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Genome and Hormones: Gender Differences in Physiology Invited Review: Cardiovascular protective effects of 17β-estradiol metabolites

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Dubey, Raghvendra K., and Edwin K. Jackson. Invited Review: Cardiovascular protective effects of 17β-estradiol metabolites. *J Appl Physiol* 91: 1868–1883, 2001.—17β-Estradiol (estradiol), the most abundant endogenous estrogen, affords cardiovascular protection. However, in a given cohort of postmenopausal women, estradiol replacement therapy provides cardiovascular protection in only a subset. The reasons for this variable action can only be understood once the mechanisms by which estradiol induces its cardiovascular protective effects are known. Because most biological effects of estradiol are mediated via estrogen receptors (ERs) and the heart and blood vessels contain both ER-α and ER-β, the prevailing view is that ERs mediate estradiol-induced cardiovascular protection. However, recent findings that estradiol protects against vascular injury in arteries of mice lacking either ER-α or ER-β seriously challenges this concept. Thus other non-ER mechanisms may be operative. Endogenous estradiol is enzymatically converted to several nonestrogenic metabolites, and some of these metabolites induce potent biological effects via ER-independent mechanisms. Therefore, it is conceivable that the cardiovascular protective effects of estradiol are mediated via its endogenous metabolites. On the basis of the evidence cited in this review, the cardiovascular protective effects of estradiol are both ER dependent and independent. The purpose of this article is to review the evidence regarding the cardiovascular protective effects of estradiol metabolites and to discuss the cellular, biochemical, and molecular mechanisms involved.

estrone; methoxyestradiol; catecholestradiol; hydroxyestradiol; mitogenesis; menopause; vascular smooth muscle; endothelium; cardiac fibroblasts

THE PROTECTIVE EFFECTS OF ovarian function on the cardiovascular system are thought to be largely mediated by 17β-estradiol (estradiol, the major ovarian estrogen). However, in vivo, estradiol is converted to both estrogenic and nonestrogenic metabolites, some of which are known to possess biological activities. Hence, the cardiovascular effects of estradiol not only reflect the biological effects of estradiol per se but also those of its biologically active metabolites. To comprehend the mechanisms by which estradiol induces its protective effects on the cardiovascular system, it is a prerequisite to understand the diverse pathways by which estradiol can directly and indirectly influence the cardiovascular system.

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Estradiol induces diverse biological effects in various tissues and/or organs, and many of these effects are mediated via a direct interaction of estradiol with estradiol receptors (ERs) that activate the expression of a specific set of genes. Some of the ER-dependent biological effects of estradiol can also be induced via nongenomic mechanisms and via ERs in cell membranes. Ample evidence also suggests that estradiol can induce biological effects via ER-independent mechanisms. Because estradiol is metabolized to estrogenic and nonestrogenic metabolites and these estradiol metabolites possess biological activity, it is feasible that estradiol metabolites play an important role in influencing the cardiovascular system; this hypothesis is the focus of the present review.

METABOLISM OF ESTRADIOL

Estradiol is synthesized mainly by the granulosa cells of the developing ovarian follicles and involves the sequential conversion of androstenedione to estrone by aromatase and the subsequent conversion of estrone to estradiol by 17β-hydroxysteroid dehydrogenase (17β-HSD; Fig. 1). Also, as shown in Fig. 1, testosterone generated from androstenedione by the action of 17β-HSD can be converted to estradiol by aromatase. In addition to the classical steroidogenic tissues (placenta and ovary), 17β-HSD is widely distributed in a large number of other tissues (e.g., adipose tissue, skin, vaginal mucosa, endometrium, breast, liver) as well as in blood vessels (discussed later). It is well established that the biological activity of estradiol in hormone-sensitive tissues is actively regulated by the interconversion of estradiol to the less active hormone, estrone, by 17β-HSD.

As shown in Fig. 1, estradiol is converted into multiple metabolites via diverse pathways. Elimination of estradiol is largely mediated via its conversion into nonestrogenic water-soluble metabolites that are excreted in urine or feces. Estradiol is largely metabolized via oxidative metabolism (to form multiple hydroxylated metabolites such as 2- and 4-hydroxyestradiol) (63), glucuronidation (to form glucuronide conjugates) (101), sulfatase action (to form sulfates) (101), esterase action (to form fatty acid esters) (101), and *O*-methylation of catecholestradiols (to form *O*-methylated catecholestradiols) (101). For details, see reviews by Rosselli et al. (80) and Zhu and Conney (100, 101).

Oxidative Metabolism (NADPH-Dependent Hydroxylation)

Oxidative metabolism of estradiol involves the metabolic conversion of estradiol by several cytochrome *P*-450 enzymes (CYP450s; Refs. 63, 101). The CYP450s are a superfamily of monoxygenases that are involved

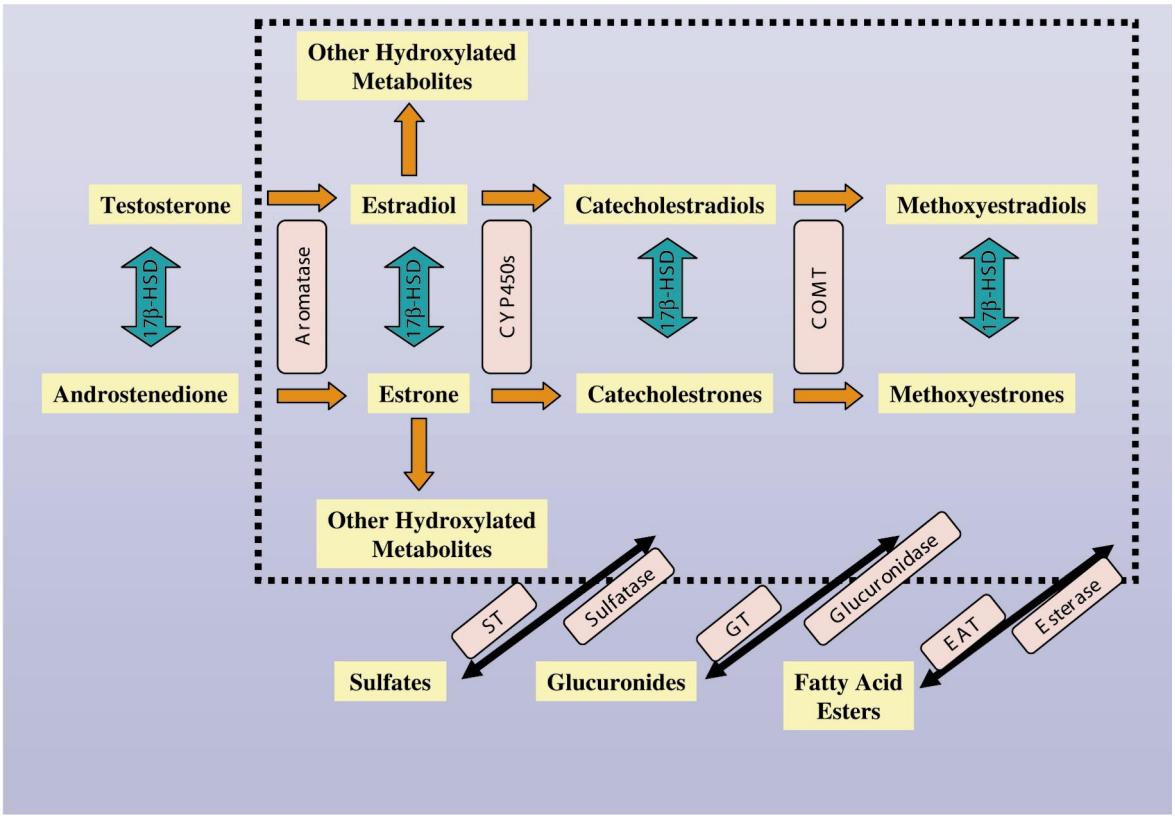

Fig. 1. Schematic representation of the multiple mechanisms via which 17β-estradiol is metabolized in humans. CYP450, cytochrome *P*-450; 17β-HSD, 17β-hydroxysteroid dehydrogenase; ST, sulfotransferase; GT, glucuronosyltransferase; EAT, ester acyltransferase; COMT, catechol-*O*-methyltransferase. Dotted line represents cellular membrane.

in the metabolism of both exogenous and endogenous compounds and that catalyze NADPH-dependent oxidative metabolism of estrogens to multiple hydroxylated metabolites. Many isoforms of CYP450s exist; however, the isozymes that metabolize steroids such as estradiol are CYP1, CYP2, and CYP3 (63, 101). Although metabolites of estradiol are less active estrogens and are water soluble and excreted in the urine, some estradiol metabolites have significant growth regulatory effects (101). The importance of estradiol metabolism is illustrated by the findings that inhibition of CYP450 enzymes by cimetidine increases estradiol levels and high doses of cimetidine may cause gynecomastia (35).

