Estrogen signaling and cardiovascular protection: what can we learn after the Women's Health Initiative?

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*Coronary heart disease, heart failure, stroke, or intermittent claudication.
WHY ARE WOMEN PROTECTED?

NEXT AT HARVARD...

WHY DOES ASPIRIN PREVENT HEART ATTACKS IN MEN BUT NOT WOMEN?

BECAUSE WOMEN DON'T LIKE SCIENCE?
The X chromosome mosaicism theory

According to the heterogametic sex hypothesis, the lack of a second X chromosome in male may lead to lower cardiovascular protection.
The hormonal theory

Estrogen protects females mammals at a cellular level, so that the incidence and severity of cardiovascular disease in females – whether they are rats, dogs or humans – will generally be lower than in males.
The Nurses’ Health Study

Risk Factors for Cerebral Hemorrhage in the<br>Controlled Hypertension<br>Amanda G. Thrift, PhD, John J. McNeil, PhD; Andrew<br>Geoffrey A. Donnan Group<br>the Department of Epidemiology and Biostatistics<br>Hospital (A.G.T., J.J.M., A.M., S.B.) and<br>Repatriation Hospitals, Waverley, Australia<br><br>Journal of Women's Health<br>The Nurses' Health Study: 20-Year Contribution to the<br>Understanding of Health Among Women

Annals of Internal Medicine<br>Postmenopausal Hormone Use and Coronary Heart Disease Events in the Nurses’ Health Study
A Prospective, Observational Study

American Family Physician<br>What's Different for Coronary Artery Disease in Women?
Menopause Is Associated With Endothelial Dysfunction in Women

Taddei et al, Hypertension. 1996;28:576
Ovariectomy also induces hyposensitivity to endothelium-dependent vasodilator ACh in women. Estrogen replacement therapy restored the hyposensitivity to ACh.

Virdis et al, Circulation. 2000;101:1158
SITES OF ESTROGEN ACTIONS

ERα expression in mice artery

Cardiovascular
Estradiol corrects endothelium-dependent relaxation to Ach and Bk in OVX- SHR.
eNOS activity and gene expression is decreased by ovariectomy in SHR. Estradiol treatment restores eNOS activity/expression

* p<0.05 vs OE
# p<0.05 vs OVX

Superoxide generation in SHR arterioles in vivo in situ

Estradiol

- Relaxing Factors ↑
- Constricting Factors ↓
- Anti-Oxidant ↑
- Cholesterol ↓
- RASS ↓
- Antimitogenic Effects ↑

KIDNEY
- Glomerular Remodeling
- Glomerulosclerosis
- Renal Arteriolar Remodeling

BLOOD VESSEL
- Vascular Remodeling
- Vasoconstriction
- SMC Growth
- ECM Deposition
- Endothelial Damage

HEART
- Cardiac Remodeling
- CF/MC Growth
- ECM Deposition

Cardiovascular Disease
Risks and Benefits of Estrogen Plus Progestin in Healthy Postmenopausal Women

Principal Results From the Women’s Health Initiative Randomized Controlled Trial

Writing Group for the Women’s Health Initiative Investigators

JAMA, July 17, 2002 – Vol. 288, No. 3
Women’s Health Initiative (WHI)

Coronary Heart Disease

- HR, 1.29
- 95% nCl, 1.02-1.63
- 95% aCl, 0.85-1.97

Stroke

- HR, 1.41
- 95% nCl, 1.07-1.85
- 95% aCl, 0.86-2.31
Estrogen Replacement Therapy:
before and after the Women’s Health Initiative (WHI)

Before WHI

Estradiol

Relaxing Factors ↑
Constricting Factors ↓
Anti-Oxidant ↑
Cholesterol ↓
RASS ↓
Antimitogenic Effects ↑

↓ Cardiovascular Disease

After WHI

Estradiol

Venous thromboembolism ↑
Stroke ↑
Coronary heart disease ↑
Cholesterol Ø

↑ Cardiovascular Disease
Risks and Benefits of Estrogen Plus Progestin in Healthy Postmenopausal Women

Principal Results From the Women’s Health Initiative Randomized Controlled Trial

Writing Group for the Women’s Health Initiative Investigators

JAMA, July 17, 2002 – Vol. 288, No. 3
WHI?

