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Potential Vascular Actions of 2-Methoxyestradiol

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Abstract

2-Methoxyestradiol (2-ME) is a biologically active metabolite of 17 β -estradiol that appears to inhibit key processes associated with cell replication *in vitro*; it may have potent growth-inhibitory effects on proliferating cells, including smooth muscle cells and endothelial cells and may be antiangiogenic. Because of these potential roles for 2-ME, its lack of cytotoxicity and its low estrogenic activity, we hypothesize that 2-ME could be a valuable therapeutic molecule for prevention and treatment of cardiovascular diseases. Whether 2-ME is as efficacious *in vivo* as it is *in vitro* at modulating vascular processes remains controversial. Here we discuss recent developments regarding mechanisms by which 2-ME might regulate vascular activity and angiogenesis and speculate on the therapeutic implications of these developments.

Pharmacokinetics of 2-Methoxyestradiol

2-Methoxyestradiol (2-ME) is a major endogenous metabolite of estradiol formed via the sequential conversion of estradiol to 2-hydroxyestradiol (2-HE) and 2-ME by cytochrome P450s (CYP450s) and catechol-O-methyltransferase (COMT), respectively (Figure 1) [1]. Both CYP450s and COMT are ubiquitous enzymes responsible for oxidative metabolism and catechol methylation, respectively, of endogenous and exogenous molecules. Hence, many tissues that produce estradiol or are exposed to estradiol may generate 2-ME, although organs synthesizing estradiol (e.g., ovary) would be the most active in this regard. While tissue levels of 2-ME are unknown, reported plasma concentrations in men, non-pregnant women and pregnant women are 10 to 35 pg/ml, 18 to 138 pg/ml and 216 to 10,690 pg/ml, respectively [2,3]; however, one should view these values as tentative until confirmed by state-of-the-art mass spectrometry. Although rapidly cleared via hydroxylation, demethylation can reconvert 2-ME to 2-HE [4] (Figure 1b).

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2-Methoxyestradiol Receptors

The receptors that mediate the biological effects of 2-ME remain ill-defined. 2-ME has little or no affinity for classical estrogen receptors (ERs), but does bind to tubulin (IC₅₀ of 2 μ M) [2]. Moreover, 2-ME binds to an uncharacterized 92 kDa protein [5]. Clearly, the identification of 2-ME receptors is highly pertinent to the development of 2-ME analogues.

Potential Effects Of 2-Methoxyestradiol

Cardiovascular and Renal Protection

In vitro (rat and human cells) and *in vivo* studies (rat models) suggest that 2-ME induces cardiovascular and renal protective actions (Figure 2) by inhibiting abnormal cellular growth in vascular smooth muscle cells, cardiac fibroblasts and glomerular mesangial cells that contribute to vasoocclusive disorders, cardiac hypertrophy and glomerulosclerosis, respectively [2,4,6,7,8].

Plasma Cholesterol

2-ME may influence plasma lipid levels in a beneficial manner [2,4,8]. Both 2-ME and 2-HE significantly reduce cholesterol levels in rats, including genetically-obese ZSF1 rats [2,4] (Figure 2). Interestingly, a recent study found that 2-ME reduces atherosclerotic lesion formation in female apolipoprotein-E deficient mice [8].

Inflammation

Invasion of tissues by monocytes/macrophages also contributes to vascular disease. *In vitro* experiments show that 2-ME inhibits the motility, migration and adhesion of circulating breakpoint cluster region-abelson (BCR-ABL) transformed cells to fibronectin [9] suggesting that 2-ME inhibits the ability of circulating inflammatory cells to adhere to and infiltrate vascular lesions (Figure 2). Indeed, evidence shows that 2-ME inhibits adhesion of monocytes to aortic endothelial cells, a prerequisite for atherosclerosis [10]. 2-ME also inhibits hypoxia inducible factor-1 α (HIF-1 α), a transcription factor that mediates inflammation [11]. Hence, 2-ME may protect against atherosclerosis by inhibiting key inflammatory processes in the vascular wall.

