

Author



Amin Boroujerdi is interested in understanding the effects of drugs on the human body. In the summer of 2002, he began his intellectual exploration of the subject under the guidance of Dr. Krause and Dr. Duckles in the Pharmacology Department of UCI's College of Medicine. Amin researched the novel and exciting topic of the nongenomic effects of estrogen on cerebral vessels. While conducting the project, he learned the importance of critical thinking and data analysis. Amin believes that this type of hands-on training will help him realize his goal of attaining a Ph.D. in pharmacology. When he is not running experiments, Amin enjoys reading, snowboarding, surfing, and visiting museums.

Key Terms

- ◆ Cardiovascular Disease
- ◆ Cerebral Blood Vessels
- ◆ Estrogen
- ◆ Hormone Replacement Therapy (HRT)
- ◆ Nitric Oxide (NO)
- ◆ Phosphatidylinositol 3-Kinase (PI3K)
- ◆ Protein Kinase B (Akt)

Estrogen Exposure Rapidly Elevates Nitric Oxide Production in Cerebral Vessels

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Biological Sciences

Abstract

The hormone estrogen plays a significant role in the regulation of cardiovascular function. Acute effects of estrogen result in dilation of cerebral blood vessels by increasing levels of nitric oxide (NO). The hypothesis of this project is that estrogen acts without transcription to activate certain kinases, such as phosphatidylinositol-3 kinase (PI3K) and protein kinase B (Akt). This leads to increased activity of the enzyme that produces NO. Production of NO was measured in isolated cerebral blood vessels from ovariectomized female rats. Estrogen was found to increase cerebrovascular NO production within 5 min and to peak after 30 min. Immunoblot analysis of vessels treated with estrogen showed increased levels of the phosphorylated state of Akt (p-Akt) and endothelial nitric oxide (p-eNOS). Immunoprecipitation studies showed that estrogen receptor alpha (ER α) is complexed with eNOS in the vessels. These results suggest that physiological levels of estrogen can rapidly increase NO levels in cerebral vessels through activation of the PI3K/Akt/eNOS pathway. These findings provide a better understanding of the effects of this hormone, which is important for the potential treatment for cardiovascular diseases.

Faculty Mentor



The effects of estrogen on brain blood vessels and stroke is a timely women's health issue. Although protective actions of estrogen have been demonstrated, recent clinical trials with combination hormone replacement therapy did not show a benefit. Amin's study identifies a new mechanism by which the major estrogen, 17 β -estradiol, affects the endothelial layer lining cerebral blood vessels. Whereas estrogen has been considered a nuclear regulator of gene expression, recent data suggest the hormone also may act rapidly to alter intracellular signaling. He tested this theory in rat cerebral blood vessels and found estrogen rapidly increased production of the vasodilator nitric oxide. While conducting research, Amin developed one-on-one relationships with graduate and postdoctoral students and faculty. He sharpened his analytical thinking skills, was awarded fellowships, and presented his work at campus symposia and a national professional meetings. Amin's exposure to biomedical research resulted in his pursuit of a Ph.D. in pharmacology.

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Introduction

In the past decade much research has focused on the potentially beneficial effects of estrogen on cardiovascular disease, the leading overall cause of death in the United States (Grady et al., 1992). For women, the risk of heart disease and stroke increases significantly after menopause, which is marked by estrogen deficiency. Hormone replacement therapy (HRT) is a treatment regimen designed to remedy menopausal estrogen deficiency. The potential role of HRT in the prevention of cardiovascular diseases remains controversial; some clinical studies show beneficial effects, while others have contradictory results (Grady et al., 1992). However, estrogen has a number of properties that could protect against cardiovascular disease including the ability to upregulate the expression of endothelial nitric oxide synthase (eNOS) protein in the endothelial cells of the blood vessel. This protein plays a significant role in the production of nitric oxide (NO), causing vasodilation and preventing clotting from occurring in the blood vessels (Mendelsohn and Karas, 1999). Estrogen's effect on the cardiovascular system is mediated through estrogen receptors. To date, two estrogen receptors have been identified, ER α and ER β (Chambless et al., 2000). Estrogen receptors are ligand-activated transcription factors. When estrogen binds to these receptors, it alters the expression of a number of target genes, including that for the production of eNOS. This mechanism accounts for the long term genomic effects of estrogen exposure on the cerebral blood vessels (Mendelsohn, 2000).

