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Mechanisms of Cerebrovascular Protection: Estrogen, Inflammation and Mitochondria

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Abstract

Investigation of estrogen action reveals a multitude of diverse effects. This brief review focuses on the impact of estrogen on the vasculature, with particular emphasis on the cerebral circulation. Three major actions of estrogen are discussed: enhancement of vasodilator capacity, suppression of vascular inflammation, and increase in mitochondrial efficiency. In both humans and animals, estrogen increases vasodilator tone, an effect dependent on a functional endothelium. Two distinct mechanisms are involved: increase in eNOS mRNA and protein and phosphorylation of eNOS via the PI-3 kinase/Akt pathway. Both effects are mediated by estrogen receptors (ER), but through two pathways, ER-mediated nuclear gene transcription and cell membrane-associated ERs, respectively. Estrogen also increases function of other endothelium-dependent vasodilators. Estrogen suppresses vascular inflammation through an NF- κ B-dependent effect. The inflammatory response has also been shown to vary significantly during the estrous cycle of rodents. Emerging information shows that estrogen increases mitochondrial biogenesis and decreases superoxide production. Suppression of mitochondrial superoxide production by 17 β -estradiol in cerebral blood vessels is mediated by the ER- α receptor and not dependent on increased Mn superoxide dismutase activity. Estrogen treatment also increases protein levels for a number of components of the electron transfer chain, as well as levels of transcription factors that regulate mitochondrial function. All of these actions of estrogen could be important in mediating vascular protection, especially in the cerebral circulation. Furthermore, given the potential of mitochondrial DNA damage to contribute to pathophysiology and aging, mitochondrial protective effects of estrogen might contribute to the longer average lifespan of women.

Keywords

Gonadal Steroids; Mitochondria; Vascular Protection; Vascular inflammation

Introduction

Investigation into the effects of gonadal steroids, including estrogen, originally focused, quite naturally, on their impact on reproduction. However, especially over the last 15 years, it has become increasingly understood that gonadal steroids also exhibit a great number of actions that could be considered non-reproductive in nature (Turgeon *et al.* 2006; Miller & Duckles, 2008). One could argue that all effects of gonadal steroids may ultimately affect reproductive capacity. However, as the average human life span continues to lengthen beyond the reproductive years, the important impact of gonadal steroids on a host of

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Conflict of Interest

There is no conflict of interest.

physiological and pathological processes becomes an ever more important concern for human health (Harman *et al.* 2005).

Some of the most well studied “non-reproductive” effects of gonadal steroids concern actions on the vascular system. Again, physiological adaptations of the vasculature are well known to be critical in all aspects of reproduction, and the role of gonadal steroids in regulating these functions is clear. However, beyond these specific actions, it is increasingly evident that gonadal steroids have profound influences on normal vascular physiology as well as pathophysiological processes. This brief review focuses on only one of the gonadal steroids, 17 β -estradiol, the most potent naturally occurring estrogen in humans (Loose-Mitchell & Stancel, 2001). However, other gonadal steroids, such as testosterone and progesterone, also are known to have important effects on vascular function (Liu *et al.* 2003; Krause *et al.* 2006; Turgeon *et al.* 2006).

Enhanced Vasodilator Capacity

One of the most consistently observed effects of estrogens in experimental animals and humans is an increase in vasodilator tone (Miller & Duckles, 2008). This effect requires a functional endothelium and has been shown in both humans and animal models to primarily depend on endothelial nitric oxide synthase (eNOS) (Duckles & Miller, 2010). As is true for a number of other hormones, both genomic and non-genomic mechanisms are involved in increasing eNOS activity (Duckles & Miller, 2010). For example, in rodents in vivo treatment with physiological levels of 17- β estradiol increases eNOS mRNA and protein levels in cerebral arteries (McNeill *et al.* 1999; Geary *et al.* 2001; Stirone *et al.* 2003). Estrogen receptor alpha (ER α) appears to be the major mediator of this effect, as 17 β -estradiol did not increase eNOS in ER α knockout mice (Geary *et al.* 2001).

Besides this effect of estrogen on eNOS gene transcription, a form of ER α localized to caveolae in the plasma membrane is responsible for increased phosphorylation of eNOS on serine 1177, enhancing NO production (Hisamoto & Bender, 2005; Levin, 2009). This effect of estrogen is mediated by phosphatidylinositol 3-kinase/Akt and has been demonstrated both in vitro and in vivo (Williams *et al.* 1992; Stirone *et al.* 2005a; Li *et al.* 2007).

