It was curiosity that initially brought Nevine to learn about the research possibilities available to her as a UCI undergraduate. This curiosity quickly grew into a deep interest, which has since guided her toward her ultimate goal of becoming a physician. Nevine delights in the research experience as a whole. As she says, “You will never get a more hands-on experience than the one research offers you. It’s the best way to apply what you have learned in class.” Apart from her studies, Nevine enjoys reading, playing badminton and tennis, and learning about different cultures.

**Key Terms**
- Cyclooxygenase-1 (COX-1)
- Enzymes
- OE
- OVX
- Prostacyclin
- Vasodilation
- Vessels

**Abstract**

Estrogen is known to have cardiovascular protective effects, but the mechanisms by which this protection is mediated are not clear. This study investigates the hypothesis that estrogen increases the production of prostacyclin (PGI₂) by blood vessels in the brain. PGI₂ is released from endothelial cells to cause smooth muscle vasodilation and to inhibit blood clot formation. Blood vessels were isolated from the brains of ovariectomized female rats (OVX) and ovariectomized female rats treated with estrogen (OE) to compare levels of three key enzymes involved in synthesizing PGI₂: phospholipase A₂ (cPLA₂), cyclooxygenase (COX-1), and prostacyclin synthase (PGI₂-S) which lead to, and thus regulate the levels of, PGI₂ formation. Estrogen treatment did not alter levels of the first enzyme in the pathway, cPLA₂. However, estrogen did significantly increase protein levels of the rate-limiting enzymes COX-1 and PGI₂-S, which would lead to increased production of PGI₂. Thus, estrogen may protect against stroke, in part by elevating PGI₂ levels in brain blood vessels.

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Introduction

The occurrence of cardiovascular disease is higher in men than premenopausal women (Lerner and Kannel, 1986), but increases in women after menopause (Kannel et al., 1976). Moreover, epidemiological data indicate that incidence of stroke in premenopausal women is one-fifth that in men. These differences might be due to the hormone estrogen, implicating it as vasoprotective.

Previous studies suggest that 17ß-estradiol (E2), the main form of estrogen, mediates cardiovascular protective effects via the promotion of endothelial-derived vasodilator synthesis and release. A recent study has found that chronic E2 treatment of ovariectomized rats causes a dose-dependent increase of endothelial nitric oxide synthase (eNOS) protein expression in the cerebral microvasculature. The E2 treatment also increases eNOS activity as compared to controls (McNeill et al., 1999). The ultimate consequence of eNOS induction is an enhanced production of nitric oxide, a potent anti-thrombotic and vasodilatory substance.

Moreover, estrogen has also been shown to promote the production of another potent vasodilator, prostacyclin (PGI2), in pulmonary, uterine, umbilical, and aortic endothelial cells in vitro (Jun et al., 1998; Vagnoni and Magness, 1998; Muck et al., 1992; Makila et al., 1982; Myers et al., 1996). Cyclooxygenase (COX-1) is a key enzyme for the production of various prostaglandins (such as prostacyclin), and thromboxanes from arachidonic acid. COX-1 is inhibited by aspirin-like drugs, probably accounting for its anti-inflammatory effects. While the reaction catalyzed by COX is the rate-limiting step in the production of PGI2, two other enzymes, phospholipase (cPLA2) and prostacyclin synthase (PGI2-S), are important in the pathway that leads to the production of prostacyclin (Figure 1).

Functional studies on isolated cerebral arteries have shown greater endothelial-related vasodilation following estrogen treatment (Geary et al., 2000a; Geary et al., 2000b). A portion of this response is blocked by COX inhibitors, suggesting increased levels of PGI2 are involved. The Krause lab recently demonstrated that estrogen increases production of PGI2 in isolated rat cerebral microvessels. However, there are three different enzymes in the pathway that lead to prostacyclin synthesis (Figure 1). Therefore, the present study was designed to further elucidate the effect of estrogen on prostacyclin synthesis by investigating the hypothesis that estrogen increases functional PGI2 activity in cerebral blood vessels by increasing levels of the enzymes cPLA2, COX-1, and PGI2-S.