Endothelial Function

2-HE improves endothelium-dependent relaxation in obese ZSF1 rats [2,4]. In vascular endothelial cells, both 2-HE and 2-ME induce COX-2 expression leading to production of prostacyclin, a vasoprotective molecule [12]. Moreover, in coronary artery endothelial cells, 2-ME and 2-HE inhibit the synthesis of endothelin-1, a vasoconstrictor associated with vasoocclusive disorders [7]. In rat aortic segments, 2-ME counteracts phenylephrine-induced contraction in the presence, but not absence, of endothelium [13], and nitric oxide synthase (NOS) inhibitors block this effect, suggesting that 2-ME abrogates vascular contraction via endothelium-dependent NO production [13]. Additionally, 2-ME increases redistribution [14] and expression [15] of endothelial NOS (eNOS), resulting in localized NO production within the plasma membrane, potentially contributing to endothelium-dependent relaxation [16]. 2-HE and 2-ME are also potent anti-oxidants (more potent than vitamin E and estradiol) [2,12], so they may likewise potentiate the vasodilatory activity of NO by preventing its oxidation. Detailed electron microscopic studies provide evidence that 2-ME prevents structural damage of vascular endothelial cells during preeclampsia [15]. Additionally, both 2-HE and 2-ME prevent low density lipoprotein (LDL) oxidation [2,7] and may protect endothelial cells against free radicals and oxidized LDL-induced injury. *In vitro* at concentrations ≥ 100 nM, 2-ME is antiangiogenic [17] and induces apoptosis in proliferating endothelial cells; whether this occurs *in vivo* remains unknown. Additional studies using

COMT knockout mice and/or pharmacological inhibitors of 2-ME are required to address this issue.

The putative ability of 2-ME to enhance endothelial vasodilation may contribute to several beneficial actions of 2-ME. 2-ME may protect against systemic hypertension [18], pulmonary hypertension [19,20], pre-eclampsia [15], renal disease [7,21] and ischemia-induced brain injury [22]. Because an abnormal endothelial barrier function is associated with these diseases, 2-ME could help preserve endothelial barrier function. For example, superfluous production of soluble Fms-like tyrosine kinase-1 (sFLT-1) disrupts the endothelial barrier in capillaries [23], and 2-ME reduces circulating sFLT-1 levels in mice with pre-eclampsia [15]. Furthermore, hypoxic conditions are associated with endothelial barrier disruption, and 2-ME inhibits hypoxia induced sFLT-1 production and HIF-1 α expression in cultured cells [15]. Further studies are warranted to investigate the effects of 2-ME on endothelial barrier function.

Vascular Smooth Muscle Cell (VSMC) Proliferation

Migration, proliferation, and extracellular matrix production by VSMCs contributes to the pathophysiology of vascular diseases such as atherosclerosis, restenosis and neointimal hyperplasia [4]. In cultured human and rat aortic VSMCs, estradiol metabolites differentially inhibit migration, proliferation and collagen synthesis in the following order of potency: 2-ME > 2-HE > 4-ME $\geq$ estradiol [24,25]. Subjecting cells to the ER α antagonist ICI 182780 or to ER antisense constructs does not block the growth inhibitory effects of catecholestradiols and/or methoxyestradiols on VSMCs [7,25], suggesting ER α -independent effects.