The type of hydroxylated estradiol metabolite generated by CYP450s depends on the estradiol position hydroxylated. Because multiple hydroxylation sites are available on estradiol, multiple hydroxylated metabolites are formed by CYP450s. For example, hydroxylation of estradiol at C-2, C-4, C-6, C-7, C-11, C-14, C-15, C-16, and C-17 results in the formation of 2-hydroxyestradiol, 4-hydroxyestradiol, 6α- and 6β-hydroxyestradiol, 7α- and 7β-hydroxestradiol, 11β-hydroxyestradiol, 14α-hydroxyestradiol, 15α-hydroxyestradiol, 16α-hydroxyestradiol (estriol), and 17α-hydroxyestradiol, respectively. Similar to estradiol, estrone can be hydroxylated at multiple positions and generate multiple hydroxylated estrone metabolites (101). Some hydroxylated metabolites of estradiol and estrone possess potent biological activities and may be involved in the etiology of cancer in reproductive tissues (101). Of the various catecholestrogens, the 2- and 4-hydroxyestradiols appear to be the most biologically active and may play a prominent physiological role in reproductive tissues, the kidney, the brain and nervous system, the heart, and the vasculature (100, 101).

O-Methylation

After hydroxylation, 2- or 4-hydroxyestradiol is rapidly methylated via enzymatic *O*-methylation to more lipophilic and nonestrogenic methylated products (100). The *O*-methylation of catecholestradiols is largely catalyzed by the enzyme catechol-*O*-methyltransferase (COMT), an enzyme that is present in large amounts in several tissues, including reproductive organs, liver, kidney, and the blood vessels (61). COMT is mostly a cytosolic enzyme. Apart from methylating catecholestradiols, COMT is also responsible for the methylation of catecholamines (61). Of all the endogenous estradiol metabolites, the 2- and 4-methoxyestradiols and methoxyestrones are the most biologically active and are involved in the biology and pathophysiology of estradiol-induced cancers (100, 101).

Esterification and Conjugation

As shown in Fig. 1, estradiol, estrone, and their hydroxylated metabolites can be further metabolized to form multiple glucuronidated metabolites, keto-metabolites, fatty acid esters, sulfated metabolites, and dehydrogenated metabolites, some with potent biological activities (see Ref. 101 for detailed list of endogenous estradiol and estrone metabolites). The enzymes sulfotransferase, glucuronosyltransferase, and acyltransferase are responsible for converting estradiol, estrone, and their hydroxylated metabolites to sulfates, glucuronides, and fatty acid esters, respectively, and some of these metabolites possess potent biological activities. Moreover, these metabolites serve as a pool for the slow release of estradiol, estrone, or their hydroxylated metabolites locally within tissues. Indeed, sulfotransferase, glucuronosyltransferase, and esterases can reconvert the estradiol and estrone sulfates, glucuronides, and fatty acid esters back to estradiol, estrone, and their hydroxymetabolites (101).

EVIDENCE THAT ESTRADIOL METABOLITES ARE BIOLOGICALLY ACTIVE

2-Hydroxyestradiol, a weak ligand for ER, may regulate multiple mechanisms in reproductive tissues, including growth of cancer cells and generation of prostaglandins in the uterus during pregnancy (6, 80, 101). 2-Hydroxyestradiol also attenuates catabolism of catecholamines by inhibiting COMT activity, and this may modulate the neurophysiological and pharmacological effects of catecholamines within the heart and vasculature (6, 80, 101). Moreover, 2-hydroxyestradiol affects the interaction of dopamine with its receptors (80, 101). Additionally, 2-hydroxyestradiol is a potent antioxidant and thereby protects membrane phospholipids and cells against peroxidation (31).

Similar to 2-hydroxyestradiol, 4-hydroxyestradiol induces several important biological effects. Although it is not the dominant metabolite formed by the liver, 4-hydroxyestradiol is a major metabolite formed in some extrahepatic tissues, including the pituitary, kidney, myometrium, and mammary gland (100, 101). Moreover, CYP450 enzymes responsible for the metabolism of estradiol to 2- and 4-hydroxyestradiol are expressed in the heart (76, 91, 92) and the vasculature (74, 90). In contrast to estradiol, 4-hydroxyestradiol binds with low affinity to ER; however, its dissociation rate from the receptor is much lower than that observed for estradiol (80). Within the renal system, 4-hydroxyestradiol has been shown to stimulate tumor growth in Syrian hamsters (101) and uterotrophic effects in rats (27, 101). 4-Hydroxyestradiol is more efficacious than estradiol in inducing progesterone receptor expression in the rat pituitary (80). Similar to 2-hydroxyestradiol, 4-hydroxyestradiol also acts as a cooxidant and increases the formation of prostaglandins from arachidonic acid within the uterus during pregnancy (80). 4-Hydroxyestradiol prevents inactivation of catecholamines by inhibiting COMT activity and thereby regulates the neurophysiological and pharmacological effects of catecholamines on the central nervous system (61, 101).

In contrast to 2-hydroxyestradiol, 4-hydroxyestradiol induces carcinogenic effects (27, 101). In fact, recent studies provide evidence for reduced 2-hydroxyla-

tion and increased 4-hydroxylation of estradiol in subjects with cancer, suggesting that 2-hydroxyestradiol or its methylated metabolite (2-methoxyestradiol) may be anti-carcinogenic, whereas 4-hydroxyestradiol and its metabolite 4-methoxyestradiol may be carcinogenic (53, 80, 100). Because abnormal growth of cells is a hallmark for atherosclerosis, glomerulosclerosis, cardiac hypertrophy/remodeling, and cancer, it is feasible that the catechol and methoxy metabolites of estradiol may play an important role in regulating cell growth within the vessel wall and the heart; this possibility is discussed further.

PROTECTIVE EFFECTS OF ENDOGENOUS ESTRADIOL METABOLITES ON THE CARDIOVASCULAR SYSTEM: ROLE OF GENOMIC VS. NONGENOMIC AND RECEPTOR VS. NONRECEPTOR MECHANISMS

The fact that endogenous metabolites of estradiol possess biological activity suggests that they could influence the cardiovascular system. Estradiol is known to induce protective effects on the cardiovascular system, and most of the research to define the mechanisms involved focus on conventional ER-mediated pathways. Much less attention is given to alternative mechanisms, which may involve ER-independent actions of estradiol via its endogenous metabolites (26, 27, 100). Indeed, although vascular endothelial and smooth muscle cells and cardiac cells (myocytes, fibroblasts) express ER-α and ER-β (23, 40), accumulating evidence suggests that the cardiovascular effects of estradiol may be mediated, at least in part, via ER-independent mechanisms. The role of ERs has been previously reviewed (27, 66). Hence, the focus of this review is to provide evidence supporting an active role of endogenous estradiol metabolites in protecting the cardiovascular system. In this regard, Table 1 summarizes the mechanisms discussed in this review by which endogenous estradiol metabolites may protect the cardiovascular system.

Effects of Endogenous Estradiol Metabolites on the Vasculature

The vascular wall is not static, and components of these structures dynamically increase, decrease, or reorganize in response to physiological and pathological stimuli. Although multiple cellular and biochemical processes are involved in vascular remodeling, smooth muscle cells (SMCs) and advential fibroblasts (52) are critical cell types mediating dynamic changes in vessel wall structure (30). In vascular remodeling, SMCs and fibroblasts undergo one or more of four basic processes: cell growth involving hypertrophy or hyperplasia, cell migration involving the immigration of cells from one locale to another in the vascular wall, modulation of the amount and types of extracellular matrix (ECM), and apoptosis, which provides an important means of population control for cells (30). Vascular endothelial cells also play a critical role in maintaining homeosta-

Table 1. *Potential mechanisms via which endogenous metabolites of estradiol may protect the cardiovascular system*

Vasoactive Molecules and Intracellular Signals	Influence of 2-Hydroxyestradiol and 2-Methoxyestradiol on Synthesis or Effects	Cardiovascular Effects of the Vasoactive Molecules and Intracellular Signals
Nitric oxide (cNOS)	+	Vasodilatation; inhibition of SMC and CF growth
Prostacyclin	+	Vasodilatation; inhibition of SMC and CF growth
cAMP	+*	Vasodilatation; inhibition of SMC and CF growth
Endothelin-1	−	Vasoconstriction; induces SMC and CF growth
Catecholamines and tyrosine hydroxylase	−	Vasoconstriction; induces SMC and CF growth
Leukotriene	−*	Vasoconstrictor; potent inflammatory and chemotactic
Leukemia inhibitory factor	+*	Inhibits injury-induced neointima formation and inhibits hypercholesterolemia-induced fatty streak formation
Collagen	−	Matrix deposition and vascular/cardiac remodeling
Adhesion molecules	?	Induce cell adhesion, migration; implicated in cardiovascular remodeling
Mitogens and cytokines PDGF, bFGF, IGF-1, FCS, ET-1, insulin	−	Induce SMC and CF growth, associated with cardiovascular remodeling
Free radicals	−	SMC and CF mitogen, EC damage (atherosclerosis/myocardial infarction) LDL and VLDL oxidation, cell-membrane phospholipid peroxidation
Cholesterol and lipids	−	SMC and CF mitogen, EC damage, cardiovascular disease
Diabetic factors (glucosuria, glycated-Hb, polydipsia, polyuria)	−	Major contributors to cardiovascular disease
MAP kinase	−	Intracellular signal for SMC and CF mitogenesis
Tubulin polymerization	−	Essential for cell division and mitogenesis in SMC and CF
Ca^{2+}-calmodulin	−	Important signalling mechanism for mitogenesis
Cell cycle regulatory proteins		A prerequisite for the progression of cells from metaphase to anaphase
Cdc2	+	
Cyclin B (degradation)	−	

cNOS, constitutive nitric oxide synthase; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; IGF-1, insulin-like growth factor-1; ET-1, endothelin-1; Hb, hemoglobin; SMC, smooth muscle cells; CF, cardiac fibroblast; EC, endothelial cells; LDL and VLDL, low- and very-low-density lipoproteins. +, Induction/positive; −, inhibition; ?, unknown. *In noncardiovascular cells.

sis by generating a battery of both growth-inhibitory and growth-stimulatory factors, as well as relaxing and contracting factors. Consequently, endothelial damage or dysfunction often leads to increased SMC migration, proliferation, and ECM synthesis (30).

