The Effects of Estrogen on Prostacyclin Synthetic Pathway in Rat Brain Vessels

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Author

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.

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 (PGI_2) by blood vessels in the brain. PGI_2 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 PGI_2: phospholipase A_2 ($cPLA_2$), cyclooxygenase (COX-1), and prostacyclin synthase (PGI_2-S) which lead to, and thus regulate the levels of, PGI_2 formation. Estrogen treatment did not alter levels of the first enzyme in the pathway, $cPLA_2$. However, estrogen did significantly increase protein levels of the rate-limiting enzymes COX-1 and PGI_2-S, which would lead to increased production of PGI_2. Thus, estrogen may protect against stroke, in part by elevating PGI_2 levels in brain blood vessels.

Key Terms

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

Faculty Mentor

Estrogen is a promising, but still controversial treatment for stroke. In this research project, Nevine contributed significantly to our understanding of the way in which the hormone estrogen alters blood circulation in the brain. Using a rat model, she found that estrogen treatment increases levels of the enzymes that produce prostacyclin in brain blood vessels. Prostacyclin could help to reduce both the risk of and brain damage resulting from stroke since it dilates arteries to increase blood flow and inhibits blood clot formation. This project also exemplifies the win-win opportunity provided by faculty-mentored undergraduate research. Nevine gained valuable biomedical research experience, as well as one-on-one faculty guidance to help her reach her goal of entering medical school. Our laboratory also benefited from the careful work of a bright and energetic student.

Diana Krause
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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 (PGI_2), 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 PGI_2, two other enzymes, phospholipase ($cPLA_2$) and prostacyclin synthase (PGI_2-S), are important in the pathway that leads to the production of prostacyclin (Figure 1).

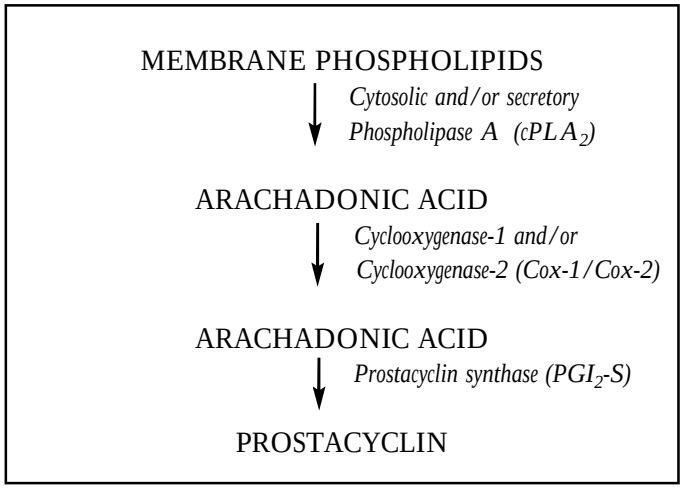

Figure 1
Pathway of prostacyclin biosynthesis (*enzymes in italics*).

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 PGI_2 are involved. The Krause lab recently demonstrated that estrogen increases production of PGI_2 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 PGI_2 activity in cerebral blood vessels by increasing levels of the enzymes $cPLA_2$, COX-1, and PGI_2-S.

Materials and Methods

In Vivo 17b 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 OE 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.

Cerebrovasculature 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 GSI5R 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-centrigued 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-

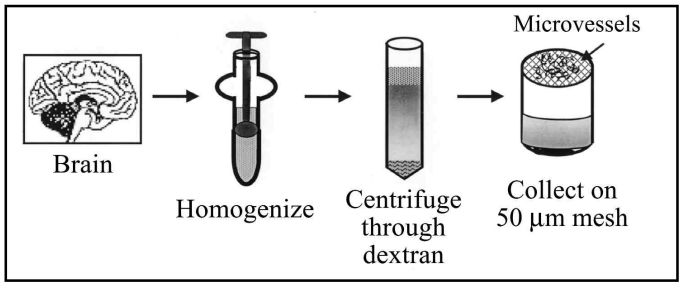

Figure 2
Vessel isolation and preparation.

tran (same process). All pellets were then collected, re-suspended 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 pepstatin, 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, $cPLA_2$, or PGI_2-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 electroblotting, 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-$cPLA_2$ (1:100), or mouse polyclonal anti-PGI_2-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 $cPLA_2$ 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 $cPLA_2$ antibody at 110 kDa, which corresponds to the molecular weight of $cPLA_2$ protein (Figure 3). For each Western Blot, similar amounts of OVX and OE lysate were

Table 1
Effect of animal treatment on serum concentrations of 17β-estradiol, body weight, and uterine weight

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

Values are means ± S.E. *Significantly different than ovariectomized female (P<0.001).

