunit I of COX IV. Colocalization is most strikingly at one end of the cell (denoted by the arrow) and in the thin perinuclear region.

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Estrogen increases cytochrome c in cerebrovascular mitochondria

Cytochrome c is critically involved in energy production, apoptosis and ROS production in mitochondria. To determine if *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 (Figure 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 17ß-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 to 18 hr). Cytochrome c protein is significantly elevated after 18 hr of E_2 exposure.

To determine if 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 (Figure 3B). We previously demonstrated that cerebrovascular estrogen receptors stimulate PI-3 kinase/Akt signaling as well as increase endothelial nitric-oxide (NO) production (McNeill et al., 2002; Stirone et al., 2005). Since both PI-3 kinase activation as well as NO 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

(Figure 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-α 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 if 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 (Figure 4A) and nuclear-encoded subunit IV COX IV (Figure 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 if these effects correlated with a functional change in enzyme activity. Indeed, measurement 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 (Figure 5A and 5B).

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Estrogen increases Nuclear Respiratory Factor-1 protein expression in cerebral blood vessels

Nuclear Respiratory Factor-1 (NRF-1) is a transcriptional regulator of nuclear-encoded mitochondrial proteins and its induction has been demonstrated to increase protein levels for a wide range of mitochondrial genes (Kelly et al., 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 Figure 6, chronic 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 chronic *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 (Figure 7A-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 due to E_2 stimulation of the electron transport chain could possibly shunt ROS production towards increased hydrogen peroxide. To test this hypothesis, we measured hydrogen peroxide production in mitochondria freshly isolated from OVX and OE cerebral blood vessels

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(Michelakis et al., 2002). However, as shown in Figure 7D, succinate-driven mitochondria produce significantly less hydrogen peroxide in E_2-treated vessels vs OVX controls.

Discussion

The present study reveals a novel and important protective mechanism exerted by estrogen in the vasculature. We found estrogen modulates mitochondrial function in cerebral blood vessels resulting in greater energy production capacity 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 previously showed estrogen treatment enhances endothelial-dependent dilation and increases the levels and activity of eNOS (Geary et al., 1998; McNeill et al., 2002; Stirone et al., 2005). Estrogen also suppresses 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 as well as rapid activation of cellular kinase pathways (Orshal and Khalil, 2004; Turgeon et al., 2004, Stirone et al., 2005). We now show vascular mitochondria are a target for estrogen action. The presence of mitochondrial estrogen receptors and effects on mitochondrial gene expression suggest 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 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 2 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-α is classically known 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 non-vascular 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; Felty and Roy, 2005; Yang et al., 2004). Estrogen response elements have been found in the D-loop, master regulatory region and within the structural genes of the mtDNA (Demonacos et al., 1996). A previous study showed 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 previously reported immunoblot analysis of cerebral vessel lysate that reveals multiple forms of ER-α 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-α is detected, and its levels are also increased in mitochondria from OE animals. It has been reported that 66 kDa ER-α has superior ability to bind DNA and alter nuclear transcription vs other ER-α subtypes (Li et al., 2003). Thus, it is likely that ER-α binds mtDNA and increases transcription in cerebral vessels, as recently shown in MCF-7 cells using human recombinant 66 kDa ER-α (Chen et al., 2004b).

While 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 thought to be a key nuclear transcription regulator responsible for increasing the transcription of nuclear-encoded mitochondrial genes (Kelly et al., 2004). Induction of NRF-1 has been demonstrated to increase cytochrome c protein and a wide range of other nuclear-encoded mitochondrial proteins (Kelly et al., 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. Importantly, all these effects were obtained using physiological levels of E_2 both *in vivo* and *in vitro*.

It is well established that mitochondrial energy production declines 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, metabolic and neurological syndromes (Lowell and Shulman, 2005; Smeitink et al., 2001; Wallace, 2001; Wilson et al., 2004). 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. Surprisingly, 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 Complex 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 as 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 the cytosol, where 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 as well as a reduction in mitochondrial ROS production. Cytochrome c may play a crucial role in these protective effects. Our data indicate 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 declines in bioenergetics and ROS production may contribute to the longer life span of females.

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FOOTNOTES

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Figure Legends

Figure 1. (A) Effect of E_2 on ER-α 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 to OVX (N=4), *P≤0.05. (B) Co-localization of the mitochondrial protein subunit I of COX IV and ER-α by laser scanning confocal microscopy in the smooth muscle of a pial artery from an intact female artery. Dual-staining used an ER-α antibody (green) and an antibody to COX IV subunit I (red). The merged image shows co-localization (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 co-localization of ER-α with COX IV subunit I (yellow) at one end of the cell and in a thin perinuclear region surrounding the nucleus (asterisk). Only ER-α (green) is present in the nucleus.