Although estradiol induces vasoprotective effects by multiple mechanisms, including alterations in plasma concentrations of lipoproteins [decrease in low-density lipoprotein (LDL) levels, decrease in oxidized LDL formation, increase in high-density lipoprotein levels], hemostatic factors, glucose, and insulin, the two most important effects of estradiol in the cardiovascular system are modulation of vascular tone and inhibition of vascular growth (27).

Effects on estrogen metabolites on vascular tone. Increased vascular tone is associated with cardiovascular disease, and increased vascular tone is in part due to decreases in endothelium-dependent and endothelium-independent vasodilation. Estradiol induces vasodilatory effects on the vasculature via both genomic and nongenomic mechanisms that cause generation of vasodilatory agent [such as nitric oxide (NO), cGMP, cAMP, adenosine, and prostacyclin] and alterations in ion channel activity (27).

In contrast to estradiol, little is known regarding the effects of endogenous estradiol metabolites on vascular tone. Similar to estradiol, perfusion of the uterine artery with 2-hydroxyestradiol and 2-hydroxyestrone induces concentration-dependent vasodilation (78). Because 2-hydroxyestradiol has little or no binding affinity to ERs, the vasodilatory effects of 2-hydroxyestradiol most likely are mediated via ER-independent mechanisms, a concept consistent with the observation that the vasodilatory effects of 2-hydroxyestradiol are observed within 60 min (78). Generation of vasodilatory agents may mediate the vasodilatory effect of 2-hydroxyestradiol. For instance, 2-methoxyestradiol, a methylated product of 2-hydroxyestradiol, induces NO synthesis in cultured bovine carotid artery endothelial cells (93). Because 2-hydroxyestradiol is rapidly metabolized to 2-methoxyestradiol by COMT (100) and COMT is highly expressed by coronary artery endothelial cells (99), it is feasible that the vasodilatory effects of 2-hydroxyestradiol are mediated via 2-methoxyestradiol-induced NO synthesis. Interestingly, in addition to inducing NO synthesis, 2-methoxyestradiol also alters the membrane localization pattern of NO synthase (93); however, the significance of this altered localization remains unclear. In contrast to the above observations, both 2-hydroxyestradiol and 2-methoxyestradiol do not induce NO synthase activity and NO levels in glomerular endothelial cells (96), suggesting that the cell type may be critical. Further studies are needed to determine the effects of hydroxyestradiols and methoxyestradiols on NO synthesis in a wide variety of vascular beds.

Estradiol induces the production of cAMP (22), an endogenous vasodilator. Although the effects of estradiol metabolites on cAMP formation in vascular cells are not known, 2-methoxyestradiol increases cAMP synthesis by 240% in MCF-7 cells (58). Therefore, it is feasible that 2-methoxyestradiol induces a similar effect in vascular cells, and this possibility needs to be investigated.

In addition to increasing NO and cAMP synthesis, 2-methoxyestradiol also induces prostacyclin synthesis by human endothelial cells (83). The effects of 2-methoxyestradiol on prostacyclin synthesis are mimicked by other endogenous estradiol metabolites, including estrone, 2-methoxyestrone, and 16α-hydroxyestrone. Moreover, compared with estradiol, methoxyestradiols are more potent in inducing prostacyclin synthesis (83). This suggests that estradiol metabolites can induce vasodilatory effects and may be partly responsible for estradiol-induced relaxation in vivo.

The above findings provide evidence that endogenous metabolites of estradiol, in particular catecholestradiols and methoxyestradiols, can induce the synthesis of vasodilatory molecules and can protect the vasculature by influencing vascular tone and blood flow via this mechanism. Whether these metabolites induce both endothelial-dependent and/or endothelial-independent vasodilation is presently unclear. In this regard, recent findings from our laboratory suggest that 2-hydroxyestradiol induces and/or improves endothelium-dependent relaxation. In obese ZSF1 rats (which suffer from genetic obesity, diabetes, hypertension, hyperlipidemia, left ventricular dysfunction, and renal disease) treated chronically (6 mo) with 2-hydroxyestradiol, the vasodilatory effects of acetylcholine (endothelium-dependent vasodilator), but not sodium nitroprusside (endothelial-independent vasodilator), in mesenteric vascular beds preconstricted with ANG II and methoxamine are significantly enhanced compared with untreated ZSF1 rats (43). These findings suggest that 2-hydroxyestradiol enhances acetylcholine-induced vasodilation largely by increasing the release of endothelium-derived relaxing factor (EDRF). Because 2-hydroxyestradiol is rapidly metabolized to 2-methoxyestradiol and 2-methoxyestradiol induces the synthesis of vasodilatory substances such as NO (93) and prostacyclin (83), it is feasible that these molecules contribute to the increased vasodilatory effects of 2-hydroxyestradiol; however, further studies are required to delineate the mechanisms involved.

Recent findings suggest the involvement of calcium-dependent translocation of endothelial NO synthase (eNOS) from cell membrane to the nucleus (38). Therefore, NO release by 2-methoxyestradiol may involve eNOS in the plasma membrane. Indeed, immunostaining studies demonstrate that 2-methoxyestradiol-induced NO synthesis in endothelial cells is accompanied by translocation of eNOS (93). Alternatively, the antioxidant effects of 2-hydroxyestradiol and 2-methoxyestradiol may potentiate the activity of NO released under basal conditions. In this regard, it is well established that both 2-methoxyestradiol and 2-hydroxyestradiol are antioxidants that are more potent than either estradiol or vitamin E (62, 82, 88, 89). Indeed, estradiol increases rat aorta EDRF activity in the absence of changes in eNOS gene expression (7), and this increase in EDRF is associated with decreased gener-

ation of O_2^- in response to estradiol and could account for the enhanced EDRF-NO bioactivity and decreased peroxynitrite release (7, 30). Hence, the catecholestradiols and methoxyestradiols may induce NO synthesis via a similar mechanism.

Estradiol can also induce vasodilation by influencing membrane fluidity and ion channel activity (e.g., maxi-K and voltage-dependent L-type calcium channels; Ref. 27). However, whether the catecholestradiols and methoxyestradiols mediate their vasodilatory effects by influencing ion-channel activities remains unknown.

Estradiol reduces blood pressure in various animal models (14, 16, 50, 84), and the modulatory effects of estradiol on vascular tone may be responsible for estradiol-induced effects on blood pressure. Whether endogenous metabolites of estradiol influence blood pressure remains unknown. However, in a recent study, treatment of hypertensive, obese ZSF1 rats with 2-hydroxyestradiol for 26 wk significantly reduced mean arterial blood pressure from 133 ± 6 mmHg in controls to 122 ± 5 mmHg in 2-hydroxyestradiol-treated rats (43). The blood pressure-lowering effect of estradiol may be mediated by direct effects of estradiol on ion channel activity or increased synthesis of vasodilatory substances such as NO, cGMP, cAMP, adenosine, and prostacyclin (27). Alternatively, endogenous metabolites of estradiol may lower blood pressure by reducing the synthesis of vasoconstrictors such as ANG II and endothelin-1 (ET-1) or by interfering with the synthesis of and decreasing the plasma levels of catecholamines (55, 75, 77, 81). Indeed, we have recently shown that both 2-hydroxyestradiol and 2-methoxyestradiol inhibit ET-1 synthesis by coronary artery endothelial cells (28).

Effects on vascular growth. Estradiol plays a key role in regulating vascular growth. Within the reproductive organs, estradiol is responsible for the cyclic angiogenesis of microvessels, whereas, in the coronary arteries and other conduit vessels (e.g., carotid artery, aorta), estradiol protects against vascular injury induced by mechanical trauma, chronic allograft rejection, and hypercholesterolemia.

In vivo studies conducted in several species and using various models (balloon injury-induced neointima formation, allograft-induced dysplasia, cholesterol/lipid-induced atherosclerosis, and vascular narrowing-induced neointima formation; see Refs. 27 and 79 for review) provide evidence that estradiol prevents vascular remodeling processes leading to neointima formation. However, the exact mechanisms involved remain unclear. Evidence suggests that the inhibitory effects of estradiol on vascular remodeling processes leading to occlusive disorders are mediated via multiple pathways involving interactions with a variety of growth factors, cell types, and biochemical/molecular mechanisms (27). Because estradiol metabolites are biologically active (100, 101), it is feasible that the antimitogenic effects of estradiol are mediated via its metabolites acting on vascular SMCs and endothelial cells.

