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Vascular Actions of Estrogens: Functional Implications

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***Abstract*——The impact of estrogen exposure in preventing or treating cardiovascular disease is controversial. But it is clear that estrogen has important effects on vascular physiology and pathophysiology, with potential therapeutic implications. Therefore, the goal of this review is to summarize, using an integrated approach, current knowledge of the vascular effects of estrogen, both in humans and in experimental animals. Aspects of estrogen synthesis and receptors, as well as general mechanisms of estrogenic action are reviewed with an emphasis on issues particularly relevant to the vascular system. Recent understanding of the impact of estrogen on mitochondrial function suggests that the longer lifespan of women compared with men may depend in part on the ability of estrogen to decrease production of reactive oxygen species in mitochondria. Mechanisms by which estrogen increases endothelial vasodilator function, promotes angiogenesis, and modulates autonomic function are summarized. Key aspects of the relevant pathophysiology of inflammation, atherosclerosis, stroke, migraine, and thrombosis are reviewed concerning current knowledge of estrogenic effects. A number of emerging concepts are addressed throughout. These include the importance of estrogenic formulation and route of administration and the impact of genetic polymorphisms, either in estrogen receptors or in enzymes responsible for estrogen metabolism, on responsiveness to hormone treatment. The importance of local metabolism of estrogenic precursors and the impact of timing for initiation of treatment and its duration are also considered. Although consensus opinions are emphasized, controversial views are presented to stimulate future research.**

I. Introduction

Scientific investigation into the nonreproductive cardiovascular actions of estrogen has waxed and waned over several decades. However, the field has been rejuvenated by a number of governmental initiatives, controversial outcomes of several large clinical trials (Hulley et al., 1998; Nair and Herrington, 2000; Rossouw et al., 2002), the growing public interest in "safer," more "bioidentical" hormones, and the interest in personalized, sex-based medicine (pharmacogenomics). Furthermore, validation of controversial mechanisms of action of sex steroids, identification of novel effects of estrogen such as a regulator of mitochondrial function, and development of new theories of treatment efficacy based on further analyses of data from various observational and clinical trials (Salpeter et al., 2004, 2006; Grodstein et al., 2006; Hsia et al., 2006b; Clarkson, 2007; Manson et al., 2007) support the possibility that hormonal therapies may be viable options to prevent some chronic conditions of aging.

With these emerging areas of science in mind, in this review we take an integrative approach toward understanding the effects of estrogen on regulation of vascular reactivity, angiogenesis, atherosclerosis, and thrombosis in an aging population. Information regarding steroid synthesis and receptors will be discussed briefly only to provide sufficient background information upon which to build the other discussion. Interactions of estrogen with other hormones, although an important consideration, are insufficiently understood and will not be included. Effects of estrogen on cardiac function, a growing field of investigation, also will not be included as the topic is sufficiently complex as to warrant a separate review. In other areas, such as changes in vascular function during hormonal transitions in puberty, information is scant. In all sections, consensus of understanding will be emphasized, and areas requiring more research will be identified.

II. Estrogen Synthesis and Receptors

A. Estrogenic/Androgenic Balance

The biosynthesis of gonadal steroids is understood well and explicated clearly in textbooks (Loose-Mitchell and Stancel, 2001). Only a few key points relevant to the current discussion of the vascular effects of estrogen merit mention here. Testosterone is a key intermediate in both women and men, being converted to estrogen by the action of aromatase and to the more potent androgen, dihydrotestosterone, by 5-α reductase. In women estradiol is the main form of circulating estrogen, and circulating levels of testosterone are relatively low. In men, testosterone is the principal circulating androgen, and circulating estrogen levels are much lower than in women.

A key point, though, is that circulating levels of hormones may not reflect those at the tissue level, as both aromatase and 5-α reductase can be found in a number of tissues, including blood vessels (Gonzales et al., 2007). For example, in bone, testosterone is converted to 17β-estradiol by aromatase; estradiol then acts locally to promote mineralization and prevent osteoporosis. In fact, mutations of genes encoding either aromatase or estrogen receptor α result in altered bone phenotype in men (Smith et al., 1994; Carani et al., 1997). 5-α Reductase in the prostate converts testosterone to the more potent androgen, dihydrotestosterone, a critical step for effective promotion of prostate growth and function (Steimer, 2003). Administration of an aromatase inhibitor to young men resulted in a decrease in endothelial vasodilator function, assessed by flow-mediated dilation of the brachial artery (Lew et al., 2003), providing evidence that conversion of testosterone to estradiol may contribute to regulation of the peripheral circulation in men. In women, evidence suggests that the relationship among circulating concentrations of free 17β-estradiol, free testosterone, and sex hormone-binding globulin may be more predictive of changes in carotid intimal thickening than concentrations of any of these hormones alone (Karim et al., 2008). Despite these few examples,

the complexities of gonadal steroid hormone metabolism and local variation are still not well understood, particularly with respect to the nonreproductive effects of gonadal steroids, including vascular effects. However, with the growing therapeutic use of inhibitors of gonadal steroid metabolism including aromatase inhibitors and inhibitors of 5-α reductase, particularly in women with a history of breast cancer, a better understanding of possible side effects, especially with long-term use, is essential (Nabholtz and Gligorov, 2006; Pritchard and Abramson, 2006). Nevertheless, more information is needed to illuminate how local balance between levels of androgens and estrogens influences cardiovascular function in both males and females and how imbalances may contribute to sex differences in the pathophysiology of cardiovascular disease.

B. Receptors for Estrogen

Evidence suggests that there are at least three, and possibly four, distinct receptors for estrogen: two ligand-activated transcription factors [estrogen receptor (ER[1]) α and ERβ], one G protein-coupled receptor, GPER (also referred to as GPR30), and a third, less well defined putative receptor, termed ER-X (Toran-Allerand, 2004). Most evidence for the existence of ER-X comes from studies of the brain; therefore, this putative receptor will not be further discussed in this review focusing on the vasculature.

1. Ligand-Activated Transcription Factors. The first estrogen receptor was discovered and described in the late 1950s; this remained the status quo until the discovery of a second estrogen-sensitive, ligand-activated transcription factor, named ERβ (Kuiper et al., 1996). The ER described initially was then termed ERα. Both ER forms have been localized to the vasculature in both endothelial and smooth muscle cells (Mendelsohn and Karas, 1999). A single case of a man with a disruptive mutation of ERα has given some insight into the nonreproductive effects of this receptor (Sudhir et al., 1997a). This 31-year-old man was of tall stature because of incomplete epiphyseal closure and had decreased bone mineral density. It is interesting that this phenotype is similar to that seen in men with mutations resulting in aromatase deficiency, as mentioned in section II.A, (Rochira et al., 2002; Jones et al., 2006). This individual also had early coronary arterial atherosclerosis and endothelial dysfunction, with no detectable flow-mediated dilation in the brachial artery (Sudhir et al., 1997b), thus providing additional support to the hypothesis that estrogen through receptor operated mechanisms regulates peripheral arterial function.

ERα and ERβ are both members of the nuclear hormone receptor superfamily and are encoded by distinct genes with different chromosomal locations (Dahlman-Wright et al., 2006). These receptors function as ligand-activated transcription factors to produce so-called genomic effects but may also act through additional mechanisms (see section III.B). Like other members of this superfamily, one gene may result in multiple proteins and diverse responses (Zhou and Cidlowski, 2005). Mechanisms for this diversity include epigenetic changes, specifically methylation, of the genes encoding these receptors, multiple isoforms of each receptor as a consequence of alternative RNA splicing, and multiple sites of translation initiation of receptor mRNA (Post et al., 1999; Ying et al., 2000; Lewandowski et al., 2002; Hirata et al., 2003). In addition, post-translational modifications may lead to alterations in both protein stability and function. Methylation of genes for both ERα and ERβ are associated with atherosclerotic tissue, and methylation of the gene for ERβ increases with passage of isolated smooth muscle and endothelial cells, thus implicating this process in receptor responsiveness with aging (Post et al., 1999; Ying et al., 2000).

There is emerging evidence that types of receptor isoforms vary from tissue to tissue and from species to species. This may account for considerable functional diversity, but this emerging field has not yet matured enough to give clear insights into implications for the actions of estrogen on a particular organ system, such as the vasculature.

Estrogen receptor-null mice have provided insights into the distinct roles of ERα and ERβ (Couse and Korach, 1999; Dupont et al., 2000). Two distinct ERα-disrupted mice have been developed. The first, αERKO$_{CH}$, involved disruption of key domains in the receptor protein; however, a transcriptionally active form of ERα truncated for the A/B domain can still be found in these mice in low amounts (Lubahn et al., 1993; Couse et al., 1997). Subsequently, a second mutant mouse (αERKO$_{ST}$) was generated, fully lacking ERα (Dupont et al., 2000). Disruption of ERα is not lethal; instead animals develop normally, with a life span comparable to that of their wild-type littermates (Lubahn et al., 1993). Females and males of both αERKO types are infertile (Couse and Korach, 1999; Dupont et al., 2000). Consistent with observations in humans, endothelium-dependent vasodilatation is reduced in these animals (Rubanyi et al., 1997).

Knockouts of ERβ (βERKO) (Krege et al., 1998) also survive to adulthood and exhibit distinct phenotypes compared with αERKO mice. Knockouts of both ERα and ERβ have also been developed (Dupont et al., 2000). As with any scientific method, a number of caveats must be taken into account when interpreting results from

[1] Abbreviations: ER, estrogen receptor; GPER, activation function, GPR30; ERKO, estrogen receptor knockout, AF, activation function; ICI 182,780, fulvestrant; SERM, selective estrogen receptor modulator; PPT, propylpyrazole triol; DPN, diarylpropionitrile; NO, nitric oxide; eNOS, endothelial nitric-oxide synthase; ROS, reactive oxygen species; SOD, superoxide dismutase; CEE, conjugated equine estrogen; COMT, catechol-*O*-methyltransferase; SWAN, Study of Women Across the Nation; WHI, Women's Health Initiative; KEEPS, Kronos Early Estrogen Prevention Study; TNF, tumor necrosis factor; IL, interleukin; PAD, peripheral arterial disease; HSP, heat-shock protein.

studies of transgenic animals, especially animals such as the ER knockouts available to date that are not conditional mutants. Nonconditional knockout mice go through the full process of development in the absence of ER, so phenotypic changes may be caused either by a change in developmental processes or by the absence of the receptor in the mature animal. These and other issues have been well summarized elsewhere (Couse and Korach, 1999). Nevertheless, when used appropriately and together with other complementary approaches, it is clear that genetically modified mice can be very useful tools in understanding the distinct roles of the two ERs in cardiovascular function, especially with aging and with fluctuations in endogenous hormonal milieu.

Levels of ERα and ERβ appear to be differentially regulated by estrogen itself. However, regulation of estrogen receptor may be dependent upon the tissue and duration of estrogen treatment. In cultured ovine endothelial cells, short treatment (2 h) down-regulated but longer exposure for 6 h increased expression of ERα while down-regulating ERβ (Ihionkhan et al., 2002). Chronic in vivo treatment with physiological levels of 17β-estradiol also up-regulates ERα protein in cerebral blood vessels (Stirone et al., 2003b). Compared with blood vessels from ovariectomized rats, levels of several ERα isoforms are higher in vessels from intact females and ovariectomized rats treated with estrogen. In contrast, expressions of ERα, ERβ, and GPR30 are reduced by 17β-estradiol in endothelium-denuded arteries but not veins derived from humans with atherosclerosis (Haas et al., 2007). ERα is up-regulated in endothelial cells of pigs after ovariectomy and down-regulated after treatment with oral estrogenic products. However, ERβ is somewhat more resistant to regulation by these manipulations (Okano et al., 2006). If estrogen receptor levels are indeed regulated by estrogen itself, this could have major implications for interpretation of human studies of the cardiovascular effects of hormone therapy (Arnal and Bayard, 2002).

Other hormones and growth factors can regulate ERs as well. In vascular cells growth factors have been shown to activate ERα in the absence of ligand, an effect that occurs via a mitogen-activated protein kinase-independent pathway (Karas et al., 1998). Progesterone can also affect levels of ER, and progesterone receptor A has been shown to function as a ligand-dependent transrepressor of other steroid receptors, including ER (Edwards, 2005). The physiological and pathophysiological implications of these effects related to changes in the ratio of expression of ERα to ERβ have not yet been fully clarified.

A number of polymorphic sites of both ERα and ERβ gene loci have been identified in humans (Rosenkranz et al., 1998; Gennari et al., 2005; Dahlman-Wright et al., 2006). In the case of ERβ, two tightly linked polymorphisms have been associated with risk of myocardial infarction in women, with the rs1271572 polymorphism variant T allele associated with increased risk and the rs1256049 variant associated with decreased risk (Rexrode et al., 2007). There were no significant relationships found in men. In the case of ERα, several polymorphisms have been associated with an increased ability of hormone replacement therapy to increase levels of high-density lipoprotein cholesterol in postmenopausal women (Herrington et al., 2002a). Associations between ERα polymorphisms have also been shown for risk of myocardial infarction and stroke in men (Shearman et al., 2003, 2006; Schuit et al., 2004; Shearman, 2006). Systolic and mean arterial pressures of older men were higher in the TC and C/C genotypes of 30T/C compared with TT genotypes (Hayashi et al., 2007).

Studies of women have not been as consistent in showing these relationships, as a variety of confounding factors, including menopausal status and use of drugs for contraception and hormone therapy, make it more difficult to analyze data in women, requiring larger numbers of subjects (Shearman, 2006). However, in one study of older women, significant differences in arterial stiffness as reflected in brachial-ankle pulse-wave velocity were found among those with 401T/C and 30T/C polymorphisms of ERα (Hayashi et al., 2007). These findings underscore the impact of estrogen on cardiovascular function. They also highlight the likelihood that estrogen may play a protective role against cardiovascular disease in men as well as in women (Shearman, 2006). However, more work is needed to explore the physiological and pathophysiological impact of estrogen receptor polymorphisms with the etiology of diseases in aging animals and humans.

2. G Protein-Coupled Estrogen Receptor. Besides acting via ERα and ERβ, there is a long history of observations demonstrating that estrogen also acts via plasma membrane receptors (Hasbi et al., 2005). Although considerable controversy remains concerning the mechanism of these so-called nongenomic actions of estrogen (see section III.B), one candidate receptor is the GPER. Originally identified as an orphan G protein-coupled receptor, GPR30; this protein was later shown to be localized to the endoplasmic reticulum and to specifically bind estrogen. This receptor was then named GPER. Binding of estrogen results in intracellular calcium mobilization and synthesis of nuclear phosphatidylinositol 3,4,5-triphosphate when GPER is expressed in COX7 cells (Hasbi et al., 2005; Revankar et al., 2005) or in a breast cancer cell line (Revankar et al., 2005; Thomas et al., 2005). Activity of adenyl cyclase was also increased in HEK293 cells transfected with GPER (Thomas et al., 2005). Regarding the relevance of GPER to vascular function, there are no definite conclusions as yet, although the receptor has been identified in human internal mammary arteries and saphenous veins (Haas et al., 2007). As discussed in section III.B, there are a number of competing mechanisms to account for non-

genomic actions of estrogen, and those relevant to vascular function have not yet been clearly elucidated.

