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Genomic and non-genomic regulation of PGC1 isoforms by estrogen to increase cerebral vascular mitochondrial biogenesis and reactive oxygen species protection

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Abstract

We previously found that estrogen exerts a novel protective effect on mitochondria in brain vasculature. Here we demonstrate in rat cerebral blood vessels that 17β-estradiol (estrogen), both *in vivo* and *ex vivo*, affects key transcriptional coactivators responsible for mitochondrial regulation. Treatment of ovariectomized rats with estrogen *in vivo* lowered mRNA levels of peroxisome proliferator-activated receptor-γ coactivator-1 alpha (PGC-1α) but increased levels of the other PGC-1 isoforms: PGC-1β and PGC-1 related coactivator (PRC). In vessels *ex vivo*, estrogen decreased protein levels of PGC-1α via activation of phosphatidylinositol 3-kinase (PI3K). Estrogen treatment also increased phosphorylation of forkhead transcription factor, FoxO1, a known pathway for PGC-1α downregulation. In contrast to the decrease in PGC-1α, estrogen increased protein levels of nuclear respiratory factor 1, a known PGC target and mediator of mitochondrial biogenesis. The latter effect of estrogen was independent of PI3K, suggesting a separate mechanism consistent with increased expression of PGC-1β and PRC. We demonstrated increased mitochondrial biogenesis following estrogen treatment *in vivo*; cerebrovascular levels of mitochondrial transcription factor A and electron transport chain subunits as well as the mitochondrial/ nuclear DNA ratio were increased. We examined a downstream target of PGC-1β, glutamate-cysteine ligase (GCL), the rate-limiting enzyme for glutathione synthesis. *In vivo* estrogen increased protein levels of both GCL subunits and total glutathione levels. Together these data show estrogen differentially regulates PGC-1 isoforms in brain vasculature, underscoring the importance of these coactivators in adapting mitochondria in specific tissues. By upregulating PGC-1β and/or PRC, estrogen appears to enhance mitochondrial biogenesis, function and reactive oxygen species protection.

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Keywords

Cerebral blood vessels; Estrogen; Glutamate-cysteine ligase; Mitochondria; Peroxisome proliferator-activated; receptor-gamma coactivator-1 (PGC-1); Glutathione

1. Introduction

We have identified a novel protective mechanism of estrogen in the cerebral circulation: estrogen alters mitochondria to increase efficiency of energy production while decreasing levels of reactive oxygen species (Duckles et al., 2006; Stirone et al., 2005b). Mitochondrial dysfunction and reactive oxygen species production play central roles in the pathophysiology of cardiovascular disease and aging (Davidson and Duchen, 2007; Madamanchi et al., 2005; Ramachandran et al., 2002; Yu et al., 2012). Thus it is critical to determine the mechanisms by which estrogen influences cerebrovascular mitochondria.

Mitochondrial biogenesis and function are regulated by master transcriptional coactivators. They coordinate expression of numerous downstream effectors from both nuclear and mitochondrial genomes so that cells can adapt to variable energy demands (Handschin and Spiegelman, 2006; Scarpulla, 2011). A family of nuclear co-activators, the PGC-1 family, has emerged as dominant regulators of oxidative metabolism (Handschin and Spiegelman, 2006; Scarpulla, 2011). Moreover, dysfunction of PGC-1 coactivators contributes to diseases such as diabetes, obesity, cardiomyopathy and neurodegeneration (Handschin and Spiegelman, 2006). We previously found that one key PGC-1 effector, nuclear respiratory factor 1 (NRF-1), is increased in brain blood vessels following estrogen treatment *in vivo* (Stirone et al., 2005b). Thus we hypothesized estrogen affects mitochondrial function by altering PGC-1 master regulators.

All three PGC-1 family members: PGC-1α, PGC-1β and PRC, influence mitochondrial function and mitochondrial biogenesis. Most is known about PGC-1α, extensively studied in skeletal muscle and brown fat, where it strongly promotes energy production and mitochondrial biogenesis (Liang and Ward, 2006; Lin et al., 2005). Additionally, in skeletal muscle, PGC-1α promotes fiber-type switching from glycolytic to oxidative, and in brown fat it increases electron transport uncoupling and adaptive thermogenesis.

Less is known regarding the other two members of the PGC-1 family, however several significant differences between PGC-1α and PGC-1β have emerged (Handschin and Spiegelman, 2006; St-Pierre et al., 2003). The degree to which mitochondrial electron transport is coupled to ATP production differs depending on which PGC-1 isoform is dominant. PGC-1α has been demonstrated to produce more uncoupled mitochondria (St-Pierre et al., 2003). PGC-1β appears to have a stronger influence over antioxidant proteins, such as manganese superoxide dismutase (MnSOD) and glutathione synthetic enzymes (St-Pierre et al., 2003). Interestingly, target genes associated with PGC-1β correlate well with the estrogen-induced changes we previously described: increased mitochondrial efficiency with decreased ROS (Stirone et al., 2005b). We hypothesized that regulation of PGC-1β may underlie estrogen-mediated changes in cerebrovascular mitochondria.

Few studies have explored the role of PGC-1 coactivators in vascular tissue. However, the high mitochondrial content of cerebrovascular endothelium suggests PGC-1 function may be particularly important in brain vessels. We recently demonstrated that levels of PGC-1α and PGC-1β in mouse cerebral endothelial cells were altered after ovariectomy (Kemper et al., 2013; Stirone et al., 2005b). We hypothesized estrogen would restore the effect of ovariectomy on PGC-1 coactivators in brain blood vessels. In the current study, we provide the first evidence that estrogen differentially regulates PGC-1 isoforms in vascular tissue to improve mitochondrial reactive oxygen species management and ATP production involving genomic and non-genomic pathways.

2. Materials and methods

2.1. In vivo treatments

All animal procedures were conducted in an AAALAC-accredited facility in accordance with protocols approved by the UC Irvine Institutional Animal Care and Use Committee. Two groups of Fischer 344 female rats (3 months old, Charles River Laboratories) were used: ovariectomized implanted with a placebo (OVX), or ovariectomized and treated with estrogen, by implanting a silastic tube containing 17β-estradiol subcutaneously (OVX+E) as previously described (Geary et al., 1998; Ospina et al., 2004; Stirone et al., 2003). After 3 weeks of treatment, animals were anesthetized by isoflurane and killed by decapitation. We have previously demonstrated that levels of estrogen in OVX+E animals are within the physiological range and significantly higher than OVX (Geary et al., 1998; McNeill et al., 2002; Stirone et al., 2005b). The physiological relevance of the OVX+E treatment was further validated by expected effects on body and uterine weight. Body weights were 184±3 g for OVX and 161±3 g for OVX+E ($P < 0.05$). Uterine weights were 37±2mg for OVX and 120±5mg for OVX+E ($P < 0.05$).

2.2. Cerebral blood vessel isolation

Blood vessels were isolated from whole brain homogenates by centrifugation through 15% dextran and collection on a 70 μm mesh as described previously (McNeill et al., 2002; Stirone et al., 2005b). This preparation contains a mixture of arteries, arterioles, capillaries, veins, and venules.