Abnormal growth of SMCs is one of the key processes responsible for neointima formation. Multiple factors (circulating, neurogenic, autocrine-paracrine, and blood flow related) influence growth of SMCs directly or in concert with each other (30). Cells respond to changes in external stimuli by activating a variety of signal transduction pathways, which culminate in stereotypical responses, such as proliferation, growth arrest, hypertrophy, differentiation, or apoptosis (30, 79, 94). A major pathway via which external stimuli induce proliferative or hypertrophic growth is the mitogen-activated protein (MAP) kinase cascade (94). To date, three different MAP kinase cascades have been characterized, i.e., the extracellular signal-regulated kinases (ERKs), the p38 MAP kinase, and the stress-activated protein kinase (SAPK) or c-Jun NH_2-terminal kinase (94). The MAP kinase pathways play an integral role in mediating the proliferative response of several growth factors on SMCs (30, 94).

In cultured rat aortic SMCs, endogenous metabolites of estradiol differentially inhibit FCS-induced DNA synthesis, proliferation, and collagen synthesis and in the following order of potency: 2-methoxyestradiol > 2-hydroxyestradiol > estradiol ≥ 4-methoxyestradiol (26, 71). In contrast, estrone, estriol, 16α-hydroxyestrone, 2-hydroxyestrone, and 4-methoxyestrone are significantly less potent and inhibit FCS-induced increases in DNA synthesis, cell proliferation, and collagen synthesis only at high concentrations (>1 μM), which are not attained physiologically (26). The fact that serum contains a battery of growth factors [platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), epidermal growth factor, insulin-like growth factor (IGF)-1, ANG II, ET-1, and so forth] implicated in the pathophysiology of vasocclusive disorders suggests that the growth inhibitory estradiol metabolites may protect against the vasocclusive actions of multiple growth factors by inhibiting signal transduction pathways critical to SMC growth. Indeed, preliminary data from our laboratory provide evidence that 2-hydroxyestradiol and 2-methoxyestradiol are more potent than estradiol in inhibiting SMC growth induced by PDGF, ET-1, ANG II, and IGF-1 (23, 26). Importantly, 2-hydroxyestradiol also inhibits free radical (peroxyl radical)-induced growth of aortic SMCs (31). The fact that free radicals mediate the growth effects of several mitogens, including ANG II (39), and play a key role in the atherosclerosis process suggests that 2-hydroxyestradiol may abrogate the vascular remodeling process in part via an antioxidant mechanism. Similar to 2-hydroxyestradiol, peroxyl radical-induced growth of SMCs is also inhibited by 2-methoxyestradiol, and, compared with 2-hydroxyestradiol, 2-methoxyestradiol is a more potent antimitogen (26). The antimitogenic effects of 2-hydroxyestradiol or 2-methoxyestradiol are not blocked by ICI-182780 (26), an ER-receptor antagonist, suggesting that the antimitogenic effects of 2-hydroxyestradiol and 2-methoxyestradiol are mediated via an ER-independent mechanism.

In addition to proliferation, the migration of SMCs from the media into the intima contributes to the vascular remodeling process associated with athero-

sclerosis and injury-induced neointima formation (30). Similar to estradiol, both 2-hydroxyestradiol (31) and 2-methoxyestradiol (8, 98) inhibit PDGF-BB-induced migration of rat and human aortic SMCs. Moreover, compared with estradiol, the metabolites are more potent in inhibiting SMC migration (8). Furthermore, the inhibitory effects of 2-hydroxyestradiol and 2-methoxyestradiol on SMC migration are not blocked by the ER antagonist ICI-182780 (8, 98), suggesting that these estradiol metabolites inhibit SMC migration via an ER-independent mechanism. Similarly, 2-hydroxyestradiol, but not estrone, abrogates free radical (peroxyl radical)-induced migration of SMCs (31), and this effect is associated with its inhibitory actions on the peroxidation of acidic membrane phospholipids (phosphatidylinositol and phosphatidylserine), which are known to regulate cell migration (45).

With regard to the mechanisms via which 2-hydroxyestradiol and 2-methoxyestradiol inhibit SMC proliferation, our recent findings suggest that both 2-hydroxyestradiol and 2-methoxyestradiol inhibit PDGF-BB-induced MAP kinase activity in both rat and human aortic SMCs (8, 26). We also find that FCS-induced MAP kinase activity is inhibited by 2-hydroxyestradiol and 2-methoxyestradiol. Compared with estradiol, 2-hydroxyestradiol and 2-methoxyestradiol are more potent in inhibiting both PDGF- and FCS-induced MAP kinase activity in SMCs (26), and ICI-182780 does not block the inhibitory effect of 2-hydroxyestradiol and 2-methoxyestradiol, suggesting that the effects of these metabolites on the MAP kinase pathway are ER independent (26).

Although 2-hydroxyestradiol and 2-methoxyestradiol induce their antimitogenic effects in part by inhibiting the MAP kinase pathway, most likely other mechanisms are also operative. For instance, 2-hydroxyestradiol and 2-methoxyestradiol inhibit the mitogenic effects IGF-1, a weak activator of MAP kinase that induces SMC migration by stimulating phosphatidylinositol turnover, diacylglycerol formation, intracellular calcium flux (12), and protein kinase C (PKC) activity (30). Because the mitogenic effects of IGF-1 and bFGF on SMC are associated with activation of PKC (30, 94), it is feasible that the inhibitory effects of these estradiol metabolites are in part mediated via downregulation of PKC activity; however, direct evidence in this regard is lacking.

Our previous study indicates that 2-hydroxyestradiol blocks the mitogenic effects of peroxyl radicals on SMC proliferation and migration (31) and that 2-hydroxyestradiol selectively prevents the peroxidation of acidic membrane phospholipids (phosphatidylinositol and phosphatidylserine) in SMCs (31). Peroxidation of phospholipids activates ERK1 and ERK2, induces the expression of c-*fos* and c-*jun* oncogene proteins, increases activator protein-1 DNA binding activity, and induces nuclear factor-κβ. The acidic phospholipids protected by 2-hydroxyestradiol are known to selectively activate PKC activity, and oxidized phosphatidylinositol induces cell migration (45). Hence, it is possible that 2-hydroxyestradiol prevents SMC growth by regulating PKC activity by selectively preventing peroxidation of phosphotidylinositol and phosphatidylserine.

Compared with 2-hydroxyestradiol, 2-methoxyestradiol is a more potent inhibitor of SMC growth and MAP kinase activity (26). However, in addition to inhibiting MAP kinase activity, 2-methoxyestradiol binds to tubulin and interferes with tubulin polymerization (17). Because polymerization of tubulin plays a key role in cell division as well as in the maintenance of cell structure and cell migration, it is possible that the growth-inhibitory effects of 2-methoxyestradiol are linked to its effects on tubulin polymerization. Indeed, recent findings from our laboratory provide evidence that 2-methoxyestradiol inhibits tubulin polymerization in SMCs (Dubey et al., unpublished observations). In the same vein, 2-methoxyestradiol inhibits calcium-calmodulin activity (3), induces Cdc2, and prevents the degradation of cyclin B, a prerequisite for the progression of cells from metaphase to anaphase (3). Finally, recent data from our laboratory (unpublished observations) provide evidence that 2-methoxyestradiol inhibits its PDGF-BB-induced expression of cyclin D in SMCs. The above findings provide evidence that 2-methoxyestradiol can interfere with pathways key to the progression of the cell cycle and cell growth.

In addition to the antimitogenic effects mediated via the direct interaction of 2-hydroxyestradiol and 2-methoxyestradiol with SMCs, these endogenous metabolites may also inhibit SMC growth indirectly by modulating the synthesis of growth-inhibitory and growth-promoting factors. In this regard, our studies show that both 2-hydroxyestradiol and 2-methoxyestradiol inhibit the release of ET-1 by ANG II, thrombin, FCS, and tumor necrosis factor-α from coronary artery endothelial cells (28). Because ET-1 is a potent SMC mitogen, it is conceivable that these endogenous metabolites of estradiol may protect the vasculature from occlusive disorders by downregulating ET-1 synthesis. Importantly, 2-hydroxyestradiol and 2-methoxyestradiol are more potent than estradiol in inhibiting ET-1 synthesis (28). In contrast, estrone, estriol, and estrone sulfate are only weak inhibitors (28). These findings suggest that 2-hydroxyestradiol and 2-methoxyestradiol are the prominent estradiol metabolites that possess potent anti-vasocclusive activities.

2-Methoxyestradiol also upregulates NO synthesis (93), and NO inhibits SMC proliferation and migration (19, 29, 36). Hence, it is feasible that increased production of NO may contribute to the inhibitory effects of 2-methoxyestradiol on SMC growth. However, direct evidence for the above possibility is lacking. Additionally, 2-methoxyestradiol stimulates the synthesis and/or release of prostacyclin (83) and cAMP (58), both of which are potent inhibitors of SMC growth, and may contribute to the antimitogenic effects of 2-methoxyestradiol on SMC growth.