Recent studies have suggested a second mechanism by which acute estrogen exposure in cerebral blood vessels leads to NO production. This occurs by activation of eNOS through the phosphatidylinositol-3 kinase (PI3K)/protein kinase B (Akt) pathway. This mechanism, which has been demonstrated in cell culture (Ebner et al., 2000), may mediate rapid or nongenomic effects of estrogen in intact blood vessels. The PI3K/Akt pathway leads to phosphorylation of eNOS and subsequent activation of the enzyme. The proposed signal transduction pathway starts when estrogen binds to ER α , leading to the activation of PI3K, which has a regulatory subunit p85 α attached to the ER α . This interaction then causes an increase in activity of Akt, which is a downstream effector of PI3K activation (Stirone et al., 2002). The activation of Akt has been shown to directly phosphorylate eNOS at amino acid serine 1177 (Fulton et al., 2002). This phosphorylated state of eNOS leads to increased catalytic activity and ultimately the production of NO, which acts as a vasodilator. This study is aimed at testing the hypothesis that estrogen can act acutely on intact

blood vessels to stimulate NO production through nongenomic mechanisms. Greater knowledge of the mechanism of the effects of estrogen in the cardiovascular system can ultimately lead to more sophisticated estrogenic drugs to treat cardiovascular diseases.

Materials and Methods

In vivo Treatment

Ovariectomized (OVX) Fischer female rats three to four months old were anesthetized with CO₂ and then decapitated one month after ovariectomy. Cerebral vessels were immediately isolated from the brains and stored at -80 °C for future use. This study was approved by the Institutional Animal Care and Use Committee (IACUC) of UCI, under protocol #1999-2048.

Cerebral Vessel Isolation

Five brains were pooled together and gently homogenized in a Dounce tissue grinder in a solution of ice-cold phosphate-buffered saline (PBS). The homogenate was then centrifuged at 720 g for 5 min at 4 °C. The pellet was then resuspended and layered over a solution of 16% dextran and centrifuged at 4,500 g for 20 min at 4 °C. This process produced three layers in the centrifuge tubes. The bottom layer contained a pellet of blood vessels and was put aside for later use. The top layer, containing the supernatant, was discarded. The middle layer, composed of cerebral tissue, was extracted from the centrifuge tube and resuspended in PBS. It was layered again over 16% dextran solution and centrifuged at 4,500 g for 20 min. This allowed more blood vessels to pellet at the bottom of the tube. Pellets obtained from the first and second dextran centrifugation were combined in cold PBS, and the blood vessels were collected and washed over a 50 μ m mesh. The blood vessels obtained were either used immediately for NO assays or lysed for immunoblot analysis. In the latter case, vessels were glass homogenized at 4 °C in lysis buffer, composed of 50 mM B-glycerophosphate, 100 μ M NaVO₃, 2 mM MgCl₂, 1 mM EGTA, 0.5% Triton X-100, 20 μ M pepstatin, 20 μ M leupeptin, 0.1 U/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Vessels were incubated in this buffer for 20 min on ice and then centrifuged for 10 min at 4,500 g at 4 °C. The supernatant was collected and then immediately used or stored at -80 °C for future use.