III. General Mechanisms of Action

A. Effects on Gene Transcription

ER, members of the nuclear receptor superfamily, use a conserved DNA binding domain to interact with specific hormone response elements in the genome and influence gene transcription. Such effects, often referred to as "genomic," were those originally described for nuclear receptors, although, as discussed in section III.B, there is considerable evidence for other ("nongenomic") mechanisms of action for many members of this receptor family, including ERα and ERβ. A major emerging theme in understanding the diverse actions attributed to these proteins involves their ability to adopt multiple states dependent on the nature of the bound ligand. Each ligand can induce a different conformation of the receptor; as a consequence distinct sets of coactivators and coreceptors may be recruited to the receptor-transcription complex, resulting in distinct effects (Heldring et al., 2007).

Similar to other members of this receptor family, ERs include structurally and functionally distinct domains, which are highly conserved during evolution (Nilsson et al., 2001). The most conserved of these domains is the DNA-binding domain, which is involved in DNA recognition and binding. A second domain, the ligand-binding domain, occurs in the COOH-terminal. Two distinct transcriptional activation functions, AF1 and AF2, recruit a variety of coregulatory proteins to the DNA-bound receptor (Matthews and Gustafsson, 2003). AF1 is localized to the N-terminal region, whereas AF2 is localized to the conserved ligand-binding domain and relies on an agonist ligand-induced protein conformation. Depending on the cellular and promoter context, AF1 and AF2 act either independently or synergistically in regulating gene expression. Adding to the complexity of estrogen action, the pattern of genes modulated by ERα and ERβ also depends on the status of other cellular signaling pathways (Heldring et al., 2007).

Unlike other members of the nuclear receptor family, the ligand binding cavity of ERs accommodates a wide range of structurally different compounds, including metabolites of estrogen and even environmental contaminants referred to as endocrine disruptors (Heldring et al., 2007). Several different types of ER antagonists have been distinguished (Hall and McDonnell, 2005). ICI 182,780 (fulvestrant) opposes the actions of estrogen in all tissues and does not distinguish between the two types of ER. In contrast selective estrogen receptor modulators (SERMs) show tissue-specific actions, acting either as antagonist or agonist, depending on the cell type. An early example was the use of tamoxifen for treatment of estrogen-dependent breast cancer. Tamoxifen is used therapeutically in the tumor cell as an antagonist of the estrogen receptor; however, tamoxifen also increases bone density, acting like an estrogen agonist (Love et al., 1992). Whereas agonists, such as 17β-estradiol, induce a conformation of the ligand-binding domain that promotes coactivator binding, the bulky side chains of SERMs prevent the agonist-induced conformation (Dahlman-Wright et al., 2006). Thus, by blocking AF2, SERMs act as antagonists in cells depending mainly on this route for activity. However, in some tissues the second transcriptional activation function, AF1, may be active, and SERMs may act as agonists in this case. Another contributor to the tissue specificity of estrogen receptor ligands appears to be variation from tissue to tissue of other ER-interacting proteins, termed coactivators and corepressors (Hall and McDonnell, 2005). Although we have some understanding of the complex mechanisms by which ERs act, their effects vary significantly depending on the tissue context, suggesting considerable potential for further development of selective therapeutic agents.

As mentioned, a number of SERMs have been developed, including tamoxifen, raloxifene, and others still in clinical development. Recently, a large study investigated the effect of raloxifene on cardiovascular disease in postmenopausal women (mean age: 67.5 years) (Barrett-Connor et al., 2006). Compared with placebo, raloxifene had no effect on the risk of primary coronary events but was associated with an increased risk of fatal stroke and venous thromboembolism. As with large human trials on hormone replacement therapy, the advanced age of this patient population could alter the response to estrogenic compounds (Harman et al., 2005b). However, it also may be that other SERMs might have a more positive cardiovascular impact.

In contrast to the nonselective ER agonist, 17β-estradiol, selective synthetic agonists such as propylpyrazole triol (PPT) and diarylpropionitrile (DPN) have been developed (Harrington et al., 2003). These compounds distinguish between ERα and ERβ and may help to distinguish actions of distinct ERs (α, β, nuclear, or non-nuclear) in experimental settings. PPT shows 400-fold selectivity in binding to ERα compared with ERβ (Stauffer et al., 2000). In contrast, DPN is selective for ERβ, although its selectivity is not as great as that of PPT (Meyers et al., 2001). Only a few studies have used these selective compounds to investigate the nature of estrogen receptors mediating vascular effects (Bolego et al., 2006). Even fewer have used concentrations of these substances that are truly selective, that is, in the nanomolar range (Harrington et al., 2003). For example, only the selective ERα agonist, DPN, induces acute NO-dependent vasodilation (Bolego et al., 2005). Likewise, in small mesenteric arteries from female mice, PPT increased flow-mediated relaxation; interestingly, there was no effect of PPT in arteries from males (Douglas et al., 2008). These studies using selective ER agonists support the conclusion that ERα is the principal form

involved in mediating the actions of estrogen on vascular function. However, much more work on the roles of estrogen receptor subtypes, including a more in-depth investigation of all vascular estrogenic actions, is clearly warranted.

B. Rapid Effects

In contrast to actions of estrogen mediated by the genomic mechanism described in section III.A, estrogen can also produce effects within a time span of seconds or minutes, too short to be mediated by the "classic" mechanism involving transcriptional activation of genes (Revelli et al., 1998; Hammes and Levin, 2007). These rapid, extranuclear actions have also been described for a number of other steroid hormones, including progesterone and aldosterone (Wehling, 1997). Activation of signaling pathways, besides modulating protein function, can also influence gene expression and thus protein levels. Therefore, the term, nongenomic, does not accurately describe such extranuclear actions; "membrane-initiated steroid signaling" and "nuclear-initiated steroid signaling" have been suggested as alternatives (Hammes and Levin, 2007). Interestingly, studies of evolution suggested that, in ancient lineages, an ER homolog is not responsive to estrogen but, instead, acts in a constitutive manner to activate transcription, even though estrogen also has important effects on reproduction in these animals (Thornton et al., 2003; Keay et al., 2006).

Although extensive investigations have focused on uncovering the mechanism of these rapid effects of estrogen, a consensus has yet to be reached. Two of the major alternatives include effects of classic ERs at the plasma membrane or a distinct membrane-associated receptor (Hasbi et al., 2005; Revankar et al., 2005; Hammes and Levin, 2007). Some of the evidence for and against these two mechanisms has been detailed elsewhere (Moriarty et al., 2006; Hammes and Levin, 2007). Key points of the controversy will be summarized here. There is considerable evidence that ERs can associate with the plasma membrane, although the particular isoform(s) of ERs remain in doubt, and there may be variability in expression among cell types. In several cell types, ERs associate with caveolae and large protein complexes. This association with caveolae, where a number of other signaling molecules also are found, is thought to promote efficient signaling. By these associations, estrogen appears to trigger a number of intracellular signaling pathways, including mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt, activation of ion channel fluxes, generation of G protein-coupled receptor-mediated second messengers and stimulation of growth factor receptors (Moriarty et al., 2006).

Much of the current investigation of a distinct membrane receptor has focused on GPER (GPR30) (see section III.B.2 and Hasbi et al., 2005). This receptor is widely distributed in the brain as well as in peripheral tissues (Owman et al., 1996), but there is, as yet, little evidence for a functional role in the vasculature. In COS7 cells and some cancer cell lines, GPR30 was exclusively localized to the endoplasmic reticulum (Revankar et al., 2005). Activation by estrogen caused mobilization of intracellular calcium and increased synthesis of phosphatidylinositol 3,4,5-trisphosphate in the nucleus. Others have reported that GPR30 is localized to the plasma membrane (Hasbi et al., 2005). Alternatively, it has been suggested that GPR30 functions only in collaboration with ERα, perhaps serving to assemble a signal complex essential to rapid estrogen signaling (Hammes and Levin, 2007). At any rate, an understanding of the possible role of G protein-coupled receptors in estrogen effects on the vasculature awaits further investigation.

One of the best-described rapid actions of estrogen is the ability to stimulate endothelial nitric-oxide synthase (eNOS) in vascular endothelial cells. Current knowledge of the mechanism of this response also has been reviewed recently (Hisamoto and Bender, 2005; Moriarty et al., 2006), so only key points will be summarized here. In general, ERs associated with the plasma membrane interact with a variety of scaffolding proteins, perhaps varying among cell types. These molecules include striatin (Lu et al., 2004) and Src-homology and collagen homology adapter protein. Furthermore, lipid modifications of ER appear to be important, including palmitoylation (Acconcia et al., 2005). ERs are targeted to lipid rafts; in endothelial cells, ER-centered protein complexes associate with caveolae. ERα, in particular, interacts with caveolin-1, an important structural protein in caveolae, and this interaction is essential for localization of ER to the plasma membrane in endothelial cells (Chambliss et al., 2000). Through this mechanism, estrogen activates eNOS via phosphatidylinositol 3-kinase/Akt, leading to phosphorylation of eNOS on serine 1177, enhancing NO production. This mechanism leads to the well described rapid effect of estrogen to enhance endothelial-dependent vasodilator responses mediated by NO, an effect that has been demonstrated both in vitro and in vivo (Williams et al., 1992; Stirone et al., 2005a; Li et al., 2007).

The complexity of understanding the mechanisms of estrogen signaling has become even more apparent by recent investigations into the relationships among multiple signaling pathways initiated by membrane estrogen receptors and changes in transcription mediated by estrogen response element-containing genes (Edwards, 2005; Vasudevan and Pfaff, 2007). Studies in several different cell types have demonstrated that membrane-initiated cell signaling by estrogen can potentiate nuclear-initiated estrogen signaling. A number of kinase cascades as well as calcium channels appear to be implicated in this transcriptional potentiation. Furthermore, the participation of these different intracellular signaling pathways may occur either in parallel or in

series, and the convergence of membrane-initiated estrogen effects to influence transcription may involve protein-protein interactions and protein translocation as well as phosphorylation of proteins (Vasudevan and Pfaff, 2007). As stated most clearly by Vasudevan and Pfaff (2007), "The novel idea that genomic transcription by hormones, i.e., ligand-dependent transcription at hormone response elements, can be affected by membrane-initiated signal transduction events initiated by cognate or noncognate ligands is a paradigm shift in nuclear receptor biology." Future studies will be necessary to clarify the functional impact of these complex interactions among signaling pathways, both membrane- and nuclear-initiated, in vascular function.

Intracellular pathways also are activated by acute application of estrogen to isolated vascular smooth muscle. The physiological consequence in most cases is relaxation of vascular rings and inhibition of proliferation in cultured smooth muscle cells (see section V.). In arterial smooth muscle, relaxations result from increased efflux of calcium involving activation of cyclic guanylate cyclase and inhibition of ATP-sensitive K^+ channels and Ca^{2+}-activated K^+ channels (Kleppisch and Nelson, 1995; Quayle et al., 1995; White et al., 1995; Prakash et al., 1999). One caveat of these experiments is the high concentrations of estrogen often needed to elicit a relaxation. However, these studies reinforce the concept that there are immediate cellular effects of estrogen in either endothelial or smooth muscle cells that alter the internal milieu of the cell, resulting in altered responsiveness to subsequent stimuli (Miller et al., 2002; Haas et al., 2007).

C. Post-Transcriptional and Translational Modulation of Proteins/Enzymes

In addition to direct estrogen receptor-regulated gene transcription, estrogenic substances may facilitate the transport of RNA from the nucleus to the cytoplasm (Thampan, 1985; Jacob et al., 2006), may influence protein expression indirectly through regulation of mRNA stability in the cytoplasm, and may regulate the rate of gene transcription of enzymes required for post-translational modification of proteins by glycosylation, phosphorylation or methylation. Therefore, post-transcriptional regulation of gene expression by estrogen modifies the cellular proteome and phenotype at all levels of protein processing.

1. RNA Stability. Concentrations of mRNA in a cell represent the sum of production through gene transcription and degradation, providing a local and rapid (nongenomic) mechanism to control protein concentration. That is, decreased stability of mRNA provides a mechanism for rapid termination of production of a protein, whereas increased stability provides a means to prolong the expression of a gene. The biochemical details of modulation of mRNA stability are reviewed elsewhere (Kracht and Saklatvala, 2002; Ing, 2005) and involve, in part, transcriptional regulation of estrogen-regulated mRNA stabilizing factor (Kawagoe et al., 2003). For the purpose of this review, it is important to emphasize that estrogen may autoregulate the stability of mRNA for its own receptor in some tissues (Saceda et al., 1989; Adams et al., 2007). Therefore, differences in efficacy of SERMs may reflect differences in the ability of the SERM-bound receptor complex to alter estrogen receptor expression. Furthermore, within a single tissue, estrogen may stabilize some mRNA while destabilizing others. Some of the anti-inflammatory effects of glucocorticoids are explained by their effects on mRNA stability (Kracht and Saklatvala, 2002). However, the anti-inflammatory effects of estrogen on mRNA stability have not been investigated in the same way and may provide insight into regulation of growth factors and cytokines involved with estrogenic modulation of angiogenesis (Kracht and Saklatvala, 2002; Fieber et al., 2006), infection-induced inflammation (Batty et al., 2006; Zhong et al., 2006), glucose metabolism (Totary-Jain et al., 2005), lipoproteins (Srivastava et al., 1992), hypoxia (Fieber et al., 2006; Fish et al., 2007), shear stress (Sokabe et al., 2004), and immunity (Mestas et al., 2005).

