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Equine Estrogens Impair Nitric Oxide Production and Endothelial Nitric Oxide Synthase Transcription in Human Endothelial Cells Compared With the Natural 17β-Estradiol

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Abstract—Conjugated equine estrogen therapy is the most common hormone replacement strategy used to treat postmenopausal women. However, the ability of an individual conjugated equine estrogen to modulate NO production and, therefore, to induce cardiovascular protection is largely unknown. The effects of equine and naturally occurring estrogens on NO generation were evaluated in human aortic endothelial cells by measuring in vivo NO production, as well as NO synthase (eNOS) activity and expression. The transcriptional activity on the eNOS gene was determined by the ability of estrogen receptors (α and β) to activate the eNOS promoter and induce transcription. Docking and molecular dynamics simulations were used to study structural features of the interaction between estrogenic compounds and estrogen receptor-α. After 24 hours of incubation, we found that estrone upregulated NO production almost as effectively as estradiol by increasing eNOS activity and expression. However, the effect of equine estrogens (equilin, equilenin, and their metabolites) were marked decreased. eNOS promoter activity by equine estrogens was 30% to 50% lower than the naturally occurring estrogens. Computational analysis of estrogen molecules revealed that position 17 and the saturation of estrogenic compounds in ring B are important determinants for estrogen receptor-α transcriptional activity. Equine estrogens increase NO production less effectively than naturally occurring estrogens, partially because of their lesser ability to activate the eNOS promoter and induce transcription. Differences in NO production by different estrogens may account for the differences in cardiovascular benefits achieved by the distinct estrogen replacement therapies. **(*Hypertension*. 2010;56:405-411.)**

Key Words: 17β-estradiol ■ conjugated equine estrogens ■ NO ■ endothelium ■ eNOS transcription

Despite a wealth of observational studies and laboratory research suggesting that estrogen replacement therapy (ERT) holds promise to prevent cardiovascular disease in postmenopausal women,[1] the Women's Health Initiative Trial reported negative results for primary prevention of cardiovascular disease by estrogen.[2] These surprising results have raised concerns over the interpretation of the study, given the beneficial effects predicted from observational studies of ERT[3,4] and from studies in animal models of cardiovascular disease.[5–7] Several explanations have been put forth to explain the discrepancy between these studies and the clinical ERT trials. One limitation is that most ERT studies in the United States have used only 1 estrogen regimen, conjugated equine estrogens (CEEs) at 0.625 mg/day, whereas the majority of animal studies and several clinical studies studied 17β-estradiol (E_2), the most abundant circulating estrogen found in humans.

CEEs represent a family of diverse estrogenic molecules that are derived from the urine of pregnant mares. The main components are estrone (E_1) and equilin, with smaller amounts of E_2, equilenin, 17α-dihydroequilin, 17β-dihydroequilin, 17α-dihydroequilenin, and 17β-dihydroequilenin (Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org). Despite their natural origin, equine estrogens are not considered bioidentical to naturally occurring estrogens in women (ie, E_2, E_1, and estriol) and, therefore, may not provide effects comparable to E_2. In fact, a recent literature review of bioidentical and nonbioidentical hormones used in ERT has associated hormone therapy using bioidentical estrogen to more beneficial cardiovascular effects compared to the use of CEE and some synthetic hormonal therapies.[8]

The cardiovascular effects of estrogen and, more specifically, E_2 have been extensively studied and primarily associated with the modulation of NO production. E_2 has been shown to increase eNOS mRNA expression in a number of vascular beds.[9–11] E_2 is known to increase NO by a mechanism that partially involves estrogen receptor (ER) binding and increased endothelial NO synthase (eNOS) gene expression.[9,10,12] Although the molecular mechanisms by which E_2

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From the Experimental Cardiology (L.N., M.H., A.P.D.), Institut d'Investigacions Biomèdiques August Pi i Sunyer, Barcelona, Spain; Computer-Assisted Drug Design Laboratory (J.S., M.P.), Research Unit on Biomedical Informatics, Institut Municipal D'investigacio Medica-University Pompeu Fabra, Barcelona, Spain; Center for the Study of Sex Differences in Health, Aging, and Disease (K.S.), Georgetown University, Washington, DC.

Correspondence to Ana Paula Dantas, Experimental Cardiology Division, Institut d'Investigacions Biomèdiques, August Pi i Sunyer (IDIBAPS), C/Casanova 143 Lab, IDIBAPS 404, 08036 Barcelona, Spain. E-mail adantas@clinic.ub.es

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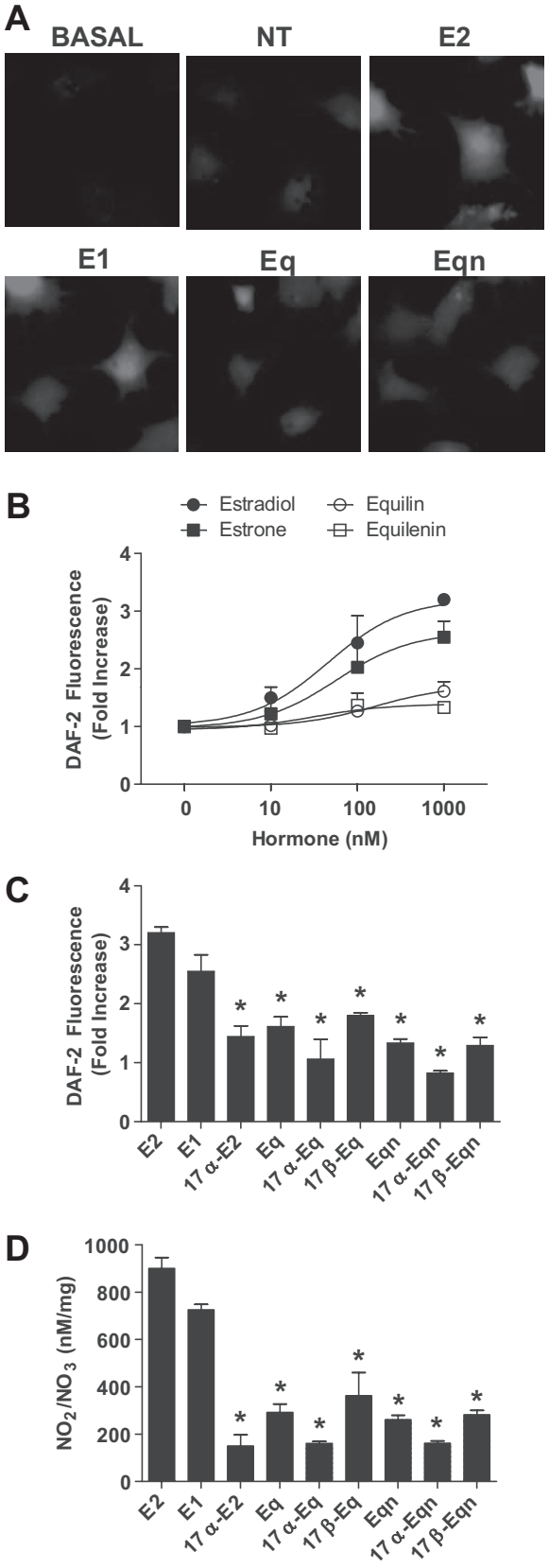

