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17β-Estradiol increases endothelial nitric oxide synthase mRNA copy number in cerebral blood vessels: quantification by real-time polymerase chain reaction

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Abstract

The enzyme endothelial nitric oxide synthase (eNOS) plays a critical role in the maintenance of vascular tone. The mechanism by which estrogen increases eNOS function remains controversial. We demonstrate here using real-time polymerase chain reaction (PCR) and immunoblot analysis that in vivo estrogen treatment leads to a 100% increase in eNOS messenger RNA (mRNA) copy number and increases eNOS protein levels by 47% in mouse cerebral blood vessels. These data suggest that estrogen can modulate eNOS at the transcriptional level in blood vessels in vivo.

Keywords: Estrogen receptor; Nitric oxide (NO); Blood vessel; Endothelium

1. Introduction

Maintenance of endothelial nitric oxide synthase (eNOS) protein levels and activity are critical to vascular function, as diminished nitric oxide (NO) availability contributes to systemic hypertension, endothelial dysfunction and atherosclerosis (Shaul, 2002). Effects on eNOS are believed to mediate some of the vascular protective effects associated with estrogen treatment (McNeill et al., 1999; Hodgin et al., 2002). However, the mechanisms by which in vivo estrogen treatment modulates eNOS function are not established.

It is now established that chronic in vivo estrogen exposure substantially elevates eNOS protein levels in rat cerebral blood vessels, but the mechanism behind this process is not understood (McNeill et al., 1999; Geary et al., 2001). In cultured human endothelial cells, estrogen can increase eNOS mRNA and protein via estrogen receptors (Hayashi et al., 1995; Kleinert et al., 1998) without affecting mRNA stability. It is therefore likely that chronic estrogen exposure specifically increases eNOS protein levels in cerebral blood vessels through a receptor-mediated increase in transcription and translation of the eNOS gene. However, data produced in cultured cells remain controversial as signal transduction may not accurately represent in vivo conditions and must therefore be validated in intact vascular tissue. In the present study, we provide evidence that chronic estrogen treatment in vivo increases eNOS mRNA copy number and eNOS protein levels in mice cerebral blood vessels.

2. Materials and methods

2.1. In vivo treatments and cerebral vessel preparation

All animal procedures were approved by the U.C. Irvine Institutional Animal Care and Use Committee. Ovariectomized (OVX) C57BL/6 female mice were obtained from Charles River Laboratories and, in some cases, implanted with a 17β-estradiol pellet (OE) as described previously (Geary et al., 2000). Implants were left in place for 4 weeks; all animals were then anesthetized by CO_2 and killed by decapitation. We have previously demonstrated by this procedure that serum estrogen levels in ovariectomized animals treated with estrogen are within the physiological range (Geary et al., 2000). Body weights were 20 ± 0.8 g

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for ovariectomized females and 19 ± 1 g for estrogen-treated ovariectomized females. Dry uterine weights were 20 ± 10 mg for ovariectomized females and 100 ± 10 mg for estrogen-treated ovariectomized females. Four brains from each animal treatment group were pooled, and blood vessels were isolated from whole brain as described previously (Stirone et al., 2003; Geary et al., 2001). This preparation contains both pial and intraparenchymal vessels.

2.2. Real-time PCR

Isolated vessels were resuspended in RNAlater reagent (Ambion) to stabilize and protect RNA. Blood vessel total RNA was isolated using an RNAqueous phenol-free RNA isolation kit (Ambion) according to the protocol provided by the manufacturer. RNA yield from pooled vessels was 3.4 ± 0.6 μg (mean ± S.E., $n = 12$). The remaining RNA was immediately placed in a −80 °C freezer. Four hundred nanograms of RNA was used for reverse transcriptase polymerase chain reaction (PCR) in a final volume of 20 μl. Moloney murine leukemia virus reverse transcriptase (PowerScript RT) was from Clontech. The reaction was carried out as described previously (Chu et al., 2002), except the reaction mixture was incubated at 42 °C for 90 min and stored at −20 °C until use.

Primers and probe were synthesized by PE Biosystems. Sense primer: (nucleotides 1419 to 1437, spanning exons 10 and 11 corresponding to the human sequence) 5′-CCTTCCGCTACCAGCCAGA-3′. Antisense primer: (1523 to 1500, spanning exons 11 and 12) 5′-CAGAGATCTTCACTGCATTGGCTA-3′. Dual labeled probe: (1467 to 1497, within exon 11) 5′-6-carboxy fluorescein-CAGGCATCACCAGGAAGAAGACCTTTAAGGA-6-carboxy tetramethylrhodamine-3′. Primers were designed to hybridize only with cDNA as described previously (Chu et al., 2002).

To control for RNA input and reverse transcription efficiency, 18S ribosomal RNA (18S rRNA) in each RNA sample was quantified using a kit purchased from PE Biosystems (TaqMan ribosomal control reagents No. 4308329). DNA standards used in real-time PCR increased in 10-fold intervals from 20 to 2×10^6 molecules for mouse eNOS and from 300 to 3×10^8 molecules for 18S rRNA.