In addition to the ability of estrogen to enhance endothelial production of NO, estrogen also affects production of other endothelial factors, and the relative importance of these various endothelial-dependent factors and the impact of estrogen may vary among vascular beds. For example, chronic treatment with estrogen increases prostacyclin synthesis in rodent cerebral arteries as well as ovine fetal pulmonary arterial endothelium by elevating levels of both cyclooxygenase-1 as well as prostacyclin synthase (Jun *et al.* 1998; Ospina *et al.* 2002; Sherman *et al.* 2002). In contrast, in rat skeletal muscle arteries treated with a NOS inhibitor, estrogen treatment increased vasodilation mediated by endothelial-dependent hyperpolarizing factor (EDHF) (Huang *et al.* 2001).

The functional consequences of increased release of NO and other vasodilator factors from the vascular endothelium have been demonstrated both in vitro and in vivo (Duckles & Miller, 2010). In women, a host of studies have shown that estrogen increases plasma concentrations of NO as well as increasing reactive hyperemia; these effects can also be seen through the menstrual cycle, reflecting an estrogenic effect (Miller & Mulvagh, 2007). Estrogenic effects are not limited to females. When young men receive an aromatase inhibitor, there is a decrease in endothelial vasodilator function, as determined by flow-mediated brachial artery dilation (Lew *et al.* 2003). This study supports the contention that estradiol may contribute to regulation of the peripheral circulation in men.

Studies of isolated arteries from ovariectomized animals with or without estrogen administration have also shown significant changes in vascular reactivity in a variety of vascular beds. In both coronary and cerebral vascular beds as well as in the aorta, chronic exposure to estrogen decreases vascular tone in an endothelium-dependent manner (Wellman *et al.* 1996; Geary *et al.* 1998; Widder *et al.* 2003; Duckles & Krause, 2007). Blockade with inhibitors of NOS or other endothelial-dependent dilators, such as prostacyclin, confirm that estrogen treatment enhances vascular vasodilator capacity through increased production of NO, prostacyclin or EDHF, depending on the particular vascular bed (Ospina *et al.* 2003; Li *et al.* 2004).

Suppressed Vascular Inflammation

Both pro- and antiinflammatory effects of estrogen have been reported in the literature, but, in animal models, anti-inflammatory effects of estrogen on the vasculature have clearly been demonstrated (Miller & Duckles, 2008). In ovariectomized rats, treatment with estrogen suppresses cerebral vascular inflammatory responses to treatment with either interleukin-1 β (IL-1 β) or lipopolysaccharide (Ospina *et al.* 2004; Sunday *et al.* 2006). A similar effect has also been reported in cultured brain endothelial cells (Galea *et al.* 2002). It is well known that NF κ B is an important transcription factor involved in activation of this inflammatory pathway (Catley *et al.* 2003). Therefore, NF κ B DNA binding was measured to determine if estrogen acts through this pathway. After treatment with IL-1 β , NF κ B DNA binding was significantly increased in cerebral blood vessels from ovariectomized rats. However, in vessels from rats treated with 17- β estradiol, the increase induced by IL-1 β was significantly depressed (Ospina *et al.* 2004), supporting the conclusion that estrogen acts, at least partially, by suppressing NF- κ B. Interestingly the magnitude of cerebral vascular inflammatory responses also varies significantly through the estrous cycle, with the greatest inflammatory response observed during estrus, when circulating progesterone is high and estrogen is low (Sunday *et al.* 2006). Interestingly, the anti-inflammatory effect of estrogen diminishes in older female animals (Miller *et al.* 2004; Sunday *et al.* 2007).

Increased Mitochondrial Efficiency, Reduced Oxidative Stress

Mitochondria, having evolved from bacterial symbionts, contain their own DNA (mtDNA), RNA and protein synthesis systems (Wallace, 2005; Miller and Duckles, 2008). The majority of mitochondrial proteins are encoded by the nuclear genome, with the exception of thirteen proteins encoded by mtDNA. Oxidative phosphorylation is the process by which mitochondria burn dietary calories, using oxygen to release chemical energy from food. The body uses this chemical energy, in the form of ATP, to perform work and to maintain body temperature. Five distinct protein complexes (I through V) located in the mitochondrial inner membrane carry out the process of oxidative phosphorylation. An important by-product of energy production by oxidative phosphorylation is the production of reactive oxygen species (ROS) within the mitochondrial matrix. ROS generated within the mitochondria can affect mitochondrial enzymes, mtDNA, as well as the cell in general. Mitochondrial ROS can cause heritable damage (damage to mtDNA) that can be perpetuated as cells divide, producing a progressive age-related change in function. Mitochondrial ROS production will increase with excess fuel supply as well as with decreased energy utilization. Thus oxidative stress will result in inactivation of iron-sulfur centers in key mitochondrial enzymes, such as aconitase, and also with an increased mutation rate of mtDNA.