Immunoblot Analysis

The lysates obtained by cerebral vessel isolation were used for immunoblot analysis to determine the presence and relative amounts of certain proteins of interest. In all immunoblot experiments, equal amounts of protein (50 μ g

in SDS sample buffer) were used for each sample. The protein concentration was determined using the Bicinchonic Acid Protein Assay Kit (Pierce, Rockford, IL). The sample lysate and the biotinylated molecular mass standards (Bio-Rad, Hercules, CA) were boiled for 4 min and loaded in lanes of an 8% polyacrylamide gel (Novex, San Diego, CA). The proteins were separated by SDS-PAGE. After the electrophoresis process, the proteins on the gel were then transferred onto a nitrocellulose membrane (Amersham, Piscataway, NJ) and subsequently incubated overnight at 4 °C in blocking buffer (PBS, 0.1% Tween 20, and 6.5% non-fat dry milk). The membrane was then incubated with primary antibodies at a dilution of 1:200 (Akt, p-Akt, PI3K, eNOS, and p-eNOS, Santa Cruz Biotechnology, Santa Cruz, CA; PA1-308, Affinity BioReagents, Golden, CO) for 3 hr. The membranes were washed five times for 5 min with T-PBS (0.1% Tween 20 in PBS) and incubated in secondary antibody (1:10,000) for a 1-hr period. Goat anti-rabbit IgG horseradish peroxidase (HRP) was used for Akt, p-Akt, and PI3K and goat anti-mouse IgG HRP was used for eNOS and p-eNOS proteins. The section of the membrane with the biotinylated molecular weights was isolated from the rest of the membrane and incubated in streptavidin-HRP at a dilution factor of 1:7,500 (Sigma, St. Louis, MO). After the second incubation, both sections of the membrane were washed five times for 5 min with T-PBS. The membrane was incubated with enhanced chemiluminescence reagent (Amersham, Piscataway, NJ) for 1 min. During this time period, the chemiluminescence reagent reacted with the HRP, which caused the protein of interest to illuminate. The membrane was then exposed on hyperfilm. The densitometric analysis program UN-SCANT (Silk Scientific, Orem, UT) was used to determine the concentration of these bands.

NO Quantitation Kit

The NO Quantitation Kit (Active Motif, Carlsbad, CA) provided a sensitive assay based on the Griess reaction for measuring NO production using nitrate and nitrite determination. To measure and compare levels of NO, nitrate must all be converted into nitrite. A nitrite standard curve was prepared. Cerebral blood vessels were divided equally, and incubated for 5, 15, 30, and 60 min at 37 °C in the presence of PBS solution or 10 nM estradiol in incubation wells. The fluid surrounding these vessels was removed, and 70 μ l was assayed using the kit. The nitrite was converted into a purple-colored azo compound by the Griess reagent, and its absorbance was read on a spectrophotometer at 540 nm.

Immunoprecipitation

Immunoprecipitation was used to evaluate the presence of a particular target protein within a lysate. The volume cor-

responding to 100 μ g of protein from the sample lysate was added to a volume corresponding to 5 μ g of a particular polyclonal antibody. This was gently mixed for 1 hr at 4 °C. Then 50 μ l of Protein G Sepharose (50% slurry) was added to the sample lysate antibody complex. This was again gently mixed for 1 hr at 4 °C. The sample lysate was centrifuged at 12,000 g for 20 sec, and the pellet was washed by resuspension and centrifuged three times with 1 ml lysis buffer and once with washing buffer. The final pellet was suspended in 30 μ l of sample buffer and then heated to 95 °C for 3 min. The beads were removed by centrifuging at 12,000 g for 20 sec and discarded. The supernatant was analyzed by SDS-PAGE.

Statistical Analysis

Data were analyzed using one way ANOVA with Newman Keuls post-hoc analysis. Statistical significance was set at $p < 0.05$. All values were expressed as mean \pm standard error mean.