Figure 2. Effect of chronic *in vivo* E_2 treatment on cytochrome c in mitochondrial and cytosolic fractions by Western blot analysis. Mean data are represented as fold difference vs. OVX mitochondrial fraction. (N=7). *P≤0.05

Figure 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 hr in the absence and presence of different inhibitors ICI-

182,780 (ICI), LY294002 (LY), or L-NAME. Mean values are presented as fold difference vs. 18 hr vehicle control. (E alone, N=12; all others, N=4) *P≤0.05.

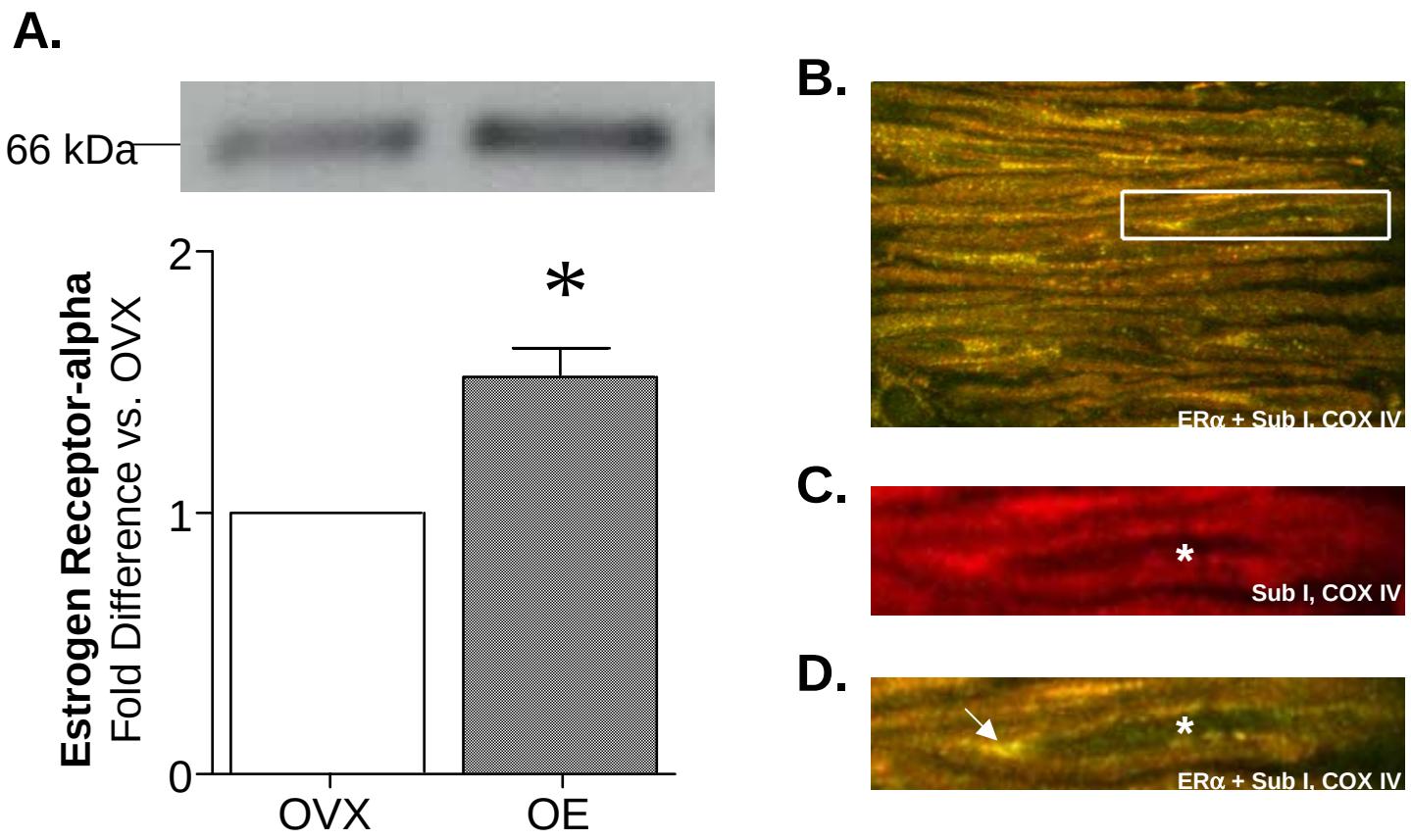
Figure 4. Effect of *in vivo* E_2 exposure on Complex IV subunits I and IV from cerebral vessel mitochondria probed by Western blot analysis. Mean values are expressed as fold difference vs. OVX. (N=4) *P≤0.05.