Recent studies have demonstrated that estrogen can increase mitochondrial biogenesis and alter mitochondrial function (Duckles *et al.* 2006; Klinge, 2008; Mattingly *et al.* 2008; Chen *et al.* 2009). Using a variety of approaches, estrogen has been shown to significantly suppress mitochondrial production of ROS in cerebral blood vessels and endothelial cells

(Stirone *et al.* 2005b; Razmara *et al.* 2008; Guo *et al.* 2010). Measurement of mitochondrial superoxide using Mitosox dye demonstrates a decreased rate of superoxide production in human brain microvascular endothelial cells treated with 10 nM 17 β -estradiol compared to vehicle control cells (Razmara *et al.* 2008). These effects of 17 β -estradiol were prevented by pre-treatment with an estrogen receptor antagonist and mimicked by an ER α agonist, but not an agonist of ER β receptors.

Two other measurements lead to the same conclusion. The first of these involves measurement of the activity of mitochondrial aconitase, which acts as a functional indicator of the level of ROS inside the mitochondrial matrix. Aconitase, one of the components of the tricarboxylic acid cycle, has an iron sulfur core which, when oxidized by ROS, results in decreased enzyme activity. Thus measurement of mitochondrial aconitase activity is one indicator of the level of mitochondrial ROS. In human brain microvascular endothelial cells treated with 17 β -estradiol, mitochondrial aconitase activity is significantly greater than in vehicle-treated cells (Razmara *et al.* 2008). After treatment with a reducing agent, this difference in mitochondrial aconitase activity disappears, supporting the conclusion that changes in aconitase activity represent oxidation of the enzyme rather than alterations in number of enzyme molecules, phosphorylation, or other modifications that could also lead to alterations in enzyme activity. The second independent measurement of mitochondrial ROS is assessment of the mitochondrial production of hydrogen peroxide, the product of superoxide metabolism by superoxide dismutase. Using mitochondria isolated from cerebral blood vessels of ovariectomized rats, succinate-driven mitochondria were shown to produce significantly less hydrogen peroxide in cerebral vessels from estrogen-treated rats compared to vessels from ovariectomized controls (Stirone *et al.* 2005b). In vivo estrogen treatment significantly increases levels of Mn superoxide dismutase, with no effect on glutathione peroxidase or catalase (Stirone *et al.* 2005b). However, a decrease in mitochondrial hydrogen peroxide can not be accounted for by an increase in Mn superoxide dismutase. Thus, results of these three independent measurements all support the contention that estrogen reduces the rate of superoxide production in cerebrovascular mitochondria.

Estrogen also influences a number of other aspects of mitochondrial function. Estrogen treatment, either in vivo or in vitro increases protein levels for a number of components of the electron transfer chain, most notably cytochrome c and both subunit I and subunit IV of complex IV (Stirone *et al.* 2005b). Since subunit I is encoded by mtDNA, while subunit IV is encoded by nuclear DNA, these findings suggest that estrogen may affect transcription of both mitochondrial and nuclear DNA. This underscores the importance of coordination between nuclear and mtDNA in order to maintain appropriate mitochondrial function under a variety of environmental circumstances (Scarpulla, 2008).

Estrogen treatment has also been shown to increase the activities of mitochondrial complex IV and citrate synthase, rate limiting steps in electron transport and the citric acid cycle, respectively (Stirone *et al.* 2005b). In order to further understand how estrogen affects mitochondrial regulation, key transcription factors known to control mitochondrial regulation were measured (Figure 1). Nuclear respiratory factor (NRF)-1 is a key transcriptional regulator of nuclear-encoded mitochondrial genes (Kelly & Scarpulla, 2004). Therefore, it is intriguing that levels of NRF-1 protein significantly increased in cerebral vessels isolated from estrogen-treated rats compared to ovariectomized controls (Stirone *et al.* 2005b). NRF-1 is regulated by a family of nuclear co-activators, including peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1 α , PGC-1 β , and PGC-1 related coactivator (PRC) (Kelly & Scarpulla, 2004). Measurement of mRNA for these three co-activators demonstrated that PGC-1 α levels were lower, but expression of both PGC-1 β and PRC was higher in cerebral vessels from estrogen-treated rats compared to ovariectomized controls (Stirone *et al.* 2010, unpublished data).). These findings support the idea that

estrogen, possibly acting through the PGC-1, NRF-1 pathway is an important modulator of mitochondrial function, increasing mitochondrial efficiency while decreasing production of ROS.