Figure 1. NO production in HAECs by distinct estrogens: (A) representative images of diaminofluorescein (DAF)-2 diacetate fluorescence in HAECs at basal level, before estrogen treatment (NT) and after 24-hour treatment with naturally occurring estrogen and the main equine estrogens; (B) dose response, shown as relative green fluorescence intensity after estrogens stimulation; (C) maximum increase in green fluorescence intensity induced by the different estrogens studied; (D) nitrite/nitrate (NO_2/NO_3) concentration (in nanomoles per milligram of protein) in HAEC media after estrogen stimulation: 17β-estradiol (E2); estrone (E1); 17α-estradiol (17α-E2); Equilin (Eq); 17β-equilin (17β-Eq); 17α-equilin (17α-Eq); Equilenin (Eqn); 17β-equilenin (17β-Eqn); 17α-equilenin (17α-Eqn). Data are plotted as the mean±SEM derived from 3 to 4 independent experiments. *$P<0.05$ vs E_2.

can potentially lead to cardiovascular protection are well described,[1] relatively little is known about how equine estrogens affect human ER function and regulation of estrogen-sensitive genes. Clearly, more information is needed on the mechanisms trigged by the components of CEE and their effects on the cardiovascular system. This study aims to compare the ability of equine and naturally occurring estrogens to modulate NO, a key regulator of vascular function. Furthermore, we analyzed estrogen-mediated activation of ER subtypes ER-α and ER-β and their ability to increase eNOS transcription. To understand how structural changes in the estrogen molecule may alter ER transcription, we used computational modeling to analyze ER-α structures in a complex with estrogens.

Methods

Methods used for NO detection and measurement of mRNA expression and transcriptional activity by estrogens are detailed in the online Data Supplement at http://hyper.ahajournals.org. Computational modeling to analyze ER-α structures in an estrogen complex used Molecular Dynamics and quantitative structure-activity relationship systems.

Results

We found an important disparity in the modulation of NO production by E_2 and by estrogens found on CEE. We observed that E_1 exhibited a slightly lower potential to increase NO production compared with E_2, whereas all of the other estrogens were significantly less effective (Figure 1). When human aortic endothelial cells (HAECs) were loaded with diaminofluorescein 2 diacetate and excited with light at 495 nm, we could observe a weak green fluorescence under basal conditions (ie, no estrogen or carbachol treatments) that increased by a factor of $\approx$2.5 after carbachol (1 μmol/L) stimuli in the NT group (no estrogen treatment; Figure 1A). Treatment with E_2 and E_1 caused a significant dose-dependent, saturable increase in green fluorescence relative to NT, indicating an increase in intracellular NO (Figure 1B). On the other hand, the equine estrogens (Eq, Eqn, and their metabolites) and the 17α-reduced form of E_2 (17α-E_2) were markedly less effective (Figure 1C). A similar pattern of estrogen-induced NO production was found when we measured NO_2/NO_3 in cell culture media (Figure 1D).

Our next steps were to determine whether a difference in the modulation of eNOS expression/activity could account for the disparity in NO production induced by naturally occurring and equine estrogens in HAECs. As shown in Figure 2A, E_2 induces a dose-dependent increase of eNOS activity. A similar response pattern was observed in eNOS activity when cells were treated with the physiological estrogen E_1 but was markedly decreased with equine estro-

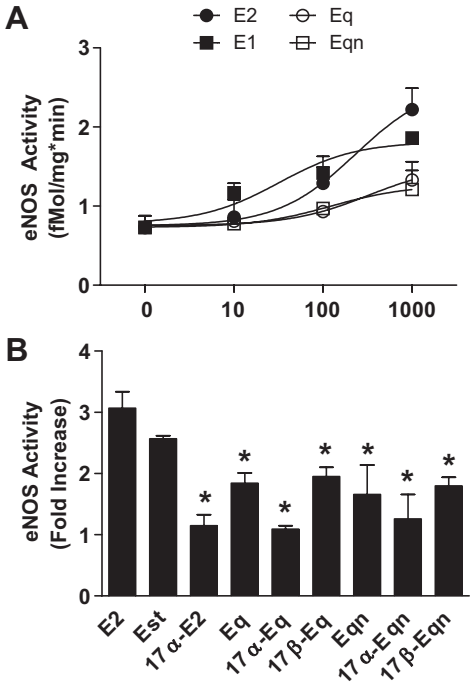

Figure 2. Effects of distinct estrogens on eNOS activity in HAECs. Dose-response effect on eNOS activity induced by naturally occurring and major equine estrogens (A) and maximal eNOS activity induced by these estrogens (B). 17β-estradiol (E2); estrone (E1); 17α-estradiol (17α-E2); Equilin (Eq); 17β-equilin (17β-Eq); 17α-equilin (17α-Eq); Equilenin (Eqn); 17β-equilenin (17β-Eqn); 17α-equilenin (17α-Eqn). Data are plotted as the mean±SEM derived from 3 to 4 independent experiments in triplicate. *$P<0.05$ vs E_2.