Real-time PCR was performed as described previously using the ABI Prism 7700 Detection System (PE Biosystems) (Chu et al., 2002). A standard curve was established in C_T (the threshold cycle number at which the initial amplification becomes detectable by fluorescence; defined as $\Delta Rn = 0.1$ in our experiments), vs. copy number of ssDNA (equivalent to cDNA after reverse transcription), and the copy number of cDNA was determined for each sample as an approximation of mRNA copies. For 18S rRNA PCR, a 2-pg equivalent of total RNA after RT was used because of its great abundance. Quantification of eNOS mRNA was expressed as copy number per nanogram of total RNA and also as the ratio of eNOS to 18S rRNA.

2.3. Western blot analysis

Microvessel samples were homogenized in lysis buffer as described previously (Stirone et al., 2003). Lysate protein concentrations were determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Equivalent amounts of microvessel protein from each sample were loaded onto 8% Tris–glycine gels (Invitrogen) and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Various amounts of recombinant bovine eNOS (Cayman) were loaded adjacent to experimental lanes on the gel to generate a standard curve for eNOS. Membranes were incubated overnight at 4 °C in blocking buffer (phosphate-buffered saline containing 1% Tween-20 [T-PBS] and 6.5% nonfat dry milk), then incubated for 3 h at room temperature with a monoclonal mouse anti-eNOS antibody (#130020, Transduction Laboratories), rinsed with T-PBS for 30 min, and incubated with anti-mouse secondary antibody conjugated to horseradish peroxidase (Transduction Laboratories). Membranes were rinsed with T-PBS for 30 min, incubated with electrochemiluminescence reagent (Amersham) for 1 min, and exposed to Hyperfilm (Amersham). Blots were probed for α-actin to verify equal loading of protein on the gel. Software for electrophoresis analysis,

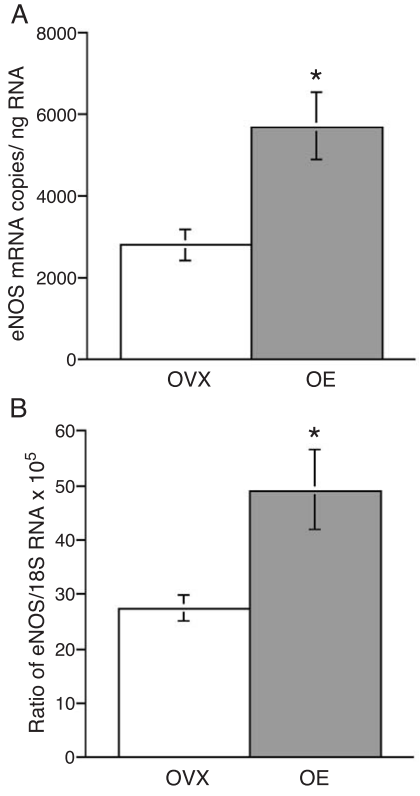

Fig. 1. (A) Number of molecules of eNOS mRNA per nanogram of total RNA in cerebral blood vessels of ovariectomized (OVX) and estrogen-treated (OE) mice ($n = 6$). (B) Ratio of eNOS mRNA copy number to 18S rRNA in cerebral blood vessels of OVX and OE mice ($n = 6$). Values are mean ± S.E.; *indicates $P \leq 0.05$.

UN-SCAN-IT (Silk Scientific), was used for densitometric analysis of immunoreactive bands.

2.4. Statistical analysis

Differences between groups were determined using a paired Student's *t*-test. Values were mean ± S.E. In all cases, statistical significance was set at $P \leq 0.05$.

3. Results

3.1. Real-time PCR

In cerebral blood vessels of mice chronically exposed to physiological estrogen levels for 1 month, eNOS mRNA copy number per nanogram of total RNA was significantly increased as compared to cerebral vessels of ovariectomized mice (Fig. 1A). In estrogen-treated animals, the ratio of eNOS/18S rRNA was also higher than that of ovariectomized animals (Fig. 1B). The real-time PCR reactions were linear over six orders of magnitude of starting cDNA standards, with a detection sensitivity of 20 molecules (data not shown).

3.2. Western blot

Densitometric analysis of eNOS immunoreactive bands from cerebral blood vessel lysates indicates a significant increase in eNOS protein levels in estrogen-treated animals relative to ovariectomized controls (Fig. 2B). In addition, a linear standard curve was generated for each blot using known concentrations of bovine recombinant eNOS protein (Fig. 2A). This curve was used to validate that the densities of experimental bands were within the linear range of film sensitivity. Normalizing eNOS band density values from mouse vessel lysates to values obtained from these standard curves, an estimate of eNOS protein concentration in the vessel lysates was determined. Using this method, eNOS protein was found to be significantly greater (47%) in vessels from estrogen-treated animals vs. ovariectomized animals (data not shown).