2. Post-Translational Modification of Proteins. Signaling cascades and phosphorylation of mitogen kinases and Akt initiated by binding of estrogen to membrane receptors as outlined in section III.B. is not usually discussed in terms of mechanisms by which estrogenic substances affect post-translational modification of proteins. Yet regulation of enzymes, which in turn affect biological half-life of other enzymes, cofactors, or receptors, may represent a more integrated approach to understanding how estrogen, and perhaps other sex steroids as well, influence vascular responses to cytokines, hormones, or environmental stimuli such as hypoxia. For example, reversible, covalent attachment of small ubiquitin-like modifiers, a process known as sumoylation, or phosphorylation of steroid receptor coactivators affects estrogen receptor binding and subsequently estrogen receptor-mediated gene transcription. Added to cells in culture, 17β-estradiol coordinates both phosphorylation and sumoylation of some coactivators through nongenomic mechanisms yet to be determined. Thus, post-translational modification of proteins, in this case, coregulators by estrogen, affects the ability of estrogen to initiate transcription of genes with estrogen receptor response elements (Wu et al., 2006). Some other post-translational modifications of enzymes by estrogen identified in nonvascular tissue are presented in Table 1. Except for work assessing regulation of nitric-oxide synthase/nitric oxide by estrogen in endothelial cells (Hayashi et al., 1995; Kleinert et al., 1998; Sumi et al., 2001; Okano et al., 2006) and superoxide dismutase in vascular smooth muscle cells (Strehlow et al., 2003a), other evidence supporting these concepts is derived from studies of nonvascular cells such as neurons, glia, cancer cell lines, and liver cells (for review, see Ing, 2005). Therefore, the

TABLE 1
Post-translational actions of estrogen

Action	Physiological Consequences	Reference
Decreased turnover of growth factor-induced ornithine decarboxylase	Increased cell proliferation	Huber and Poulin, 1996
Activated secretion of MMP-7	Increased paracellular permeability	Gorodeski, 2007
Altered synthesis of glycosyltransferases	Altered half-life of glycoprotein hormones	Ulloa-Aguirre et al., 2001
Increased phosphorylation of telomerase	Increased cell proliferation	Kawagoe et al., 2003
Increased expression of propyl hydroxylase domain 1	Decreased cellular sensitivity to hypoxia	Tian et al., 2006
Increased protein binding to mRNA for AT_1 receptors	Decreased expression of AT_1 receptors	Wu et al., 2003
Coordination of phosphorylation and sumoylation of steroid receptor coactivators	Cell specific control of ligand-dependent nuclear transcription	Wu et al., 2006

AT_1, angiotensin II receptor type 1; MMP, matrix metalloproteinase.

ability of estrogen to regulate degradation of mRNA and inactivate proteins causing vascular injury or to stabilize mRNA and maintain proteins required for vascular repair requires further study in vascular cells of male and female animals.

IV. Mitochondria

Estrogenic actions on mitochondrial function may contribute to the ability of estrogen to modulate a variety of age-related diseases, including endothelial and vascular dysfunction. Mitochondrial reactive oxygen species (ROS) are produced as a by-product of oxidative phosphorylation. ROS can affect mitochondrial lipids, proteins, and DNA (Wallace, 2005). In particular, accumulation of ROS-induced mitochondrial DNA mutations over the lifespan is thought to contribute to the pathophysiology of a number of age-related diseases and to be a major cause of aging itself. Thus, an impact of estrogen on mitochondrial function might explain the longer lifespan of women as well as contribute to the ability of estrogen to protect against a variety of age-related diseases (Wallace, 2005; Duckles et al., 2006).

Estrogen can profoundly affect mitochondrial function in vascular endothelium (Stirone et al., 2005b) as well as in other cell types (Kim et al., 2006; Pedram et al., 2006; Yager and Chen, 2007). An important cellular target is the cerebral microvasculature, which comprises the blood-brain barrier. Because of the relatively high energy demands of these specialized endothelial cells, cerebral vascular endothelium contains more mitochondria than endothelium in other vascular beds (Nag, 2003). Tissues with high metabolic activity would be predicted to produce higher levels of mitochondrial superoxide as a by-product of oxidative phosphorylation (Wallace, 2005) and be particularly subject to age-related disease. Indeed, mitochondrial ROS production may contribute to neurodegenerative diseases such as Parkinson's, Alzheimer's, and Huntington's (St-Pierre et al., 2006). Functional changes in the blood-brain barrier or other aspects of cerebrovascular function would also contribute to the pathophysiology of these age-related diseases of the brain.

Estrogen affects mitochondrial function through increasing oxidative phosphorylation, while at the same time decreasing mitochondrial superoxide production (Stirone et al., 2005a; Duckles et al., 2006; St-Pierre et al., 2006) (Fig. 1). Exposure of ovariectomized rats to estrogen increased activities of both citrate synthase and complex IV, key rate limiting steps in the tricarboxylic acid cycle and electron transport chain, respectively, in cerebrovascular mitochondria. Corresponding increases in key related proteins after estrogen treatment include cytochrome *c* and subunits I and IV of complex IV. We were surprised to find that these indices of increased capacity for oxidative phosphorylation were associated with decreased ROS production after estrogen treatment. Levels of mitochondrial production of both superoxide and hydrogen peroxide were decreased after estrogen exposure. Chronic in vivo exposure to estrogen also increased levels of manganese superoxide dismutase but did not affect levels of glutathione peroxidase or catalase (Stirone et al., 2005b).

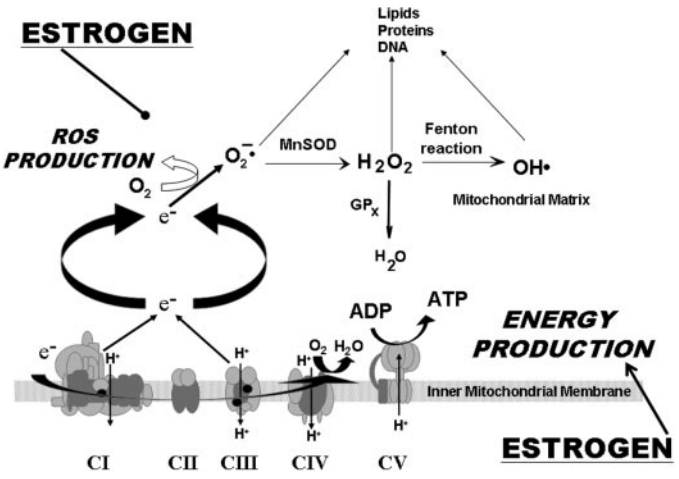

FIG. 1. Schematic diagram of the current hypothesis concerning the impact of estrogen treatment on mitochondrial function. Estrogen appears to promote energy production (oxidative phosphorylation) while decreasing mitochondrial generation of ROS. The oxidative phosphorylation system is composed of five enzyme complexes, within the inner mitochondrial membrane. Activity of this system generates an electrochemical gradient across this membrane, which leads to production of ATP. At the same time, electrons leaking into the mitochondrial matrix interact with oxygen, resulting in superoxide production. Superoxide is then metabolized by manganese superoxide dismutase (MnSOD); the resulting H_2O_2 is reduced to water by glutathione peroxidase-1 (GPx1). H_2O_2 can also be converted by the Fenton reaction to the highly reactive hydroxyl radical ($OH^{\bullet}$). ROS in the mitochondrial matrix target lipids, proteins, and mitochondrial DNA. Adapted from Duckles et al. (2006).

The mitochondrial targets for estrogen are not known but estrogen could act by influencing either nuclear or mitochondrial-encoded genes or both. Indeed, levels of nuclear respiratory factor-1, a key master regulator of nuclear-encoded mitochondrial genes, increased after estrogen treatment (Stirone et al., 2005b). However, this mechanism does not rule out a direct effect of estrogen on the mitochondrial genome as estrogen receptors are found in mitochondria (Chen et al., 2004; Yang et al., 2004; Stirone et al., 2005b). Improved understanding of mechanisms by which mitochondrial and nuclear genomes are coordinated to maximize mitochondrial function will provide a better understanding of the impact of estrogen on energy production.

Consistent with the effect of estrogen on mitochondrial function, a number of genes for mitochondrial proteins encoded by either nuclear or mitochondrial DNA are regulated by either ERα or ERβ (O'Lone et al., 2007). In aorta from wild-type ovariectomized female mice, estrogen treatment both up- and down-regulated a number of genes involved in mitochondrial function. However, in ERα knockout mice, a larger number of genes involved in mitochondrial function were down-regulated by estrogen treatment, implying that ERα predominantly down-regulates genes involved in the electron transport chain (O'Lone et al., 2007) and enhanced expression of antioxidants. However, as noted by the authors, these findings in aorta are not necessarily consistent with studies of other tissues, vascular endothelial cells, or even other blood vessels (O'Lone et al., 2007). Clearly, more studies on the effects of estrogen treatment on mitochondrial function will be essential to sort out these important and complex effects.

By decreasing mitochondrial production of ROS even while sustaining robust oxidative phosphorylation, estrogen would decrease the rate of accumulation of mitochondrial DNA mutations over the lifespan. By this mechanism, estrogen would protect against age-related disease, but one would not predict that estrogen would be able to reverse accumulated mutations of mitochondrial DNA. This mechanism of estrogen's effects has important consequences for the timing of estrogen treatment. Thus, administration of estrogen after a significant period without estrogen exposure, would only protect against future mitochondrial damage but would not reverse accumulated damage during estrogen-free periods. Such a mechanism might have contributed to the lack of effect of estrogen on cardiovascular disease in recent large trials of estrogen replacement therapy, in which subjects entered the study an average of 10 years past menopause (Harman et al., 2005a,b).

Mitochondrial production of ROS also plays a key role in oxidative stress (Madamanchi and Runge, 2007), so one would predict that estrogen may also have an important impact on vascular oxidative stress. Besides mitochondria, ROS can emanate from a number of sources, including nicotinamide adenine dinucleotide oxidase, xanthine oxidase, lipoxygenase, or nitric-oxide synthase uncoupling (Madamanchi et al., 2005) Although excess ROS production is proposed to be an initiating factor in vascular pathophysiology, lower levels of ROS can also serve important signaling functions in the vasculature. When ROS production remains low enough that mechanisms of ROS destruction are not overwhelmed, controlled activation of signaling pathways by ROS may be maintained (Gutierrez et al., 2006; Lyle and Griendling, 2006).

In addition to the mitochondria, estrogen also suppresses ROS through other mechanisms. For example, estrogen treatment reduces angiotensin II-induced free radical production in vascular smooth muscle cells (Strehlow et al., 2003a) and decreases NADPH-stimulated superoxide production by mouse cerebral arteries (Miller et al., 2007a). Estrogen also suppresses strain-increased NADPH oxidase activity and intracellular generation of ROS in human umbilical vein endothelial cells (Juan et al., 2004). Furthermore, in vascular smooth muscle cells estrogen treatment increases protein levels of both manganese superoxide dismutase (SOD) and extracellular SOD by increasing transcription rate. There was no effect of estrogen on copper-zinc SOD, glutathione peroxidase, or catalase. Likewise, treatment of ovariectomized rats with estrogen increased levels of manganese SOD protein in cerebral blood vessels, but did not change levels of catalase or glutathione peroxidase (Stirone et al., 2005b).

Oxidative stress may influence blood flow in humans. For example, in estrogen-deficient postmenopausal women, whole leg blood flow was reduced compared with that in premenopausal women. Because blood flow increased only in the postmenopausal women after administration of an antioxidant, ascorbic acid, it was concluded that different levels of oxidative stress contributed to differences in blood flow between the two groups (Moreau et al., 2007). However, the disparate ages of the pre- and postmenopausal groups and the lack of direct demonstration of an effect of estrogen per se make it difficult to draw conclusions regarding how estrogen contributes to these processes; nevertheless, it is clear that much more remains to be learned about the impact of estrogen on vascular oxidative stress and the implications for pathophysiology.

V. Physiological Consequences

In sections III and IV, intracellular mechanisms by which estrogenic compounds affect gene transcription and translation, protein synthesis and oxidative metabolism were described. In this section, the integrated consequences of these activities will be discussed.

A. Vascular Responsiveness

1. Arteries. In general, the most consistent effect of estrogen treatment on vascular responsiveness reported from a large number of studies conducted on isolated

arteries, experimental animals, and humans is vasodilation or suppression of vascular tone. In evaluating these effects of estrogen one needs to consider that sex differences, per se, may not simply be reflective of differences in levels of circulating hormones. As described in section II.A, tissue localization of key enzymes responsible for testosterone metabolism may result in local tissue levels of estrogen or dihydrotestosterone that exceed circulating levels. Thus, the best way to evaluate effects of estrogen in intact organisms is to administer the hormone in gonadectomized animals. Indeed, cardiovascular effects of estrogen can be seen in both male and female gonadectomized animals (McNeill et al., 1999; Geary et al., 2000; Bolego et al., 2005), and there is evidence in humans that estrogen contributes to the regulation of vascular function in males (Lew et al., 2003).

The most prominent effects of estrogen on vascular reactivity are mediated through direct effects on endothelial function (Miller and Mulvagh, 2007), but studies of very high concentrations of estrogen may show additional, nonphysiological effects. A plethora of studies in humans have clearly demonstrated that estrogen promotes vasodilation through an eNOS-dependent mechanism (Miller and Mulvagh, 2007). These include demonstration of an estrogen-stimulated increase in plasma concentrations of NO, increases in reactive hyperemia after estrogen treatment, and changes through the menstrual cycle reflective of an estrogenic effect. Interestingly, age influences flow-mediated vasodilation in women. In one study acute responses of postmenopausal women to estrogen (18 h after placement of a transdermal patch) declined with age (Sherwood et al., 2007). Likewise, postmenopausal women receiving either acute estrogen (within 1 h of sublingual administration) or chronic estrogen (3 months oral administration) all demonstrated increases in flow-mediated dilation, but this increase was significantly greater in women who were less than 5 years past menopause compared with women more than 5 years past menopause (Vitale et al., 2008). Furthermore, for women more than 5 years past menopause, flow-mediated vasodilation increased significantly more in women who had received estrogen treatment in the past compared with those who had not. These findings support the idea that, in the absence of estrogen, endothelium-dependent release of NO is reduced, and the ability of estrogen to increase this response is abrogated the longer an individual is without estrogen exposure. Whether this abrogation involves epigenetic regulation of estrogen receptors (see section II.B.1) or other mechanisms remains to be determined.

In both coronary and cerebral vascular beds and in the aorta, chronic exposure to estrogen, either endogenous or by estrogen treatment in ovariectomized female rodents, decreases vascular tone in an endothelium-dependent manner (Wellman et al., 1996; Geary et al., 1998; Widder et al., 2003; Duckles and Krause, 2007). These effects have been shown to depend on an increase in NO production resulting from a genomic effect to increase levels of eNOS (McNeill et al., 1999; Stirone et al., 2003a) as well as more rapid effects to increase NO production (Knot et al., 1999; Stirone et al., 2005a). Because the abilities of estrogen to alter vascular reactivity and increase eNOS levels are absent in ERα knockout mice (Geary et al., 2001) and are mimicked by selective estrogen receptor agonists (Widder et al., 2003), modulation of NO is most likely through ERα. Similar findings have been made in skeletal muscle arterioles, in which flow-induced dilation was greater in female than in male rats and was increased by estrogen in ovariectomized females (Huang et al., 1998). This effect of estrogen to modulate the regulation of wall shear stress was also shown to depend on enhanced endothelial NO release.

An endogenous substance, 27-hydroxycholesterol, inhibits the ability of estrogen to increase endothelial release of NO (Umetani et al., 2007). This inhibition occurred for both transcription-mediated and nontranscription-mediated effects of estrogen on NO production. The importance of this endogenous factor was demonstrated by measuring changes in vascular NO synthase after various manipulations that altered circulating levels of 27-hydroxycholesterol. Interestingly, 27-hydroxycholesterol had estrogenic effects on nonvascular cells. Thus, this substance exhibits a SERM-like effect, acting as an antagonist in the vasculature, but an agonist in other tissues.