gens and 17α-E_2 (Figure 2B), indicating that different estrogens have distinctly different effects on eNOS activation. The major mechanism involved in estrogen-induced increase in NO availability includes transcriptional stimulation of eNOS gene expression. To determine whether different estrogens regulate eNOS transcription differently, we initially used quantitative real-time PCR to assess the effects of estrogen treatments on steady-state eNOS mRNA expression. Figure 3A shows a representative experiment demonstrating that E_2 treatment increased eNOS mRNA levels in HAECs by a factor of ≈2.5 compared with NT. Similar to responses seen with eNOS activity, E_1 increased eNOS transcription as effectively as E_2, whereas equine estrogens and 17α-E_2 were markedly less effective.

The best-characterized mechanism for E_2 modulation of eNOS transcription involves ER binding to the eNOS promoter region and activating transcription, which results in increased eNOS expression and activity in endothelial cells.[10] In this regard, we performed a promoter/luciferase reporter gene analysis to determine ER transcriptional activity. In agreement with previous studies, our data show that ER-α is the main ER subtype responsible for estrogen activation of eNOS promoter and transcription. We observed that, although physiological estrogens (E_2 and E_1) induce an ≈10- to 15-fold increase on eNOS promoter activity through ER-β, induction by ER-α activation was ≈2 to 3 times higher still (Figure 3B).

Computational modeling analysis of ER-α interaction with estrogenic series E_2, E_1, and 17α-E_2 highlights the importance of position 17 (the only structural difference between these 3 estrogens) to NO production: in order of potency, E_2 (17-β-hydroxyl)>E_1 (17-keto)>17α-E_2 (17-α-hydroxyl). With the E_2 and ER-α complex, there is a stable $His524^T$-419 interaction during binding of hydrogen (position 17, E_2) with $His524^T$ (ER-α; Figure 4A). In contrast, the structure of the E1 complex (Figure S1) shows how the keto group on position 17 cannot establish a favorable interaction with His524 when the imidazole ring is present in the T-tautomer. Instead, the E_1 keto group forms a hydrogen bond to His524 when present as $His524^{\Pi}$ tautomer (Figure 4B, inset). In the case of 17α-E_2, neither the final complex

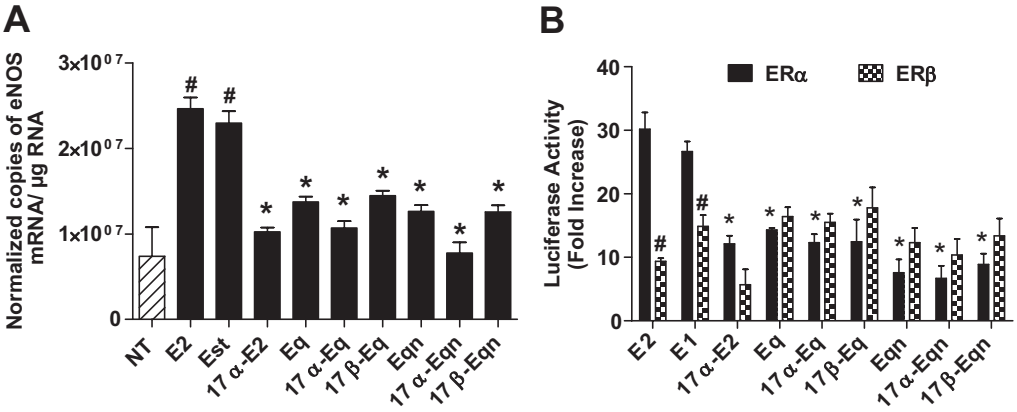

Figure 3. Transcriptional activity of ERs on eNOS gene by different estrogens. A shows the number of copies of eNOS mRNA per microgram of RNA in HAEC before (NT) and after treatment with different estrogens (1 μmol/L). eNOS copy number was quantified by comparison with a standard curve and normalized to the 18S copy number, which was used as an endogenous reference gene. B, Transcriptional activity of ERα and ERβ on eNOS promoter. COS-7 cells were transiently cotransfected with eNOS promoter-luciferase plasmid and ERα (solid bar) or ERβ (patterned bar) cDNA. eNOS promoter-dependent luciferase activities were determined and plotted as fold increase activation over the luciferase activity of COS-7 cells treated with vehicle only. 17β-estradiol (E2); estrone (E1); 17α-estradiol (17α-E2); Equilin (Eq); 17β-equilin (17β-Eq); 17α-equilin (17α-Eq); Equilenin (Eqn); 17β-equilenin (17β-Eqn); 17α-equilenin (17α-Eqn). Data are plotted as the mean±SEM derived from 3 to 4 independent experiments in triplicate. *$P<0.05$ compared to E_2; #$P<0.05$ compared to NT (A) or compared to ERα (B).