Materials and Methods

In Vivo 17ß Estradiol Animal Treatment

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, Irvine, under protocol #99-2048. Two groups of rats were compared: ovariectomized female rats (OVX) and ovariectomized females treated with estrogen (OE). Gonadectomies were performed on 3-month-old Fischer 344 rats (Harlan Sprague-Dawley) anesthetized with ketamine 90 mg/kg and xylazine 10 mg/kg IP. In O E animals, hormone treatment was started at the time of gonadectomy by implanting hormone-filled silicone elastomer capsules subcutaneously. Capsules remained in place until the animal was killed. Animals were killed by decapitation four weeks after gonadectomy, and brains were immediately frozen at -80 °C. Uteri of the animal were collected and weighed dry. Blood was collected and serum levels of estradiol were determined by radioimmunoassay.

Cerebrovascular Isolation

Cerebral vessels were isolated from rat brain parenchyma, according to the protocol of McNeil et al. (1999, Figure 2). Briefly, four brains were pooled, homogenized gently with a Dounce tissue grinder in ice-cold phosphate-buffered saline (PBS, 0.01 M, pH 7.4), and centrifuged (using a Beckman GS-5R swinging bucket rotor) at 2000 g for 10 min at 4 °C. The supernatant was then discarded, and the pellet was washed by re-suspension in PBS and re-centrifuged 3 times, 5 min each at 2000 g. The pellet was re-suspended in PBS, gently layered on top of a 17% dextran (35,000 MW) solution and centrifuged at 5500 g for 20 min. The middle layers were collected, rewashed, and re-suspended with dex-
tran (same process). All pellets were then collected, resuspended in PBS, layered over dextran, and centrifuged at 5500 g for 20 min. The final pellet was poured over a nylon mesh screen (50 µm) and washed extensively with a strong stream of cold PBS. The blood vessel fraction, containing arteries, arterioles, veins, venules, and capillaries was collected from the top of the screen and stored at -80 °C.

**Tissue Lysis and Protein Content Determination**

Vessel samples were glass homogenized in a lysis buffer consisting of 50 mM β-glycerophosphate, 100 µM sodium orthovanadate, 2 mM magnesium chloride, 1 mM EGTA, 0.5% Triton X-100, 1 mM DL-dithiothreitol, 20 µM peptatin, 20 µM leupeptin, 0.1 U/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride, then incubated on ice for 20 min. Samples were centrifuged at 5000 g for 10 min at 4 °C and the supernatant collected and frozen at -20 °C. Protein content was determined by a modification of the Bradford method.

**SDS-PAGE/Western Blot**

Samples from each animal group (OVX and OE) were run in duplicate side by side. Between 30 and 50 µg of vessel protein was loaded onto 8% tris-glycine gels and separated by SDS-PAGE. Next, 50 mg of RAW 264.7 macrophage or 25 mg of NIH/3T3 fibroblast or human endothelial whole cell lysates were loaded as positive controls for COX-1, cPLA₂, or PGI₂-S blots, respectively, and biotinylated molecular weight markers (Bio-Rad) were loaded on each gel as well. After electrophoretic separation, proteins were transferred to a nitrocellulose membrane by electroblothing, and membranes were incubated overnight at 4 °C in blocking buffer (0.01 mM PBS containing 1% Tween-20 [T-PBS] and 6.5% nonfat dry milk). Membranes were then incubated with one of three primary antibodies: mouse polyclonal anti-COX-1 (1:350), mouse polyclonal anti-cPLA₂ (1:100), or mouse polyclonal anti-PGI₂-S (1:100) in blocking buffer for 3 h at room temperature (RT). The blots were rinsed six times, 5 min each in T-PBS. Membranes were then incubated with the secondary antibody anti-mouse IgG-horseradish peroxidase (1:7,500) in blocking buffer for 1 h at RT, after which they were washed 5 x 5 min in T-PBS at RT. Membranes were rinsed with T-PBS six times for 5 min each. Membranes were exposed to electrochemiluminescence (ECL) reagent for 2 min. They were then exposed and developed.

**Data Analysis**

Densitometric quantification was performed using the computer-based image analysis program, UN-SCAN-IT, and statistical analysis determined by the student’s t-test.

**Results**

Animal subjects (rats) were examined by the use of preliminary determinants to ensure that results were pertinent to the hypothesis. Therefore, when the animals were euthanized, OE animals showed significantly lower values of body weight, higher levels of estrogen in the blood, and bigger uterine weights in comparison to OVX rats (Table 1).