In addition to effects of estrogen on endothelial production of NO, there is substantial evidence that estrogen affects production of other endothelial factors including products of cyclooxygenase. For example, chronic treatment with estrogen increased prostacyclin synthesis in small-caliber cerebral arteries and ovine fetal pulmonary arterial endothelium by elevating levels of cyclooxygenase-1 as well as prostacyclin synthase (Jun et al., 1998; Ospina et al., 2002; Sherman et al., 2002), resulting in a shift from cyclooxygenase-dependent vasoconstriction to vasodilation after estrogen treatment (Ospina et al., 2003). In rat mesenteric arteries, estrogen suppressed vasoconstriction, which was dependent on activity of prostaglandin H synthase (Davidge and Zhang, 1998). With the use of transfected cultured ovine endothelial cells; estrogen activated the human COX-1 promoter, a response mediated by either ERα or ERβ (Gibson et al., 2005). Interactions between NOS- and cyclooxygenase-dependent pathways and the effects of estrogen have been highlighted by several studies. Comparison of rat mesenteric arteries after ovariectomy or ovariectomy with estrogen treatment showed that estrogen increased the NO component of endothelium-dependent dilation, while decreasing the cyclooxygenase component (Case and Davison, 1999). Interactions among endothelial factors are highlighted by studies of cerebral vessels from mice with dysfunc-

tional NOS. In cerebral vessels from control mice treated with estrogen, eNOS was up-regulated, but there were no effects of estrogen treatment on cyclooxygenase-1, production of prostacyclin, or constriction to indomethacin. In contrast, in animals with dysfunctional NOS, either eNOS knockouts or animals treated chronically with a NOS inhibitor (Li et al., 2004), all three parameters were enhanced after estrogen treatment. Emphasizing the diversity of endothelial function in different vascular beds in arteries from skeletal muscles of rats treated with a NOS inhibitor, estrogen treatment increased vasodilatation mediated by endothelium-derived hyperpolarizing factor (Huang et al., 2001).

The myriad of effects of estrogen on vascular function are highlighted by changes in gene expression in aortas of ovariectomized wild-type or ER knockout mice after treatment with estrogen (O'Lone et al., 2007). Four clusters of genes were identified, showing that ERα and ERβ regulate distinct sets of genes with little overlap between the two receptor types. ERα was responsible for most of the increases in gene expression caused by estrogen in wild-type aortae, whereas ERβ generally decreased expression of a different set of genes. As mentioned in section IV, one of the most striking effects of estrogen was to modulate sets of genes involved in mitochondrial function, with both ERα and ERβ modulating different genes. Estrogen treatment also modulates cellular ROS production, by regulating both proteins involved in mitochondrial respiratory chain complexes and oxidoreductase gene sets. As pointed out by the authors, findings in mice aortae may only reflect vascular effects of estrogen in this specialized large artery dominated by smooth muscle cells, with estrogen acting mainly to reduce cell proliferation.

Whereas much basic science work has been directed to understanding how 17β-estradiol affects vascular function, a common clinically prescribed product, conjugated equine estrogen, contains metabolites of estrogen, estrone, and estrone sulfate (Kikuchi et al., 2000). Estrone sulfate must be hydrolyzed to estrone to enter cells. Estrone increases production of nitric oxide and prostacyclin in endothelial cells (Kikuchi et al., 2000; Lippert et al., 2000; Rauschemberger et al., 2008) and also increases proliferation of cultured rat smooth muscle cells (Rauschemberger et al., 2008). However, both estrone sulfate and estrone have a null effect on proliferation and migration of cultured human aortic smooth muscle cells (Dubey et al., 2000) but suppress transcription of promitogenic factors such as platelet-derived growth factor, interleukin-1, and interleukin-6 (Kikuchi et al., 2000). Reasons for these discrepancies among studies and between functional assays and molecular tests are not clear. However, efforts to better define conditions that affect responsiveness of various tissues to metabolites of estrogen are warranted, given that the relationship among estradiol, free estradiol, and estrone may relate to changes in development of carotid intimal hyperplasia (Karim et al., 2008). There is considerable genetic variation in expression of human hydroxysteroid sulfotransferase, and the biological activity of the enzyme may relate to the number of copies of the gene (Hebbring et al., 2007; Ji et al., 2007).

2. Veins. In contrast to what is known about the effects of estrogen on arteries, information regarding estrogenic effects on veins is scant. This lack of information is somewhat surprising in light of the well known adverse side effect of venous thrombosis in women using estrogenic treatments. As is observed in arteries, acute application of 17β-estradiol in vitro caused concentration-dependent, endothelium-dependent decreases in tone in rings of femoral veins derived from female pigs. These endothelium-dependent relaxations to 17β-estradiol were mediated by NO, but potassium channel activation seemed to contribute to the relaxation only in veins derived from gonadally intact females (Bracamonte et al., 2002a). These relaxations were not inhibited by the estrogen receptor antagonist ICI 182,780, suggesting, perhaps, involvement of receptor(s) or mechanisms other than the classic ERα and ERβ. In support of this concept, 17α-estradiol also caused relaxation of veins in the presence and absence of the endothelium as did the SERM raloxifene (Bracamonte et al., 2002a,b). Hormonal status of the animal (gonadally intact or ovariectomized) influences the relative contribution of endothelium-derived NO and potassium channels as causal to the relaxations to both 17β-estradiol and raloxifene. In contrast to these results are observations that acute application of 17β-estradiol does not cause endothelium-independent relaxations of human saphenous veins derived from persons with atherosclerosis (Haas et al., 2007). Several factors may contribute to these discrepancies. First, the saphenous vein is a muscular, cutaneous, thermoregulatory, innervated vein compared with deep veins, which are less muscular and not innervated in the same way. Most of the veins were derived from older (64 years of age) males, and because hormonal status affects both expression of estrogen receptors and signaling cascades, these may not be representative of veins from women or individuals without atherosclerosis. Finally, although, thrombosis may occur in these superficial veins, it is not usually associated with estrogen treatments but with other conditions such as cancer. Clearly, additional information is needed in regard to differences in estrogen responsiveness of veins from various anatomical locations and how these responses relate to development of venous embolitic disease among individuals of differing ages, hormonal status, or disease conditions.

With use of venous occlusion plethysmography, infusion of bradykinin caused a greater increase in the diameters of dorsal hand veins in postmenopausal women after 6 months of treatment with oral conjugated equine estrogen (CEE) and progestin compared with untreated

women (Ceballos et al., 2000). This effect was lost when treatment was stopped. Therefore, chronic menopausal hormonal treatment seems to increase endothelium-dependent responses in cutaneous veins of women as in arteries. The use of this technique to monitor changes in venous responsiveness has not been assessed in other clinical hormone treatment trials, and it may be useful in assessing differences among women in response to such treatments or perhaps to evaluate endothelial function in other populations as they age.

Critical to the development of thrombus is interaction of platelets with the venous wall. The in vitro response of porcine veins to autologous platelets was dependent upon the sex and hormonal status of the animal such that addition of platelets caused greater contraction of veins from ovariectomized animals than from those with intact ovaries (Lewis et al., 2001). These contractions to the autologous platelets reflect both hormonal modulation of the venous wall and the platelets themselves. However, if one platelet-derived product, ADP, was added to the veins, endothelium-dependent relaxations were not reduced by indomethacin in veins from ovariectomized animals as they were in those derived from gonadally intact animals (Lewis et al., 2001). These observations suggest that the presence of ovarian hormones affects endothelial production of inhibitory prostanoids in veins as in arteries. Indeed, if ovariectomized animals were treated for 4 weeks with either oral 17β-estradiol or raloxifene, endothelium-dependent relaxations to ADP were increased compared with those in untreated ovariectomized animals, but these relaxations were mediated by both NO and an inhibitory prostanoid only in veins from estradiol-treated animals. In contrast, endothelium in veins from raloxifene-treated animals produced a contractile prostanoid, most likely thromboxane (Lewis et al., 2006). Thromboxane stimulates platelets to aggregate and, thus, may contribute to a procoagulant phenotype in response to this SERM, which is known to increase the incidence of venous thrombosis in women (Barrett-Connor et al., 2002, 2006). As the venous wall is a key component of Virchow's triad required for the initiation of the thrombus (Bracamonte and Miller, 2001), more work is needed to understand how various estrogenic products affect both the endothelium and smooth muscle of veins to develop products with arterial protection but limited venous risks. Varicose veins represent a venous disorder that is associated with increases in circulating estrogen (Vin et al., 1992; Ciardullo et al., 2000). However, causality of this condition is complicated by various genetic components and physical factors such as obesity. Despite the numerous studies of estrogen modulation of collagen formation in skin (Verdier-Sevrain et al., 2006), little is known about how estrogens affect the extracellular matrix of the venous wall, which leads to formation of tortuousities.

B. Angiogenesis

Angiogenesis, the formation of new blood vessels from existing blood vessels, requires several steps including degradation of existing vascular basement membrane, proliferation and migration of endothelial cells into tubular structures in the tissue, and formation of new matrix around neovessels. In ovulating women, estrogenic regulation of these processes is evidenced by neovascular development in the uterus. However, these processes are essential in nonreproductive tissue for wound healing, repair of damaged organs, restoration of blood supply to ischemic tissue and tumor growth (Cid et al., 2002; Rubanyi et al., 2002). Estrogen regulates enzymes involved in formation of matrix including the matrix metalloproteinases and plasminogen activators, which may be responsible for rendering complex atherosclerotic plaques unstable (Cid et al., 2002; Jones et al., 2003). Growth factors and adhesion molecules necessary for angiogenesis include fibroblast growth factor-2, vascular endothelial growth factor, nitric oxide, and various integrins required for cell attachment (Cid et al., 2002; Rubanyi et al., 2002). Although estrogenic compounds increase proliferation of endothelial cells in vitro and in vivo in the vicinity of an endothelial lesion (Garnier et al., 1993; Banerjee et al., 1997; Krasinski et al., 1997), circulating endothelial-progenitor cells derived from the bone marrow may be a critical source of endothelial cells involved in maintaining and repairing damaged vascular lining, in angiogenesis to ischemic tissue, and in the formation of new blood vessels or vasculogenesis (Takahashi et al., 1999; Quraishi and Losordo, 2007).

There is little information regarding how the number and characteristics of colony-forming progenitor cells in circulating blood change across the life span in both males and females. In reproductively competent individuals, the number of hematopoietic progenitor cells was greater in males than in females, but the variability in the numbers of colony-forming cells was higher in females than in males, suggesting that sex steroids modulate hematopoiesis and perhaps other progenitor cells in the bone marrow (Horner et al., 1997). Indeed, loss of ovarian hormones in animals and humans reduced the number of circulating bone marrow-derived endothelial progenitor cells, whereas estrogenic treatment of ovariectomized animals and postmenopausal women increased their number (Goldschmidt-Clermont, 2003; Strehlow et al., 2003b; Bulut et al., 2007). Estrogens slowed the senescence of these cells through increased telomerase activity and increased their proliferation through activation of ERα (Imanishi et al., 2005a,b,c; Hamada et al., 2006; Masuda et al., 2007). Collectively, these effects would lead to rapid repair of vascular wounds by increasing endothelial regrowth with release of endothelium-derived factors, such as nitric oxide, which are inhibitory to smooth muscle proliferation, therefore reducing development of intimal hyperplasia

(Krasinski et al., 1997; Strehlow et al., 2003b; Schmidt-Lucke et al., 2005). The pivotal contribution of ERα in the formation and regulation of endothelial-progenitor cells may explain in part the accelerated formation of atherosclerosis and adverse outcomes in men with disruption and/or polymorphism of the ERα (*ESR1*) gene in men (Sudhir et al., 1997a,b; Ferrero et al., 2003; Schuit et al., 2004). In the future, it will be important to identify how populations of bone marrow-derived progenitor cells change with age and with gonadal hormone treatments in men and women. Studies also are needed to better understand how other progenitor and pleiotropic cells in the bone marrow, adventitia, and adipose tissue are influenced by aging and hormonal interventions (Lewis et al., 1997; Oparil et al., 1999; Mao et al., 2005; Hong et al., 2007; Stringer et al., 2007) to develop cell-based therapies for revascularization of ischemic tissue and for tissue engineering.

C. Vascular Consequences of Estrogenic Modulation of Autonomic Function

The autonomic nervous system is essential for homeostatic control of heart rate and blood pressure. ERα and ERβ are distributed throughout the central nervous system, except in the cerebellum of both male and female animals (Herbison et al., 2000; Kelly et al., 2005; Vanderhorst et al., 2005). ERα seems to be the predominant receptor subtype in the brain, although there are some differences in expression of each receptor between the sexes and between ovariectomized female animals and those treated with estrogen (Vanderhorst et al., 2005). Identification of ERβ in some earlier studies, however, may have been influenced by the specificity of the antibody used for immunological localization (Razandi et al., 2004).

Ligand stimulation of estrogen receptors in neurons activates nongenomic and genomic intracellular pathways similar to those described for endothelial and smooth muscle cells (McEwen, 2001; Kelly et al., 2005) (see section III). Estrogenic immunoreactive fibers colocalize with adrenergic (tyrosine hydroxylase-positive)-, cholinergic (vesicular acetylcholine transporter-positive)-, and serotonergic-positive cells. In addition to affecting the pituitary/gonadal axis controlling reproductive function, behavior, response to stress, and body temperature, estrogenic mediated neuronal activity affects heart rate, blood pressure, and sleep. Therefore, it is not surprising that disturbances in heart rate variability (palpitations) and hypertension, temperature regulation, and sleep are symptoms of the estrogen deplete state of menopause. Furthermore, central effects on appetite and activity may promote weight gain and lethargy associated with menopause in some women and, thus, could be considered as a potential physiological component of "life-style risk factors" for cardiovascular disease.