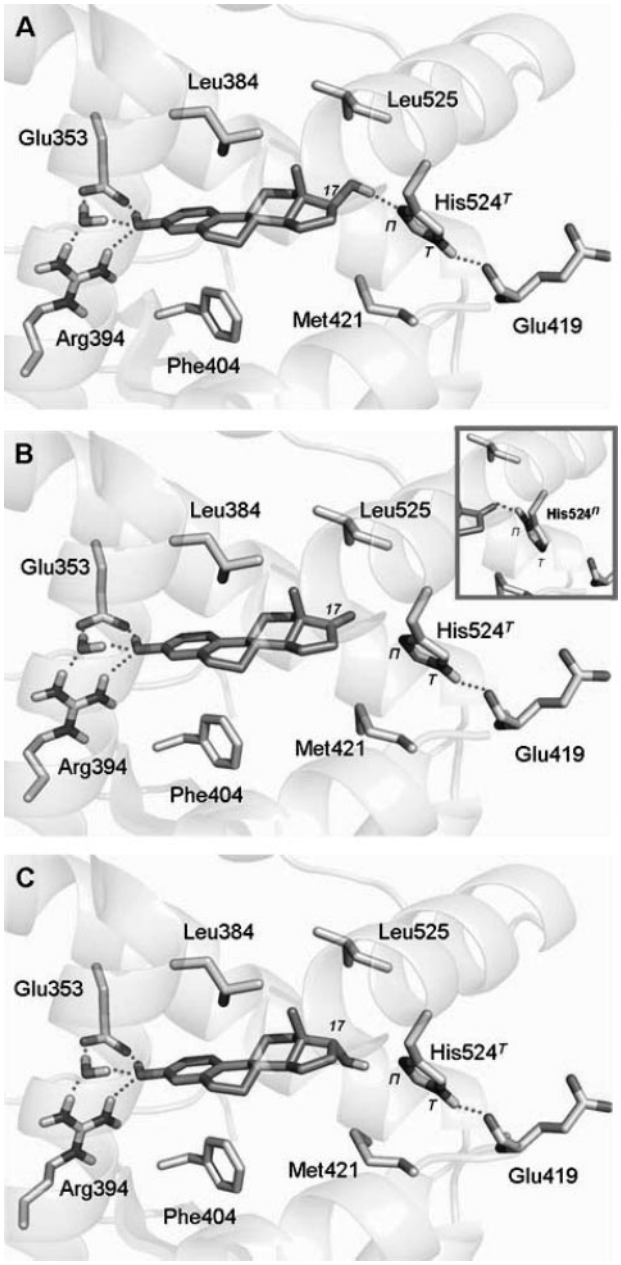

Figure 4. Role of chemical moiety and stereochemistry on position 17. ER-α in complex with estrogenic compounds: (A) E_2 establishes a hydrogen bond to $His524^T$ tautomer while maintaining the stabilizing $His524^T$-Glu419 interaction; (B) E_1 lacks the hydrogen bond with $His524^T$ tautomer but can establish such interaction with the Π-tautomer (inset) at the expense of the stabilizing $His524^T$-Glu419 interaction; (C) 17α-E_2 is not able to establish any hydrogen bond with $His524^T$.

structure nor the short molecular simulation results reveal any stable interaction between the α-hydroxyl group and His524 (Figure 4C).

Changes in chemical moiety/stereochemistry at position 17 cannot fully account for differences in NO production. Therefore, we further analyzed how modification of ring B saturation (the only structural difference between equine estrogens and E_2 and E_1) accounts for differential ER-α activity. When we compared E_2, 17β-Eq, and 17β-Eqn, we observed an inverse correlation between ring B saturation and the flexibility of the estrogen molecule. In this context, E_2 containing a saturated ring B adopts a half-chair conformation and maintains it during 200-picosecond simulation (Figure 5C). The more rigid 17β-Eq (ring B with 2 double bonds) and 17β-EQN (ring B with 3 double bonds) prefers a more planar conformation (Figure 5C). However, despite these existing conformational differences, Figure 5A shows that all 3 of the compounds establish a very similar interaction with ER-α. In contrast, calculation of the p*K*a value of the phenolic group on ring A by Strategic Promotion of Ageing Research Capacity Chemical Reactivity Model demonstrates that lower ring B saturation evokes higher acidity (E_2 [p*K*a: 10.54]<17β-EQ [p*K*a: 10.41]<17β-EQN [p*K*a: 9.91]), an effect that could interfere with estrogenic interaction with Glu353 on ER-α. As illustrated in Figure 5B, a deprotonated and, therefore, more acidic estrogen compound cannot establish an interaction with Glu353 on ER-α.

Discussion

These studies are the first to show that equine estrogens are individually less effective in modulating NO production and eNOS expression in HAECs than the naturally occurring estrogens E_2 and E_1. CEEs represent the most common form of ERT in the United States for postmenopausal women seeking relief from symptoms associated with estrogen deficiency, as well as for the prevention of osteoporosis.[13] Although estrogen has been largely described as cardioprotective, the specific effects of CEEs on cardiovascular function are not well established and remain rather controversial. Few basic studies have described similarities between the CEE mixture and E_2 in exerting potentially beneficial effects on cardiovascular function.[14] In contrast, others have described differences in E_2 efficacy compared with CEE in modulating NO production in porcine endothelial cells and platelets.[15]

It is commonly accepted among basic scientists and physicians that treatment with CEE cannot be considered as a replacement of estrogen during menopause, because CEE consists of a complex mixture lacking the main physiological estrogen in women (ie, E_2).[16,17] Moreover, the CEE mixture not only contains estrogenic compounds but also progestins and, importantly, androgens that may interfere with the beneficial effects of estradiol.[17,18] A recent review of clinical data from published ERT studies showed that beneficial effects from replacement therapy using CEE are not comparable to those observed when bioidentical estrogens and those most physiologically similar to humans are used.[8] If these negative effects of therapy with the CEE mixture are derived from the type of estrogen or the presence of progesterone and androgens in its composition is largely unknown. In these studies, we have hypothesized that each estrogenic component on the CEE mixture may not only differentially activate the 2 ER subtypes and transcription but also act differently to modulate cardiovascular function. Contrary to our data, a study by Wingrove et al[19] showed that equine estrogens do increase eNOS protein expression in cultured human coronary endothelial cells to levels 1.5 to 2.0 times higher than E_2. Differences in the tissue and vascular bed studied may be a