2.3. Western blot analysis

Whole vessel lysates were prepared as previously described (Stirone et al., 2005b). Equal amounts of protein were loaded in each lane of a 4–12% Bis–Tris or 16% Tris–glycine gel (Invitrogen, Carlsbad, CA) and separated by SDS-polyacrylamide gel electrophoresis. Samples from each of the experimental groups were always run together on the same gel for comparison. Proteins were then transferred to PVDF Immobilon-FL (Millipore, Burlington, MA) membranes, incubated in blocking buffer and treated with primary antibodies: PGC-1α, FoxO1 (forkhead transcription factor), p-FoxO1 (SER-256), (Santa Cruz Biochemicals, Santa Cruz, CA); PCC-1β (Aviva Systems Biology, San Diego, CA); α-actin (Sigma, St. Louis, MO); nuclear respiratory factor-1 (NRF-1; a gift from the Scarpulla laboratory); TFAM (Aviva Systems Biology, San Diego, CA), glutamate-cysteine ligase modulatory domain (GCLm), gift from Luderer Lab, described in (Thompson et al., 1999)

and catalytic domain (GCLc, NeoMarkers, Fremont, CA); and MitoProfile Total OXPHOS Rodent Antibody Cocktail (Mitosciences/Abcam, Eugene, OR). Appropriate secondary antibodies were used, and the bands were visualized using either enhanced chemiluminescence reagent and Hyperfilm (GE Healthcare, Piscataway, NJ) or LICOR Biosystems Odyssey. UN-SCAN-IT software (Silk Scientific, Orem, UT) was used for densitometric analysis of immunoreactive bands on film, and Odyssey software was used for quantification of fluorescent bands. To assess protein loading of the gel, α-actin protein levels were determined for each lane in a blot and used to correct values for bands of interest on the same blot. Corrected optical densities were used for statistical analysis. Figures are plotted as actin ratios or as fold difference from one experimental group, obtained by normalizing values from each blot.

2.4. Ex vivo vessel experiments

In some cases, freshly isolated cerebral blood vessels from OVX female rats were pre-equilibrated and treated with 17β-estradiol in PBS at 37 °C as described previously. The concentration of 17β-estradiol used in the current study was selected based on concentration response studies from previous work (McNeill et al., 2002; Stirone et al., 2005b). For these *ex vivo* experiments, vessels were incubated in either 10 nM 17β-estradiol encapsulated in 2-hydroxy-propyl-β-cyclodextrin (Sigma Chemical, St. Louis, MO) or an equivalent concentration of 2-hydroxy-propyl-β-cyclodextrin alone (vehicle control, Sigma Chemical, St. Louis, MO) at 37 °C in 95% O2/5% CO_2 for 6–18 h. In some experiments, the estrogen receptor antagonist ICI-182,780 (1 μM; Tocris, Ellisville, MI), PI-3 kinase inhibitor LY294002 [2-(4-morpholinyl)-8-phenyl-1(4 H)- benzopyran-4-one hydrochloride] (10 μM; Calbiochem, San Diego, CA), or the eNOS inhibitor, L-NAME [N^G-nitro-L-arginine-methyl ester] (100 μM; Sigma) were added to the vessels for a 30 min pre-equilibration period and then maintained during the 17β-estradiol or vehicle treatment. Following incubation, vessels were lysed for western blot analysis.

2.5. Quantitative real-time PCR

RNA extraction from cerebral blood vessels was performed using Trizol reagent according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA), and purified after RNAse-free DNase (Roche, Mannheim, Germany) treatment with RNeasy columns (Qiagen, Valencia, CA). First strand cDNA synthesis was then performed using the SuperScriptTM kit (Invitrogen), and qRTPCR performed using the FastStart DNA Master SYBR Green kit in a Light Cycler (Roche). The mRNA levels of PGC-1α, PGC-1β, and PRC were normalized to actin, and the results compared to a standard curve. Oligonucleotide forward and reverse primers used for rat PGC-1α were (5′-cagtcaagctgtttttgacgac-3′ and 5′-cggagagttaaaggaagagcaa- 3′), rat PGC-1β (5′-gtacagctcattcgctacatgc-3′ and 5′-ccagaagttcccttaggatgg-3′), rat PRC (5′-tgacaactcggtgaataccttg-3′ and 5′-ggtgaggttgggtaagcagtag-3′) and actin (5′-cactggcatcgtgatggact- 3′ and 5′-caggaaggaaggctggaaga-3′). The specificity of PCR products was controlled by sequencing, and the relative amounts of transcripts were calculated on the basis of crossing point analysis. Results from three independent amplifications for each sample, analyzed in duplicate, were combined for statistical analysis.

2.6. Mitochondrial/nuclear DNA ratios

DNA was extracted from cerebral blood vessels using Gentra Puregene Tissue Kit according to the manufacturer's recommendations. qPCR measurements of the ratio between nuclear DNA marker tubulin-specific chaperone A (TBCA) and the mitochondrial DNA marker 12S rRNA were computed using the ΔCt method (Livak and Schmittgen, 2001). Oligonucleotide forward and reverse primers used for TBCA were (5′-tgtcccacctgtttgttctct-3′ and 5′-ggtccttggacacctagacg-3′) and 12S rRNA (5′-ctcaagacgccttgcctagc-3′ and 5′-tcgtatgaccgcggtggct-3′) (Arduini et al., 2011). Crossing points for each sample were computed as an average of wells amplified in triplicate on an ABI 7000 machine using SYBR Green (Fermentas/Fisher Scientific).

2.7. Total glutathione assay

Total glutathione was extracted from isolated blood vessels of OVX and OE animals by homogenization in cold TESSB buffer (20 mM Tris, 1 mM EDTA, 20 mM sodium borate, 2 mM serine, pH 7.4) (White et al., 2003) and protein precipitation by 5% sulfosalicylic acid, a procedure which does not precipitate small peptides, such as glutathione, as previously described (Tsai-Turton and Luderer, 2005). Total glutathione was measured using an enzymatic recycling assay modified for use in a microplate reader (Tsai-Turton and Luderer, 2005).

2.8. Statistical analyses

All data values are given as mean±SEM. Statistical differences were determined by Student's *t*-test, or where appropriate, oneway ANOVA with repeated measures followed by the Newman– Keuls post-hoc analysis. In all cases, statistical significance was set at $P \leq 0.05$.

3. Results

3.1. Estrogen differentially regulates PGC-1 isoforms

In order to determine if estrogen treatment modulates expression of PGC-1 isoforms, we performed quantitative real-time PCR experiments using total RNA isolated from cerebral blood vessels of OVX and OVX+E rats. PGC-1α mRNA was significantly reduced in OVX +E blood vessels compared to those of OVX animals (Fig. 1A). In contrast, *in vivo* estrogen treatment significantly increased levels of both PGC-1β (Fig. 1B) and PRC (Fig. 1C) mRNA in cerebral blood vessels.

3.2. Estrogen decreases PGC-1α protein in vivo and ex vivo

We previously found that estrogen increases expression of nuclear-encoded mitochondrial proteins in cerebral vessels (Stirone et al., 2005b), so we were surprised to see that PGC-1α mRNA was decreased by estrogen treatment. We then verified whether estrogen also influences the level of PGC-1α protein. As shown in Fig. 2A, western blot analysis of cerebral vessels from OVX and OVX+E animals revealed that chronic *in vivo* treatment with estrogen also resulted in a significant down-regulation of PGC-1α protein (44±10% decrease *vs*. OVX, $n=4$; *, $P < 0.05$).