1. Hypertension, Sympathetic Tone, and Stress. Hypertension is a major cause of cardiovascular morbidity and mortality in postmenopausal women (Thom et al., 2006). Estrogen should reduce development of hypertension through peripheral actions such as up-regulation of endothelium-derived vasodilator factors with simultaneous down-regulation of vasoconstrictor factors, such as endothelin-1 (Barber et al., 1996; Barber and Miller, 1998; Best et al., 1998; Dubey et al., 2001), inhibition of the renin-angiotensin system by reducing transcription of angiotensin-converting enzyme in endothelial cells (Brosnihan et al., 1994; Gallagher et al., 1999), and down-regulation of angiotensin 1 receptors (Nickenig et al., 1998). Another potential pathway involved in the etiology of hypertension is the production of 20-hydroxyarachidonic acid by cytochrome P450a monooxygenase, which shows an androgen sensitivity (Holla et al., 2001; Capdevila et al., 2007). Depletion of estrogen with a concomitant increase in androgens would, therefore, reduce local inhibitory signals while increasing procontractile signals at the vascular wall, leading to increased peripheral resistance and blood pressure in the absence of concomitant decreases in sympathetic tone. In addition to these direct effects on the vascular wall, in general, withdrawal of estrogen increases sympathetic tone as measured by increases in peripheral sympathetic neuronal activity and circulating levels of norepinephrine resulting in increased blood pressure, especially in the presence of a stressor (Saab et al., 1989; Owens et al., 1993; Vongpatanasin et al., 2001; Liu et al., 2003; Wyss and Carlson, 2003; Fernander et al., 2004). Whether estrogen, when injected directly into the brain, increases or decreases sympathetic tone depends upon the specific nuclei that are stimulated (Saleh and Connell, 2007). Increased sympathetic tone may result from reduction of inhibitory effects mediated by ERβ as deletion of the gene for this receptor in mice resulted in a hypertensive phenotype (Zhu et al., 2002). In the brain, ERβ seems to be localized in cardiovascular centers with inhibitory neurons (Blurton-Jones and Tuszynski, 2002). Thus, estrogen depletion would be associated with withdrawal of inhibitory tone such as that imparted by the parasympathetic system, thereby increasing peripheral resistance and lowering heart rate variability. Decreased heart rate variability was observed in women after oophorectomy compared with age-matched women who underwent hysterectomy with conservation of the ovaries. Heart rate variability was restored in the oophorectomized women after 3 months of estrogen therapy, although the type of estrogen therapy was not identified in this study (Mercuro et al., 2000). It remains to be resolved whether the type of treatment (oral or transdermal) or formulation (conjugated equine estrogen, 17β estradiol, estrone, or estrone in combination with testosterone or progestogens) would be critical in defining the overall effectiveness of modulating autonomic activity (Matthews et al., 2001; Vongpatanasin et al., 2001; Liu et al., 2003; Matthews et al., 2005).

The type of estrogenic treatment and parameters measured to ascertain estrogenic effects on blood pressure may be important in explaining discrepancies in changes in blood pressure reported in various clinical trials (Felmeden and Lip, 2000; Ashraf and Vongpatanasin, 2006). To date, none of the large scale clinical trials have evaluated blood pressure responses or heart rate variability with estrogen treatments relative to an individual's ability to metabolize or respond to a particular estrogen. Genetic variability in estrogen receptors and the ability to metabolize estrogen may be critical factors that could help to differentiate central autonomic effects of estrogen from those responses occurring at the level of the blood vessel wall in the systemic circulation. In clinical trials of hormone treatment, blood pressure is usually measured under resting conditions. However, variation in blood pressure, as would occur over the course of 24 h in response to various stimuli (exercise and stress), may be critical in evaluating estrogenic effects on sympathetic control (Saab et al., 1989; Kaplan et al., 1996; Mercuro et al., 2000; Vongpatanasin et al., 2001) as estrogenic treatment of menopausal women reduced increases in pituitary-adrenal hormones, blood pressure, and pulse pressure induced by mental task induced stress (Matthews et al., 2001). Because postmenopausal women are at risk for hypertension (Felmeden and Lip, 2000; Thom et al., 2006), in the future, it will be critical to evaluate the effects of various combination estrogenic products and their route of administration on blood pressure control.

2. Metabolism of Adrenergic Neurotransmitter and Regulation of Adrenergic Receptors. In the periphery, the sympathetic nervous system with adrenergic transmission comprises a major innervation of arteries, arterioles, and veins. In addition to modulating neuronal activity through binding to estrogen receptors, estrogen also regulates adrenergic neurotransmission through effects on catecholamine reuptake at the synaptic cleft (Hamlet et al., 1980; Ball and Knuppen, 1990; Herbison et al., 2000), genomic regulation of α-adrenergic receptors (Colucci et al., 1982; Paden et al., 1982; Herbison et al., 2000) and competes with norepinephrine for adrenergic binding sites (Hiemke and Ghraf, 1982; Paden et al., 1982; Parvizi and Wuttke, 1983; Ball and Knuppen, 1990). Furthermore, catecholestrogens (2-hydroxyestradiol and 4-hydroxyestradiol) show binding affinity for tyrosinase, affecting catecholamine synthesis, and for catechol-*O*-methyltransferase (COMT), affecting catecholamine degradation (Ball and Knuppen, 1990; Zhu, 2002) (Fig. 2). Inhibition of catecholamine reuptake at the synaptic cleft and inhibition of degradation would have the net effect of prolonging the impact of an adrenergic neuronal signal.

Furthermore, one catecholestrogen, 2-hydroxyestradiol, binds irreversibly to proteins and nucleic acids causing damage to DNA possibly providing an initiating step in breast and uterine cancers (Cavalieri et al., 1997). Thus, conversion of 2-hydroxyestradiol by COMT to 2-methoxyestradiol is considered a detoxifying step in the metabolism of estrogen. These metabolic products of estradiol also have important, but concentration-dependent effects on vascular smooth muscle and endothelial cells. At low concentrations, metabolic products of estradiol inhibit smooth muscle proliferation and endothelial proliferation and thus reduce vascular response to injury, whereas at higher concentrations they are antiangiogenic (del Pozo et al., 2004; Klauber et al., 1997). Thus, when the conversion of 2-hydroxyestradiol to 2-methoxyestradiol is absent as in cells derived from COMT knockout mice, antimitotic effects of 2-hydroxyestradiol are prevented (Zacharia et al., 2001, 2003).

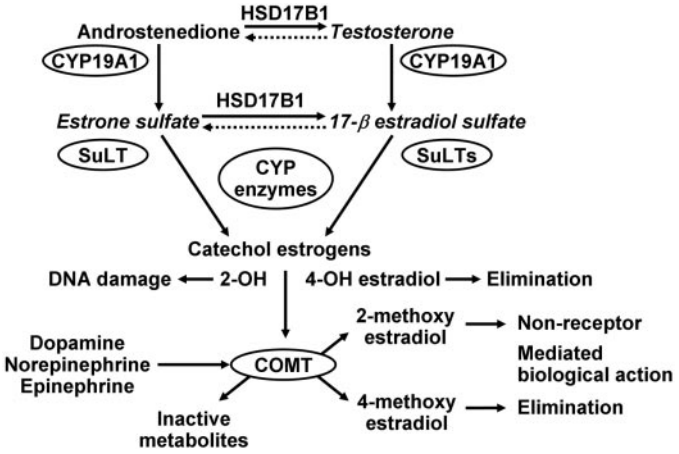

FIG. 2. Schematic diagram of the metabolic pathways involved in the synthesis and biotransformation of estrogen in the liver and extravascular tissue. Estrogen and some metabolites each have specific binding affinities for estrogen receptors. In addition, other metabolites of estrogen also have biological activities that do not require binding to the classically defined receptors. Identification of differences in copy numbers of genes and polymorphisms in cytochrome P450 enzymes will affect the efficacy of a particular estrogenic treatment as well as the biological consequence of that treatment depending on the rate of metabolism and the end product (Table 2). Competition of the catecholestrogens with adrenergic transmitters for COMT will affect the rate at which adrenergic neuronal signaling is sustained. CYP, cytochrome P450 enzymes designated by numbers; HSD17B1, 17β-hydroxysteroid dehydrogenase; SuLT, sulfatases; SULTs, sulfotransferases. Modified from Fig. 4 of Miller and Mulvagh (2007).

Because COMT is a ubiquitous enzyme, it might be expected that genetic variation in COMT would affect circulating levels of estrogens in postmenopausal women and perhaps serve as a risk factor for stress-induced cardiovascular disease. The polymorphism at codon 158 in COMT (V158M) decreases the methylation activity of the enzyme (Weinshilboum, 2006). In postmenopausal women genotyped as homozygous for the valine/valine codon (or high metabolizers, *COMTHH*), serum levels of 17β-estradiol were lower 3 h after an oral dose of estradiol valerate than in women with either the heterozygous genotype or homozygous for the methionine/methionine codon (Worda et al., 2003). Although there was no increase in overall mortality associated with COMT polymorphisms, in a population-based study of 2979 nondiabetic individuals, there was an in-

crease in nonischemic heart disease among individuals with the Met/Met and Met/Val genotypes. These authors cautiously state that these findings may be incidental (Hagen et al., 2007).

In another population study of 2507 peri- and postmenopausal women referred to a clinic for initiating estrogenic treatment, polymorphisms in COMT were associated with breast cancer occurrence but not with cardiovascular pathological conditions (Tempfer et al., 2004). As provocative as these results might be, without details regarding the phenotype of these women including other environmental risk factors (i.e., smoking status), medications including type and duration of hormonal therapy, years of follow-up, or criteria for recording an adverse event, the clinical relevance of these observations remains to be delineated. Clearly, additional analyses are needed to better understand relationships between estrogen metabolism and cardiovascular disease including interactions with other metabolic risk factors such as homocysteine and environmental estrogenic compounds such as the phytoestrogens (Zhu, 2002).

3. Variation in Vasomotor and Neuronally Mediated Symptoms of Menopause—Genetic Considerations. Menopausal symptoms are reported in populations of women from around the world (Freeman and Sherif, 2007). Despite the commonality of this occurrence, there is not universal presentation in all women of a single set of symptoms: sleep disturbances, night sweats, hot flashes, heart palpitations, irritability, and depression. Presentation of each symptom can be absent in some women and range from mild to severe in others. There is some belief that menopausal symptoms may be associated with a culturally driven negative attitude about menopause in women (Freeman and Sherif, 2007). However, it seems just as likely that the negative impact on quality of life of women experiencing symptoms may influence their attitude about menopause. As discussed in section V.C.1, receptors for estrogen are widely distributed within the central nervous system in areas of the brain controlling body temperature (preoptic hypothalamus), sleep (raphe nuclei), and heart rate (solitary tract). Furthermore, there is anatomical evidence that menopausal symptoms are autonomically driven physiological responses. Thus, a probable explanation for variation in menopausal symptoms among women might be genetic variation in genes directing estrogen metabolism (synthesis and catabolism) or estrogen receptors. Indeed, data are beginning to emerge that provide insight into the genetic variations contributing to various menopausal phenotypes.

The most extensive investigation to date into polymorphisms of genes encoding enzymes needed for estrogen metabolism and estrogen receptors as well as SNP associations with vasomotor symptoms and cardiovascular risk parameters is the Study of Women Across the Nation (SWAN). SWAN is a longitudinal observational study of women in the United States between the ages of 42 and 52 years of age who were still menstruating, who were not using exogenous hormones, and who were followed for 6 years. A strength of this study is that genotypes were analyzed from women of four ethnic/racial groups: African American, Caucasian, Chinese American, and Japanese American. Although vasomotor symptoms (hot sweats, cold sweats, and night sweats) were reported in all ethnic groups, associated genetic polymorphisms differed by race/ethnicity. In Caucasian women, vasomotor symptoms were associated more with polymorphisms in the gene for 17β-hydroxysteroid dehydrogenase responsible for conversion of estrone to 17β-estradiol (Fig. 2). In this case, lower conversion would decrease variability in 17β-estradiol levels and therefore symptoms (Crandall et al., 2006) (Table 2).

Studies that link menopausal symptoms and genetic phenotypes to cardiovascular disease progression or outcomes have yet to be conducted. However, additional information should be forthcoming with continued analysis of DNA collected from participants in large trials such as SWAN, the Women's Health Initiative (WHI), and the ongoing Kronos Early Estrogen Prevention Study (KEEPS) (Harman et al., 2005a). Linking menopausal symptoms to progression of occlusive cardiovascular disease and/or hypertension and risk for adverse outcomes such as stroke, myocardial infarction, or thrombosis is important as the current guidelines of the U.S. Food and Drug Administration for use of hormonal treatment products is for relief of menopausal symptoms and not for prevention of chronic diseases. However, evidence from women who participated in the CEE-only arm of the WHI suggests that estrogenic treatments may provide vascular protection even to women who are asymptomatic for menopausal symptoms (Manson et al., 2007). In the WHI women who had undergone a hysterectomy were randomly assigned to placebo or CEE alone. After cessation of the trial, women were contacted to participate in evaluation of coronary arterial calcium by computed tomography. Coronary calcification was

TABLE 2
Potential physiological consequences of single nucleotide polymorphisms associated with estrogen receptors and estrogen metabolism in Caucasian women

Data derived from Hagen et al. (2007), Rexrode et al. (2007), Sowers et al. (2006), and Tempfer et al. (2004).

Gene	SNPs	Consequence
ESR1	rs2234693	Ovarian aging; cognitive function
	rs9340799	Cognitive function
	rs3798577	Ovarian aging; apolipoprotein A-1
ESR2	rs1256030	Lumbar spine bone mineral density
	rs1271572	Myocardial infarction
17HSD	rs2830	Vasomotor symptoms
	rs592389	Vasomotor symptoms
	rs615942	Vasomotor symptoms
CYP1A1	rs2606345	Depressive symptoms
CYP 19	rs2414096	Diabetes mellitus
	rs2446405	Insulin sensitivity
COMT	V158M	Cancer/nonischemic heart disease?

TABLE 3
Coronary calcification in women participating in the CEE only—arm of WHI
Data derived from Tables 1 and 2 of Manson et al. (2007).

	Number of Participants[a]		Odds Ratio (95% CI)[b]
	CEE (n = 537)	Placebo (n = 527)	
CAC scores (Agatston units)			
0 (referent)[c]	299	266	1.00
>0	238	261	0.81 (0.64–1.03)
<10 (referent)[c]	348	302	1.00
≥ 10	189	225	0.73 (0.57–0.93)
<100 (referent)[c]	448	408	1.00
≥ 100	89	119	0.68 (0.50–0.93)
Vasomotor symptoms[d]			
Yes	23.3%	26.5%	
No	76.7%	73.5%	

CAC, coronary arterial calcification.
[a] Numbers of individuals who were at least 80% adherent to CEE or placebo for at least 5 years.
[b] Odds ratios were calculated for the CEE group compared with the placebo group.
[c] Referent groups are all participants within the stated CAC range.
[d] Percentage of women reporting moderate-to-severe vasomotor symptoms in each assigned group before initiation of treatment (baseline).

lower in women who had been randomly assigned to CEE compared with placebo (Table 3). Because coronary arterial calcification is considered to be a significant risk factor for future myocardial infarction (Raggi et al., 2003; Budoff et al., 2005; Hecht et al., 2006), these data support a protective vascular action of estrogen. Because approximately 75% of these women did not report having menopausal symptoms at the time they initially enrolled in the study, these data support the conclusion that menopausal estrogen treatments may benefit all women regardless of symptomatology.