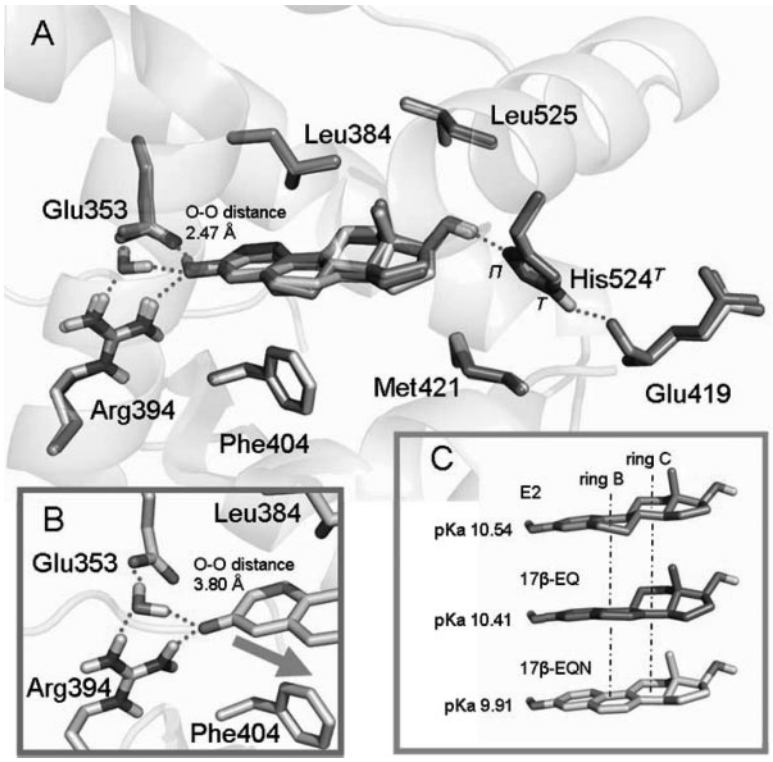

Figure 5. Role of B ring saturation on ER-α activity. A, Superimposition of the ER-α receptor complexes for E_2, 17β-EQ and 17β-EQN. B, A deprotonated estrogenic compound (negatively charged phenolate-anion) cannot establish the key interaction with Glu353. C, The degree of saturation determines the conformation and p*Ka*: E_2 adopts a half-chair conformation, whereas 17β-EQ and 17β-EQN favor a more planar ring B conformation, which also affects ring C conformation and ring A acidity.

plausible explanation for those conflicting results but also reflect the complexity of estrogen signaling. The fact that the same estrogenic molecule acts differently depending on the vascular tissue studied indicates that a simple binding affinity and ER activation do not determine the capacity of estrogen molecules to modulate gene transcription. In fact, evidence accumulated over the past decade has demonstrated that ER-mediated responses will vary, based on the nature of the estrogenic compound and a number of conditions, including the following: (1) the specific ER-bound conformational change; (2) the tissue-dependent ER subtype expression profile; and (3) the composition of coregulator molecules in a given cell.[1] A recent study of the structure-activity relationship of various estrogens showed that all of the equine estrogens present in the CEE mixture have a markedly lower transcriptional activity on specific DNA sequences, called estrogen response elements, although some of them exhibit an affinity to ER subtypes that approximate or even exceed E_2 activity.[20]

Furthermore, the effects of estrogens may also be influenced by pathophysiological conditions, such as aging and the presence of pre-existing cardiovascular disease.[21,22] Detailed examination of the data from the Women's Health Initiative indicates that early initiation of estrogen replacement produces more favorable results than the late average time of initiation used in the Women's Health Initiative studies overall.[23–25] These observations, together with observational studies, have led scientists to create the so-called "timing hypothesis." This theory states that estrogen-mediated benefits to prevent cardiovascular disease may occur only when treatment is initiated before the detrimental effects of aging or cardiovascular disease are established in the vasculature.[22] Therefore, aging of a giving organism should always be taken into account when the pharmacological and physiological responses by estrogens are determined. In this regard, to better characterize the physiological relevance of this study, measurements of NO production, eNOS activity, and eNOS gene expression were carried out in cultured HAECs obtained from 3 donors with characteristics approximately similar to patients taking ERT, that is, women 50 to 55 years old. In those cells, among all of the estrogens found in the CEE mixture, E_1 was the only one observed to resemble E_2 in its potential to enhance eNOS transcription and, therefore, to increase NO.

In general, estrogen effects are mediated by both subtypes of ER (ER-α and ER-β), members of the nuclear receptor superfamily that are encoded by 2 distinct genes (ESR1 and ESR2). Because both ER subtypes (ER-α and ER-β) are transcription factors, on agonist binding they are able to bind with high affinity to specific estrogen response elements in the promoter region of target genes and thereby transactivate gene expression.[26–28] By this mechanism, estrogen is thought to modulate expression of molecules that are crucial to the control of cardiovascular pathophysiology, including eNOS. Studies using transient transfection assays with eNOS promoter fragments have demonstrated the ability of E_2 to upregulate eNOS promoter activity in different cell lines.[10,29] ER-mediated induction of eNOS by E_2 has been demonstrated in a variety of vascular beds and is consistent with E_2-induced upregulation of eNOS promoter activity,[10] although the estrogen response element site that induces eNOS transcription is still unclear. Although the importance of ER in the control of eNOS transcription has been well described, the specific role of other estrogens' interaction with ER-α and ER-β in gene transcription and, more specifically, in the mechanisms that lead to eNOS expression, is poorly under-

stood. To address this issue, we measured the ability of naturally occurring and equine estrogens to modulate eNOS promoter activity on COS-7 cells transiently cotransfected with plasmid to express ER-α or ER-β and with reporter gene plasmids containing the DNA fragments of the eNOS promoter region. COS-7 cells were chosen because they do not constitutively express ERs and are not estrogen responsive[30] and also because previous studies showing the effects of ER-mediated upregulation of the eNOS promoter have been done in this cell line.[10]

The data from our studies on promoter activity confirm that ER-α is the estrogen mainly responsible for increasing eNOS transcription. After treatments with naturally occurring estrogens E_1 and E_2, eNOS promoter activity was $\approx$2 to 3 times higher than that induced by ER-β. Corroborating our data, studies on ER-α and ER-β knockout mice have established the role of ER-α in regulating endothelial NO synthesis. Basal levels of endothelial-derived NO have been found to be significantly lower in the vasculature of ER-α knockout mice than in wild-type animals. On the other hand, ER-β knockout mice had normal NO production,[31] suggesting that ER-α is the main ER subtype responsible for vascular protection by estrogen. Moreover, our research is the first to demonstrate that, although equine estrogens do promote eNOS transcription, these effects are significantly lower than those observed with natural estrogens (Figure 3B).