Figure 5. Effect of chronic *in vivo* E_2 exposure on enzyme activities of (A) COX IV and (B) citrate synthase in cerebral vessel mitochondria. Mean data are expressed as fold difference vs. OVX. (N=8) *P≤0.05.

Figure 6. Effect of chronic E_2 on cerebrovascular NRF-1 protein. Representative Western blot for NRF-1. Mean data are expressed as fold difference vs. OVX. (N=6) *P≤0.05.

Figure 7. Effect of chronic E_2 on mitochondrial ROS-converting proteins and hydrogen peroxide production in cerebral blood vessels. Western blots are shown for (A) MnSOD, (B) GPX-1 and (C) catalase. (D) Mitochondrial hydrogen peroxide production. Mean data are expressed as fold difference vs. OVX. (N=4, MnSOD; 5, GPX-1; 4, Catalase; 8, hydrogen peroxide production) *P≤0.05.

Figure. 1



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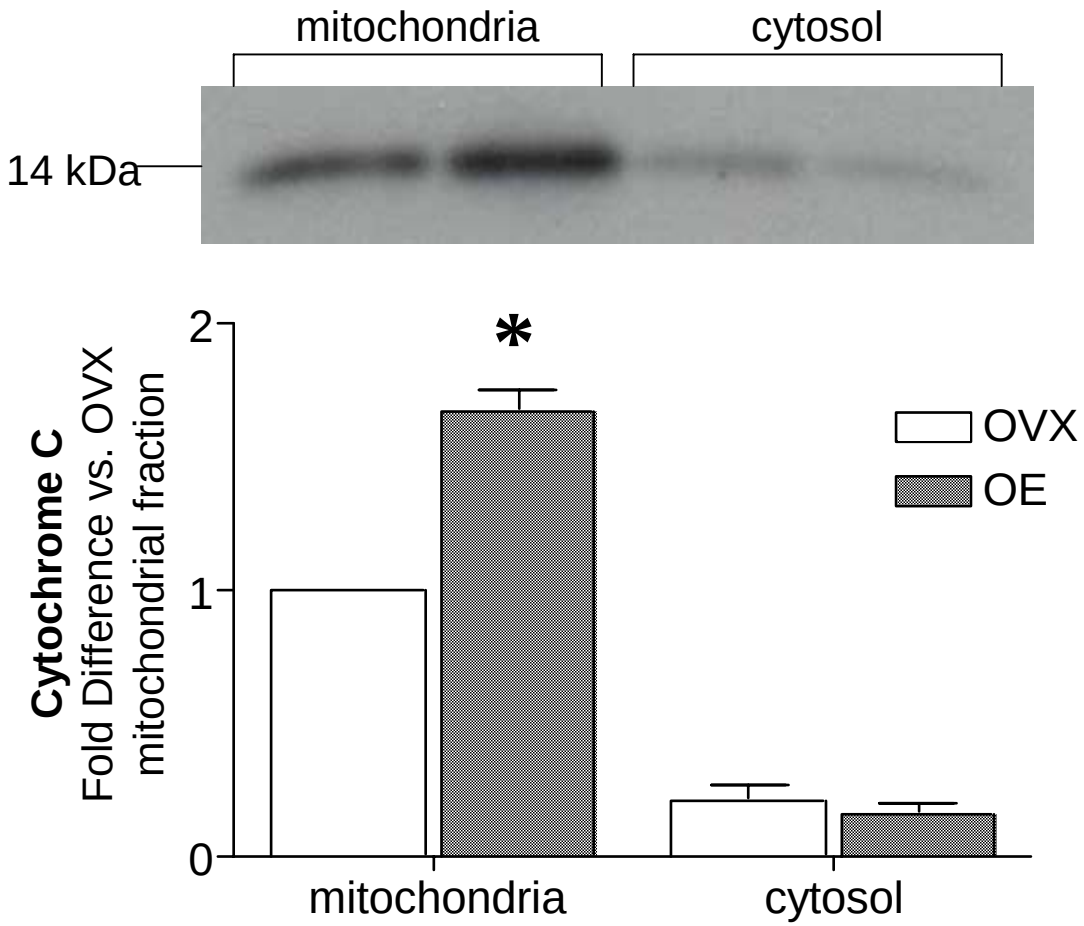