- Dose regimen
- Association of estrogen with progestins
- Administration route
- Type of Estrogen
- Average age of women beginning the trial
Type of Estrogen

“I’m in an experimental program that treats menopause with ostrich hormones. Now I only get hot flashes when I’m laying an egg.”
Conjugated Equine Estrogens
PREMARIN®

Concentration of estrogen found in Premarin®
(http://www.fda.gov/cder/news/celetterjw.htm) and corrected by average of BMI described by the clinical trials HERS, ERA and WHI.

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<th>Sodium Estrogen Sulfate</th>
<th>[Estrogen] (mg/tablet of Premarin® 0.625mg)</th>
<th>Estrogen (mg)/BMI (kg/m²)</th>
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<tr>
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<tr>
<td>17α-Estradiol</td>
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<td>9.0 x 10⁻⁴</td>
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<tr>
<td>17α-Dihydroequilin</td>
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<td>Equilenin</td>
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<td>5.2 x 10⁻⁴</td>
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<tr>
<td>Δ8,9-dehydroestrone</td>
<td>0.026</td>
<td>9.0 x 10⁻⁴</td>
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Different estrogenic molecules do not activate transcription of a given gene in the same pharmacological way as 17β-Estradiol.

The differential modulation of gene transcription is mostly dependent on how conformational changes of ER allow its binding to DNA and the recruitment of different coregulators molecules rather than ligand binding affinity.
Estrogen-mediated NO production

Estrogen effects on eNOS transcription

Normalized copies of eNOS mRNA/µg RNA

Luciferase Activity (Fold Increase)

Luciferase Reporter Vector (pTL-Luc) 4.8 kb

Amplication Plot

Normalized copies of eNOS mRNA/µg RNA

Luciferase Activity (Fold Increase)
Estrogen Receptor Signaling

Ligand → Nuclear Membrane → ERα/β → Dimerisation and Conformational Change → Transcription Modulation

- TGACCCnnnGGTCA -

Coactivators

AP1; SP1; NF-kB

Promoter region of target genes
ER Dimerization – FRET Analysis

**ERα/ERα**

**ERβ/ERβ**

---

**ERα/ERα**

**ERβ/ERβ**

---

**FRET Ratio**

- DMSO
- E2
- E1
- Eq
- 17β-Eq
- Eql
- 17β-Eq

---

**Nessun segnale FRET**

- CFP
- YFP

**Segnale FRET**

- CFP
- YFP

---

**FRET**

- 436 nm
- 480 nm
- 535 nm

---

**ER**

- α
- β

---

**480 nm**
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<td>+1</td>
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ChIP Assay for E-ER/DNA Biding

Estrogen (100nM)

Time (min)

5  15  30  45

Protein/DNA Crosslink

Sonication

I.P

Reverse Crosslink

qPCR
ChiP Analysis for ERα/DNA Interaction

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<th>15</th>
<th>30</th>
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<td>E1 (100nM)</td>
<td>![Image]</td>
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<td>Eq (100nM)</td>
<td>![Image]</td>
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<td>17β-Eq (100nM)</td>
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Fluorescence Anisotropy Analysis of ERα/β and SP1 Site Interaction

ERα

ERβ

Hormone (nM)

Anisotropy Change (mA)

Hormone (nM)

Anisotropy Change (mA)
• Molecular Modelling studies

Small structural differences are responsible for significant changes in NO production:

(i) the chemical moiety and stereochemistry at the position 17

(ii) the saturation degree of ring B
Saturation of ring B

17-β-estradiol (E2) > 17-β-equilenin (17β-Eq) > 17-β-equilenin (17β-Eq)

Flexibility

pKa 10.54

17β-EQ

pKa 10.41

17β-EQN

pKa 9.91

Glu353

Arg394

Phe404

Leu525

His524

O-O distance 2.47 Å

Mol421

Glu419
Molecules designed to express tissue-specific agonist and antagonist activities.