The analysis of our functional results, in combination with the molecular structure findings of the estrogen studies, drew our attention to the fact that molecules with a high degree of structural similarity promote very different effects on eNOS transcription. Although a number of studies have investigated the structural requirements for diverse compounds to bind with ERs,[20,32] little is known about how these structural changes alter ER-α response to transcription. X-ray crystal structure analysis of various estrogens studied indicates that estrogenic compounds with a β-hydroxyl group at position 17 of the molecule maintain a more stable hydrogen network that may favor transcriptional activity compared with estrogenic compounds that express a less favorable estrogen (E_1) or have a disrupted interaction at this site (17α-estrogens). Nevertheless, as discussed, the differences in chemical moiety/stereochemistry at position 17 cannot account for the modulation of transcription by ER-α: 17β-Eq or 17β-Eqn with a β-hydroxyl group in position 17 (identical to E_2) are transcriptionally much less active than E_2. Saturation on ring B is the major difference between E_2 and the 17β-equine estrogens, suggesting that ring B properties are also important determinants for ER-α transcriptional activity. As proposed by Hsieh et al,[33] changes in the degree of ring B saturation of estrogenic molecules could primarily affect the degree of flexibility and hydrophobicity of the ligands. It is plausible that a more flexible estrogenic molecule is able to adopt a better ligand-receptor fit than a more rigid steroid scaffold. On the other hand, a higher hydrophobic estrogen allows more effective interaction with the hydrophobic residues in the ER-α ligand–binding pocket. The specific correlation of structural modifications and transcriptional activity is more fully discussed in the online Data Supplement.

Taken together, our studies suggest that equine estrogens increase NO production less effectively than naturally occurring estrogens, as a result of their lesser ability to activate the ER-α–mediated increase of eNOS promoter activity and eNOS transcription. Chemical moiety and stereochemistry at position 17 and the degree of ring B saturation in estrogenic compounds play a significant role in ER-α transcriptional activity. However, further studies are needed to determine whether the lower eNOS transcriptional activity of the various components of CEEs played a role in the negative findings from the estrogen-alone arm of the Women's Health Initiative Study or, even more specifically, if the combination of equine estrogens with E_1 would affect NO production.

Perspectives

Cardiovascular disease is the leading cause of death for women in Western countries. Therefore, cardiovascular disease is a high priority for public health policy and an overarching women's health problem with substantial impact on morbidity, as well as mortality, especially in postmenopausal women. The data from our study aim to elucidate the molecular mechanisms underlying the differential modulation of distinct estrogen molecules that regulate vascular function. Such studies will contribute to our understanding of the controversy surrounding hormone replacement therapy and cardiovascular protection. Ultimately, an understanding of the differences between naturally occurring human estrogens and the CEEs most often used as female hormone replacement may lead to the development of improved pharmacological therapies for postmenopausal women. because women now spend, on average, one third of their lives in menopause and the incidence of Cardiovascular disease is rapidly increasing, results obtained from these studies will have wide-ranging potential impact.

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Disclosures

None.

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ONLINE SUPPLEMENT

EQUINE ESTROGENS IMPAIR NO PRODUCTION AND eNOS TRANSCRIPTION IN HUMAN ENDOTHELIAL CELLS COMPARED TO THE NATURAL 17β-ESTRADIOL.

Short Title: Equine estrogens impair NO production.

Laura Novensa[1], Jana Selent[2], Manuel Pastor[2], Kathryn Sandberg[3], Magda Heras[1], Ana Paula Dantas[1]

[1]Experimental Cardiology, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain; [2]Computer-Assisted Drug Design Laboratory, Research Unit on Biomedical Informatics (GRIB), IMIM - University Pompeu Fabra, Barcelona, Spain; [3]Center for the Study of Sex Differences in Health, Aging and Disease, Georgetown University.

Address for correspondence:
Ana Paula Dantas, PhD
Experimental Cardiology Division
Institut d'Investigacions Biomèdiques
August Pi i Sunyer (IDIBAPS)
C/ Casanova 143 Lab. IDIBAPS 404
08036 – Barcelona
Tel: +34 93 451 69 05
Fax:+34 93 451 41 48
adantas@clinic.ub.es

Methods

1. Cell Culture and Treatments

Human aortic endothelial cells (**HAEC**) from 3 women aged 50-55 were obtained from the American Type Culture Collection (**ATCC**) and Clonetics (Lonza), and maintained in culture in phenol red-free DMEM-F12 supplemented with 10% (v/v) fetal bovine serum (**FBS**) and endothelial growth factors (Lonza). COS-7 cells were obtained from ATCC and maintained in phenol red-free DMEM-F12 with 10% FBS and without endothelial growth factors. Cells were plated onto 0.2% gelatin-coated culture dishes and studied before cell confluence between passages 5 and 7. Culture medium was changed to charcoal-striped low-serum (1%) media, and incubation proceeded overnight. Both HAEC and COS-7 cells were then treated for 24h with increasing dose (10nM to 1µM) of the following estrogens (Steraloids, Newport RI): estrone (E1), 17α-estradiol (17α-E2), equilin (Eq), 17β-dihydroequilin (17β-Eq), 17α-dihydroequilin (17α-Eq), equilenin (Eqn), 17β-dihydroequilenin (17β-Eqn) and 17α-dihydroequilenin (17α-Eqn) **(Figure S1)**. All these estrogens in sulfate-conjugated form are components of the conjugated equine estrogens (Premarin, Wyeth-Ayerst) commonly used by postmenopausal women for hormone replacement therapy. The effects of these equine estrogens on NO production were compared to the effects of the major estrogen naturally produced by women, 17β-estradiol (E2) (Steraloids). Estrogens were solubilized in ethanol and stored at -20°C; the same volume of ethanol was used as vehicle control and the final concentration of ethanol did not exceed 0.1% (v/v).