Taken together, the above findings provide evidence that endogenous metabolites of estradiol, 2-hydroxyestradiol, and 2-methoxyestradiol in particular can induce antimitogenic effects on SMCs by directly and indirectly interacting with SMCs. This notion is

also supported by recent findings that 2-methoxyestradiol inhibits angiogenesis (34). Moreover, our studies show that, in intact male rats, the protective effects of estradiol on neointima formation are significantly enhanced when estradiol is administered with quercetin (inhibitor of 2-hydroxyestradiol catabolism; Ref. 26) to elevate circulating 2-hydroxyestradiol levels (32).

Similar to SMCs, estradiol also inhibits the growth of adventitial fibroblasts (52). Because infiltration by and abnormal growth of adventitial fibroblasts are known to contribute to the vascular remodeling process (52), it is feasible that estradiol may protect against vasoocclusive disorders via this mechanism. However, whether endogenous metabolites of estradiol also have a similar antimitogenic effect remains unknown and needs to be investigated.

Estradiol metabolites directly interact with endothelial cells and influence their growth and function. The growth regulatory effects of several endogenous estradiol metabolites on endothelial cells are concentration dependent. In this regard, low concentrations (physiological range; 10–100 nM) of endogenous estradiol metabolites (2-hydroxyestrone, 2-methoxyestrone, 2-hydroxyestradiol, 2-methoxyestradiol, 2-hydroxyestriol, 2-methoxyestriol, 4-hydroxyestrone, 4-methoxyestrone, 4-hydroxyestradiol, 4-methoxyestradiol, estrone, and estriol) significantly induce proliferation of cultured vascular endothelial cells (56), whereas, at higher concentrations ($\geq$100 nM; pharmacological concentrations), 2-hydroxyestrone, 2-hydroxyestradiol, 2-methoxyestradiol, and 16α-hydroxyestrone inhibit endothelial cell proliferation. The above findings suggest that physiological concentrations of endogenous estradiol metabolites may be helpful in promoting endothelial cell growth following vascular injury and may protect against occlusive disorders associated with endothelial damage.

2-Methoxyestradiol induces the synthesis of NO (93) and prostacyclin (83) from endothelial cells. Because these factors play a key role in maintaining homeostasis by inhibiting platelet aggregation and adhesion to endothelial cells, 2-methoxyestradiol may indirectly protect endothelial cells against platelet-induced injury. In addition, both 2-hydroxyestradiol and 2-methoxyestradiol are potent antioxidants (more potent than vitamin E and estradiol; Refs. 62, 82, 88, 89), which effectively prevents the oxidation of LDL to ox-LDL (82, 89), a molecule known to injure/damage endothelial cells. Because free radicals are generated at sites of cell injury and in response to cytokines (30), it is feasible that antioxidant effects 2-hydroxyestradiol and 2-methoxyestradiol may also prevent endothelial cell apoptosis by scavenging free radicals, which are known to induce apoptosis (10). Taken together, 2-hydroxyestradiol and 2-methoxyestradiol may protect vascular endothelial cells against injury and maintain homeostasis. The detailed biochemical and molecular mechanisms by which these metabolites induce endothelial cell growth remain undefined and need to be investigated.

The contention that physiological concentrations of 2-methoxyestradiol induce protective effects on the vascular endothelium is indirectly supported by our recent findings that in genetically obese ZSF1 rats, which suffer from cardiovascular complications, administration of 2-hydroxyestradiol, a precursor of 2-methoxyestradiol, selectively improves endothelium-dependent relaxations (43). Although these findings do not provide evidence for a direct role of 2-methoxyestradiol, they nevertheless provide evidence that endogenous metabolites of estradiol (e.g., 2-hydroxyestradiol) can positively influence endothelial function and may protect against vasocclusive disorders associated with cardiovascular disease.

Although low concentrations of 2-methoxyestradiol induce endothelial cell growth, pharmacological concentrations are potent antiangiogenic agents. The biochemical mechanisms by which 2-methoxyestradiol inhibits endothelial cell growth are partially defined. 2-Methoxyestradiol induces apoptosis in actively growing, but not confluent, endothelial cells (97). Consistent with the findings of Fostis et al. (34), a recent study by Yue et al. (97) demonstrates that apoptotic effects of 2-methoxyestradiol on vascular endothelial cells are mediated via the SAPK pathway and Fas expression. The SAPK pathway is a family of novel kinases that bind to the c-Jun transactivation domain and phosphorylate Ser-63 and Ser-73. In contrast to the MAP kinases, the SAPKs are weakly activated by growth factors but strongly activated by cellular stress (e.g., ultraviolet light, heat shock, protein synthesis inhibitors). Downregulation of SAPK with bFGF, IGF, and forskolin only partially abrogates the apoptotic effects of 2-methoxyestradiol. This suggests that pathways other than SAPK are also involved in mediating the apoptotic effects of 2-methoxyestradiol. In this context, evidence exists for a role for Bcl-2, Fas protein, and β-galactosidase in mediating the apoptotic effects of 2-methoxyestardiol. In addition to inducing apoptotic effects, 2-methoxyestradiol inhibits the migration and proliferation of endothelial cells. 2-Methoxyestradiol induces its antimitogenic and anti-migratory effects by disrupting microtubules and by reducing bFGF-induced increases in urokinase-type plasminogen activator activity (34).

Effects of Endogenous Estradiol Metabolites on Cardiac Fibroblasts

Abnormal growth of cardiac fibroblasts within the left ventricles importantly contributes to the cardiac remodeling process associated with hypertension, myocardial infarction, and reperfusion injury. Cardiac fibroblasts comprise 60% of the total heart cells and contribute to pathological structural changes in the heart by undergoing proliferation, depositing ECM proteins, and replacing myocytes with fibrotic scar tissue (21). In women, the incidence of heart disease is increased following menopause, and estradiol substitution therapy may induce protective effects in this regard. Although multiple mechanisms are involved, the hypothesis that the cardioprotective effects of estradiol are mediated via estradiol metabolites is untested. Our

studies show that 2-hydroxyestradiol and 2-methoxyestradiol are more potent than estradiol in inhibiting serum-induced proliferation and collagen synthesis in rat cardiac fibroblasts (21). In contrast to 2-hydroxyestradiol and 2-methoxyestradiol, other metabolites of estradiol such as estrone, estriol, and estrone sulfate are ineffective and inhibit cardiac fibroblast growth only marginally at high concentrations (21). These findings suggest that hydroxy and methoxy metabolites of estradiol are biologically active and can prevent cardiac processes known to induce heart disease.

We also observed that the antimitogenic effects of estradiol and its 2-hydroxy and 2-methoxy metabolites are enhanced, rather than inhibited, by the partial ER-antagonist 4-hydroxytamoxifen, suggesting that these effects are mediated via ER-independent mechanisms (21). This contention is further supported by our recent observation that the antimitogenic effects of estradiol, but not 2-hydroxyestradiol and 2-methoxyestradiol, on cardiac fibroblasts are blocked only by high (50 μM) concentrations of ICI-182780, which also inhibits the metabolism of estradiol to 2-hydroxyestradiol (26).

Effects of Endogenous Estradiol Metabolites on Vasoactive Factors Associated with Cardiovascular Disease

Modulation of circulating growth factors. Although endogenous metabolites of estradiol directly influence the growth and function of vascular SMCs and endothelial cells, they may also indirectly influence cardiovascular cells by altering the synthesis of circulating factors. In vitro studies demonstrate that 2-hydroxyestradiol and 2-methoxyestradiol induce the synthesis of antivasocclusive factors such as NO, prostacyclin, and leukemia inhibitory factor [a factor that inhibits injury-induced neointima formation (68) and hypercholesterolemia-induced fatty streak formation (67) as well as upregulates LDL receptors and lowers serum cholesterol levels (67)]. Recent studies from our laboratory provide evidence that 2-hydroxyestradiol and 2-methoxyestradiol inhibit the secretion of ET-1 (28), a potent constricting factor with mitogenic activity that is implicated in the pathophysiology of cardiovascular disease. Moreover, catecholestradiols inhibit leukotriene synthesis (1) and lower cholesterol/lipid levels (57). Finally, recent findings from our laboratory provide evidence that 2-hydroxyestradiol has antidiabetic effects and improves all measures of glucose control, including glucosuria, polyuria, polydipsia, glucose tolerance, and glycated hemoglobin levels. Because diabetes is associated with cardiovascular disease, the antidiabetic effects of 2-hydroxyestradiol may induce protective effects on the cardiovascular system (43).

Antioxidant effects and interaction with lipids. The role of lipids in inducing cardiovascular disease is well established in postmenopausal women. Ovarian dysfunction at the onset of menopause and increased incidence of coronary artery disease are associated with increases in LDL levels, decreases in high-density lipoprotein levels, and decreases in estradiol levels (70). These findings suggest that, under physiological conditions, interactions between hormones and cholesterol/lipids importantly contribute to maintaining vascular health.