Polymorphisms in the gene for aromatase, which is required for production of both estrone and 17β-estradiol have been associated more with metabolic cardiovascular risk factors including insulin sensitivity and diabetes than with vasomotor symptoms (Table 2) (Sutton-Tyrrell et al., 2005; Crandall et al., 2006; Lo et al., 2006). Interestingly, polymorphisms in estrogen receptors were not consistently associated with blood lipids in women participating in SWAN as was reported in women from the WHI (Herrington and Howard, 2003; Sowers et al., 2006). In the future, analysis of testosterone and sex hormone-binding globulin may be useful in identifying subgroups of asymptomatic women who might receive a cardiovascular benefit from menopausal estrogen treatment, perhaps reflecting effects of estrogens at the level of the vascular wall depending on local gonadal steroid metabolism (Karim et al., 2008).

VI. Estrogenic Effects in Pathophysiology

A. Inflammation

Inflammation is a stereotypic response of tissues to injury (Kracht and Saklatvala, 2002). Because many cardiovascular diseases including atherosclerosis are thought to have an inflammatory etiology (Libby, 2002), some discussion regarding estrogenic effects on inflammation is warranted.

Literature concerning the effect of estrogens on inflammatory responses seems contradictory, with both proinflammatory and immunosuppressive effects reported. In animal models, anti-inflammatory effects have been clearly reported, but, in humans, estrogens are thought to have proinflammatory effects in chronic autoimmune diseases. The situation is further complicated by findings that estrogen metabolites produced by first-pass hepatic metabolism may have proinflammatory effects as well, causing differences in response depending on the route of administration. Further complications in understanding this field and its impact on vascular disease include the number of different ways in which inflammation is defined, the variety of stimuli, acute or chronic, used to initiate tissue injury, and differences in endpoints used to define the inflammatory response. Detailed and comprehensive reviews of the general topic of estrogenic regulation of inflammatory processes address some of these points and provide excellent diagrams and tables of specific actions of estrogens on regulation of specific cytokines and leukocytes associated with innate and acquired immunity (Stork et al., 2004; Straub, 2007). However, for the purposes of this review, a few points are important to emphasize to facilitate future research into estrogenic regulation of inflammation associated with vascular disease.

Pathogens, specifically Gram-negative bacteria, initiate inflammation through binding of bacterial lipopolysaccharides to Toll-like receptors on the surface of vascular cells. Transient or sustained release of cytokines such as tissue necrosis factor-α (TNF-α) or several of the family of interleukins (IL-1, IL-6, IL-17, and others) sustains the inflammatory response through intracellular signaling cascades. These signaling cascades result in both transcriptional and translational modification of receptors, chemokines, and enzymes, including nitric-oxide synthases and matrix metalloproteinases (Stork et al., 2004; Straub, 2007). Inflammatory responses may have either positive or negative consequences, depending in part on the time frame of occurrence. For example, expression of adhesion molecules on the surface of dam-

aged cells with subsequent release of cytokines increases blood flow to the damaged area, serving a protective effect. Recruited leukocytes phagocytize damaged cells and/or invading organisms. In terms of negative effects, however, cytokines released from these cells may also facilitate regrowth of damaged tissue and secretion of extracellular matrix that can, for example, form fibrous scarring characteristic of fibrous plaque. Indeed genetic polymorphisms in Toll-like receptors may be protective against cardiovascular disease while rendering the individual more susceptible to infection (Arbour et al., 2000; Kiechl et al., 2002; de Kleinj and Pasterkamp, 2003; Miller et al., 2004b; Zwaal et al., 2005). Evaluation of Toll-like receptor polymorphisms has not yet been conducted for women participating in hormone treatment trials.

Many of the intracellular signaling cascades affected by Toll-like receptors and interleukin molecules are common to those stimulated by surface estrogen receptors (see section III). Therefore, it might be expected that estrogenic treatments would modulate inflammatory responses to infectious pathogens. However, the relationship of subclinical infection to progression of cardiovascular disease has not been considered in clinical studies of hormone treatments, for example, in relation to periodontal disease (Ford et al., 2007). However, the most consistent reports of estrogenic modulation of inflammation in vascular tissue of animals involve activation of cytokine pathways of inflammation. Estrogenic abrogation of inflammation initiated by TNF-α is perhaps the most consistent of these effects and may be critical for linking infection to cardiovascular disease as TNF-α increases transiently even with low levels of lipopolysaccharide challenge (Jayachandran et al., 2007). Interestingly, in rats estrogen has been shown to suppress vascular inflammatory responses to IL-1β and lipopolysaccharide treatments, and this response has also been shown to vary through the estrous cycle (Galea et al., 2002; Ospina et al., 2004; Sunday et al., 2006). An intriguing result is that this anti-inflammatory effect of estrogen is lost in older female animals (Miller et al., 2004a; Sunday et al., 2007).

Vascular sensitivity to infection-associated inflammation in relationship to integrity of specific estrogen receptors has not been studied directly and may provide insight into identifying individuals susceptible to infection and to cardiovascular disease with aging. For example, as discussed elsewhere in this article, in response to vascular injury, deficiencies in ERα lead to accelerated development of atherosclerosis in men (Sudhir et al., 1997b; Shearman et al., 2003), and estrogen treatments do not reduce the vascular response to injury after endothelial denudation in mice deficient in this receptor (Pare et al., 2002). ERα mediates estrogenic abrogation of some but not all cytokine-induced expression of cell adhesion molecules in endothelial cells (Cid et al., 1994; Caulin-Glaser et al., 1996; Chen et al., 1999). Alternatively, ERβ mediated the estrogenic abrogation of TNF-α-induced inflammation in cultured smooth muscle cells (Xing et al., 2007).

In transfection experiments, ERβ modulates expression of ERα (Hall and McDonnell, 1999). Therefore, differential expression of estrogen receptors in either endothelial or smooth muscle cells and in blood elements interacting with the vascular wall may be critical in defining how various estrogenic products modulate an inflammatory response because various estrogenic products such as SERMs, CEE, or estrogen metabolites such as estrone, estriol, and estrone sulfate do not bind to estrogen receptors with equal affinity (Ensrud et al., 2006; Hsia et al., 2006b). Furthermore, as some inflammatory cytokines may regulate both expression and activity of steroid sulfatase and sulfotransferases, metabolism of estrogen at the level of the vascular wall may be critical in establishing pro- or anti-inflammatory outcomes (Nakamura et al., 2003; Hebbring et al., 2007).

An individual's ability to metabolize estrogen may be critical in determining whether estrogenic treatments, oral or transdermal, are beneficial. Oral 17β-estradiol is metabolized in the liver and oral CEE, which contains a variety of estrogen metabolites is also modified in the liver. Thus, the relationship among circulating 17β-estradiol, estrone, and estrone sulfate will vary with an individual's ability to metabolize the initial product (Hebbring et al., 2007). Effects of estrogen on all parameters of inflammation are dose-dependent (see Straub, 2007, for review), and metabolism of 17β-estradiol as well as estrone sulfate can occur within vascular cells (Nakamura et al., 2003; Dubey et al., 2004). Metabolic products of 17β-estradiol have various biological activities. Therefore, effective dosing may vary, depending on which product has the greatest effect on a given parameter of the inflammatory process and an individual's ability to metabolize the estrogen (Dubey et al., 2000). For example, in response to an interarterial challenge of amyloid-β in ovariectomized rats, adherence of leukocytes to both mesenteric arterioles and venules was reduced in rats treated with oral CEE compared with those treated with oral 17β-estradiol. Furthermore, inhibition was dose-dependent, such that leukocyte adhesion was reduced with increases in the dose of oral 17β-estradiol. Therefore, interpretation of effects of estrogen on this inflammatory response depends both on formulation and dose of the estrogenic product (Thomas et al., 2003). The effective dose of estrogen may also be dependent upon the cytokine milieu. For example, interleukin 1β may regulate expression of enzymes that affect local production of 17β-estradiol by inhibiting sulfatase and stimulating expression of estrogen sulfotransferase (Nakamura et al., 2003).

Oral estrogenic preparations are considered to be proinflammatory because hepatic metabolism of 17β-estradiol produces proinflammatory metabolites in higher concentrations than might be produced by more slowly absorbed

transdermal products (De Lignieres et al., 1986; Seed et al., 2000; Vehkavaara et al., 2001; Lacut et al., 2003; Strandberg et al., 2003; Brosnan et al., 2007). However, inflammation in clinical studies is defined by changes in circulating levels of cytokines, for example C-reactive protein, P-selectin, TNF-α, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and fibrinogen. Such measurements do not identify the cell of origin of the cytokine/protein or take into account kinetics of their production, biological half-life, or degradation. Furthermore, in many clinical trials, these measurements represent two time points (before and after intervention) and are related to changes in risk factor profile rather than to measurable physical changes in the vascular wall or disease progression (Stork et al., 2004; Miller et al., 2007b). In contrast, in studies using experimental animals, effects of estrogen on vascular inflammation evaluate structural changes in the vascular wall including infiltration of leukocytes or expression of adhesion molecules or secretion of enzymes, such as matrix metalloproteinases. Changes in these parameters are linked to a physiological consequence such as cell proliferation, migration, or receptor expression, showing that estrogenic treatments reduce infiltration of leukocytes to arteries after endothelium denudation and cytokine-induced gene transcription in smooth muscle (Chen et al., 1996; White et al., 1997; Oparil et al., 1999; Tolbert et al., 2001; Wang et al., 2005; Xing et al., 2007). Therefore, studies are needed to better define estrogenic effects on specific parameters of the inflammatory process associated with progression of vascular disease in humans. These studies will require longitudinal assessment of soluble factors as well as evaluation of vascular anatomy and immunocompetence.

Most studies comparing oral to transdermal preparations of estrogenic treatments on plasma markers of inflammation have evaluated transdermal preparations of 17β-estradiol (Seed et al., 2000; Chen et al., 2001; Vehkavaara et al., 2001; Sendag et al., 2002; Strandberg et al., 2003; Girdler et al., 2004; Stevenson et al., 2004). Few studies have examined transdermal preparations of estrogen metabolites, for example, estriol (Mishra et al., 2006), or transdermal preparations of compounded formulations of estriol and estrone sulfate. These latter preparations have gained popularity as more natural, bioidentical formulations, but data supporting their superiority over other estrogenic formulations are scant in regard to specific measures of vascular physiology.

In addition to modulation of production of inflammatory proteins produced by the liver or direct effects on the vascular wall, estrogen may also modulate inflammatory responses indirectly through the hypothalamic-pituitary-adrenal axis, including stimulating release of corticotropin-releasing hormone from the hypothalamus and corticosteroids from the adrenal glands. Activation of the hypothalamic-pituitary-adrenal axis associates hormonally mediated events to centrally mediated events and to the manifestation of depression, stress, and inflammation (Kaplan et al., 1996; Kelly et al., 2005). In general, estrogen treatment suppresses the stress response to challenges such as intra-arterial injection of interleukin 1β, hemorrhage, and hypoxia and to emotional challenges such as noise and psychosocial factors (Smith et al., 1995; Kaplan et al., 1996; Buller et al., 1999; Dayas et al., 2000) albeit through different neuronal pathways. Links between stress and estrogenic treatments have implications in understanding sex-based differences in susceptibility to infection, chronic inflammatory conditions, and hypertension (Critchlow et al., 1963; Matthews et al., 1995; Kaplan et al., 1996; Dayas et al., 2000; Cutolo et al., 2002; Fernander et al., 2004) and should be considered as potential physiological risk factors for cardiovascular disease in women in addition to the usual psychosocial risk factors such as marital status, level of education, and income.

B. Atherosclerosis

Changes in vascular anatomy characterizing atherosclerotic lesions occur over decades. Although there is a large body of evidence that estrogen affects the vascular wall, the molecular mechanisms of vascular responsiveness to sex steroids during different stages of development of atherosclerosis are not clear. Because the response of cells in the nonatherogenic artery may not be the same as those in developing plaque, both the timing and nature of interventions to reduce the rate of these changes should matter when one is considering the question of whether estrogenic treatments provide protection against cardiovascular disease. This unifying hypothesis has emerged from numerous evaluations of data from preclinical, observational, epidemiological, and prospective clinical trials (Clark, 2006; Schnatz, 2006; Shapiro, 2006; Clarkson, 2007; Hodis and Mach, 2007; Rossouw et al., 2007). For example, if estrogenic therapy was initiated within the first 10 years of menopause, the odds ratio for adverse cardiac events was reduced (Grodstein et al., 2006; Hsia et al., 2006b). This reduction was independent of the type of menopause, that is, natural or surgical (Mack et al., 2004; Grodstein et al., 2006; Hsia et al., 2006b; Rocca et al., 2006; Manson et al., 2007). However, the same consistent pattern was not observed for stroke risk (see section VI.C). These observations in humans suggest that arteries of different anatomical origin may have different susceptibilities to stimuli initiating and sustaining atherosclerotic and other pathological processes (Moreau et al., 2002).

1. Peripheral Arterial Disease. Few studies have evaluated effects of estrogenic treatments on the incidence of PAD. In women with existing cardiovascular disease participating in the Heart and Estrogen/progestin Replacement Study, the combined hormone treatment did not reduce the incidence of PAD (Hsia et al., 2000; Grady et al., 2002). Likewise in older, but generally more healthy women participating in the WHI, estrogenic treatments also did not reduce the rate of PAD, but the overall incidence was low (Hsia et

al., 2004, 2006a). However, in studies where femoral arterial diameter and anatomy were evaluated by ultrasound, the intimal to medial ratio was lower in treated versus untreated menopausal women (Moreau et al., 2002, 2003; Naessen and Rodriguez-Macias, 2006). It remains to be determined whether or not timing of initiation of estrogenic treatments impacts development of PAD independent of other risk factors such as smoking, diabetes and hypertension (Hsia et al., 2000).

2. Carotid Intimal Medial Thickness. In experimental animals, estrogenic treatments consistently reduced development of carotid intimal medial thickness after a mechanical injury or atherosclerotic diet (Foegh et al., 1995; Chen et al., 1996; Karas et al., 1999; Oparil et al., 1999). In humans, a consequence of carotid arterial atherosclerosis, the incidence of ischemic stroke, was not reduced in women participating in the WHI (Rossouw et al., 2002, 2007), but no assessment of carotid anatomy was performed in these women. However, as with the femoral arteries, in studies in which the carotid arteries have been evaluated by ultrasound, a consistent reduction in carotid intimal medial thickness is observed in postmenopausal women using estrogenic treatments compared with those who do not (Sator et al., 1998; Deneke et al., 2000; Hodis et al., 2001; Mihmanli et al., 2002; Moreau et al., 2002; Takahashi et al., 2004; Karim et al., 2008). A direct testing of the timing hypothesis of estrogen intervention on progression of carotid intimal medial thickness is ongoing in the Early versus Late Intervention Trial with Estradiol (clinical trial NCT00114517). The Early versus Late Intervention Trial with Estradiol will compare the effect of oral 17β-estradiol (oral 1 mg/day) on the rate of change of carotid intimal medial thickness in women less than 6 years past menopause to women who are more than 10 years past menopause. Effects of oral conjugated equine estrogen (0.425 mg/day) and transdermal 17β-estradiol (50-μg weekly patches) on both progression of carotid intimal medial thickness and coronary calcification are being evaluated in women who are within 3 years of menopause in KEEPS (clinical trial NCT00154180). Therefore, within the next 5 years, data will be available to allow evaluation of three different estrogenic formulations on the same outcome of disease progression, i.e., carotid intimal medial thickness, in an age spectrum spanning two decades of menopause. These studies will provide valuable evidence regarding efficacy of products that may affect a risk for ischemic stroke (see section VI.C.).