Results

NO production was found to increase in cerebral vessels acutely exposed to 17 β estradiol for 5, 15, 30, and 60 min (Figure 1). Levels of NO in the vessel media were increased by approximately 50% after a 30-min incubation. NO production that was stimulated by estradiol was blocked by the PI3K inhibitors wortmannin and LY294002, which confirms the importance of PI3K to nongenomic NO production. Levels of NO in the presence of inhibitor wortmannin were shown to be equivalent to the levels present in

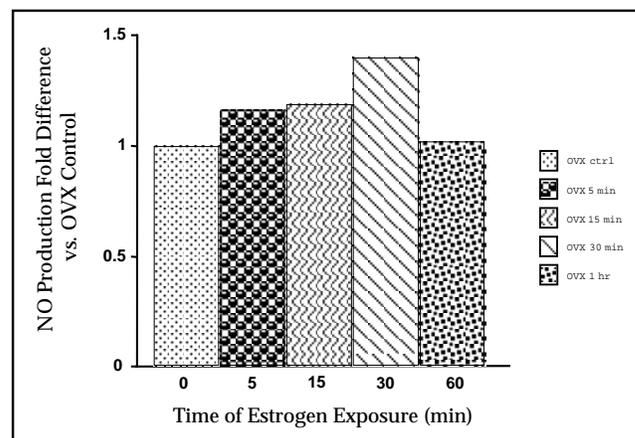


Figure 1
Cerebral blood vessels were incubated with 10 nM 17 β estradiol for 5, 15, 30, or 60 min. The medium was assayed for nitrites, reflecting release of NO. This release in the presence of estrogen is expressed as fold-difference over NO released by vessels in the absence of estrogen (OVX ctrl); n=2.

cerebral vessels unexposed to estradiol. The effects of estradiol were also blocked by the estrogen receptor antagonist ICI 182,780 (Figure 2), implicating the importance of estrogen receptors in the regulation of NO nongenomically. Western blot for Akt in cerebral vessels revealed a single

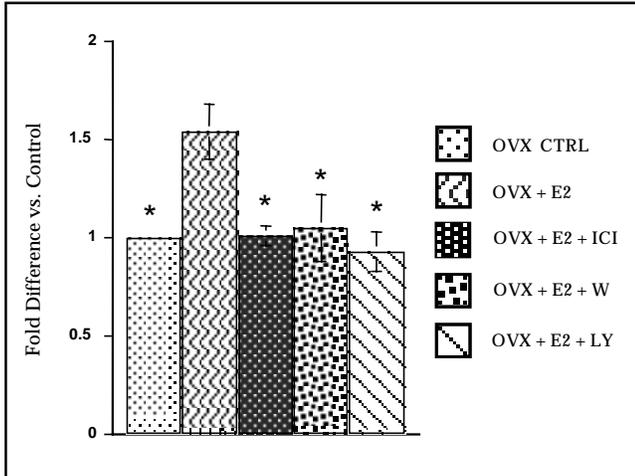


Figure 2
Effects of acute exposure to estrogen in cerebral blood vessels incubated with 10 nM 17 β estradiol, inhibitors of PI3K, wortmannin, and LY294002 for 30 min. Medium was assayed for nitrites, reflecting release of NO. * $p < 0.05$ compared to OVX female rats treated with E2; n=8 for E2, n=6 for ICI, n=3 for W and LY294002.

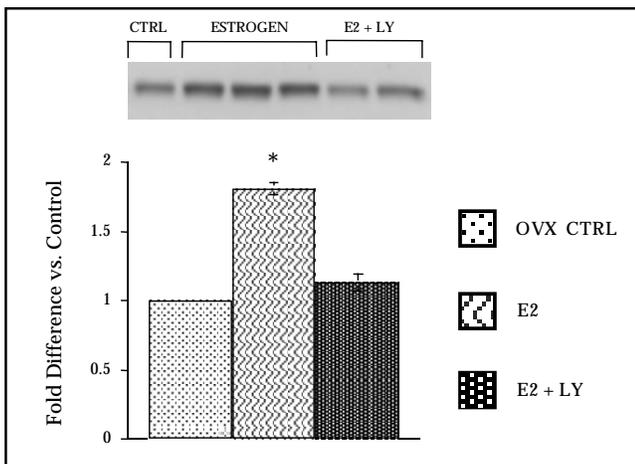


Figure 3
Representative Western blot showing p-Akt present in cerebral blood vessel lysates. The blot was probed for p-Akt serine 473, and bands were detected at a molecular weight of 50 kDa. Blood vessels were incubated for 30 min in the absence and presence of 10 nM 17 β estradiol. The PI3K inhibitor, LY294002, was added in some samples. * $p < 0.05$ compared to ctrl; n=3.