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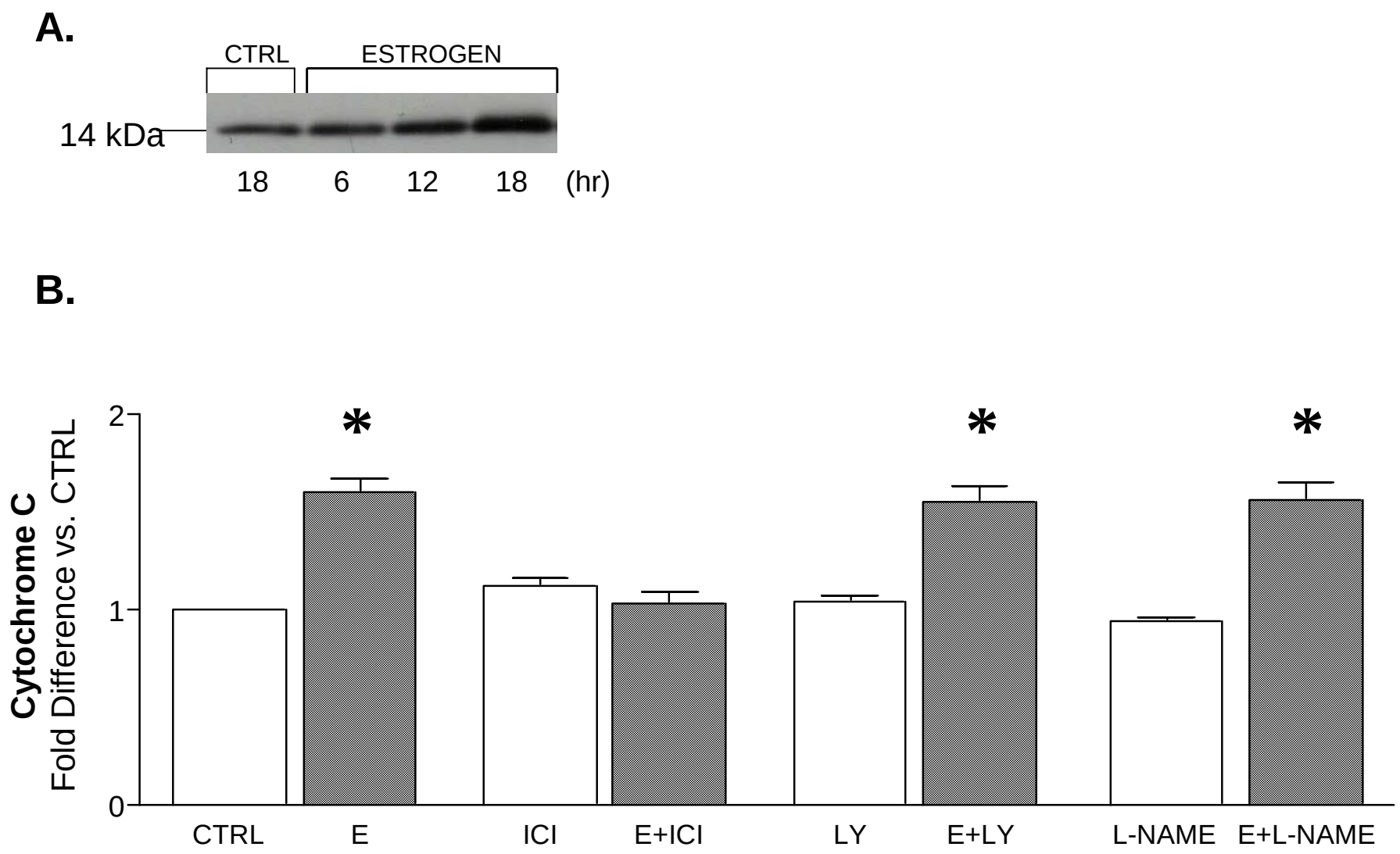