3. Coronary Arterial Calcification. Coronary calcification, a predictor of future adverse cardiovascular events, can be present in early menopausal women who do not present with the usual risk factors for cardiovascular disease (Rumberger et al., 1994; Hodis et al., 2001; Hecht et al., 2006). Estrogenic treatments reduce coronary arterial calcification in postmenopausal women (Budoff et al., 2005; Mackey et al., 2005; Manson et al., 2007). And as discussed above (section V.C.3), reduced calcification was observed with CEE treatment even in women who did not experience menopausal symptoms (Manson et al., 2007) (Table 3). These data support a cardiovascular protective effect of CEE in women for whom estrogenic treatments would not be prescribed under the current practice guidelines. Mechanisms by which estrogen reduces calcification are multifactorial but most likely include modulation of cell differentiation (Fitzpatrick et al., 2003; Abedin et al., 2004; Anderson et al., 2004; Rzewuska-Lech et al., 2005), genetic variation related to bone matrix proteins and osteoblast/clast activation (Doherty et al., 2003), and perhaps susceptibility to infection by calcifying nanoparticles (Miller et al., 2004b). Much remains to be learned about the contribution of specific estrogen receptors in arterial calcific processes. However, ERβ is prominent in coronary arterial plaque, and polymorphisms in the gene for ERβ were associated with myocardial infarction in women (Christian et al., 2006; Rexrode et al., 2007). Intracellular processes mediated by this receptor that contribute to or limit calcification are not known.

4. Endothelial Dysfunction and Other Modalities to Assess Cardiovascular Risk in Menopausal Women. Modulation of quantity of intimal hyperplasia and arterial calcification reflect long-term effects of estrogen on components of the vascular wall. However, effects of estrogen on endothelial function are noted within 3 days of ovariectomy in rabbits and within months in humans (Gisclard et al., 1988; Lieberman et al., 1994; Bush et al., 1998). Because endothelial dysfunction may reflect the earliest stages of disease processes (Hamburg et al., 2004; Feletou and Vanhoutte, 2006), evaluation of endothelial function relative to menopausal age may provide another diagnostic modality to identify women who might receive a cardiovascular benefit from estrogenic treatments. In a prospective study that stratified women by age since menopause, forearm vasodilatation, as an indication of endothelial function, increased after 3 months of oral estrogen treatment (estradiol valerate, 1 mg/day) in all women. However, the magnitude of the increase diminished with age past menopause (Vitale et al., 2008). In women who had used estrogenic treatments before the study, the effect of aging was diminished. These results confirm the hypothesis that endothelial function diminishes with age, that estrogen maintains endothelial function, and that even temporary use of estrogen may delay the detrimental impact of aging on endothelial function. However, longitudinal, prospective studies are needed to provide additional data regarding the rate of change of endothelial function with age and to determine the duration of the "carryover" effect of estrogen on endothelial function once treatment is stopped (Hynes and Duckles, 1987; Seals et al., 2006; Sherwood et al., 2007). This latter point is especially important, given the current prescribing rec-

ommendation to use estrogen treatments for the shortest period of time to relieve menopausal symptoms.

Additional parameters, including endothelial function, are needed for cardiovascular risk stratification in early menopausal women as the standard characterization of risk using parameters of hypertension, plasma lipids, and smoking status (i.e., Framingham Risk Score) does not adequately predict risk in this group of women (Shaw et al., 2006; Lakoski et al., 2007; Miller et al., 2007b; Sherwood et al., 2007). The search for a set of blood biomarkers has not yielded a reliable indicator of early disease (Redberg et al., 2000; Kullo et al., 2003, 2006). Because coronary calcification increases the risk for future adverse events (Raggi et al., 2003; Desai et al., 2004; Budoff et al., 2005), identifying women at risk by inexpensive screening modalities, such as assessing arterial calcification using mammograms, may provide an additional, inexpensive way to stratify risk for women. However, additional studies are needed to confirm in newly menopausal women the relationship between breast and coronary calcification that has been found in older women (Maas et al., 2004; Kataoka et al., 2006; Rotter et al., 2008).

Another potential diagnostic tool to evaluate risk in early menopausal women may be analysis of blood-borne microparticles (or microvesicles). Microparticles are formed during activation and apoptosis of activated cells. These circulating spheres of membrane range in size from 0.1 to 1 μm and carry surface signature molecules of their cell of origin, varying with specific disease conditions including various cardiovascular diseases (Boulanger et al., 2006; Lynch and Ludlam, 2007). Few studies have evaluated microparticle populations in asymptomatic populations. Expression of phosphatidylserine on microparticles varied among early, asymptomatic menopausal women being screened for KEEPS (Miller et al., 2008). The level of expression was not associated with the usual risk factors for cardiovascular disease such as plasma lipids, high-sensitivity C-reactive protein, body mass index, blood pressure, or smoking status. However, in this group of women, the quantities of endothelium- and platelet-derived microparticles expressing phosphatidylserine were significantly and positively correlated with subclinical coronary disease defined by a coronary calcification score >50 Agatston units (M. Jayachandran, R. D. Litwiller, W. G. Owen, J. A. Heit, T. Behrenbeck, S. L. Mulvagh, P. A. Araoz, M. Budoff, S. M. Harman, and V. Miller, submitted). Therefore, analysis of populations of microparticles may provide insight into cell-cell interactions in early disease processes. With refinement and standardization of methods to detect and analyze populations of microparticles, this approach may help identify early disease processes in otherwise asymptomatic populations. Development of such new, easily accessible markers of the progression of vascular disease would aid in understanding the impact of a variety of therapies, including gonadal steroid hormones.

C. Stroke

Ischemic stroke is uncommon in women before menopause and increases substantially as women age, leading to the premise that women are protected early in life by reproductive hormones (Barrett-Connor and Bush, 1991; Wenger et al., 1993). However, findings of recent clinical trials have been surprising in not showing protective effects of hormonal therapy in reducing the incidence of stroke. Careful analysis of data from the WHI demonstrates that daily administration of CEE alone in women without a uterus did not protect against ischemic stroke (Hendrix et al., 2006). In the youngest group of women (50–59 years) the cumulative hazard ratio for ischemic stroke was 1.09, whereas this ratio was 1.72 in women aged 60 to 69. Thus, it is clear that treatment with CEE increased the risk of ischemic stroke in a group of generally healthy postmenopausal women in whom a decrease in coronary calcification was recorded. This study makes it clear that CEE, not the medroxyprogesterone given to women with a uterus, was responsible for the increased risk of stroke in the WHI as a whole (Rossouw et al., 2002). Nevertheless, it is also clear that sex and hormonal status are important factors in the pathophysiology of many diseases, including ischemic stroke. For example, a recent study of randomized low-dose aspirin for the primary prevention of cardiovascular disease in women demonstrated that aspirin lowered the risk of stroke without affecting the risk of myocardial infarction (Ridker et al., 2005). This result is significantly different from earlier findings in men, again raising the importance of the variable of sex in understanding the pathophysiology of cerebrovascular disease.

In animal studies, that treatment with the natural estrogen, 17β-estradiol, protects against cardiovascular disease and neuronal damage of experimental ischemic stroke. For example, administration of 17β-estradiol consistently decreased lesion size in rodent ischemic stroke (Alkayed et al., 2000; McCullough and Hurn, 2003). A number of possible explanations for the discrepancy between recent clinical trials and animal studies have been offered. These include the possibility that the oral hormone replacement therapy regimen used in recent studies may not be the most advantageous for positive cardiovascular effects. In contrast to animal studies where 17β-estradiol was administered i.p. or s.c., in human trials, CEE was administered orally. Thus, another possibility is that some of the various, little-studied estrogenic compounds in the CEE preparation were deleterious (Turgeon et al., 2004). Although animal studies have demonstrated untoward effects of medroxyprogesterone (Sunday et al., 2006), analysis of the CEE-only arm of the WHI appears to rule out this explanation (Barrett-Connor and Stuenkel, 1999; Turgeon et al., 2004; Hendrix et al., 2006). However, it is particularly notable that, in the Heart and Estrogen/Progestin Re-

placement Study, only women with existing coronary disease were studied (Hulley et al., 1998), whereas in the WHI, the mean age of women at initial screening was 63 years, and more than 65% of the women were older than 60 years of age (Rossouw et al., 2002). If 17β-estradiol is protective but cannot reverse preexisting vascular disease, then perhaps hormone replacement therapy has not been administered early enough in menopause in these studies to be effective (Naftolin et al., 2004).

What do we know about the actions of 17β-estradiol on cardiovascular function that might explain how this hormone could have powerful protective effects against cardiovascular disease and stroke but not be effective against existing disease? The nonreproductive effects of estrogen on the cardiovascular system may have protective effects in stroke, including beneficial effects on lipid metabolism, increased vascular endothelial production of NO and prostacyclin, promotion of endothelial cell growth and angiogenesis and suppression of inflammatory responses. Furthermore, if individuals lack exposure to estrogen for a period of time, progression of atherosclerosis attributable to changes in expression of estrogen receptors, an unfavorable lipid profile, and consequent endothelial dysfunction may not be reversed by subsequent estrogen administration. However, actions of estrogen on vascular mitochondrial function may provide additional explanations. Changes in mitochondrial function with age would not be reversible. Ischemic stroke, with a much greater incidence in older individuals, is an age-related disease; thus, it makes sense to hypothesize that age-related mitochondrial changes contribute to the pathophysiology of ischemic stroke. If estrogen is protective, then treatment of women well past menopause who have not been continually exposed to estrogen would not be protected from stroke. In fact, it would be quite possible that, through other mechanisms, such as prothrombotic actions, estrogen might even increase stroke incidence, as was seen in clinical trials of CEE. Current understanding of the biological actions of estrogen on cerebral blood vessels and the cerebral microvesicles that support neuronal function is incomplete.

There is increasing recognition that the health of neurons during ischemic stroke depends on local microvessels and supporting cells such as astrocytes (del Zoppo, 2006; Iadecola et al., 2006). Together, these elements comprise what is referred to as the "neurovascular unit" because of the close association and interaction among cerebrovascular cells, astrocytes, and neurons. Perivascular neurons appear to communicate to blood vessels through astrocytic processes to adjust local blood flow (Zonta et al., 2003; Hamel, 2006). Astrocytes also provide metabolic support for neurons, using energy substrates supplied by the microvessels (Pellerin and Magistretti, 1994). Whereas stroke studies in animals have traditionally focused on neuronal survival mechanisms, it is becoming apparent that the neurovascular unit must be protected from ischemic injury to improve stroke outcome (del Zoppo, 2006; Iadecola et al., 2006). This complexity is challenging for the researcher because each cell type within the unit has varying responses and coping strategies during the progression of stroke injury and recovery.

Three key cell types of the neurovascular unit (neurons, astrocytes and endothelial cells), are highly metabolic, requiring energy to maintain ion pumps and transporters critical to the proper functioning of the brain. With particular relevance to this review, cerebrovascular endothelial cells are highly metabolic compared with other vascular beds, containing more mitochondria than other types of endothelium (Nag, 2003). Cerebral endothelial cells have the unique function of maintaining the blood-brain barrier, a critical site of injury during ischemic stroke that leads to vasogenic edema. Furthermore, vascular dysfunction during stroke compromises local blood flow, contributing to the evolution of brain injury. The responses to ischemic stroke of the various components of the neurovascular unit and the timing of these responses are different. For example, in models of transient ischemia/reperfusion, the peak of superoxide production, as measured by hydroethidine oxidation, was seen in neurons after 1 h reperfusion but not until 4 h in endothelial cells (Kim et al., 2002). αB-crystallin, a small heat-shock protein (HSP), is induced transiently in neurons of the peri-infarct (penumbra) region at 4 h after ischemia/reperfusion but does not appear in astrocytes until 2 to 4 days later (Piao et al., 2005). Another heat-shock protein, HSP70, is expressed only in endothelial and glial cells, but not in neurons in the ischemic core. In the penumbra, HSP70 is also expressed in metabolically stressed neurons (Sharp et al., 2000; Kokubo et al., 2003). Early expression of matrix metalloproteinase-9 and vascular endothelial growth factor after ischemia/reperfusion is associated with microvessels, causing degradation of the vascular matrix and blood-brain barrier leakage (Zhao et al., 2006). However, 7 to 14 days after stroke these factors in the peri-infarct zone are primarily involved in neurovascular remodeling and repair (Zhang et al., 2002; Zhao et al., 2006). More work is needed to understand the effect of estrogen on all components of the neurovascular unit, and the mechanisms by which estrogen may affect the progression of ischemic injury to develop useful therapeutic interventions (Bushnell et al., 2006).

D. Migraine

In section VI.C, the concept of the neurovascular unit was introduced. The neurovascular unit is also implicated in the etiology of migraine. Although migraines may be considered an "essentially benign condition" (Bousser, 2004; Bousser and Welch, 2005), they certainly have a negative impact on quality of life for the migraineurs. Migraines express as pain and are associ-

ated with vasodilatation of cerebral and meningeal arteries. They are classified as occurring with or without a visual aura, thus implicating different neuronal involvement between the two types of migraines (Bousser, 2004; Wessman et al., 2004; Bousser and Welch, 2005; Brandes, 2006; MacGregor et al., 2006). Indeed, individuals who experience aura can be biochemically differentiated from those who do not (Ferrari, 1992).

Migraines show a 3-fold-greater prominence in women compared with men (Brandes, 2006; Martin and Behbehani, 2006). In some women, migraines may be associated with the menstrual period, ameliorated by pregnancy, and diminished at menopause and may worsen with menopausal hormone treatment. These observations suggest that fluctuations in estrogen levels, especially a decrease, may be a precipitating factor in migraines without aura (Bousser, 2004; Wessman et al., 2004; MacGregor et al., 2006). However, differences in circulating levels of estrogen were not observed between women with and without menstrual migraine. Urinary excretion of estrone-3-glucuronide was more than 2-fold higher in women with migraine than in those who did not experience migraine, suggesting that the ability to metabolize estrogen may relate to development of migraine (MacGregor et al., 2006). In-depth analyses related to estrogen metabolism among women who experience migraines, with or without aura, and women who do not, need to be conducted. In particular, production of catecholestrogens, perhaps influencing production and disposition of adrenergic neurotransmitters, could participate in neuronally induced cerebral vasospasm (Ferrari, 1992; Martin and Behbehani, 2006).