band at the expected molecular weight of 50 kDa. Levels of Akt protein were equivalent in cerebral vessels acutely exposed to estrogen and unexposed control vessels. However, levels of the p-Akt were higher in cerebral vessels acutely exposed to estrogen versus the control. This shows that acute estrogen exposure plays a significant role in increasing the p-Akt protein. When estrogen treated cerebral vessels were also treated with the PI3K inhibitor wortmannin (data not shown) and LY294002 (Figure 3), levels of p-Akt remained similar to levels observed in control vessels. eNOS and p-eNOS were also detected on Western blots as a band at a molecular weight of 135 kDa. While levels of eNOS protein were equivalent in cerebral vessels that were both acutely exposed and underexposed to estrogen, levels of p-eNOS were higher in cerebral vessels exposed to estrogen (Figure 4). The p85 α regulatory subunit of PI3K was shown to co-immunoprecipitate with ER α (data not shown). eNOS synthase was shown to co-immunoprecipitate with ER α . ER α was also shown to co-immunoprecipitate with eNOS synthase (Figure 5).

Discussion

The results obtained from this experiment support the hypothesis that acute estrogen exposure results in increased NO production in cerebral blood vessels through the non-genomic PI3K/Akt/eNOS pathway. Past studies have shown that chronic estrogen exposure specifically increases eNOS protein levels in cerebral blood vessels through genomic mechanisms involving a receptor-mediated increase in transcription and translation of the eNOS gene (Mendelsohn, 2000). In the present experiment, the time periods in which estrogen affected the production of NO are considered too short to be accomplished through a genomic mechanism involving transcription and translation of the eNOS gene. This result suggests that the short-term effects of estrogen on eNOS activity is based on a non-transcriptional action of estrogen through the cellular signaling pathway. This rapid effect appears to be mediated through estrogen receptors since the estrogen receptor inhibitor ICI 182,780 blocked the effects of estrogen on NO. In addition, this estrogen receptor mechanism appears to act through PI3K because the inhibitors of PI3K, wortmannin and LY294002 blocked the effects of estrogen. This finding supports the idea that the acute effects of estrogen on NO production are dependent on the PI3K/Akt pathway.

The kinase Akt plays a significant role in activating eNOS. It has been shown to directly phosphorylate eNOS at the amino acid serine 1177 (Fulton et al., 2002). Immunoblot

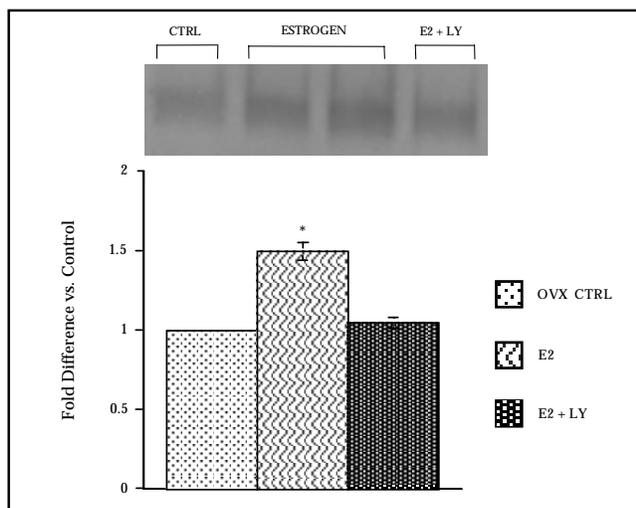
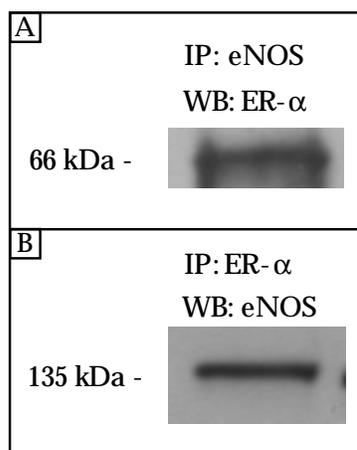