2. Plasmid construction and transfection

The eNOS-luciferase (eNOS-Luc) plasmid was generously provided by Dr Santiago Lamas (Centro de Investigaciones Biológicas, CSIC, Madrid, Spain). A fragment corresponding to the 5'-flanking regulatory region of the human eNOS gene at positions from -1910 to +48 was obtained by PCR, using a human genomic clone (AF032908) as template and the following primers: (upper 5'-GGGGTACCTCACCTGAGGCTAGGAGT-3' and lower, 5'-CCGGTACCTGGGCCACGCTCTTCAAG-3'). PCR product was cloned into the *Kpn*I site of the promoterless luciferase reporter vector pGL3-Basic and sequenced to confirm correct orientation. Human full-length ERα (Ultimate ORF IOH34665) and ERβ (Ultimate ORF IOH10714) were purchased from Invitrogen. The ERα and ERβ open reading frames were cloned into the entry vector pENTR221 and subcloned into a pcDNA3.2/V5-DEST for high expression of ERs in transfected cells, using the LR recombination reaction according to the manufacturer's instructions (Invitrogen). The recombinant vectors pcDNA3.2/V5-ERα or pcDNA3.2/V5-ERβ or the empty (control) vector pcDNA6.2/V5 were cotransfected with eNOS-Luc and β-gal vectors directly into COS-7 cells using FuGENE 6 reagent, following the supplier's instructions.

3. Nitric Oxide Generation in HAEC

Because of its short half-life, current opinion is that assessment of NO generation should include more than one detection method. We used two approaches to determine estrogen-induced NO production in human aortic endothelial cells (HAEC): 1) intracellular nitrosation of NO-sensitive fluorochrome DAF2-DA; and 2) concentration of NO metabolites (NO_2/NO_3) in cell culture media. Briefly, preconfluent HAEC grown in 6-well cell culture plates were loaded with DAF-2 DA (final concentration 5 µM, 30 min, 37°C), rinsed three times with culture media, and kept in the dark at 37°C with a warming stage on an inverted microscope (Axiovert 2000, Carl Zeiss Inc). DAF2-DA fluorescence was assessed basally at excitation/emission wavelength of 495/515 and after challenging with Carbachol (1µM) to increase NO production, with readings taken 5 min after exposure to the agonist. Images were recorded by Axiovision 4.6 software (Zeiss Imaging) and increase of green fluorescence intensity by the distinct estrogens was measured with Image J (National Institutes of Health). Basal fluorescence was subtracted from each group and data was expressed as a fold increase following carbachol stimulation. The levels of NO metabolites (NO_2/NO_3) after carbachol stimulation were determined in the cultured media from HAEC by a commercial fluorimetric assay (Calbiochem) following the supplier's instructions.

4. eNOS activity

Measurements were performed in HAEC grown in 60-mm dishes as previously described[1]. Following estrogen treatments, HAEC was lysed and protein concentration determined with BCA protein assay kit (Pierce). For each sample, an equal amount of protein (20 µg) was incubated (37°C for 60 minutes) with assay mixture containing Tris-HCl 50 mmol/L, tetrahydrobiopterin 6 µmol/L, FAD 2 µmol/L, FMN 2 µmol/L+NADPH 10 mmol/L, L-arginine/L-[^{3}H]arginine 100 mmol/L (5 µCi/mL), $CaCl_2$ 6 mmol/L, and calmodulin 0.1 µmol/L. The reaction was stopped with 400 µL of ice-cold stop buffer (HEPES 50 mmol/L, EDTA 5 mmol/L; pH 5.5), which chelates the calcium required by eNOS and, consequently, inactivates eNOS. Cation-exchange resin (500 µL) (Dowex, Na^+ form, equilibrated with HEPES 50 mmol/L; pH 5.5) was added to each reaction mixture to remove the excess L-arginine. The aliquots were placed in spin cups, centrifuged for 1min at 12 000g, and flow-through collected in scintillation vials containing 4mL of scintillation liquid. Radioactivity was quantified in a beta counter during 1 min. and data was expressed as fMol per mg of protein per min.

5. eNOS mRNA expression by Real-time PCR

Total cellular RNA was isolated from cell cultures with RNeasy minicolumn kit (Qiagen), as described[2]. Total RNA from each preparation was reverse transcribed in the presence of MuLV reverse transcriptase, oligo(dT) and random hexamers (Applied Biosystems) according to manufacturer recommendations. The amount of cDNA for eNOS was quantified on an ABI 7500 Real-Time PCR System using the TaqMan methodology (Applied Biosystems) and eNOS-specific primers and probe were inventoried with FAM- H00167166 (Applied Biosystems).

As endogenous control for RNA input and reverse transcription efficiency, 18S ribosomal RNA was quantified and multiplexed in each RNA sample using inventoried TaqMan ribosomal control reagents (VIC - No.4310875, Applied Biosystems). Measurements were performed in triplicate on 2 µl of cDNA in 25-µl volume Taqman universal master mix (Applied Biosystems), with the following cycling parameters: 95C for 10 min, followed by 40 cycles of 95C for 15 s and 60 C for 1 min. Number of eNOS transcript copies was established by comparison to a calibration curve based on known amounts of cDNA from untreated HAEC and expressed as copy number per µg of total RNA normalized by the ratio of eNOS mRNA to 18S rRNA[3].

6. Reporter gene for ER transcriptional activity

To investigate ERα and ERβ transactivation activity on eNOS promoter, COS-7 cells were cotransfected with 1 µg of reporter gene plasmid eNOS-Luc, 0.5 µg of either pcDNA3.2/V5-ERα or pcDNA3.2/V5-ERβ constructs, and 10 ng of β-galactosidase (β-gal) plasmid (Promega) as control for transfection. At 48h, transfected cells were washed with PBS and lysed with luciferase assay system lysis buffer; luciferase activity was measured in cellular extracts using the Promega luciferase assay system. Luciferase activity was determined as relative light units and was normalized to the corresponding β-gal activity, which was measured by the β-gal assay system (Promega). Transfection activity of estrogens was expressed as fold increase relative to the signal obtained from cell treated with vehicle.