But what are the implications of these effects of estrogen on mitochondrial function in cerebral blood vessels and perhaps other vascular beds? First of all, it is important to recognize that cerebral blood vessels are highly metabolically active, with a greater complement of mitochondria in cerebrovascular endothelium compared to other vascular beds (Nag, 2002). This reflects the unique role of cerebral endothelial cells in maintaining the blood-brain barrier, as well as the importance of the cerebral vasculature in maintaining autoregulation of blood flow as well as the exquisite match of local blood flow to brain metabolic activity. Highly metabolically active cells are particularly subject to progressive mitochondrial and cellular damage from oxidative stress due to the increased production of mitochondrial ROS, as described above. Therefore, the ability of estrogen to preserve endothelial function, particularly in the brain, could significantly influence the occurrence and course of a number of age-related neurological diseases, including Parkinson's, Alzheimer's and stroke (Girouard & Iadecola 2006).

Further implications of the effect of estrogen on mitochondrial function relate to the well-known links between cardiovascular disease, age, exercise and diet, and the rising importance of the metabolic syndrome in developed nations (Eckel *et al.* 2005). As described briefly above, one important common link between age, exercise and diet that could explain rapid changes in incidence of some diseases, including cardiovascular, lies in the mitochondria (Wallace 2005). This concerns the impact of ROS produced by mitochondrial oxidative phosphorylation on mitochondrial DNA, resulting in the inevitable accumulation of mtDNA mutations over time, especially in highly metabolically active cells. This is the foundation for the mitochondrial theory of aging. Since both lack of exercise and an abundant diet will increase mitochondrial ROS production, mitochondria link aging, lack of exercise and abundant diet. The ability of estrogen to promote mitochondrial efficiency while decreasing ROS production provides one explanation for the longer life span of women (Duckles *et al.* 2006). If estrogen decreases mitochondrial ROS production, the rate of aging and developing pathophysiology may be delayed in women exposed to estrogen. This protection would no longer be present after loss of ovarian function (Parker *et al.* 2005).

Conclusion

This brief review has focused on the effects of estrogen to increase endothelial-dependent vasodilation, suppress vascular inflammation and increase mitochondrial efficiency and decrease mitochondrial ROS production in the cerebral circulation. All of these actions of estrogen may have profound effects on the pathophysiology of cardiovascular disease.

Endothelial, or vascular, dysfunction, and the loss of endothelial vasodilator production (Grundy *et al.* 2004), has been associated with hypertension, atherosclerosis, diabetes and other diseases as well as with normal aging in both humans and rodents (Duckles *et al.* 2006). The ability of estrogen to increase production of endothelial-dependent vasodilators represents one mechanism for estrogen to contribute to prevention of cardiovascular disease. Vascular inflammation is also an important part of cardiovascular pathophysiology, including the development of atherosclerosis as well as the sequelae of stroke. Thus the ability of estrogen to suppress vascular inflammation may contribute to delay of disease progression. Finally, oxidative stress is known to contribute to cardiovascular disease, and, especially in metabolically active cells, such as cerebral endothelial cells, mitochondria can be an important generator of ROS (Wallace 2005).

Thus the ability of estrogen to suppress mitochondrial ROS generation may directly alter vascular tone as well as have longer-lasting protective effects. The link between mitochondrial oxidative phosphorylation, production of ROS, damage to mtDNA, and degenerative diseases associated with aging may help us to understand why mitochondrial actions of estrogen may delay human aging and disease. Thus, all three of these mechanisms, increase in endothelial vasodilator production, suppression of vascular inflammation, and promotion of mitochondrial efficiency and suppression of mitochondrial superoxide production, may contribute to the impact of estrogen on cardiovascular disease and aging. However, given the pleiotropic nature of estrogenic effects, it may be necessary to develop agents with greater specificity in order to produce useful therapeutic interventions. A greater understanding of the nature of estrogen's effects and the underlying mechanisms will surely be critical in achieving this therapeutic goal.

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Figure 1.

The figure illustrates a hypothesis for the mechanism by which estrogen influences mitochondrial function based on current knowledge of the coordinated regulation of nuclear and mitochondrial DNA. Within the nucleus ER α bound to estrogen promotes transcription of members of the PGC-1 family of transcription factors. Increased levels then promote nuclear transcription of NRF-1. NRF-1 increases transcription of nuclear genes that encode mitochondrial proteins involved in oxidative phosphorylation, such as cytochrome C, which are then transported into the mitochondria. NRF-1 also promotes nuclear transcription of TFAM. The TFAM protein acts within the mitochondria to increase transcription of proteins encoded by mitochondrial DNA, such as subunit I of complex IV.

Abbreviations: ER α , (estrogen receptor α); PGC-1 α , (peroxisome proliferator-activated receptor-gamma coactivator); NRF-1, (nuclear respiratory factor-1); TFAM, (transcription factor A-mitochondrial).