To validate that this *in vivo* effect was a direct effect of estrogen on the cerebral blood vessels and to determine the time course by which this effect occurred, we isolated cerebral vessels from OVX animals and treated them with 17β-estradiol (10 nM) *ex vivo*. Fig. 2B shows a western blot of PGC-1α protein in isolated cerebral blood vessels exposed *ex vivo* to estrogen for 6–18 h. PGC-1α protein was significantly decreased at 18 h. As shown in Fig. 2C, addition of the estrogen receptor antagonist, ICI-182,780, fully inhibited the ability of estrogen to decrease levels of PGC-1α in the vessels. Both PI_3 kinase (PI3K) and nitric oxide (NO) have been implicated in modulating the expression of PGC-1α (Lira et al., 2010; Nisoli et al., 2004, 2003), and we have previously shown that estrogen can influence each of these two pathways in cerebral vessels (McNeill et al., 2002; Stirone et al., 2005a). Fig. 2C demonstrates that PI3K inhibition by LY294002 fully inhibited the ability of estrogen to decrease PGC-1α protein in the isolated vessels. In contrast, the NOS inhibitor, L-NAME, had no effect on estrogen-mediated down-regulation of PGC-1α.

3.3. Estrogen increases FoxO1 phosphorylation

PI3K/Akt activation has been shown to down-regulate PGC-1α through Akt phosphorylation of the forkhead transcription factor, FoxO1 (Cook et al., 2002; Daitoku et al., 2003; Hong et al., 2011). Since PI3K inhibition blocked the ability of estrogen to downregulate PGC-1α (Fig. 2C), we sought to determine if phosphorylation of FoxO1 would also be altered by the hormone. Indeed, chronic *in vivo* estrogen treatment led to a significant increase in levels of Ser-256 phosphorylated FoxO1 in cerebral vessels (Fig. 3B) with no change in total FoxO1 protein (Fig. 3A).

3.4. Estrogen increases NRF-1 protein levels in vessels ex vivo

In contrast to the effect on PGC-1α, estrogen treatment increased mRNA levels of PGC-1β and PRC. The latter two transcription factors would be expected to increase mitochondrial biogenesis through nuclear respiratory factor 1 (NRF-1) (Lin et al., 2003). We previously demonstrated that *in vivo* treatment with estrogen causes a significant increase in cerebrovascular NRF-1 protein (Stirone et al., 2005b). Therefore we used the *ex vivo* protocol to test whether direct activation of estrogen receptors in isolated blood vessels would affect levels of NRF-1 protein. We also used the PI3K inhibitor to test for involvement of the nongenomic, estrogen receptor-PI3K pathway that we showed above to be involved in down-regulation of PGC1α (Fig. 2C). Fig. 4 represents densitometric analyses of immunoblots for NRF-1 protein from cerebral vessels isolated from OVX animals and then treated *ex vivo* for 12–18 h with 10 nM 17β-estradiol in the absence or presence of the estrogen receptor inhibitor, ICI- 182,780, or the PI3K inhibitor, LY294002. NRF-1 protein was significantly elevated after 12 h of estrogen treatment and remained high after 18 h exposure. ICI-182,780 fully inhibited this effect, while LY294002 failed to attenuate the increase in NRF-1 protein caused by estrogen treatment.

3.5. Estrogen increases PGC-1β, TFAM and OXPHOS proteins

PGC-1β is known to induce NRF-1 expression. NRF-1, in turn, increases expression of many nuclear-encoded mitochondrial proteins involved with oxidative phosphorylation (OXPHOS) as well as proteins that affect mitochondrial genes (Scarpulla, 2008). One such

protein, TFAM, translocates to the mitochondria to initiate mitochondrial DNA transcription and replication (Kang et al., 2007). To further investigate whether estrogen upregulates this pathway, we compared PGC-1β and TFAM proteins in cerebral vessels from OVX and OVX+E animals. As shown in Fig. 5A and B, chronic treatment with estrogen significantly increased the levels of both proteins.

NRF-1 has also been shown to induce the expression of proteins involved in mitochondrial OXPHOS (Kelly and Scarpulla, 2004). Therefore, we compared protein levels of selected subunits from the different OXPHOS complexes in cerebral vessels taken from OVX and OVX+E animals (Fig. 6). We found that estrogen treatment significantly increased Complex I subunit NDUFB8, Complex III subunit UQCRC2 and Complex V subunit ATP5α. No significant differences were found in Complex II subunit SDHB. Previously we demonstrated that estrogen treatment increases the protein levels of Complex IV subunits as well as the activity of this complex in cerebral blood vessels (Stirone et al., 2005b).

3.6. Estrogen increases mitochondrial DNA content

Mitochondrial DNA content is a reflection of cellular mitochondrial content, and this measure is largely independent of variations in mitochondrial size (Medeiros, 2008). Increases in PGC-1 levels have been shown to increase TFAM, which subsequently increases mitochondrial DNA replication (Kang et al., 2007). In order to determine changes in mitochondrial DNA content, ratios between the nuclear DNA marker, tubulin-specific chaperone A (TBCA), and the mitochondrial DNA marker, 12S rRNA, were computed (Fig. 7). The ratio between mitochondrial and nuclear DNA was significantly higher in vessels isolated from OVX+E animals as compared to OVX. These data support the conclusion that estrogen treatment results in mitochondrial biogenesis in cerebral blood vessels.

3.7. Estrogen increases cerebrovascular glutamate-cysteine ligase (GCL) and glutathione levels

It has been demonstrated in cultured myotubes that adenoviralmediated overexpression of PGC-1β, but not PGC-1α, results in increased mRNA expression of both subunits of the rate-limiting enzyme for glutathione synthesis, glutamate-cysteine ligase (GCL) (St-Pierre et al., 2003). Given the results of our analysis of PGC-1 isoforms, namely the selective increase in PGC-1β, we sought to determine if estrogen would also change protein levels of GCL modulatory (GCLm) and catalytic (GCLc) subunits. As shown in Fig. 8A and B, *in vivo* estrogen treatment (OVX+E) significantly elevated cerebrovascular GCLc and GCLm protein levels compared to OVX. Furthermore, as shown in Fig. 8C, increase in both subunits of glutamate-cysteine ligase (GCL), the rate limiting enzyme for glutathione synthesis, correlated with an increase in total glutathione/mg protein in cerebral vessels from OVX+E animals compared to OVX. These findings support the conclusion that estrogen increases the function of the rate-limiting step for glutathione synthesis, resulting in increased cerebrovascular levels of glutathione.

4. Discussion

Appropriate regulation of mitochondrial biogenesis and function is a critical component of adaptation to external conditions and prevention of pathogenesis and age-related disorders. Thus, it is important to identify key regulators and the mechanisms that alter mitochondria in different tissues.We have demonstrated that the steroid hormone 17β-estradiol (estrogen) modulates several master transcriptional regulators of mitochondrial function, NRF-1, TFAM and PGC-1 isoforms, in brain blood vessels. The pattern of a decrease in PGC-1α together with an increase in PGC-1β, PRC, NRF-1 and TFAM appears to underlie protective effects of estrogen on cerebral blood vessel mitochondria that we previously described (Stirone et al., 2005b). These changes may reflect context-specific actions of estrogen and the particular role of vascular mitochondria in supporting the unique metabolic requirements of cerebrovascular function.

We previously demonstrated that long-term estrogen treatment *in vivo* increases NRF-1 protein levels in cerebral vessels (Stirone et al., 2005b). A subsequent report showed that, in cultured MCF7 cells, ERα directly interacts with the NRF-1 promoter, initiates its transcription and interacts directly with NRF1 protein (Mattingly et al., 2008), demonstrating several levels of genomic action of estrogen receptors in mitochondrial regulation. NRF-1 is believed to be a key nuclear transcriptional regulator responsible for regulating many nuclear-encoded mitochondrial proteins, including cytochrome c (Kelly and Scarpulla, 2004). Our data from cerebral vessels treated *ex vivo* show that estrogen can increase NRF-1 protein by 12 h, and the level of NRF-1 remains elevated at 18 h. Estrogen also elevated cerebrovascular levels of cytochrome c protein after 18 h, as determined previously under similar experimental conditions (Stirone et al., 2005b). The two time courses are consistent with estrogen acting first on NRF-1 to regulate cytochrome c expression. Estrogen-induced increases in NRF-1 and cytochrome c are both blocked by an estrogen receptor antagonist, but are unaffected by an inhibitor of PI3K. This suggests that estrogen acts through an ER to upregulate NRF-1 expression and other nuclear-encoded mitochondrial proteins in cerebral vessels.