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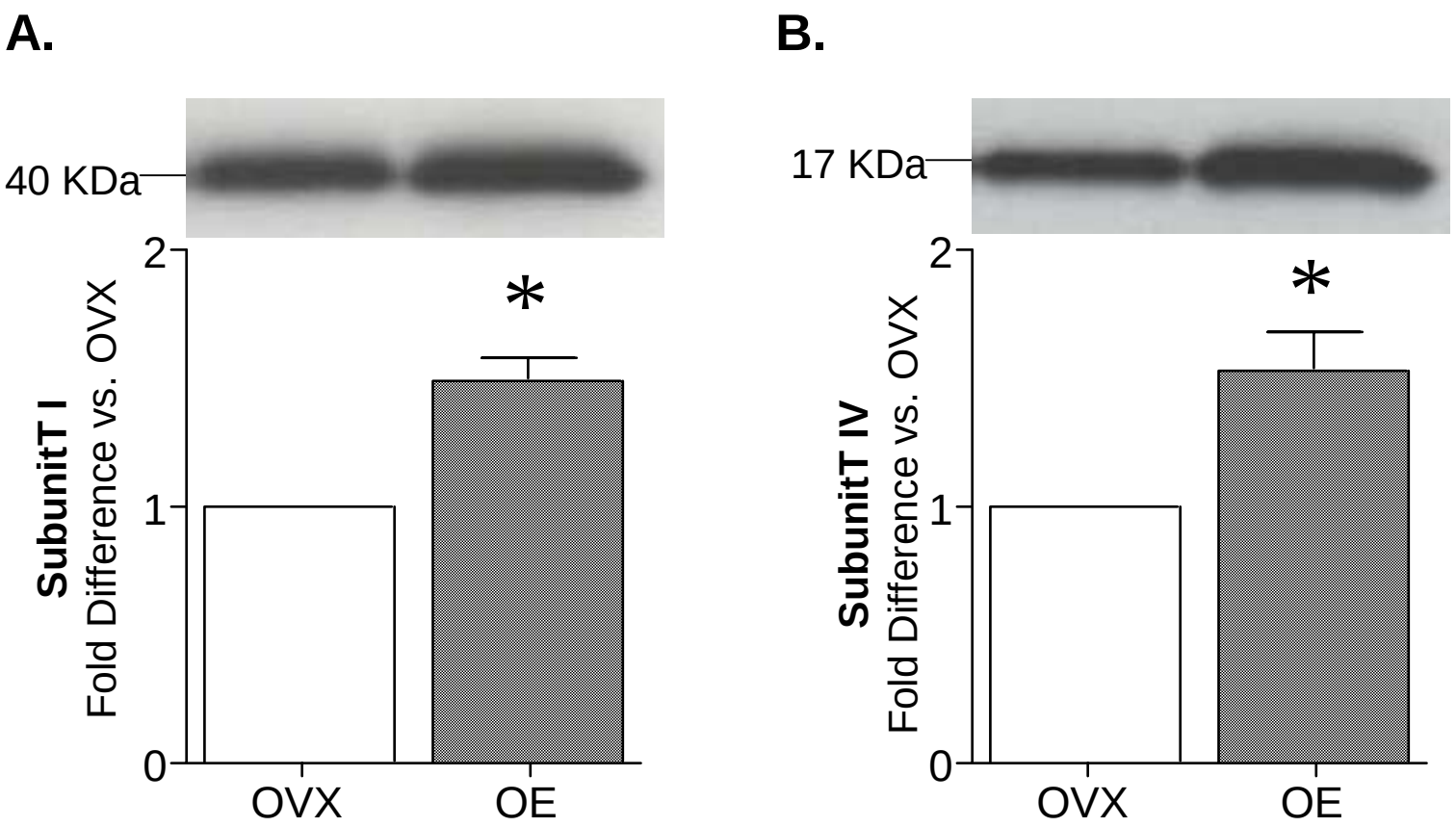