Several genetic polymorphisms are associated with familial migraine including genetic variation in ERα (G594A polymorphism of exon 8) (Colson et al., 2004, 2006; Johnson et al., 2007). As discussed above (section V.C), estrogen receptors are located within brain nuclei that innervate the cerebral vasculature as well as other nuclei regulating cardiovascular function (Martin and Behbehani, 2006). Thus, in addition to influencing adrenergic mechanisms, estrogen may also modulate central opioidergic tone, release of peptidergic transmitters from trigeminal nuclei, and the GABAergic system, perhaps modulating NO (Johnson et al., 2005; Bergerot et al., 2006; Brandes, 2006; Martin and Behbehani, 2006; Puri et al., 2006). Because ERα stimulates NO production in vascular endothelium, there might also be direct modification of migraine occurrence through this pathway. Implicating NO in the etiology of migraine are observations that platelet production of NO was greater in women with menstrual migraine than in those without (Brandes, 2006). NO released from platelets could contribute to decreases in cerebral vascular tone in vivo or reflect changes in synthesis of NO that might occur locally within the cerebral vasculature. A polymorphism (E298D) in eNOS results in decreased activity of the enzyme. This variant is associated with increased risk for cardiovascular and cerebrovascular disease. The homozygous variant was an independent risk factor for stroke in persons with migraine with aura (Borroni et al., 2006). Approximately 80% of individuals participating in this study were female, reflecting the prominence of the condition in women, suggesting that this variant is prevalent among women with migraine (Borroni et al., 2006). The association of this genetic variation in eNOS with those of ERα in a larger population remains to be determined. If the genetic variant results in decreased activity of eNOS, these results are difficult to interpret within the context that increased production of NO may trigger migraine (Thomsen and Olesen, 2001). Some evidence implicates neuronally derived NO in the etiology of migraine, but no association of migraine with genetic variation of neuronal nitric oxide synthase was found (Johnson et al., 2005; Bergerot et al., 2006; Borroni et al., 2006). More information is needed regarding estrogenic modulation of all three isoforms of nitric oxide synthase in the cerebrovascular unit.

In addition to estrogenic modulation of neuronal transmission associated with pain and endothelial NO (Rossouw et al., 2002; Martin and Behbehani, 2006; Welch et al., 2006), estrogen may induce migraine through direct effects on vascular smooth muscle cells. For example, estrogen increased the efflux of magnesium from cultured cerebral smooth muscle cells (Li et al., 2001). Indeed, magnesium is an effective treatment for migraine in some individuals (Ferrari, 1992; Martin, 2007).

The relationship between migraine and stroke should be considered, especially as related to brain ischemia. In doing so, however, it is important to try to distinguish migraine as an underlying pathological condition increasing the risk for stroke as opposed to migraine as a symptom resulting from a stroke. Most evidence indicating that migraine may be a risk factor for stroke comes from studies of incidence of ischemic stroke in young women who experience migraine with aura. Although the risk for stroke overall is low, approximately 18 per 100,000 per year, the relative risk for stroke is 3.8 to 6.2 in women who have migraine with aura compared with those who experience migraine without aura (Bousser, 2004; Bousser and Welch, 2005). The relative risk for ischemic stroke increases in this group with oral contraception use and smoking. As the former may increase the risk for thrombosis and the latter is a known risk factor for cardiovascular disease, this suggests that migraine with aura may reflect an underlying vascular pathological condition that is exacerbated by these environmental stressors (Bousser, 2004). Middle-aged (average approximately 58 years) women participating in the population-based study, Atherosclerosis Risk in Communities Study, who experienced migraine with aura also had an increased risk for ischemic stroke (Stang et al., 2005). This observation also points to an underlying pathological condition of

the neurovascular unit contributing to migraine. In the WHI, the incidence of stroke was greater even in the CEE-only arm of the study in which those randomly assigned to treatment had lower incidence of myocardial infarction and coronary calcification (Rossouw et al., 2002, 2007; Manson et al., 2007). The incidence of migraine in this cohort was not assessed. These observations point to the need to understand and differentiate factors contributing to stroke risk from those contributing to cardiac risk (Bousser and Welch, 2005; Bushnell et al., 2006).

Several chronic alterations in small arterial anatomy and function, which may not show a sex difference in frequency, predispose an individual to ischemic stroke and migraine with aura. One syndrome, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes, is associated with mutations in mitochondrial DNA (Bousser and Welch, 2005). The relationship between these mitochondrial mutations and mitochondrial pathways modulated by estrogen as discussed in section IV remains to be explored.

E. Thrombosis

The risk of venous thrombosis is a "black box" warning required by the United States Food and Drug Administration on labeling of estrogenic products. Although increased incidence of venous thrombosis has been reported in numerous clinical trials of estrogenic products in menopausal women (Scarabin et al., 1997; Herrington et al., 1998; Grodstein et al., 2001; Barrett-Connor et al., 2002; Rossouw et al., 2002; Vickers et al., 2007), little is known about what actually constitutes risk for an individual woman. Concentrations of soluble markers in the blood associated with either inflammation or proteins of the coagulation cascade including substances also associated with arterial disease such as C-reactive protein, fibrinogen, and homocysteine were higher in individuals who experienced a thrombotic event compared with those who did not (Meijers et al., 2000; Pradhan et al., 2002; van Hylckama Vlieg and Rosendaal, 2003; Eilertsen et al., 2005). Although assays for these plasma/serum markers have been clinically validated, no global assessment tool has been established to identify an "at risk" phenotypic profile for an individual woman contemplating use of estrogenic treatments.

An alternative approach to defining a procoagulant phenotype is to use genetic analysis based on that obtained from individuals who have experienced an adverse event. Perhaps the most consistent risk for estrogenic treatment and venous thrombosis is factor V Leiden. However, individuals who do not carry the mutation may be at risk for a thrombotic event, whereas those who carry it may never experience an event (Price and Ridker, 1997; Herrington et al., 2002b; Heit, 2006; Miller et al., 2006; Simon et al., 2006). The formulation of the estrogenic treatment may also be critical for increasing risk even in individuals with this mutation, as incidence of venous thrombosis was less in individuals with factor V Leiden using transdermal compared with oral estrogenic products (Scarabin et al., 2003; Straczek et al., 2005). As discussed in sections V.C and VI, the first-pass metabolism of oral products in the liver may increase inflammatory cytokines such as high-sensitivity C-reactive protein and other coagulation proteins, increasing the risk of thrombosis in susceptible individuals (Scarabin et al., 1997, 2003; Brosnan et al., 2007). Alternatively, detoxification of the catecholestrogens may affect production of prostacyclin, which acts to inhibit platelet aggregation (Needleman and Parks, 1982). Direct comparison of oral CEE and transdermal 17β-estradiol on soluble proteins and other cytokines related to coagulation and inflammation together with an estrogen metabolic profile will be measured in the KEEPS trial (Harman et al., 2005a).

Genetic variants in platelet surface receptors and estrogen receptors have been evaluated in regard to individual risk for arterial thrombotic events but without consistent findings (Alessio et al., 2007; Kjaergaard et al., 2007). No studies have evaluated an individual's genotype for enzymes that metabolize estrogen and related that to thrombotic risk. Differences in copy number of cytosolic sulfotransferase reflect the ability to metabolize estrogen (Hebbring et al., 2007). However, how this difference translates to risk for disease, i.e., thrombosis, cancer, or other conditions, remains to be determined. In the future it will be important to take a polygenomic approach to estimating thrombotic risk, including evaluation of estrogen receptors, enzymes metabolizing estrogen and receptors for environmental factors and exposure to inflammation-provoking pathogens (Arbour et al., 2000; Kiechl et al., 2002; van Hylckama Vlieg and Rosendaal, 2003; Jayachandran et al., 2007; Mari et al., 2007). Interaction of age, hormonal status, and environmental factors should also be considered. For example, a dose of lipopolysaccharide that was not lethal to reproductively competent mice became lethal in reproductively senescent mice and to a greater extent if the mice lacked ERβ (Miller et al., 2006). Additional studies are needed to exploit new models for studying thrombosis in experimental animals (VanLangevelde et al., 2005).

Contrary to evidence that an early menopause increases risk for arterial cardiovascular disease (de Kleijn et al., 2002; Hu et al., 1999; Rocca et al., 2006), data from a hospital based case-control study suggest that the risk of venous thromboembolism decreases with early menopause (Simon et al., 2006). That is, the shorter the exposure to endogenous estrogen is (an early menopause), the less the risk of a venous thrombotic event. The reason for these apparent contrary findings is unclear, underscoring the need for basic research into how hormones affect the venous wall. However, the impact of the number of pregnancies and related issues of change in vessel compliance resulting from connective

tissue reorganization and physical challenges related to obstruction of venous return also need to be considered carefully (Simon et al., 2006).

As generation of a clot, arterial or venous, requires interaction of the blood with a biochemical or mechanical lesion in the vascular wall, it is also critical to understand how estrogenic treatments affect formed elements in the blood. Platelets are the formed element in the blood critical for the generation of thrombin. Both platelets and their precursors, megakaryocytes in the bone marrow, contain estrogen receptors (van Kesteren et al., 1997; Khetawat et al., 2000; Bracamonte et al., 2002c; Jayachandran and Miller, 2003; Yang et al., 2004). Thus, changes in hormonal status, i.e., at the transition to puberty, menopause, and hormonal treatments, influence the phenotype of the circulating platelet pool (Jayachandran and Miller, 2002; Jayachandran et al., 2004, 2005a,b). In general, in studies of estrogen treatment to large animals, platelet aggregation and secretion decreased compared with ovariectomized animals. However, not all specific components of the platelet secretome were regulated the same by different formulations of oral estrogens. In particular, stimulated release of nitric oxide was greater in platelets from animals treated with 17β-estradiol than in those treated with oral CEE or raloxifene (Jayachandran et al., 2005b). Other phenotypic changes including expression of adhesion molecules, phosphatidylserine, and CD40, which allow the platelet to interact with leukocytes and endothelial cells of the vascular wall, were affected by hormonal status and implicated in progression of arterial disease (Henn et al., 1998; Schonbeck et al., 2001; Jayachandran and Miller, 2002; Prasad et al., 2003; Jayachandran et al., 2005b; Wu and Li, 2006; Cognasse et al., 2007). However, expression of these adhesion molecules is also increased with infection, thus perhaps linking environmental stimuli to thrombotic susceptibility with estrogenic treatments.

As discussed in section VI.B.4, activation and interaction of cells of the vascular wall with blood elements result in the formation of membrane-derived microparticles. Microparticles bind fibrinogen, initiating platelet microaggregation (Holme et al., 1998), and also act as carriers between cells of biochemically active molecules including tissue factor (Losche et al., 2004; Morel et al., 2004), contributing to activation of the coagulation cascade. Microparticles of endothelial origin are elevated in persons with venous thromboembolism (Chirinos et al., 2005). However, prospective studies are needed to determine whether elevation of specific populations of microvesicles define a thrombotic phenotype before an event and if so, how that phenotype may be affected by estrogenic treatment. Because circulating proteins and peptides turn over rapidly and their source usually cannot be identified, evaluation of changes relative to hormonal therapy has not provided meaningful information for identifying an "at risk" thrombotic phenotype (Schonbeck et al., 2001; Pradhan et al., 2002; Ridker et al., 2003; Andersson et al., 2005; Healy et al., 2006; Miller et al., 2008). Therefore, evaluation of cellular origin of microparticles and their functional characteristics (i.e., thrombin-generating capacity) may allow for a more consistent mechanism to define an at risk thrombotic phenotype in early menopausal women.

VII. Future Directions: Summary

Although areas needed for future research have been mentioned in each of the preceding sections, there are several points deserving particular attention.

A. Pharmacogenomics

Delivering the right drug at the right dose to the right person at the right time is a treatment goal. Genetic testing to reach this goal is already realized in prescribing the selective estrogen receptor modulator, tamoxifen, as an adjuvant treatment for breast cancer (Andersson et al., 2005; Goetz et al., 2005; Borges et al., 2006). A picture emerging from genomic data published from several large scale trials evaluating efficacy of estrogenic treatments is that, in the future, a polygenomic or genome-wide approach will be needed to determine dosing and formulations to maximize the benefit and reduce risk for women considering using these treatments. Such an approach may consider both receptors (pharmacodynamics) and pathways that metabolize the hormone (pharmacokinetics) (Weinshilboum and Wang, 2006). Thus, depending upon the ability of an individual to metabolize these chemicals, treatment with 17β-estradiol alone may not prove equally efficacious to conjugated equine estrogen, estriol, or estrone. Therefore, experiments are needed to more accurately define an estrogen metabolome relative to menopausal symptoms and disease susceptibility.

In addition, research is scant regarding long-term systemic effects of oral or transdermal products compared with sublingual or vaginal products and compounded products. It will be desirable that an interdisciplinary approach be taken in evaluating women using the various formulations to increase the evidence base for prescribing these products. That means, for example, when studies are conducted to evaluate efficacy of products to treat menopausal symptoms such as hot flashes, other parameters of cardiovascular health should be evaluated simultaneously. A limiting factor for such interdisciplinary approaches, of course, is the immediate cost of conducting the study. However, with careful design the up-front costs of such studies may outweigh the expense of additional future trials. Furthermore, increased collaborations requiring sharing of databases and distribution of banked samples to laboratories with specific expertise will be needed so that efforts are not duplicated but validated.

B. Variation of Physiological/Pathological Impact of Hormones across the Lifespan

A unifying concept emerging from the various observational and clinical trials in humans and preclinical studies in animals regarding the vascular actions of estrogenic treatments is that timing matters (Mendelsohn and Karas, 2007). As discussed throughout this review, age may be an important factor in estrogenic effects on inflammatory responses, with a loss of estrogenic efficacy in older animals. Hormonal treatment given to animals or individuals with established cardiovascular disease may initiate a different set of physiological responses compared with responses in the absence of established disease. For example, estrogen may have a disadvantageous effect in vessels with developing or established plaque compared with an advantageous effect to decrease development of atherosclerotic lesions when administered earlier in the disease progression. Thus, the ability of estrogenic treatment to prevent or slow progression of vascular remodeling or plaque formation may be limited to a narrow window of opportunity. Another aspect of the importance of timing involves the effects of estrogen on mitochondrial function. Earlier in life, the effect of estrogen to reduce mitochondrial ROS production may delay accumulation of mitochondrial DNA mutations. However, once such mutations have accumulated, estrogen may be unable to reverse this effect. Thus, actions of estrogen on mitochondrial function may only be protective, but estrogen may lack the ability to reverse existing disease processes. Questions regarding regulation of hormonal receptors, their coregulators, and other factors affecting their transcription and translation as well as changes in downstream signaling cascades before and after slow hormone withdrawal, as in natural menopause, or abrupt, as in surgical menopause, need to be addressed and may lead to novel markers of early disease processes as well as better identification of when to intervene with treatment for maximal benefit and minimal harm.

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