NRF-1 is regulated by the PGC-1 family of transcription factors (Scarpulla, 2008). In contrast to findings in other tissues and *in vitro* systems (Scarpulla, 2008), we found that changes in PGC-1α do not parallel changes in NRF-1 and cytochrome c in cerebral blood vessels. Estrogen suppression of PGC-1α is dependent on PI3K, but effects of estrogen to increase NRF1 and cytochrome c are not. This disconnect suggests that estrogen increases NRF-1 and cytochrome c by a pathway distinct from PGC-1α. We have previously demonstrated that estrogen activates PI3K and Akt in the cerebral vasculature (Stirone et al., 2005a). Our findings suggest that down-regulation of PGC-1α may be mediated through Akt phosphorylation of the forkhead transcription factor, FoxO1, as demonstrated after estrogen treatment. Phosphorylation would cause FoxO1 to translocate out of the nucleus where it can no longer activate PGC-1α, as previously demonstrated (Cook et al., 2002; Daitoku et al., 2003; Hong et al., 2011).

Because our findings suggest that PGC-1α does not mediate effects of estrogen on NRF-1 and mitochondrial function in cerebral blood vessels, we also examined the other two

PGC-1 isoforms. Estrogen treatment significantly increased mRNA levels of both PGC-1β and PRC, but suppressed PGC-1α mRNA. We recently showed that ovariectomy produces the opposite effects in freshly isolated brain endothelial cells *i.e.*, increases in PGC-1α but decreases in PGC-1β mRNA levels (Kemper et al., 2013). Results from the present study indicate that a lack of estrogen underlies the switch in PGCs caused by ovary removal.

Interestingly, while PGC-1α coactivates both estrogen receptors, PGC-1β appears selective for ERα and does not coactivate other steroid hormone receptors such as ERβ and the glucocorticoid receptor (Kressler et al., 2002). The interaction between ERα and PGC-1β also has been shown to be partially responsible for the transcriptional activity of the selective estrogen receptor modulator, tamoxifen (Kressler et al., 2007). Previous work from our laboratory has shown that ERα is the dominant receptor subtype in cerebral vessels (Kemper et al., 2013; Stirone et al., 2005b), and mitochondrial effects of estrogen in cerebrovascular endothelial cells are predominantly mediated by ERα (Razmara et al., 2008; Stirone et al., 2005b). Together, these studies suggest estrogen elevates cerebrovascular PGC-1β expression through a specific receptor.

Upon examination by western blot, we found that cerebrovascular levels of PGC-1β and TFAM both increased significantly in animals treated with estrogen. This indicates that changes in PGC-1β mRNA levels translate into changes in protein as well as increases in the downstream mitochondrial-targeted transcription factor TFAM. TFAM is generally associated with mitochondrial biogenesis. To confirm this effect of estrogen, we measured protein levels of mitochondrial OXPHOS subunits and mitochondrial/ nuclear DNA (mt/ nDNA) ratios, both markers of mitochondrial biogenesis (Medeiros, 2008). We established significant increases in subunits from complex I, III and V, as well as the mt/nDNA ratio. We previously demonstrated estrogen increases subunit expression as well as the activity of complex IV (Stirone et al., 2005b). Together, these findings strongly reinforce the hypothesis that estrogen increases mitochondrial biogenesis in cerebral blood vessels.

In addition to being an initiator of mitochondrial biogenesis, PGC-1β also alters mitochondrial function. PGC-1β produces mitochondria whose respiration is strongly coupled to ATP production, whereas mitochondria regulated by PGC-1α exhibit increases in uncoupled respiration (St-Pierre et al., 2003). Tightly coupled mitochondria are able to sustain high ATP production; but they also have increased potential for reactive oxygen species production and mitochondrial damage. To offset this risk, PGC-1β also has been shown to strongly induce mitochondrial antioxidant enzymes, such as manganese superoxide dismutase (MnSOD) and glutathione synthesizing enzymes (Handschin and Spiegelman, 2006; St-Pierre et al., 2003).

We, and others, have shown that estrogen can increase the antioxidant capacity of mitochondria. In cerebral endothelial cells and blood vessels, we previously found estrogen increases MnSOD and decreases mitochondrial reactive oxygen species production (Razmara et al., 2008; Stirone et al., 2005b). There was no effect of estrogen on the mitochondrial glutathione peroxidase isoform, GPX-1 (Stirone et al., 2005b); however, the glutathione antioxidant system is also dependent on glutathione synthesis (Lu, 2009). This was the focus of the current study, and we found estrogen treatment increased levels of both

subunits of the rate-limiting enzyme, glutamate cysteine-ligase (GCL), as well as glutathione levels in cerebral blood vessels. Glutathione plays key roles in free radical scavenging, maintaining cellular redox status and regulating cell survival in response to a wide variety of insults (Lu, 2009) and likely contributes to vasoprotective effects of estrogen. Our findings are consistent with studies in other cells and tissues that show estrogen-mediated increases in glutathione levels as well as GCL subunits (Bethea and Reddy, 2012; Lee et al., 2009; Montano et al., 2004); however the underlying mechanisms were not determined. Given that overexpression of PGC-1β, but not PGC-1α, has been shown to induce both MnSOD and GCL (St-Pierre et al., 2003), our findings in cerebral vessels are consistent with the novel hypothesis that estrogen increases mitochondrial antioxidant enzymes through PGC-1β activation. This mechanism may explain the ability of estrogen to decrease production of mitochondrial reactive oxygen species in cerebral blood vessels (Razmara et al., 2008).

Although little studied, tissue or cell-specific differences in PGC-1 isoform expression and regulation may have a profound impact on the local quantity and function of mitochondria. All PGC-1 isoforms are evolutionary well-conserved with the presence of similar domains, but overall sequence homology between these three isoforms is relatively low, suggesting distinct roles for the isoforms (Scarpulla, 2011). Indeed, both isoforms, PGC-1α and β, are expressed in numerous tissues but contain distinct binding sites activating different groups of mitochondrial genes. Transcriptional activities of both PGC-1α and β must be tightly controlled since they are extremely potent and able to interact with a large variety of transcription factors (Hong et al., 2011; Scarpulla, 2011). The mechanism of transcriptional regulation of the PGC-1 family requires further study, but recruitment of a defined set of coactivators and corepressors could also explain disparate effects. Differential regulation implies that functions of each isoform are different in order to fulfill tissue-specific metabolic requirements (Benard et al., 2006; Handschin and Spiegelman, 2006; Scarpulla, 2011). For instance, PGC-1α regulates thermogenesis in brown fat and hepatic gluconeogenesis, while PGC1-β regulates lipogenesis in liver (Lin et al., 2005; Sonoda et al., 2007). Likewise estrogen does not have the same metabolic effects in all tissues. Measures of mitochondrial parameters such as calcium loading capacity, peroxide production, and mitochondrial membrane polarization respond differently to estrogen in tissues such as brain, heart and liver (Moreira et al., 2011).