Estradiol influences the vascular effects of LDL cholesterol, and, similar to estradiol, its metabolites interact with cholesterol/lipids. In vivo studies show that administration of estradiol metabolites such as 4-hydroxyestradiol, 2-hydroxyestradiol, 2-methoxyestradiol, and 2-methoxyestrone to ovarectomized rats significantly reduces circulating cholesterol levels (57). Recent results from our laboratory provide evidence that, in genetically obese rats (ZSF1 rats), administration of 2-hydroxyestradiol reduces hypercholesterolemia (399 ± 24 vs. 247 ± 28 mg cholesterol/100 ml at 25 wk into the treatment in control vs. 2-hydroxyestradiol-treated rats, respectively; Ref. 43). These findings suggest that endogenous metabolites of estradiol can positively influence the cardiovascular system by favorably influencing cholesterol levels. The mechanisms by which 2-hydroxyestradiol and 2-methoxyestradiol induce their cholesterol-lowering effects remain undefined.

The antioxidant effects of estradiol metabolites on ox-LDL formation may play a critical role in regulating antivasocclusive effects of estradiol under pathophysiological conditions associated with hyperlipidemia and hypercholesterolemia (27, 30, 79). It is feasible that these metabolites may also prevent the oxidation of very low-density lipoproteins. Recent findings from our laboratory demonstrate that 2-hydroxyestradiol is a potent antioxidant that prevents peroxyl radical-induced peroxidation of vascular SMC membrane phospholipids and inhibits peroxyl radical-induced proliferation and migration of SMCs (31). Also, 2-hydroxyestradiol prevents the oxidation of acidic membrane phospholipids (phosphatidylinositol and phosphatidylserine), which are known to activate PKC activity and play a critical role in regulating SMC growth (31). Together, these findings suggest that the antioxidant effects of endogenous estradiol metabolites at the cell membrane level may play a role in mediating the growth regulatory and antimitogenic effects of estradiol metabolites.

Interactions with catecholamines. As shown in Fig. 1, catecholestradiols such as 2- and 4-hydroxyestradiols are metabolized to 2- and 4-methoxyestradiols via *O*-methylation by COMT. Our studies show that vascular endothelial cells, SMCs, and cardiac fibroblasts express COMT activity and are very efficient in converting catecholestradiols to methoxyestradiols (99). Importantly, COMT is also involved in the metabolism of catecholamines, which are known to induce deleterious effects on the cardiovascular system (61). Because both catecholestradiols and catecholamines share COMT for their metabolism, interactions of these compounds at COMT may play an important role in determining the effects of these molecules on the cardiovascular system. In this regard, norepinephrine, epinephrine, and isoproterenol inhibit the metabolism of 2-hydroxyestradiol to 2-methoxyestradiol (98, 99). More-

over, these catecholamines abrogate the antimitogenic effects of estradiol and 2-hydroxyestradiol, but not 2-methoxyestradiol, on SMC and cardiac fibroblast growth (98). This suggests that catecholamines block the antimitogenic effects of both estradiol and 2-hydroxyestradiol by inhibiting their metabolism to 2-methoxyestradiol. Importantly, compared with the aorta, SMCs from human coronary arteries have increased expression of COMT (99), suggesting that the increased local formation of 2-methoxyestradiol within the coronary arteries protects women against coronary artery disease. Moreover, pathological increases in catecholamines would abrogate the protective effects of estradiol/2-hydroxyestradiol.

Although COMT-mediated conversion of 2-hydroxyestradiol to 2-methoxyestradiol may induce cardioprotective effects, it would also prevent the catabolism of catecholamines by competing for COMT. Significant increases in the local levels of catecholamines within the heart or vessel wall could induce deleterious effects. Indeed, this interaction may play a role in the increases in cardiac complications following hormone-replacement therapy observed in some clinical studies (41), as well as in pregnancy-induced hypertension (15). On the other hand, catecholestradiols inhibit tyrosine hydroxylase, the rate-determining enzyme of catecholamine biosynthesis, and could decrease catecholamine content via this mechanism (75). Finally, COMT is a ubiquitous enzyme that is highly expressed in most tissues (61) and abnormally high levels of 2-hydroxyestradiol would be needed to elevate catecholamine levels. Of more importance would be the balance between the local generation of 2-hydroxyestradiol and catecholamines. Estradiol is largely metabolized to 2-hydroxyestradiol within the liver, and steady-state levels of circulating 2-hydroxyestradiol can be readily converted to 2-methoxyestradiol within the vessel wall (99). In contrast, catecholamines can be generated locally within the vessel due to innervation, and this would ensure high local levels of catecholamines. On the basis of the above findings, increased synthesis of catecholamines is more likely to abrogate the protective effects of 2-hydroxyestradiol; however, further investigation is warranted.

Effects of Endogenous Estradiol Metabolites on the Kidney

Blood pressure is directly influenced by renal function and homodynamic. Premenopausal females are protected against the progression of renal disease (85), and gender differences in renal hemodynamics (69) are well established. Evidence from epidemiological/clinical studies and from experimental models of renal injury suggest that estradiol is responsible for the resistance of kidneys in females to renal disease (85). Similar to the vasculature, damage and dysfunction of glomerular and capillary endothelial cells and abnormal growth of glomerular mesangial cells, a cell phenotypically similar to SMCs, are associated with pathogenesis of renal disease, such as glomerulosclerosis (30). The pathophysiology of the glomerular-remodeling process mainly involves increased mesangial cell proliferation, migration, and ECM production (30). These processes can be triggered by multiple factors such as increased generation of growth promoters, decreased generation of growth inhibitors, and cytokine production in response to immune reactions (30).

Estradiol has been shown to induce protective effects on the kidneys (see Ref. 27 for review); however, whether endogenous metabolites of estradiol are involved remains unclear. In a recent study, we show that 2-hydroxyestradiol and 2-methoxyestradiol inhibit mitogen-induced proliferation and collagen synthesis in human glomerular mesangial cells (96) and provide evidence that these antimitogenic effects are ER independent. We also demonstrate that, similar to SMCs, the inhibitory effects of estradiol on glomerular mesangial cell growth are induced by CYP450 inducers and blocked by CYP450 and COMT inhibitors (24). Moreover, the antimitogenic effects of 2-hydroxyestradiol are blocked by the COMT inhibitor quercetin (24). This suggests that local metabolism of estradiol to methoxyestradiol plays a key role in mediating the ER-independent antimitogenic effects of estradiol. However, in contrast to estradiol, 2-hydroxyestradiol and 2-methoxyestradiol are unable to induce NO synthesis in glomerular endothelial cells (96). Taken together, our findings suggest that endogenous metabolites of estradiol may protect against glomerular remodeling process associated with glomerulosclerosis and may indirectly protect the cardiovascular system by maintaining renal function.

EVIDENCE THAT METHOXYESTRADIOLS MEDIATE THE CARDIOPROTECTIVE EFFECTS OF ESTRADIOL

The above findings provide evidence that endogenous metabolites of estradiol that have little binding affinity for ERs can inhibit growth of both vascular SMCs and cardiac fibroblasts, processes that contribute to vascular and cardiac remodeling leading to vasocclusive and cardiac hypertrophy, respectively. Because blood vessels and the heart express ERs (23, 40), it is believed that the protective effects of estradiol are ER mediated. However, the recent finding that estradiol protects against vascular injury in mice lacking either functional ER-α or ER-β (42, 44) suggests that other mechanisms are operational. Because the hydroxy and methoxy metabolites of estradiol are potent inhibitors of SMC (26) and cardiac fibroblast growth (21), we hypothesize that the inhibitory effects of estradiol on SMC and cardiac fibroblast growth are mediated via its metabolites. In this regard, ample evidence suggests that the cardiovascular effects of estradiol could be mediated by metabolites present in the circulation as well as via the local metabolism of estradiol to catecholestradiols and methoxyestradiols in the cardiovascular tissue (Fig. 2), and evidence supporting these possibilities are further discussed.

Evidence that Circulating Estradiol Metabolites Can Influence the Cardiovascular System

The rates of urinary excretion of 2-hydroxyestradiol and 2-hydroxyestrone are 20–180 μg/24 h and 180–2,100 μg/24 h, respectively (2, 37). The plasma concentration of 2-hydroxyestrone plus 2-hydroxyestradiol is 218–420 pg/ml (18, 33). 2-Hydroxyestrone can be readily converted to 2-hydroxyestradiol. Also, the metabolites of estradiol concentrate in the membranes of various cells, including liver, brain, uterus, and follicles (49, 65), suggesting that the concentrations of estradiol metabolites within subcellular compartments may be higher than those observed in the circulation.