How does 2-ME inhibit VSMC growth? As mentioned, both 2-HE and 2-ME are potent antioxidants [4,7,12]; also, they inhibit free radical (peroxyl-radical)-induced proliferation and migration of VSMCs [26]. Flow cytometry indicates that 2-ME inhibits VSMC proliferation at both G₀/G₁ and G₂/M phases of the cell cycle [6] both *in vitro* (VSMC cultures) and *in vivo* (balloon injury-induced neointima formation in rats). For example, 2-ME downregulates hyperphosphorylated retinoblastoma protein (pRb), cyclin D₁, cyclin B₁, phosphorylated-ERK1/2 (MAPK) and phosphorylated-Akt, (all positive regulators of VSMC growth) [6] (Figure 3). In addition, 2-ME induces p27 expression (a negative regulator of VSMC growth), downregulates vascular expression of proliferating cell nuclear antigen (PCNA) and c-myc, and upregulates vascular COX-2 expression [6]. It remains unclear whether the modulatory effects of 2-ME on the cell cycle and signal transduction pathways in VSMCs contribute to 2-ME's inhibitory actions, or whether these effects are simply "bystander" manifestations, i.e., are simply a consequence rather than a cause of cell cycle arrest. Both 2-HE and 2-ME may also influence VSMCs indirectly by increasing the levels of endogenous compounds that inhibit growth (such as NO, cAMP and prostacyclin) or by decreasing the levels of endogenous compounds that stimulate proliferation (such as endothelin-1 and catecholamines) [2].

2-Methoxyestradiol Potentially Mediates Antiproliferative Effects Of Estradiol And Catecholestradiols

As mentioned, 2-ME is generated from the sequential metabolism of estradiol to 2-HE and 2-ME by CYP450s and COMT, respectively (Figure 1). VSMCs contain aromatase activity [27] and are capable of locally synthesizing estradiol [27]. Moreover, VSMCs are enzymatically equipped with CYP450 and COMT enzymes, and incubation of VSMCs with estradiol or 2-HE results in 2-ME formation [28,29]. Inasmuch as endogenous estradiol and 2-HE are metabolized to 2-ME (Figure 1), it is plausible that the antiproliferative effects of estradiol and 2-HE on VSMCs are also mediated in part by 2-ME. Furthermore, endogenous factors competing for the same pathway may compromise the beneficial effects of estradiol and 2-HE. For example, catecholamines and medroxyprogesterone (a synthetic progestin used

for combined hormone therapy with estradiol) block the conversion of estradiol and 2-HE to 2-ME and interfere with their antimitogenic effects [30,31].

ER-Independent Mechanisms Likely Contribute to the Antiproliferative Effects of Estradiol, Catecholestrodiols And Methoxyestrodiols

Ample evidence suggests that the antiproliferative actions of 2-ME and 2-HE are in part ER-independent. For example, even though 2-HE and 2-ME have little or no binding affinity for ERs, they inhibit growth of cardiovascular cells. Moreover, these antimitogenic effects are not blocked by ICI 182,780 and are present in cells cultured from ER α β double knockout mice [2,7,29,32]. However, whether the antiproliferative effects of estradiol are mediated via classical ERs or via 2-HE/2-ME-linked ER-independent mechanisms remains unclear and is discussed below.

Estradiol inhibits VSMC proliferation in injury-induced vascular lesions in mice lacking ER α [33], ER β [34] and both ER α and ER β [35], suggesting that the inhibitory effects of estradiol itself are ER-independent or involve an unidentified ER. Importantly, in ER α knockout mice [36], the inhibitory effects of estradiol on injury-induced lesion formation and VSMC proliferation are abrogated, suggesting that ER α mediates the protective effects of estradiol against lesion formation and VSMC proliferation [36]. Interestingly, compared to wild-type, injury-induced lesion formation and VSMC proliferation are also dramatically reduced in ER α knockout mice. Whether lack of robust VSMC growth in ER α knockout mice contributes to the lack of protective effects of estradiol remains unclear. Also, in studies with mice lacking both apoE and ER α , both ER α -dependent and ER α -independent mechanisms appear to account for the plaque-reducing and atheroprotective effects of estradiol [37]. Importantly, the atheroprotective effects of estradiol are not lost in mice lacking apoE and ER β [38]. Together these findings suggest that ER α -independent effects are not ER β -mediated and may involve an ER or an ER-independent mechanism. Because 2-ME inhibits both injury-induced neointima formation and atherosclerosis [6,8], it is possible that endogenous conversion of estradiol to 2-ME contributes to the antivasoocclusive effects of estradiol via an ER-independent mechanism. *In vitro* evidence for this possibility exists, in that antimitogenic effects of estradiol and 2-HE are blocked in VSMCs from COMT knockout mice [39] that express both ER α and ER β and by pharmacological inhibitors of estradiol metabolism [29]. Future studies in COMT knockout mice may help dissect the exact role of ER α and estradiol-derived 2-ME in inhibiting lesion-induced neointima formation and VSMC proliferation.