In summary, our work supports a crucial and potentially beneficial role for estrogen in the cerebral vasculature by guiding energy production and regulating the metabolism of ROS through activation of PGC-1 co-activators. Estrogen appears to act via both genomic and non-genomic receptor mechanisms to suppress PGC-1α and increase PGC-β, in order to promote biogenesis of tightly-coupled mitochondria with elevated antioxidant capacity. Elucidation of the effects of estrogen on mitochondrial function in this highly metabolically active tissue has critical implications for understanding the hormonal impact on function as well as pathophysiology of diseases of the brain and the process of aging (Duckles et al., 2006).

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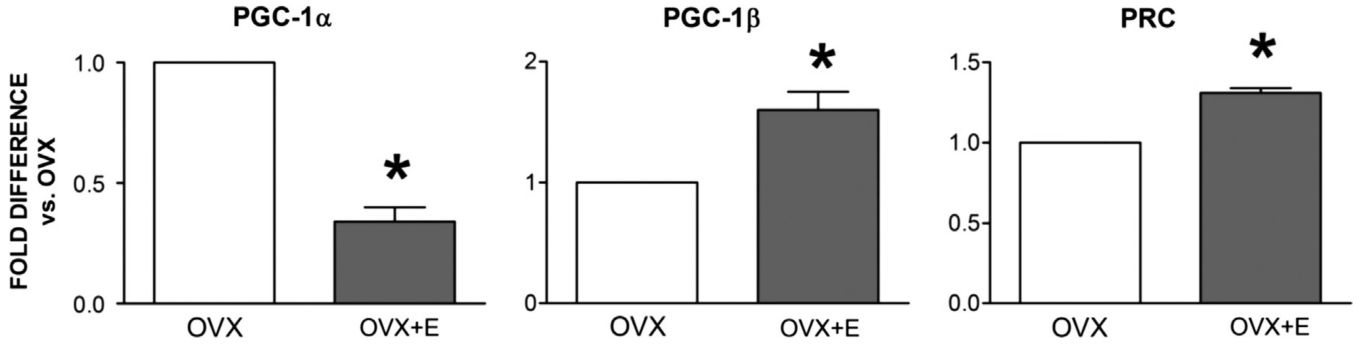

Fig. 1.

Effect of *in vivo* 17β-estradiol (OVX+E) treatment on cerebrovascular PGC-1 mRNA expression. Quantitative real-time PCR measurements for (A) PGC-1α, (B) PGC-1β and (C) PRC mRNA are shown. Data were normalized to β-actin as an internal control and mean values expressed as fold difference *vs*. OVX. N=4. *$P < 0.05$.

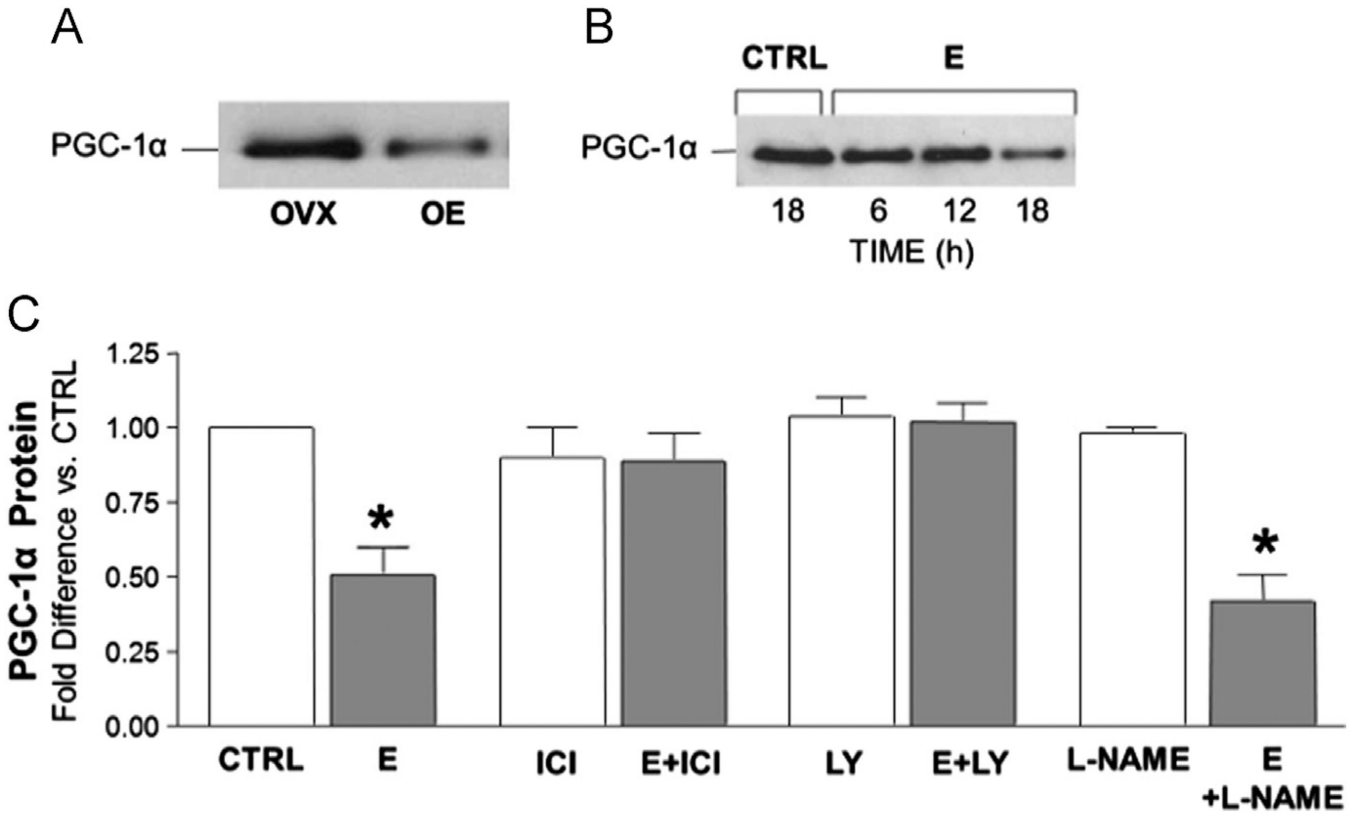

Fig. 2.

Effect of *in vivo* (A) and *ex vivo* (B, C) Estrogen treatment on PGC-1α protein levels in cerebral vessels. (A) Representative western blot showing PGC-1α protein (~91 kDa) in whole vessel lysates from OVX and OVX+E rats. (B) Time course of *ex vivo* treatment of isolated OVX vessels with 10 nM 17β-estradiol; representative blot showing PGC-1α protein. (C) Densitometric analysis for PGC-1α after 18 h *ex vivo* treatment of isolated vessels with estrogen in the absence and presence of ICI-182,780 (1 μM), LY294002 (10 μM), or L-NAME (100 μM). In all cases, band density was corrected for the loading control. Mean values are presented as fold difference *vs.* 18 h vehicle control run on the same blot. (Estrogen alone, $N=12$; all others, $N=4$) $^{*}P < 0.05$ compared to the respective condition in the absence of estrogen.