Levels of cPLA₂ protein were detected by SDS-PAGE. Vessel lysates from OVX rats and OE rats (n=4) were examined. Positive bands were detected with a specific cPLA₂ antibody at 110 kDa, which corresponds to the molecular weight of cPLA₂ protein (Figure 3). For each Western Blot, similar amounts of OVX and OE lysate were

<table>
<thead>
<tr>
<th>Animal group (rats)</th>
<th>17β-Estradiol (pg/ml)</th>
<th>Body weight (g)</th>
<th>Uterine weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovariectomized (OVX)</td>
<td>0.5 ± 0.5</td>
<td>184 ± 3</td>
<td>0.04 ± 0.002</td>
</tr>
<tr>
<td>Ovariectomized + Estrogen Treatment (OE)</td>
<td>68.9 ± 4.2*</td>
<td>165 ± 4*</td>
<td>0.25 ± 0.03*</td>
</tr>
</tbody>
</table>

Values are means ± S.E. *Significantly different than ovariectomized female (P<0.001).
run side by side. The densities of the bands were quantified and normalized to the OVX values on each blot. The results display no significant difference in the levels of cPLA\(_2\) between the two groups (p > 0.05).

For the comparison of the COX-1 enzyme level for OVX versus OE rats, five vessel preparations from each group were examined (n=5). Membranes were probed with a mouse polyclonal antibody directed towards COX-1 and bands were detected at 70 kDa, which corresponds to the molecular weight of COX-1 protein (Figure 4). Levels of COX-1 in OE animals are significantly higher than those in OVX rats (p < 0.05), showing a nearly 5-fold increase.

Finally, in comparing the levels of brain vascular PG\(_{1\_2}\)-S in cerebral vessels of OVX versus OE rats, six preparations from each group were compared (n=6). As expected, the bands on the Western Blot appeared at a molecular weight of 56 kDa for PG\(_{1\_2}\)-S (Figure 5). It was shown that there is a significant increase, nearly 7-fold, (p < 0.05) in the production of this enzyme in OE rats.

**Discussion**

Estrogen reduces brain damage in a variety of experimental models of stroke (Hurn and Macrae, 2000). Epidemiological evidence also suggests that loss of gonadal hormones after menopause increases the risk of stroke and other cardiovascular diseases. Various clinical studies have indicated that postmenopausal hormone replacement therapy helps prevent cardiovascular morbidity and mortality in the older female generation (Grodstein et al., 1996; Stampfer et al., 1991; Bush et al., 1987). On the other hand, it has been shown that estrogen and post-hormonal therapy do not reduce the overall rate of coronary heart disease events in postmenopausal women with previously established coronary disease (Hulley et al., 1998). The appropriate use and outcome of hormone replacement therapy is currently being evaluated.

Several mechanisms by which estrogen affects vascular tissue have been identified. Studies have shown that estrogen increases the level of prostacyclin production in human vascular endothelial cells and other endothelial cells (Mikkola et al., 2000). Other studies have demonstrated that prostacyclin is a key mediator of vasodilation in endothelial cells (Jun et al., 1998), thus acting as a major vasoprotective molecule. Prostacyclin would dilate vessels and therefore increase blood flow in the brain. Also, since many strokes are caused by clots that block blood flow, the clot-preventing effect of prostacyclin would also help reduce the risk of stroke. Recently it has been demonstrated that chronic in vivo 17\(\beta\)-estradiol treatment of ovariectomized rats results in an elevation of basal and arachidonic acid-stimulated prostacyclin production by cerebral blood vessels in vitro (unpublished data, Ospina, Krause, Duckles). This process of conversion of arachidonic acid to prostacyclin involves the three enzymes cPLA\(_2\), COX-1 and PG\(_{1\_2}\)-S. It was hypothesized that estrogen increases the amount of prostacyclin production by increasing levels of one or more of these three enzymes. This study verified that estrogen treatment in vivo increases levels of COX-1 and PG\(_{1\_2}\)-S, but not cPLA\(_2\), in rat cerebral blood vessels.
First, several elements were compared to ensure that the results were indeed due to the effects of estrogen. Uterine weights were measured and it was apparent that the OE uterine weight is significantly larger than that of OVX animals (p < 0.01). Moreover, analysis of blood samples by the use of radioimmunoassays indicated that the estrogen levels in the blood of OE animals are significantly higher (p < 0.01) than those of OVX animals. Body weights of OE animals are significantly lower (p < 0.01) than the body weights of OVX rats. These apparent differences are expected effects of estrogen and therefore ensure that the observed differences in levels of cPLA\(_2\), COX-1 and PG\(_{1\beta}\)-S between the OVX and OE rats are due to the effects of estrogen only.