Although 2-hydroxylation of estradiol leading to 2-hydroxyestradiol formation is a major pathway via that estradiol is metabolized, due to the rapid metabolism of 2-hydroxyestradiol and 2-hydroxyestrone, the true circulating levels of these compounds remain unclear. In this regard, it has been shown that the plasma half life of 2-hydroxyestradiol is 96 s (46) and its clearance rate is 11 times higher than estradiol (5, 46). The rapid metabolism of 2-hydroxyestradiol is catalyzed largely via its methylation by COMT, which converts 2-hydroxyestradiol to 2-methoxyestradiol (61, 101). Because 2-methoxyestradiol is more potent than 2-hydroxyestradiol in inhibiting SMC and cardiac fibroblast growth (21, 26), the rapid conversion of 2-hydroxyestradiol to 2-methoxyestradiol would further increase the effects of estradiol mediated via these metabolites. Indeed, the circulating levels of 2-methoxyestradiol plus 2-methoxyestrone are 257–339 pg/ml in normal women and 2,035–10,691 pg/ml in pregnant (37th–40th wk) women. Similarly, the levels of 2-methoxyestrone, which can be readily converted to 2-methoxyestradiol, are 4,000–6,000 pg/ml in pregnant women (50-fold higher than the concentration of estradiol). Interestingly, the levels of methoxyestradiols in premenopausal women range between 46 pg/ml (follicular phase) and 70 pg/ml (luteal phase), whereas the levels in postmenopausal women are 33 pg/ml, which are substantially lower compared with premenopausal women (11). The fact that hydroxyestradiols and methoxyestradiols are present in significant amounts in the circulation provides strong evidence that they can induce biological effects on the cardiovascular system.

The methoxy metabolites of estradiol, i.e., 2- and 4-methoxyestradiols and methoxyestrones, have low binding affinity to the classical estrogen receptors but have a higher binding affinity to sex hormone-binding globulin (SHBG) than estradiol (80, 101). This finding suggests that the methoxy metabolites of estradiol may have a longer half-life than estradiol and may be present in the circulation at much higher levels than estradiol and may contribute to the high levels of unconjugated 2-methoxyestrone (4,000 pg/ml) observed in pregnant women (6). Although increased binding to SHBG may increase the half life, it may also prevent the intracellular availability of methoxyestradiols. Indeed, compared with the biological activity of 2-methoxyestradiol observed in vitro, the doses of 2-methoxyestradiol used to inhibit tumor formation and angiogenesis in vivo are relatively high (34, 80, 101). Apart from the increased binding to SHBG, the biological effects and/or potency of the methoxyestradiols may also be influenced by catabolism (demethylation).

Although catechol and methoxy metabolites of estradiol induce protective effects on the cardiovascular system, circulating estradiol metabolites may not reflect their biological effects. Our recent studies show that, in contrast to catecholestradiols and methoxyestradiols, estrone and estriol, which are present

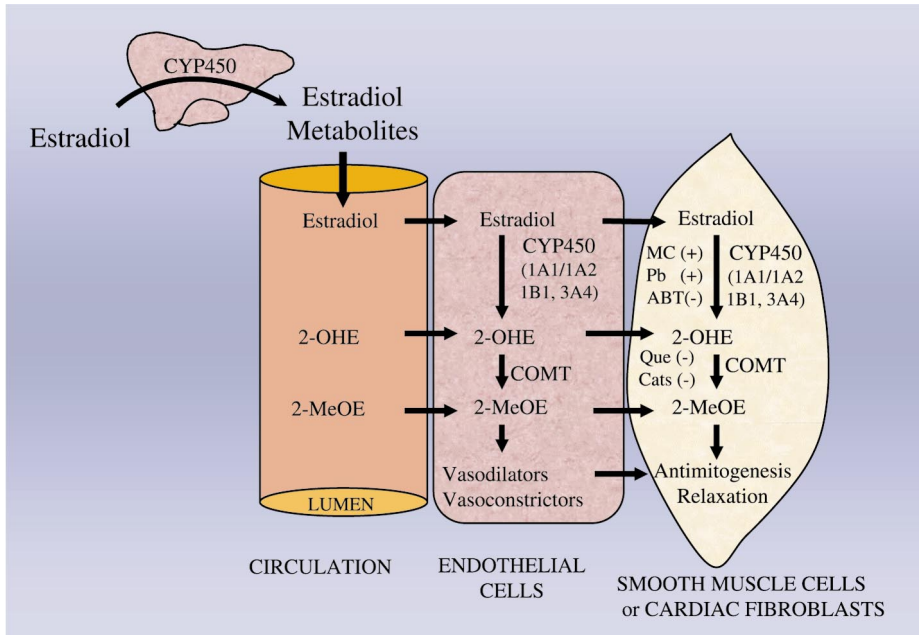

Fig. 2. Diagram showing the various pathways via which the endogenous estradiol metabolites can influence cells responsible for maintaining normal cardiovascular function. Circulating metabolites of estradiol, generated via the metabolism of estradiol by the liver, can diffuse into the endothelial cells, vascular smooth muscle cells, and/or cardiac fibroblasts. The catecholestradiols (i.e., 2- or 4-hydroxylated estradiol) can be efficiently converted to 2- and 4-methoxyestradiols by COMT and induce their biological effects. Moreover, estradiol can be taken up and metabolized in vascular endothelial cells, vascular smooth muscle cells, and cardiac fibroblasts by the CYP450 enzymes to catecholestradiols and subsequently by COMT to methoxyestradiols. The catecholestradiols (2- and 4-hydroxyestradiol) and methoxyestradiols (2- and 4-methoxyestradiol) formed in the vascular and cardiac cells or present in the circulation can induce their biological effects on the cardiovascular system by inducing vasodilatory and antimitogenic effects. MC, 3-methylcholantherene; PB, phenobarbital; ABT, 1-aminobenzotriazole; Que, quercetin; Cats, cateholamines; (−), inhibitor; (+), inducer.

in large amounts endogenously, are not effective in inhibiting SMC growth and MAP kinase activity (26). In fact, they significantly abrogate the inhibitory effects of estradiol (23), suggesting that inactive estradiol metabolites may block the biological effects of active estradiol. Indeed, this may in part explain why the use of conjugated estrogen apparently does not reduce cardiovascular risk in postmenopausal women (41).

Evidence That Local Conversion of Estradiol to Methoxyestradiols Mediates its Antimitogenic Effects Via ER-Independent Pathway

Recent studies support the concept that local metabolism of estradiol to methoxyestradiol in cancer tissues defines the final outcome on growth (100, 101). If true, the local metabolism of estradiol to methoxyestradiol may also be responsible for the antimitogenic effects of estradiol in the cardiovascular system. Indeed, vascular SMCs, vascular endothelial cells, cardiomyocytes, and cardiac fibroblasts express the key enzymes responsible for the conversion of estradiol to 2-hydroxyestradiol and 4-hydroxyestradiol (90–92). In this context, among the CYP450 isozymes (CYP1A1, CYP1A2, CYP1B1, and CYP3A4) known to metabolize estradiol to catechol estradiols, CYP1A1 (90) and CYP1B1 are expressed in vascular SMCs and endothelial cells and cardiomyocytes (90–92). Both vascular endothelial cells and SMCs, as well as cardiac fibroblasts, contain COMT and metabolize catecholestradiols to methoxyestradiols (25, 99). These findings suggest that vascular SMCs as well as cardiac fibroblasts are well equipped with enzymes to metabolize estradiol to catecholestradiols and methoxyestradiols. In addition, recent studies also provide evidence that both vascular SMCs and cardiac myocytes/fibroblasts contain aromatase activity (9, 40, 40a) and hence are capable of synthesizing estradiol endogenously. Finally, the vasculature has been shown to express arylhydrocarbon receptors (74), which are responsible for activating CYP450 enzymes, including the isoforms responsible for the metabolism of estradiol to 2-hydroxyestradiol (80), and vascular abnormalities are observed in mice lacking the AhR coactivator ARNT (27).

The above findings provide indirect evidence in support of the notion that the local metabolism of estradiol to methoxyestradiol is responsible for the antimitogenic effects of estradiol. Recent findings from our laboratory provide direct evidence in support of this mechanism (24–26). Our studies show that, in SMCs and cardiac fibroblasts, the inhibitory effects of estradiol on DNA synthesis, cell proliferation, collagen synthesis, and MAP kinase activity are enhanced by CYP450 inducers (2-methylcholantherene, phenobarbital, β-naphthoflavone; Fig. 2) and blocked by a CYP450 inhibitor (1-aminobenzotriazole, Fig. 2; Refs. 25 and 26). The fact that these agents have no binding affinity for ERs (26) suggests that their modulatory effects are not due to interactions with ERs. Catecholestradiols inhibit SMC and cardiac fibroblast growth via conversion to methoxyestradiol, as supported by the finding that the antimitogenic effects of 2-hydroxyestradiol are completely blocked by the competitive COMT inhibitor quercetin (25, 26, 98). This finding is further supported by the observation that vascular SMCs as well as cardiac fibroblasts express the cascade of enzymes responsible for the conversion of estradiol to methoxyestradiols (Fig. 2). Although these findings provide evidence that the effects of estradiol are mediated via methoxyestradiols, which methoxyestradiols (i.e., 2- or 4- or both) mediate these effects remain undefined. However, the possibility that conversion of estradiol to estrone and then to methoxyestrone is responsible for the antimitogenic effects of estradiol can be ruled out since direct addition of estrone to SMCs or cardiac fibroblasts does not inhibit cell growth (21, 23, 26). Finally, the antimitogenic effects of estradiol are blocked by specific inhibitors of CYP1A1 and CYP1B1 but not by inhibitors of CYP1A2 and CYP3A4, suggesting that the antimitogenic effects are mediated via 2- and/or 4-methoxyestradiols (25). Taken together, the above findings provide convincing evidence for a novel ER-independent pathway (as shown in Figs. 2 and 3) by which estradiol may induce its protective effect on the cardiovascular system. Thus our findings provide evidence that local metabolism of estradiol to methoxyestradiols may play a key role in mediating the protective effects of estradiol on the cardiovascular system (Fig. 3).