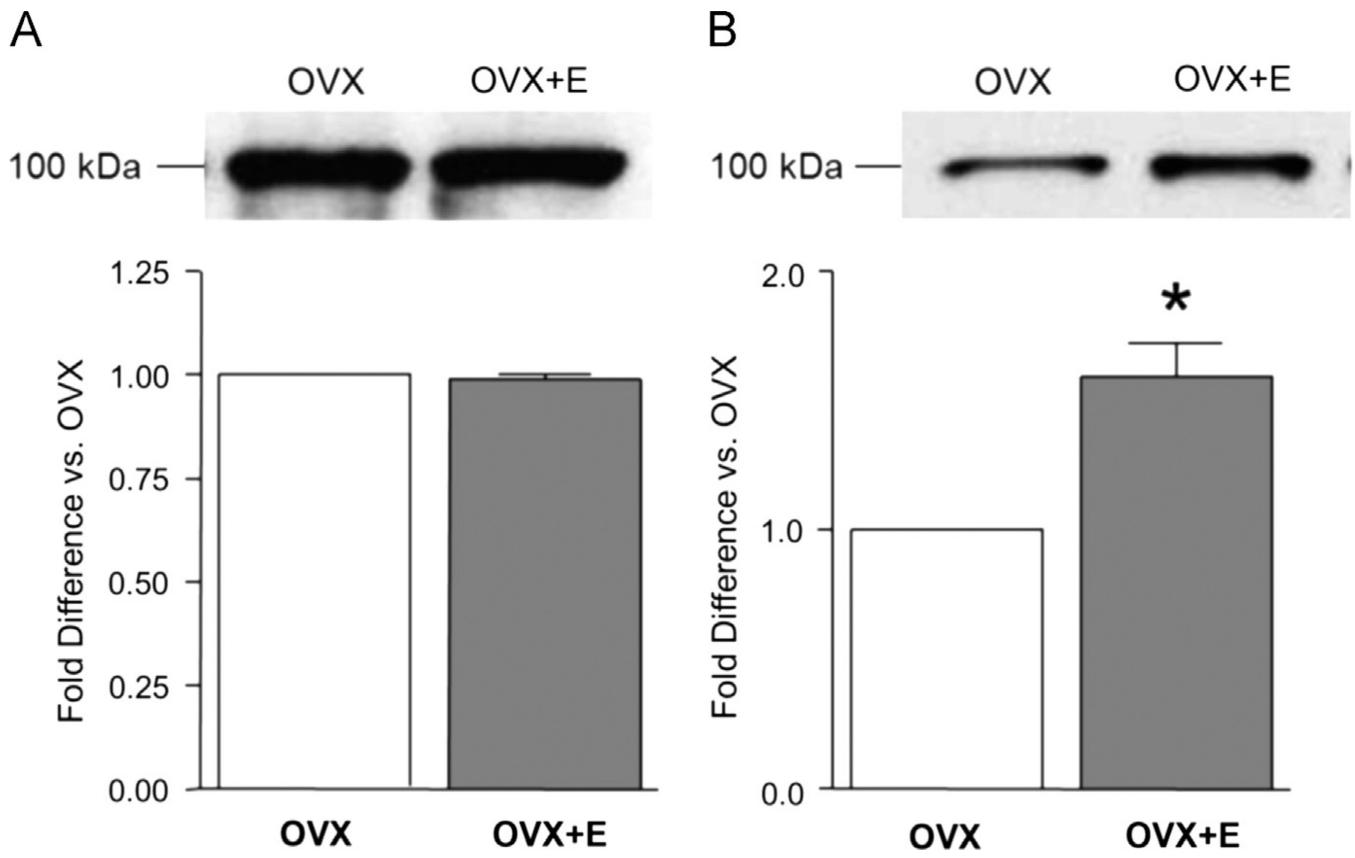

Fig. 3.

Effect of *in vivo* estrogen treatment on cerebrovascular levels of total FoxO1 protein (A) and Ser-256 phosphorylated form of FoxO1 (B) as determined by western blot and densitometric analysis. Mean data are represented as fold difference *vs*. OVX. $N=6$; $^{*}P < 0.05$.

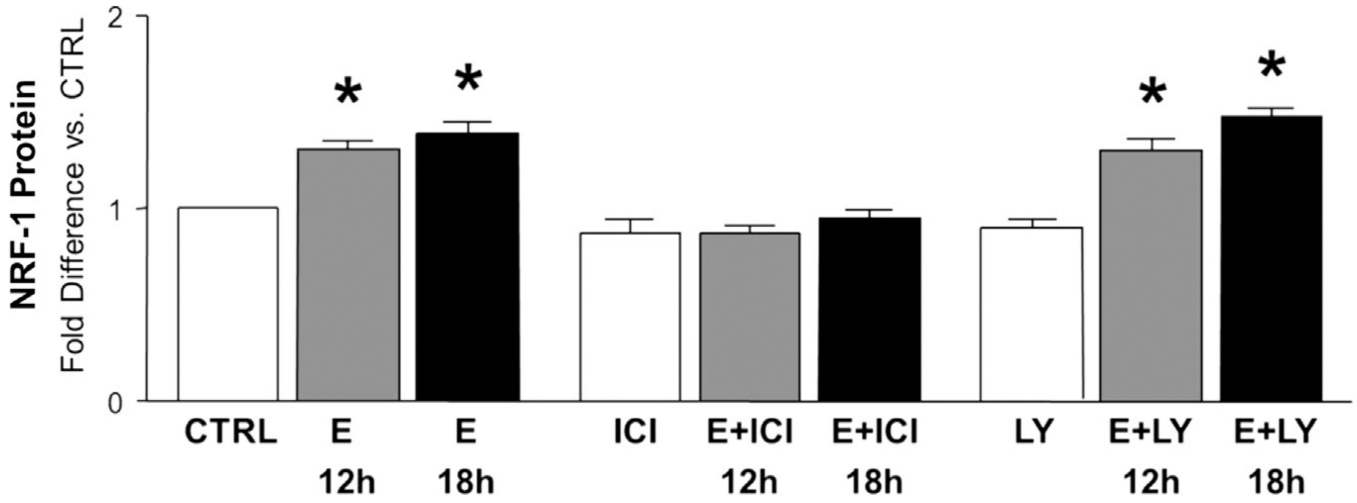

Fig. 4.

NRF-1 protein in cerebral blood vessels following *ex vivo* treatment with estrogen (10 nM) for 12 or 18 h in the absence and presence of ICI-182,780 (1 μM) or LY294002 (10 μM). Densitometric analyses of western blots are shown. Mean values are presented as fold difference *vs*. 18 h vehicle control. $N=4$. $^{*}P < 0.05$ compared to respective condition without estrogen.