Pharmacological evidence for a role of ERs in mediating inhibitory effects of estradiol in VSMCs is inconclusive. Some investigators [40], but not others [41], report that ICI 182,780 attenuates estradiol's ability to reduce injury-induced neointima formation. ICI 182,780 not only binds to ERs but also blocks the metabolism of estradiol to hydroxyestrodiols [25,29]. In VSMCs, ICI 182,780 blocks the antimitogenic effects of estradiol only at concentrations that block the metabolism of estradiol to hydroxyestrodiols [25,29]. Hence the abrogatory effects of ICI 182,780 in one study may be due to the high concentrations used, since estradiol levels are increased by more than 2-fold in rats given ICI 182,780, perhaps due to inhibition of estradiol metabolism. Because ICI 182,780 is both an ER α β antagonist and an inhibitor of estradiol metabolism, it is difficult then to clearly decipher whether it blocks the antimitogenic effects of estradiol by inhibiting ERs or by attenuating estradiol metabolism. However, results from both molecular and pharmacological studies suggest that antiproliferative effects of estradiol are mediated via both ER α -dependent and ER-independent mechanisms.

2-Methoxyestradiol and Angiogenesis

Studies by Fotsis et al. [17] demonstrate that oral administration of 2-ME inhibits angiogenesis *in vivo* and suppresses tumor growth by limiting blood supply. Antiangiogenic effects of 2-ME at high concentrations may explain its beneficial effects in pulmonary hypertension [19, 20], rheumatoid arthritis [42], tumors [43], eye diseases associated with neovascularization [44] and endometriosis [45].

The proposed mechanisms by which high concentrations of 2-ME inhibit angiogenesis are depicted in Figure 4. Similar to VSMCs (Figure 3), 2-ME interacts with endothelial tubulin dynamics and HIF-1 α to affect target proteins (e.g., VEGF) and genes involved in endothelial proliferation, apoptosis, survival and invasion, processes that importantly regulate angiogenesis [6,43,45,46].

2-Methoxyestradiol and Apoptosis

Caspase-3 cleavage assay, FACS analysis of 2-ME-treated VSMCs and terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) staining of carotid segments from rats treated with 2-ME do not support the hypothesis that 2-ME induces apoptosis in VSMCs [6]. However, some investigators report 2-ME-induced apoptosis in VSMCs [47]. Although the reasons for these disparate findings remain unclear, perhaps differences in methodologies used and experimental conditions (different treatment times and serum concentrations) are potential factors, and this potential role of 2-ME needs to be further investigated.

In endothelial cells, it appears that 2-ME induces apoptosis, reflected by increases in expression of death receptor 5 (DR5; a member of tumor necrosis factor (TNF) death receptor family) and Fas (another member of the death receptor family) [48] (Figure 4). Specifically, DR5 activation by 2-ME renders endothelial cells more sensitive to the cytotoxic activities of the DR5 ligand, TNF-related apoptosis inducing ligand (TRAIL) [48]. 2-ME-induced apoptosis requires the sequential activation of caspase-8, caspase-9, and caspase-3 [48]. Death receptors signal apoptosis by recruiting Fas-associated death domain (FADD) to the oligomerized DR complex, where it facilitates the binding and activation of procaspase-8 [49]. In endothelial cells, expression of dominant-negative FADD inhibits 2-ME induced apoptosis by approximately 75% [48], suggesting that in endothelial cells 2-ME induces apoptosis via the extrinsic pathway.