In opposition to our hypothesis, our results indicate that there is no significant difference between levels of cPLA\(_2\) in OE rats as compared to OVX rats (p > 0.05). These results indicate that the levels of cPLA\(_2\) are not affected by estrogen. One study on quail oviduct found that phospholipase A\(_2\) is induced by estrogen administration (Payard et al., 1992). However, this effect does not occur in cerebral blood vessels.

The results do, however, support the hypothesis that estrogen leads to an increase in COX-1-production, thereby leading to an increase in prostaglandin synthesis. Previous studies have shown an increase in COX-1 in pulmonary endothelial cells as a result of an increase in estrogen administration (Jun et al., 1998). Other studies have demonstrated that there is an increase in the myogenic tone of cerebral blood vessels due to COX-1-dependent mechanisms (Geary et al., 2000). This study demonstrates that there is an increase in COX-1 expression in the endothelium of cerebral arteries as a result of increased estrogen levels. If the increase in COX-1 protein results in an increased level of the final product PG\(_{1\beta}\), then vessel relaxation should be increased. At the molecular level, cDNA experiments have demonstrated that overexpression of COX-1 in a cell line enhanced PG\(_{1\beta}\) synthesis (Wu, 1995). It is plausible that an elevation of COX-1 leads to an increase in the final product because COX-1 catalysis is the rate-limiting step of this pathway (Figure 1). In endothelial cells, prostacyclin is generally the primary final product; however, the COX-1 intermediate can also lead to other prostanoids, as well as the potent constrictor thromboxane A\(_2\).

Finally, the results support the hypothesis that an increase in estrogen administration caused an increase in PG\(_{1\beta}\)-S levels, thus leading to an increase in PG\(_{1\beta}\) levels. Zou et al. (1999) demonstrated that PG\(_{1\beta}\)-S is inactivated in early-stage atherosclerotic lesions. Moreover, it was shown that PG\(_{1\beta}\)-S inactivation in atherosclerosis not only leads to decreased prostacyclin synthesis but also to accumulation of the active vasoconstrictor PGH\(_2\). No study has yet shown the effects of estrogen on PG\(_{1\beta}\)-S. This study is the first to demonstrate that estrogen increases the level of PG\(_{1\beta}\)-S protein. This effect of estrogen would also be expected to increase PG\(_{1\beta}\) synthesis since PG\(_{1\beta}\)-S is the final enzyme in the pathway of PG\(_{1\beta}\) formation.

Results support the hypothesis that estrogen increases the levels of both COX-1 and PG\(_{1\beta}\)-S protein expression in cerebral blood vessels. This increase appears to underlie previous observations that estrogen increases PG\(_{1\beta}\)-S production and COX-dependent vasodilation in isolated cerebral blood vessels (unpublished work, Geary, Ospina). Because estrogen acts on nuclear receptors to influence genomic events, it is proposed that estrogen increases expression of COX-1 and PG\(_{1\beta}\)-S. Further studies should be performed on the molecular level to examine the mRNA expression of these enzymes to determine if there is an increase in expression at the molecular level.

**Conclusion**

It was hypothesized that estrogen increases prostacyclin production by increasing levels of one or more of three enzymes involved in prostacyclin synthesis. Results indicated that estrogen treatment in vivo increases levels of COX-1 and PG\(_{1\beta}\)-S, but not cPLA\(_2\), in rat cerebral blood vessels. It is proposed that estrogen increases the level of these two enzymes because it acts on nuclear receptors to influence genomic events. Further studies should be performed at the molecular level to examine the mRNA expression of these enzymes. Discovering the specific effects of estrogen on vasodilation and thus prevention of stroke may lead eventually to an understanding of how to prevent stroke in post-menopausal women and men.

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WORKS CITED