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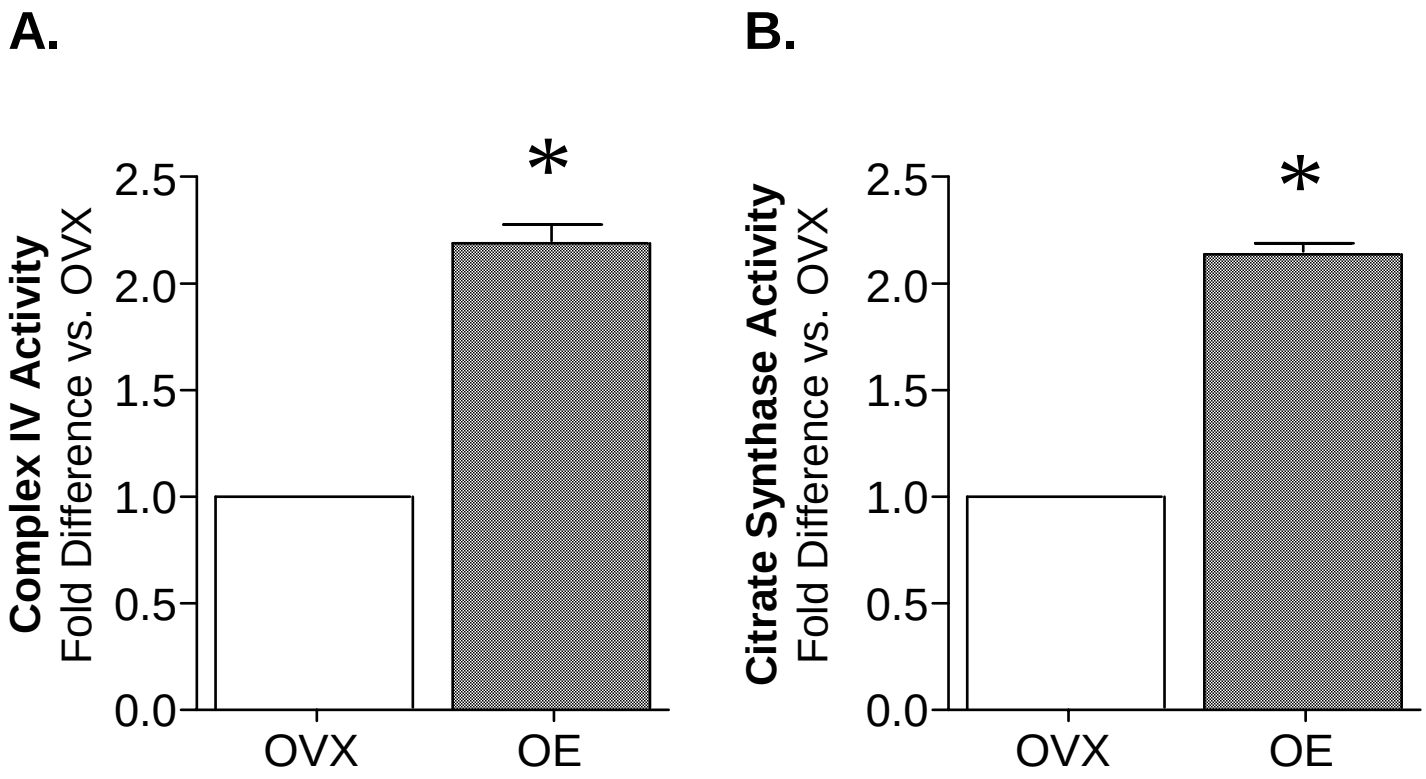

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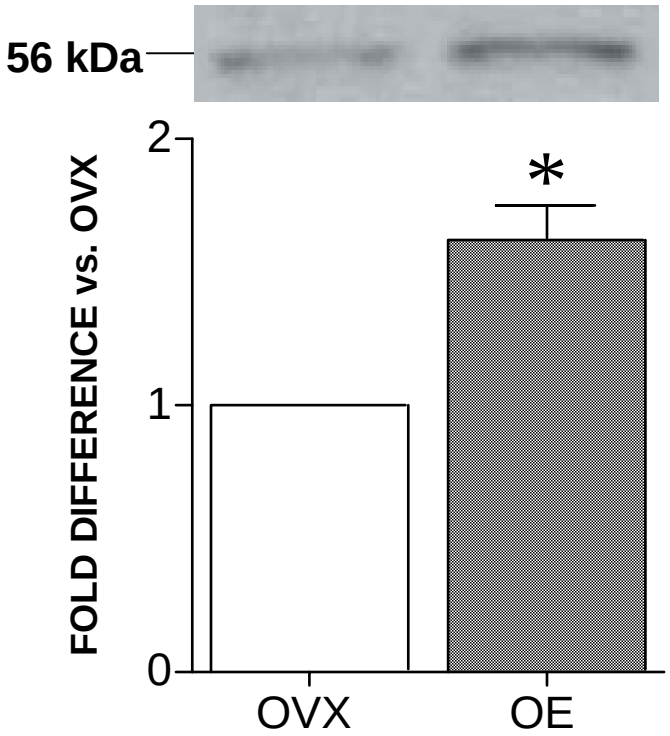

Figure. 7