The intrinsic or mitochondrial apoptotic pathway may also play a role in mediating apoptotic effects of 2-ME in endothelial cells. Treatment of endothelial cells with low concentrations of 2-ME rapidly activates c-Jun NH2-terminal kinase (JNK)/Stress activated protein kinase (SAPK) [50] and upregulates Fas, triggering programmed cell death (Figure 4). Although apoptotic effects of 2-ME are evident, whether these effects are specifically induced in pathologically-proliferating cells or in normal cells remains unknown. Finally, whether conversion of estradiol to 2-ME also induces apoptotic effects remains unknown and needs to be investigated.

Clinical Implications and Future Directions

That 2-ME potentially inhibits cell growth via common signaling pathways suggests that it may be a useful therapeutic agent for various proliferative diseases. 2-ME is also presumed to lower cholesterol [2,8], inhibit inflammatory processes associated with vascular and renal diseases and to attenuate atherosclerosis [9,10,19,20,21], angiogenesis/neovascularisation and capillary formation [17,43,44] (Figure 2). Because 2-ME inhibits key mechanisms associated with capillary leakage and angiogenesis, it may be of therapeutic use against diseases such as

pulmonary hypertension, endometriosis, pre-eclampsia, renal diseases, solid tumors and angiogenic eye disorders.

A drawback of current estrogen therapies is the increased risk of developing breast and endometrial cancers. Because 2-ME is non-toxic and potentially has both antivasoocclusive and anticarcinogenic actions, it could be used clinically to prevent cardiovascular disease in women without increasing risk of cancer. Being non-feminizing, 2-ME could also be used to treat cardiovascular disease in men. However, several issues need to be investigated. Although 2-ME may protect against disease-associated angiogenesis, it may also interfere with biological processes such as follicular development and intestinal epithelial cell growth, which are regulated by physiological angiogenesis and cell proliferation. Also, it is important to determine whether 2-ME mimics estrogenic effects in hot flushes and osteoporosis, and whether 2-ME is devoid of pro-thrombotic effects. Also, 2-ME increases thymus weight and uterine growth in mice [8], although the pathological consequences of these effects are unclear.

Another major challenge of developing 2-ME as a useful drug is overcoming its undesirable pharmacokinetic properties, i.e., its poor oral bioavailability and short half-life [3]. Future studies therefore should also focus on developing 2-ME analogs or 2-ME delivery systems that increase its bioavailability and overcome its short half-life. In order to develop more potent 2-ME analogues, the receptor via which 2-ME induces its biological effects needs to be identified. 2-ME may be less efficacious and potent *in vivo* compared with its actions *in vitro*, and nanotechnology and drug modeling will be key in resolving the pharmacokinetic/ pharmacodynamic issues that reduce the therapeutic potential of 2-ME. In this regard, modification of the molecule to target specific tissues will also be helpful to enhance its therapeutic potential and reduce adverse effects.

In conclusion, research thus far suggests that 2-ME blocks mechanisms that regulate cell proliferation and classifies this estrogen derivative as a potential therapeutic tool in proliferative diseases. Nonetheless, additional studies are warranted to address more carefully its safety and efficacy.

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Figure 1.

The endogenous formation and metabolism of 17β-estradiol (E2). (a) E2 interacts with ERα and ERβ to mediate its estrogenic actions in multiple tissues; (b) Endogenous E2 is metabolized to 2-hydroxyestradiol (2-HE) and 2-methoxyestradiol (2-ME) via the sequential actions of cytochrome P450 (CYP) and catechol-O-methyltransferase (COMT). CYP exists in multiple isoforms that are capable of metabolizing estradiol to catecholestadiols (e.g., CYP1A1, CYP1A2, CYP1B1 and CYP3A4). 2-HE has mild estrogenic actions and is rapidly metabolized to 2-ME. 2-ME has little or no affinity for ERs and mediates its biologic actions via ER-independent mechanisms. (c) 2-ME is rapidly cleared from the body via phase II conjugation enzymes involving glucuronidation and sulfation (hepatic clearance: 712 ml/min in humans). Moreover, demethylation can reconvert 2-ME to 2-HE. ER, estrogen receptor.