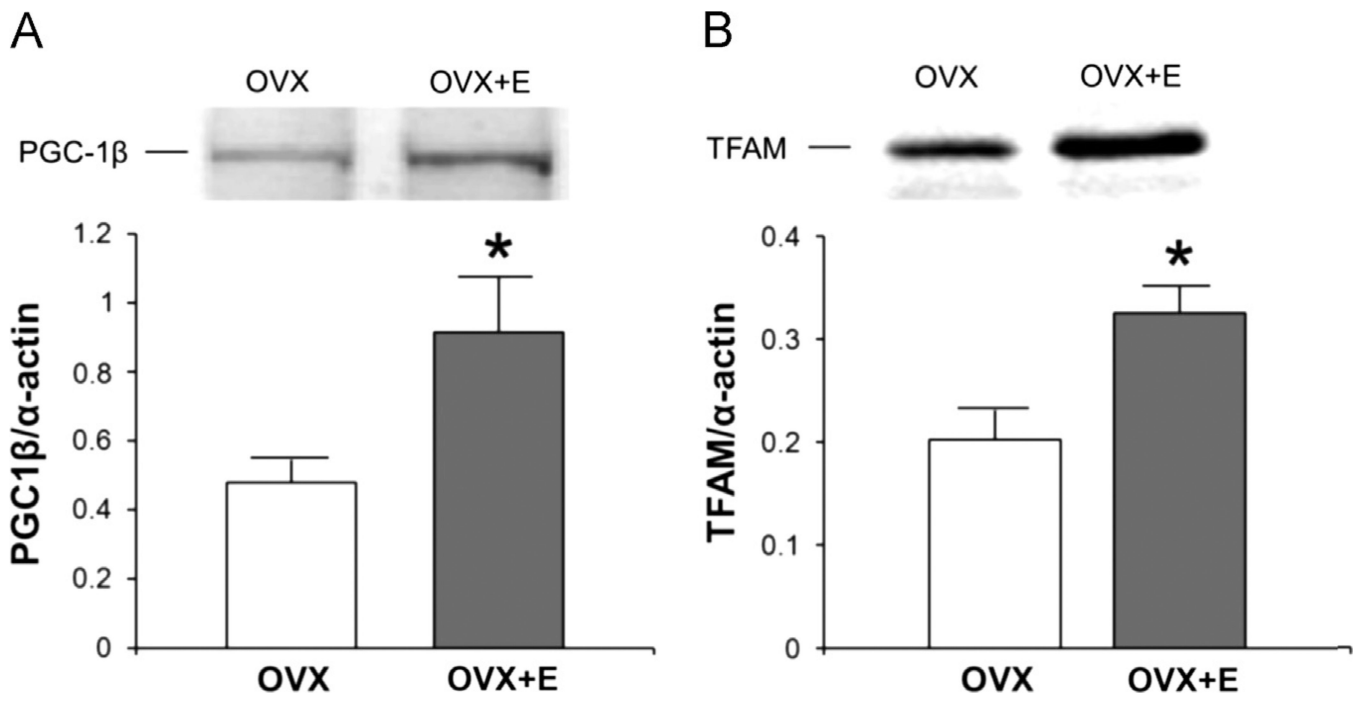

Fig. 5.

Effect of *in vivo* estrogen treatment on protein levels of (A) PGC-1β (110 kDa) and (B) mitochondrial transcription factor A (TFAM) (28 kDa) in cerebral vessels. Representative western blots and intensity analysis are shown for vessels from OVX and OVX+E rats are shown. In all cases, band density is normalized to the loading control α-actin. Values are means±S.E.M., N=5; *P < 0.05.

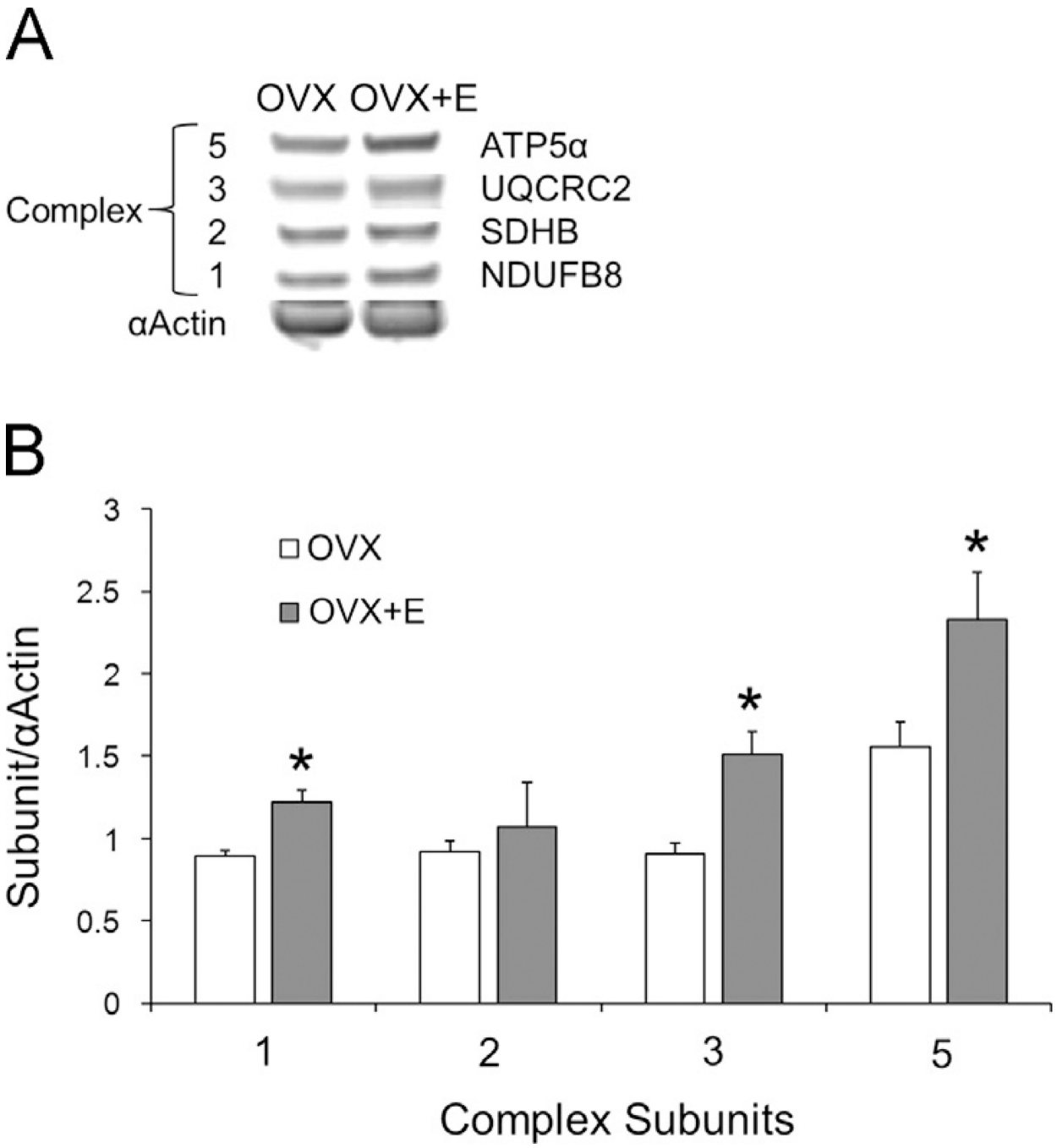

Fig. 6.

Effect of *in vivo* estrogen treatment on protein levels of mitochondrial OXPHOS subunits. (A) Representative western blot; bands have been cropped and rearranged by subunit number for clarity. Bands with corresponding molecular weights are: Complex 5 (55 kDa), Complex 3 (48 kDa), Complex 2 (30 kDa) and Complex 1 (20 kDa). (B) Intensity analysis of immunoblots for vessels from OVX and OVX+E rats is shown. Band density was normalized to the loading control α- actin in each lane. Values are means±S.E.M., *N*=6; **P* < 0.05.