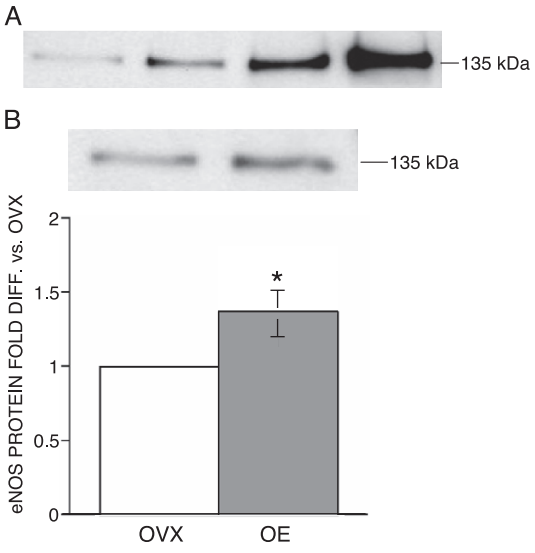

Fig. 2. (A) Western blot of increasing concentrations of bovine recombinant eNOS protein used to generate a standard curve for eNOS. (B) Cerebral blood vessels from ovariectomized (OVX) and estrogen-treated (OE) mice were probed for eNOS immunoreactivity. Protein levels are expressed as a fold difference in OE vs. OVX ($n = 9$). Values are mean ± S.E.; *indicates $P \leq 0.05$.

4. Discussion

In the present study, we have confirmed and extended our previous findings that chronic in vivo estrogen treatment increases eNOS protein levels in cerebral blood vessels. Using the method of real-time PCR to quantify eNOS mRNA levels in cerebral vessels from female mice, we demonstrate that there is a concomitant increase in eNOS mRNA copy number with increases in vessel eNOS protein. Our data indicate that there are approximately twice the number of eNOS mRNA copies in estrogen-treated mice vs. ovariectomized controls, suggesting that the elevation seen in eNOS protein in estrogen-treated animals is likely due to an increase in message for the eNOS gene.

Several studies provide evidence that estrogen can increase levels of eNOS mRNA in endothelial cells in culture without affecting mRNA stability (Hayashi et al., 1995; Kleinert et al., 1998). These studies indicated that estrogen treatment results in enhanced binding of the transcription factor Sp1, an essential coactivator protein involved in activation of the human eNOS promoter (Kleinert et al, 1998). An additional study using ovine pulmonary artery endothelial cells treated with estrogen in culture showed an increase in eNOS mRNA and protein which was blocked with the estrogen receptor antagonist ICI-182,780 {7α-[9-(4,4,5,5,5,-pentaflouropentylsulfinyl) nonyl] estra-1,3,5-(10)-triene-3, 17β-diol} (MacRitchie et al., 1997). In rat aorta, eNOS mRNA was increased with pregnancy and in vivo treatment with estrogen, but not progesterone or testosterone (Goetz et al., 1994). Thus, it is likely that the increased eNOS mRNA is due to transcriptional mechanisms, although changes in mRNA stability may also contribute.

A recent study in our laboratory demonstrated that estrogen acts directly on cerebral vessels to mediate the increases in eNOS protein thereby eliminating the hypothesis that indirect, whole body effects of estrogen are required to modulate eNOS levels (McNeill et al., 2002). This study also showed an attenuation of estrogen-mediated increases in eNOS protein levels when cerebral vessels were pretreated with the estrogen receptor partial agonist tamoxifen or antagonist ICI-182,780, validating an estrogen receptor-mediated mechanism. Furthermore, we have demonstrated

that estrogen receptor-α is present in the endothelium of cerebral vessels (Stirone et al., 2003) and that estrogen receptor-α knockout mice, after estrogen treatment, do not develop increases in eNOS protein (Geary et al., 2001).

Only one previous study has quantified eNOS mRNA in cerebral blood vessels to an absolute amount using real-time PCR (Chu et al., 2002). In that study, male and female mice 7 to 9 months of age were studied for gender differences in eNOS expression in pial arteries, but no difference was found. This result is contrary to an expected increase in females due to estrogen. However, in that study, the estrogen status of the intact female was undetermined at the time of study but likely variable due to the erratic estrus cycles associated with older females.

Though controversy remains, the mechanisms by which estrogen modulates both the protein level and activity of eNOS are proving to be multidimensional. Elevated levels of eNOS protein have been shown to correlate with increases in cerebrovascular eNOS activity (McNeill et al., 2002) and NOS-dependent dilation of cerebral arteries (Geary et al., 2000; 2001). Recent work indicates that estrogen may not only elevate eNOS protein through a classical genomic steroid receptor mechanism, but also modulates eNOS activity acutely through the activation of the phosphatidylinositol-3′ kinase/Akt pathway in vascular endothelial cells with subsequent phosphorylation of eNOS at Ser^{1177} (Haynes et al., 2000).

As the role of eNOS in cardiovascular function and health becomes more apparent, it is critical to understand the diverse mechanisms by which estrogen may mediate vascular protection through this enzyme. This requires validation of existing cell culture data in intact vascular tissue and in vivo studies. The present in vivo study in cerebral blood vessels supports previous evidence in cultured cells that estrogen exposure modulates eNOS protein levels through changes in mRNA copy number.

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