Cardiovascular Protection

- ↓ VSMC / CF Growth
- ↓ Neointima Formation
- ↓ Collagen Deposition
- ↓ Cholesterol Levels
- ↓ Atherosclerosis
- ↓ Ventricular Hypertrophy
- ↑ Endothelial Function
- ↓ Endothelial Damage
- ↓ Blood Pressure
- ↓ Vasoconstrictors
- ↑ Vasodilators

Renal Protection

- ↓ GMC Growth
- ↓ ECM Deposition
- ↓ Glomerulosclerosis
- ↓ Renal Toxicity
- ↑ Renal Function
- ↓ Metabolic Syndrome Linked Renal Damage
- ↑ Insulin Sensitivity

Anti-Inflammatory Actions

- ↓ Macrophage / Monocyte Adhesion to Endothelial Cells
- ↓ Renal Toxicity and Pulmonary Hypertension Induced Infiltration of Macrophages / Monocytes in Renal (Glomeruli), Vascular and Pulmonary Lesions

Figure 2.

The potential beneficial effects of 2-methoxyestradiol (2-ME) are shown. 2-ME induces cardiovascular protection and attenuates pulmonary hypertension by improving endothelial function and inhibiting abnormal growth of vascular smooth muscle cells (VSMCs), synthesis of extracellular matrix (ECM) proteins like collagen, infiltration of inflammatory cells (monocytes/macrophages) in vascular lesions and lowering cholesterol levels. Similar to VSMCs, 2-ME inhibits left ventricular hypertrophy by inhibiting abnormal growth and ECM synthesis/deposition by cardiac fibroblasts (CF). 2-ME reduces cardiovascular and renal damage associated with the metabolic syndrome and obesity. 2-ME may also protect against renal disease/toxicity by attenuating glomerulosclerosis/glomerular remodeling by inhibiting the abnormal growth of glomerular mesangial cells (GMC), deposition of EMC proteins like collagen by GMCs and infiltration of inflammatory cells (monocytes/macrophages). ↑ induction; ↓ inhibition.

Figure 3. Schematic representation of the cellular mechanisms in vascular smooth muscle cells (VSMCs) via which 2-methoxyestradiol (2-ME) potentially inhibits proliferation, migration and extracellular matrix (ECM) synthesis and mediates anti-vasoocclusive actions. 2-methoxyestradiol, 2-ME; positive cell cycle regulators include phosphorylated mitogen activated kinase, hyperphosphorylated retinoblastoma protein, phosphorylated-Akt, cyclin D₁, cyclin B₁; negative regulators of cell cycle include p27; HIF-1 α , hypoxia-induced factor-1 α ; ↓, inhibition; ↑, activation; ? unknown.

Figure 4.

Schematic representation of the cellular mechanisms in endothelial cells mediating the antiangiogenic and capillary barrier actions of 2-ME. (i) Disruption of tubulin dynamics by 2-ME inhibits cellular accumulation of hypoxia-induced factor-1 α (HIF-1 α), thereby inhibiting its translocation to the nucleus and resulting in inhibition of transcription and secretion of angiogenic factors like vascular endothelial growth factor-1 (VEGF-1). Decreased generation of VEGF-1 and Fms-like tyrosine kinase-1 (sFLT-1) protects against endothelial cell proliferation, migration and survival as well as endothelial barrier dysfunction. 2-ME can also induce antiangiogenic effects by causing endothelial cell apoptosis. (ii) 2-ME can generate reactive oxygen species (ROS) via activating c-Jun N-terminal kinase (JNK) or directly interacting with mitochondria to stimulate caspase-3 leading to apoptosis. (iii) 2-ME can also trigger apoptosis by upregulating the extrinsic pathway by increasing the expression of death receptor (DR5) and activating caspase-8. The collective effects of these mechanisms are protection against endothelial barrier disruption and capillary leak, inhibition of angiogenic activity and neovascularisation, and potential protection against tumor progression, plaque development, pulmonary hypertension, obesity, pre-eclampsia, renal disease, eye disease, endometriosis and rheumatoid arthritis. ↓, inhibition; ↑, increase; ----- hypothesized effects.