Evidence for Nonreceptor-Mediated Antimitogenic Effects of Estradiol

Recent studies by Iafrati et al. (42) show that estradiol induces vasoprotective effects in mice lacking ER-α, suggesting that the effects may be mediated via ER-β. The notion that ER-β mediates antivasocclusive effects of estradiol is further supported by findings by Mäkela et al. (60) who demonstrated that genistein, a phytoestrogen that binds exclusively to ER-β (>96% binding) and has negligible affinity for ER-α binding (48), inhibits balloon injury-induced neointima formation. However, this study does not provide evidence regarding whether the effects of genistein are blocked by ER antagonists. Hence, it is feasible that the effects of genistein are mediated via some alternative pathway not involving ERs. Indeed, genistein is a tyrosine kinase inhibitor and inhibits growth of cancer cells containing or lacking ERs (95). Moreover, our previous study demonstrates that the inhibitory effects of genistein on human aortic SMCs are not reversed by ICI-182780 (20). Also, Karas et al. (44) provided evidence that estradiol protects against vascular injury in mice in which ER-β is genetically disrupted. Together, these finding provide evidence that estradiol can induce vasoprotective effects via mechanisms independent of ERs. However, the possibility that ER-α may compensate for ER-β in ER-β knockout mice, and vice versa, remains an unresolved issue. The development

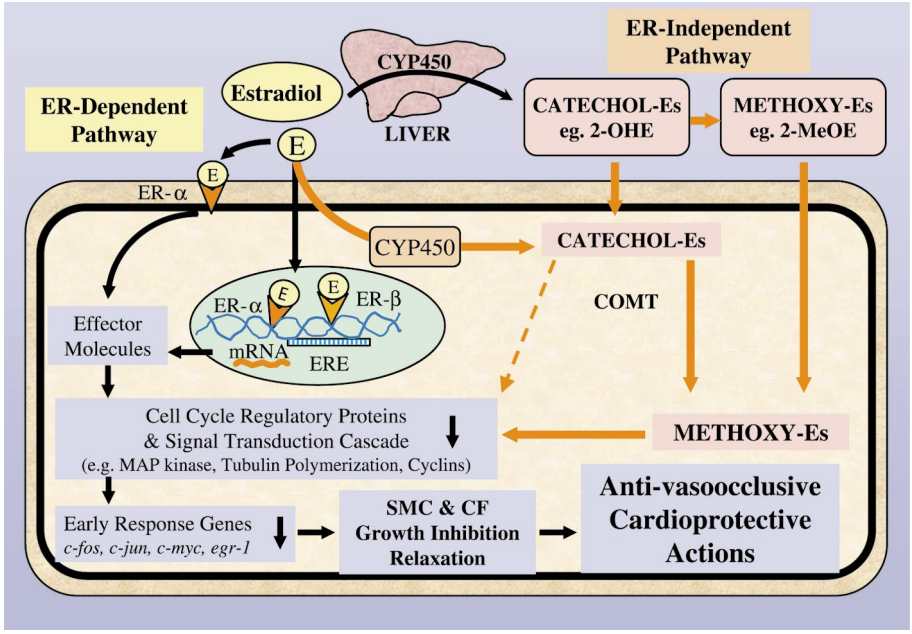

Fig. 3. Diagram depicting the estrogen receptor (ER)-dependent and ER-independent pathways via which estradiol can induce its biological effects on the cardiovascular system. Interaction of estradiol (E) with the ER-α or ER-β can trigger the estrogen response element (ERE) and upregulate the mRNA for the synthesis of specific effector molecules, which subsequently influence signal transduction mechanisms, induce antimitogenic effects on smooth muscle cells (SMC) and cardiac fibroblasts (CF), and induce protective effects on the cardiovascular system. Alternatively, metabolism of estradiol to 2- and 4-hydroxyestradiol by CYP450 and subsequently to 2- and 4-methoxyestradiol by COMT can trigger antimitogenic effects by interacting with signal transduction pathways, via ER-independent mechanisms. The ER-independent effects could also be mediated by circulating estradiol metabolites. MAP kinase, mitogen-activated protein kinase.

of mice lacking both ER subtypes would assist in further addressing this question.

Additional lines of evidence support the hypothesis that estradiol may induce cardiovascular protective effects via mechanisms not involving ERs. In this regard, increased expression of both ER subtypes occurs in the vasculature of male rats following allograft and balloon injury (54, 61). However, estradiol is unable to prevent neointima formation in nongonadectomized male rats following balloon injury (73), suggesting that mechanisms other than ERs are responsible for mediating the antivasocclusive effects. Medroxyprogesterone, a synthetic progestin that has no affinity for ERs, also abrogates the inhibitory effects of estradiol on neointima formation (51). Indeed, we and others report that pharmacological agents such as tamoxifen and 4-hydroxytamoxifen, which compete with estradiol for ERs, do not abrogate the antimitogenic effects of estradiol. Moreover, in vitro studies from our laboratory provide evidence that the estradiol metabolites, 2-hydroxyestradiol and 2-methoxyestradiol, which have minimal affinity for ERs, are several times more potent than estradiol in inhibiting SMC as well as cardiac fibroblast growth (21, 26, 31, 71). Also, the inhibitory effects of estradiol metabolites are not blocked by ICI-182780 (26). The above findings, together with the fact that SMCs and endothelial cells are capable of metabolizing estradiol (9, 98, 99), suggest that the protective effects of estradiol may be mediated via local generation of estradiol metabolites and independent of ERs.

Although the binding affinity of ICI-182780 to ERs is similar to estradiol (47, 48), 50 times higher concentrations of ICI-182780 are required to block the effects of estradiol on SMC growth (26). Moreover, studies from our laboratory provide evidence that ICI-182780, which is structurally similar to estradiol, inhibits estradiol metabolism (26). In this regard, concentrations of ICI-182780 lower than the K_i value are ineffective in blocking the inhibitory effects of estradiol, even when the estradiol-to-ICI-182780 ratio is 1:100 (26). Importantly, concentrations of ICI-182780 that block the inhibitory effects of endogenous estradiol on neointima formation in nonovarectomized rats are associated with a significant increase (more than double) in the circulating estradiol levels (4), potentially due to the inhibition of estradiol metabolism by high concentrations of ICI-182780. However, in the same study, ICI-182780 did not elevate estradiol levels in ovarectomized rats receiving estradiol and prevented the inhibitory effects of estradiol on neointima formation (4). Finally, the notion that the antimitogenic effects of estradiol are mediated via methoxyestradiol and an ER-independent mechanism is supported by the fact that the antimitogenic effects of estradiol on SMCs and cardiac fibroblasts are blocked by CYP450 and COMT inhibitors.

ESTRADIOL METABOLISM AND CLINICAL IMPLICATIONS

Although estradiol replacement therapy is known to induce protective effects on the cardiovascular system, only some postmenopausal women derive benefit. On the basis of the conventional concept that the biological effects of estradiol are ER mediated, it is postulated that the lack of protective effects may be due to a decrease in ER expression. However, this notion is not supported by the observation that the prevalence of cardiovascular disease is not increased in subjects lacking ERs (86, 87) and that there is no association between ER polymorphisms and angiographic severity of coronary artery disease (64). Hence, other factors must be involved. We hypothesize that differences in the metabolism of estradiol to hydroxyestradiols and methoxyestradiols is partly responsible for the inconsistent cardioprotective effects of estradiol. In this re-

gard, differences in metabolism can be caused by multiple factors such as diet, smoking, environmental agents, and drugs, all of which can influence CYP450 activity and therefore influence the metabolism of estradiol (101). Also, androgens and medroxyprogesterone, which abrogate the protective effects of estradiol on neointima formation (without influencing ERs), inhibit the metabolism of estradiol to 2-methoxyestradiol (13, 32). Thus the estradiol metabolizing capability of individuals varies enormously and this variation may be responsible for the inconsistent cardioprotection afforded by estradiol replacement therapy.

If the protective effects of estradiol are indeed mediated via hydroxyestradiol and methoxyestradiols, this could have significant therapeutic implications. Some of the major drawbacks of estradiol replacement therapy are *1*) only some postmenopausal women derive cardiovascular benefits from therapy, *2*) estradiol replacement therapy increases the risk of certain cancers, *3*) due to feminizing effects, estradiol replacement therapy would not be an option for most males. Because 2-methoxyestradiol is a potent anticarcinogenic agent with nonfeminizing effects, it could be used therapeutically, with no increase in risk of cancer, and could be employed in both women and men. Also, administration of 2-methoxyestradiol would directly protect the cardiovascular system without the need for metabolism, thus rendering more consistent effects. Nonetheless, future studies are needed to further investigate the role of estradiol metabolism in the cardiovascular system.

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