7. Molecular modeling analysis

This study used the X-ray Crystal Structure of ERα in complex with E2 (PDB access 1GWR). The structures of the ERα in complex with E1, 17α-E2, 17β-EQ, and 17β-EQN, for which no crystallographic structure is available, were obtained using docking simulations of these ligands into the ligand-free ERα of 1GRW. The results obtained were subjected to 200 ps molecular dynamics (MD) simulation (force field MMF94x, 300 K, time step 2 fs) and subsequent energy minimization by applying gradient minimization until the RMS gradient was lower than 0.001 kcal/molÅ (Software MOE). The diverse tautomeric states of Histone-524 (His524T: protonation of N3, His524П: protonation of N1) were generated and studied using the MOE “rotamer/tautomer explorer.”

8. Statistical Analysis

Results of one-way analysis of variance (ANOVA), followed by the Tukey test for multiple comparisons, are shown as mean ± SEM. Values were considered statistically significant at $p<0.05$.

Computational analysis of estrogen molecules

Two compounds in complex with ER have been reported as partially explaining the observed results of the modulation of ERα and DNA transcription: 17β-estradiol[4] and the CEE analogue, 17-β-methyl-17α-hydroxy-dihydroequilenin[5]. According to our experimental data, the most potent estrogen to increase NO production is E2, showing a steroidal structure with an aromatic ring A, unsaturated rings B, C, and D, and a β-hydroxyl group in position 17 **(Figure 1)**. All other estrogens studied are structurally similar to E2, differing only in (i) chemical moiety and stereochemistry at position 17 and (ii) ring B saturation **(Figure 1)**. Remarkably, these apparently small structural differences may be responsible for significant differences in NO production.

In order to understand the influence of position 17, we analysed the structures of ERα in complex with E2 (X-ray crystal structure, PDB ID 1GRW) and E1, 17α-E2 (modeled complexes). The X-ray crystal structure of the ERα in complex with E2 (PDB ID 1GWR) shows the key site of estrogen compound interactions: (i) hydrogen network of the phenolic ring A with Glutamic Acid (Glu) 353, Arginine (Arg) 394 and one molecule of water; (ii) hydrophobic stabilization of the steroid scaffold by Leucine (Leu) 384; Leu525, Phenylalanine (Phe) 404 and Methionine (Met) 421 and (iii) a hydrogen bridge from the 17-β-hydroxyl of ring D with the Histidine (His) 524^{T} tautomer **(Figure 5A in manuscript)**. Moreover, these series suggest that estrogenic compounds with the properties to interact with $His524^{T}$ and maintain the stabilizing $His524^{T}$-Glu419 interaction possess higher transcriptional activity than estrogenic compounds that express a less favourable or disrupted interaction at position 17. Compounds such as E1 with $His524^{\Pi}$ interaction but without the stabilizing $His524^{T}$-Glu419 interaction exhibit slightly lower transcriptional activity; however, this decrease is markedly lower in estrogenic compounds such as 17α-E2 with disrupted $His524^{T/\Pi}$ interaction but with a stabilizing $His524^{T}$-Glu419 interaction. The hydrogen bond of E1 with the $His524^{\Pi}$ tautomer can be expected to be energetically less favorable than that between E2 and the $His524^{T}$ tautomer, since the bond between E1 and $His524^{\Pi}$ requires breaking the stable $His524^{T}$-Glu419 interaction, which may result in slightly lower ERα activity. In the complex ERα with 17α-E2, the angle of the α-hydroxyl group at position 17 does not allow hydrogen to bond with His524 in the T- or Π-tautomeric form, thus resulting in a complete loss of hydrogen bond with the His524. This unfavorable α-hydroxyl orientation is mainly produced by the bulky residues Leu384 and Leu525, which prevent the ligand from shifting its position slightly upwards. The significance of His524 has also been shown by alanine mutation of His524, indicating its high importance in estrogens compared to antiestrogens (e.g. 4-OH tamoxifen) for ligand recognition[6].

Moreover, our studies suggest that a higher degree of ring B saturation can enhance the flexibility and hydrophobic properties of estrogen, which in turn increases transcriptional activity of ERα (i.e., transcripitional activity of E2 (one double bond) > 17β-Eq (two double bonds) > 17β-Eqn (three double bonds). Unfortunately, this association is not so straightforward. Despite the existing conformational differences between E2, 17β-Eq and 17β-Eqn, our modeling

studies reveal that all three compounds establish the same key interaction with ERα.

Furthermore, we observed that the degree of ring B saturation is likely to alter physiochemical properties such as the acidity of the phenolic group in ring A. Calculation of this pKa value for E2, 17β-Eq and 17β-Eqn demonstrates that increasing saturation at ring B increases estrogen acidity. To understand the impact of acidity on anionic ring A, we modeled ERα in complex with the deprotonated form of the more acidic form of 17β-Eqn estrogen. The obtained model indicates that anionic ring A results in a loss of the hydrogen bond with Glu353. A short 200 ps MD simulation of repulsive forces demonstrates the tendency of deprotonated 17β-Eqn to move more than 1 Å from the negatively charged Glu353 side chain, with an O-O distance increase from 2.47 Å **(Figure 5A, manuscript)** to 3.80 Å **(Figure 5B, manuscript)**. In this regard, we propose that the binding posture of a more acidic estrogen induces ERα conformation with low transcriptional activity on eNOS and is most likely responsible for the experimentally observed decrease in NO production relative to the neutral E2.

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Figure S1 – Chemical structures of the set of estrogens used in our studies. The estrogens differ structurally in chemical moiety/stereochemistry of position 17 and the saturation of ring B.