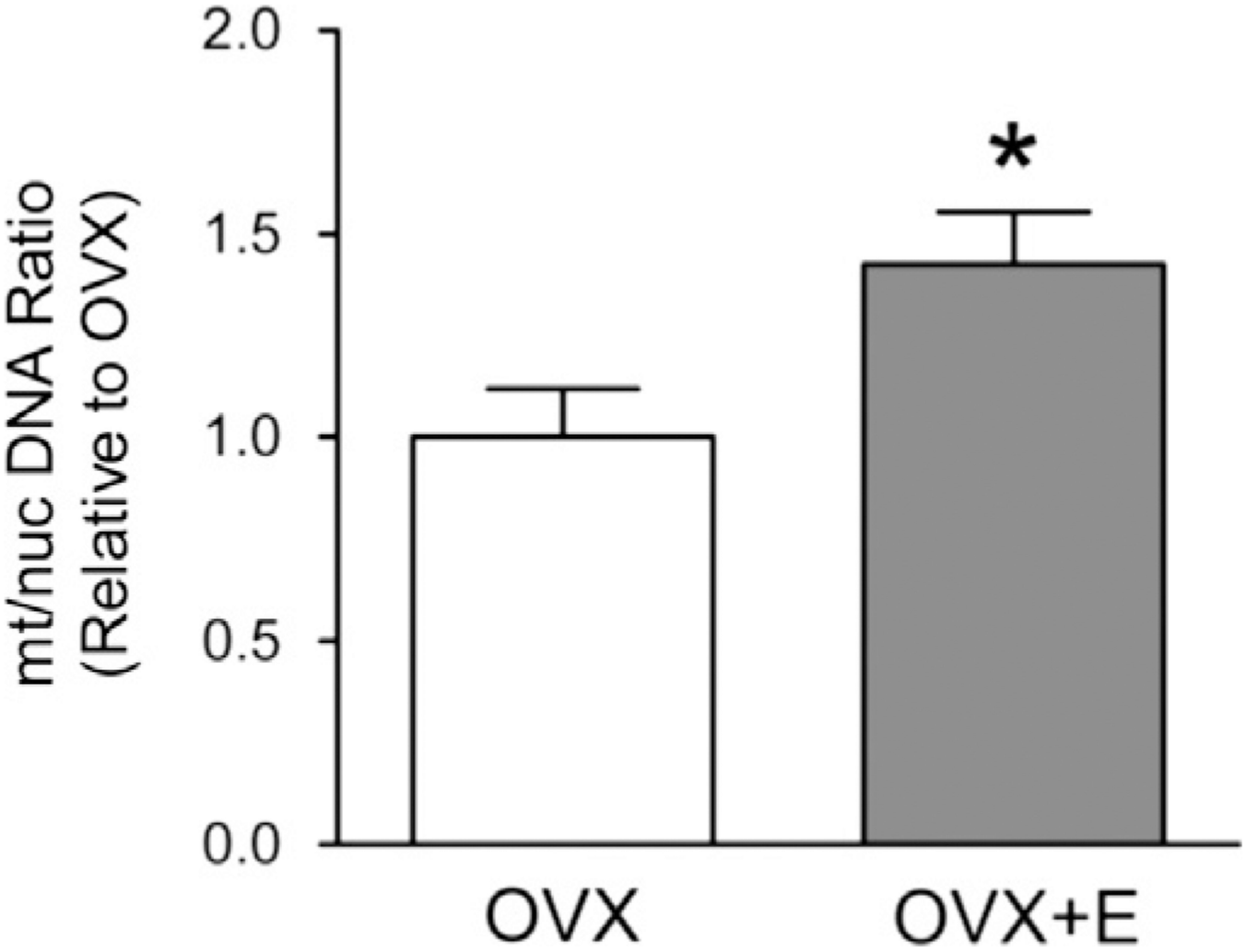

Fig. 7.

Effect of *in vivo* estrogen on mitochondrial/nuclear DNA ratios in cerebral vessels. qPCR experiments established the relative abundance of the nuclear genome marker tubulin-specific chaperone A (TBCA) and the mitochondrial DNA marker 12S rRNA. Values are fold difference relative to OVX, displayed as means±S.E.M., N=5; *P < 0.05.

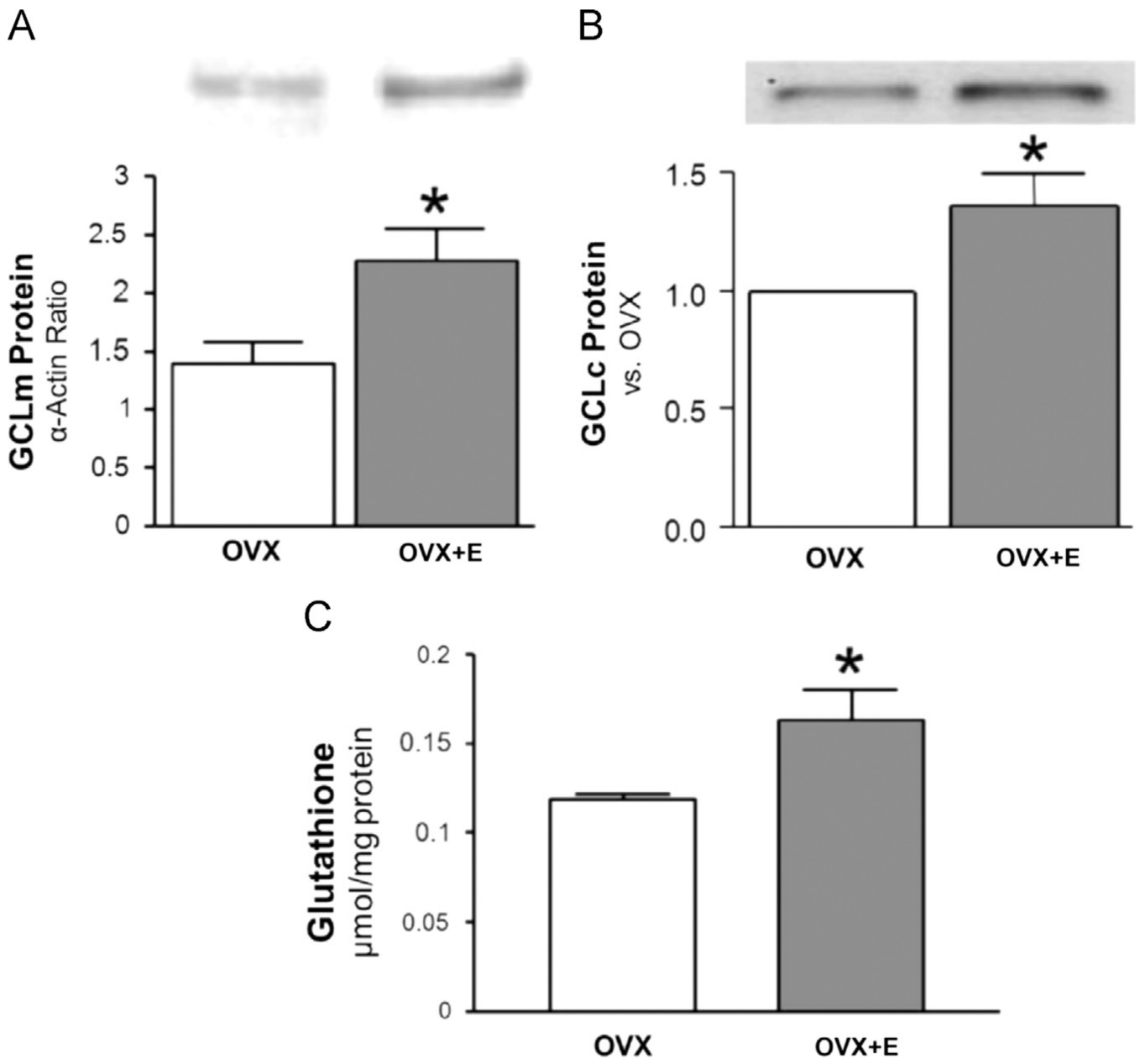

Fig. 8.

Effect of *in vivo* estrogen treatment on levels of cerebrovascular glutamyl-cysteine ligase subunits (GCL) and glutathione. (A, B) Representative western blots and band intensity analysis comparing protein levels of GCL modulatory (31 kDa) and catalytic (73 kDa) subunits in cerebral blood vessels from OVX and OVX+E animals. α-Actin was used as the loading control. Mean data are represented as fold difference *vs*. OVX. N=6; *$P < 0.05$. (C) Total glutathione levels measured in extracts of freshly isolated cerebral vessels from OVX and OVX+E rats. Values are means±S.E.M., N=4; *$P < 0.05$.