Figure 4
Representative Western blot showing p-eNOS present in cerebral blood vessels lysates. The blot was probed for p-eNOS serine 1177. Blood vessels were incubated for 30 min in the absence and presence of 10 nM 17 β estradiol. The PI3K inhibitor, LY294002, was added in some samples. * $p < 0.05$ compared to ctrl OVX; $n=3$.

Figure 5
Representative Western blot of cerebral vessel lysates from OVX rats. A: Immuno-precipitation for eNOS; probe for ER α . B: Immunoprecipitation for ER α ; probe for eNOS. Blots represent three separate experiments.



analysis showed equivalent levels of Akt in cerebral vessels treated and untreated with estrogen. Alternately, levels of p-Akt were shown to be higher in cerebral vessels treated with estrogen acutely. This result indicates that acute estrogen exposure does not increase the amount of Akt protein present within the cell. Rather, it increases the phosphorylated state of the protein. The phosphorylation of Akt activates the enzyme, which in turn leads to the phosphorylation of eNOS and, ultimately, NO production in the endothelial cells of the cerebral blood vessels. The effects of estrogen on p-Akt, however, were blocked by the PI3K inhibitor wortmannin and LY294002. Therefore, p-Akt requires PI3K since Akt is a downstream target of PI3K.

eNOS is an important enzyme that produces NO in endothelial cells causing vasodilation to occur. Estrogen now appears to regulate this enzyme through two methods. The first method is by the traditional genomic estrogen receptor pathway, which increases the amount of eNOS protein molecules, thus increasing the capability of NO synthesis. The other method is by increasing the phosphorylation of eNOS, which reduces the relative calcium requirement for eNOS activation. Previous studies have shown that eNOS activation is highly dependent on the presence of intracellular calcium (Shaul, 2002). Reducing the calcium requirements of eNOS activation would lead to an increase in NO synthesis. Levels of eNOS and p-eNOS were also measured in this experiment. There was not sufficient time to allow for an increase of eNOS molecules by the traditional genomic pathway. Levels of p-eNOS, however, were higher in cerebral vessels acutely exposed to estrogen. eNOS phosphorylation and Akt phosphorylation occur in parallel fashion during acute estrogen exposure, suggesting that eNOS is activated to p-eNOS by Akt dependent phosphorylation, which is part of the PI3K/Akt/eNOS pathway. Recent studies have shown that the phosphorylation of eNOS by the PI3K/Akt/eNOS pathway is a critical control step for NO production by endothelial cells (Fulton et al., 2002).

Another important aspect was whether ER α was physically associated with the PI3K/Akt/eNOS pathway. Certain proteins in this pathway were co-immunoprecipitated to see if they formed a complex with one another. Immunoprecipitation was performed for ER α and then probed for the p85 α regulatory subunit of PI3K using Western blotting techniques. The p85 α regulatory subunit co-immunoprecipitated with ER α . eNOS protein was shown to co-immunoprecipitate with ER α . This result was further validated when eNOS was immunoprecipitated and then probed for ER α . The ER α was also shown to co-immunoprecipitate with eNOS, which further supports the data that acute exposure of estrogen increases NO through interaction of ER with proteins involved in the PI3K/Akt/eNOS pathway.

Conclusion

In summary, this experiment demonstrated that acute estrogen exposure to cerebral blood vessels increased levels of Akt phosphorylation, eNOS phosphorylation, and the synthesis of NO, which is accomplished through the post-translational effect on the PI3K/Akt pathway. Further experiments showed that certain proteins in this pathway also co-immunoprecipitated, suggesting that these proteins interact with one another (Shaul, 2002). Future research

may examine this nongenomic pathway in greater depth and investigate the benefits it may provide for the prevention of cardiovascular diseases.

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