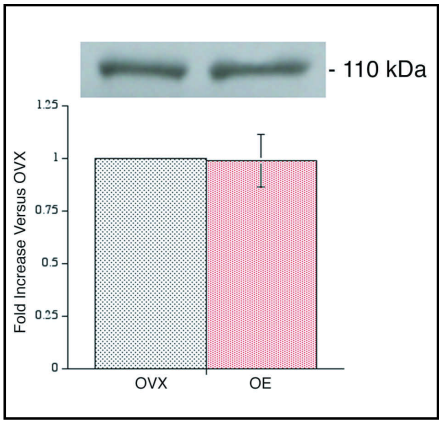

Figure 3
Western Blot of $cPLA_2$ protein in cerebral vessels from OVX and OE rats.

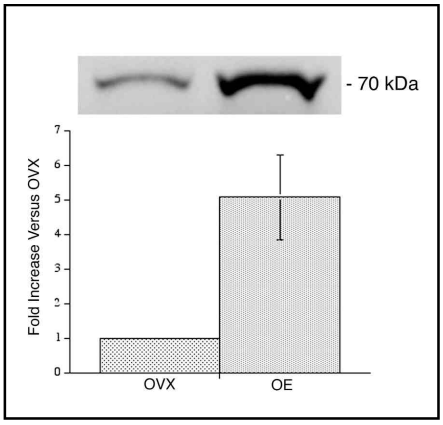

Figure 4
Western blot of COX-1 protein in cerebral vessels from OVX and OE rats. *P<0.05

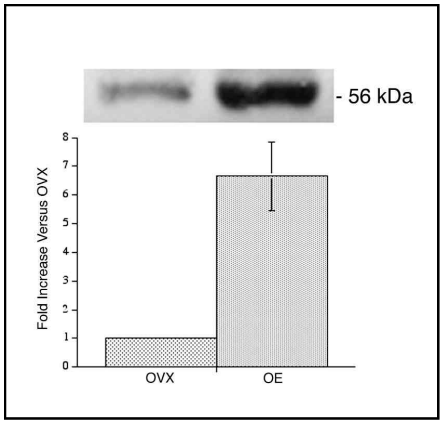

Figure 5
Western Blot of PGI_2-S protein in cerebral vessels from OVX and OE rats. *P<0.05.

Autoradiographic films containing bands were densitometrically analyzed using the computer0based image analysis program, UN-SCAN-IT.

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 PGI_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 PGI_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* 17β-estradiol treatment of ovariectomized rats results in an elevation of basal and arachadonic acid-stimulated prostacyclin production by cerebral blood vessels *in vitro* (unpublished data, Ospina, Krause, Duckles). This process of conversion of arachadonic acid to prostacyclin involves the three enzymes $cPLA_2$, COX-1 and PGI_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 PGI_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 PGI_2-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 (Fayard 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 PGI_2, then vessel relaxation should be increased. At the molecular level, cDNA experiments have demonstrated that overexpression of COX-1 in a cell line enhanced PGI_2 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 PGI_2-S levels, thus leading to an increase in PGI_2 levels. Zou *et al.* (1999) demonstrated that PGI_2-S is inactivated in early-stage atherosclerotic lesions. Moreover, it was shown that PGI_2-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 PGI_2-S. This study is the first to demonstrate that estrogen increases the level of PGI_2-S protein. This effect of estrogen would also be expected to increase PGI_2 synthesis since PGI_2-S is the final enzyme in the pathway of PGI_2 formation.

Results support the hypothesis that estrogen increases the levels of both COX-1 and PGI_2-S protein expression in cerebral blood vessels. This increase appears to underlie previous observations that estrogen increases PGI_2-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 PGI_2-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 prostecyclin synthesis. Results indicated that estrogen treatment *in vivo* increases levels of COX-1 and PGI_2-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

Bush, T.L., E. Barrett-Connor, L.D. Cowan, M.H. Criqui, R.B. Wallace, C.M. Suchindran, H.A. Tyroler, and B.M. Rifkind. "Cardiovascular Mortality and Non-Contraceptive Use of Estrogen in Women: Results From the Lipid Research Clinics Program Follow-Up Study." Circulation 75 (1987): 1102-1109.

Fayard, J.M., S. Chanal, A. Fanidi, J.F. Pageaux, M. Lagarde, and C. Laugier. "In Vivo Inhibition of Basal and Estrogen-Induced Phospholipase A2 Activities by the Triphenylethylene Antiestrogen, Tamoxifen, in Immature Quail Oviduct." European Journal of Pharmacology 216 (1992): 127-130.

Geary, G.G., D.N. Krause, and S.P. Duckles. "Estrogen Reduces Mouse Cerebral Artery Tone Through Endothelial NOS- and Cyclooxygenase-Dependent Mechanisms." American Journal of Physiology 279a (2000): H511-H519.

Geary G.G., D.N. Krause, and S.P. Duckles. "Gonadal Hormones Affect Diameter of Male Rat Cerebral Arteries Through Endothelium-Dependent Mechanisms." American Journal of Physiology 279b (2000): H610-H618.

Grodstein, F., M.J. Stampfer, J.E. Manson, G.A. Colditz, W.C. Willett, B. Rosner, F.E. Speizer, and C.H. Hennekens. "Postmenopausal Estrogen and Progestin Use and the Risk of Cardiovascular Disease." The New England Journal of Medicine 335 (1996): 453-461.

Hulley, S., D. Grady, T. Bush, C. Furberg, D. Herrington, B. Riggs, and E. Vittinghoff. "Randomized Trial of Estrogen Plus Progestin for Secondary Prevention of Coronary Heart Disease in Postmenopausal Women." Journal of the American Medical Association 280 (1998): 605-613.

Hurn, P.D., and I.M. Macrae. "Estrogen as a neuroprotector in stroke." Journal of Cerebral Blood Flow Metabolism 20 (2000): 631-652.

Jun, S.S., Z. Chen, M. Pace, P. Shaul. "Estrogen Upregulates Cyclooxygenase-1 Expression in Ovine Fetal Pulmonary Artery Endothelium." Journal of Clinical Investigations 102 (1998): 176-183.

Kannel, W.B., M.C. Hjortland, P.M. McNamara, and T. Gordon. "Menopause and Risk of Cardiovascular Disease." The Framingham Study. Ann Internal Medicine 85 (1976): 447-452.

Lerner, D.J., and W.B. Kannel. "Patterns of Coronary Heart Disease Morbidity and Mortality in the Sexes: A 26-year Follow-up of the Framingham Population." American Heart Journal 111 (1986): 383-390.

Makila, U.M., L. Wahlberg, L. Viinikka, and O. Ylikorkala. "Regulation of Prostacyclin and Thromboxane Production by Human Umbilical Vessels: the Effect of Estradiol and Progesterone in a Superfusion Model." Prostaglandins Leukotrienes Med 8 (1982): 115-124.

McNeill, A.M., N. Kim, S.P. Duckles, and D.N. Krause. "Chronic Estrogen Treatment Increases Levels of Endothelial Nitric Oxide Synthase Protein in Rat Cerebral Microvessels." Stroke 30 (1999): 2186-2190.

Mikkola, T., L. Viinikka, and O. Ylikorkala. "Administration of Transdermal Estrogen without Progestin Increases the Capacity of Plasma and Serum to Stimulate Prostacyclin Production in Human Vascular Endothelial Cells." Fertility and Sterility 73 (2000): 72-74.

Mück, A.O., Y. Guo, H. Seeger, K. Korte, and T.H. Lippert. "Production of Prostacyclin in Human Umbilical Cord Blood Vessels. Effect of Natural and Synthetic Estrogen." Journal of Gynecology 114 (1992): 414-419.

Myers, S.I., R.H. Turnage, L. Bartula, B. Kalley, and Y. Meng. "Estrogen Increases Male Rat Aortic Endothelial Cell (RAEC) PGI2 Release." Prostaglandins Leukotrienes and Essential Fatty Acids 54 (1996): 403-409.

Stampfer, M.J., G.A. Colditz, W.C. Willett, J.E. Manson, B. Rosner, F.E. Speizer, and C.H. Hennekens. "Postmenopausal Estrogen Therapy and Cardiovascular Disease." The New England Journal of Medicine 325 (1991): 756-762.

Vagnoni, K.E., and R.R. Magness. "Estrogen and Lipopolysaccharide Stimulation of Prostacyclin Production and the Levels of Cyclooxygenase and Nitric Oxide Synthase in Ovine Uterine Arteries." Biology of Reproduction 59 (1998): 1008-1015.

Wu, K.K. "Molecular Regulation and Augmentation of Prostacyclin Biosynthesis." Mediators in the Cardiovascular System: Regional Ischemia 45 (1995): 11-17.

Zou, M.H., M. Leist, and V. Ullrich. "Selective Nitration of Prostacyclin Synthase and Defective Vasorelaxation in Atherosclerotic Bovine Coronary Arteries." American Journal of Pathology 154 (May 1999): 1359-1365.