SERMs
(Selective ER Modulators)

Raloxifene

Tamoxifen
SERM-induced effects on endothelium-dependent relaxation by ACh and Bk in microvessels of OVX-SHR.
Nitric Oxide Production by SERMs

- Estradiol
- Raloxifene
- Tamoxifen

**Graph:**
- DAF-2 Fluorescence (Fold Increase) vs. (nM)
- Estradiol
- Raloxifene
- Tamoxifen

- p<0.05
- p<0.001
RUTH Trial
(Raloxifene Use for the Heart)

Coronary Events

Cumulative incidence per 1,000 women

Years

MERCED

(MEnopausia y Raloxifeno en la Cardiopatía isquémica: Efecto en la Disfunción endotelial)
### Average age of women beginning the trial

<table>
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<th>HRT</th>
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<td>HERS</td>
<td>CEEs</td>
<td>~ 67</td>
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<tr>
<td>WHI</td>
<td>CEEs</td>
<td>~ 64</td>
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<td>RUTH</td>
<td>Ralox</td>
<td>~ 67</td>
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<tr>
<td>MERCED</td>
<td>Ralox</td>
<td>~ 60</td>
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*Illustration with a cartoon: 'You’re deliberately putting yourself at risk of ill health by being over 65...’*
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.

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**THE “TIMING” HYPOTHESIS**

![Graph showing risk ratio for CVD vs. years postmenopause at randomization](image)

- Risk Ratio for CVD: $\text{HR}=0.62$
- $r = 0.994$
- $p = 0.071$

Effect of raloxifene on the incidence of the primary coronary end point (coronary death, nonfatal MI, or hospitalized ACS other than MI, whichever occurred first) by age.
Aging-associated effects on E2-mediated production of NO

Young

Aged

Aging-associated effects on E2 modulation of eNOS expression

A swap from anti-oxidant to pro-oxidant effect by E2 is observed in aged females

Anti-inflammatory effects of Estrogen: a matter of timing

→ Uterine arteries obtained from 68 women (age 41-86) at the moment of hysterectomy were cleaned, divided into three segments and cultured for 24h in tissue culture media containing 17beta-estradiol (100nM), Raloxifen (100nM) or vehicle.

→ Exclusion criterion: use of hormone replacement therapy, SERMs (Raloxifen, Tamoxifen...), chronic anti-inflammatory therapy, satins, RAS inhibitors, diabetes.

→ Multiplex, immunobead-based assay, was performed to measured 13 cardiovascular-related inflammatory biomarkers.

CVD1: MMP-9; sE-Selectin; s-ECAM; s-VCAM; t-PAI

CVD3: IFNγ; IL-10; IL-1b; IL-6; IL-8; MCP-1; TNFα; VEGF
## Pearson’s Correlation Coefficients

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<th></th>
<th>Untreated</th>
<th></th>
<th></th>
<th>Estrogen</th>
<th></th>
<th></th>
<th>Raloxifene</th>
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<td>$r^2$</td>
<td>P value</td>
<td>Summary</td>
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<td>$r^2$</td>
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<td>E-Selectin</td>
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<td>0.519</td>
<td>&lt;0.0001</td>
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<td>0.7274</td>
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(a): Analysis of Covariance (ANCOVA) reveals significant difference ($p < 0.05$) in comparison to untreated group.
Aging can be associated to a switch from a beneficial anti-inflammatory action by estrogen, at earlier stages of menopause, to a pro-inflammatory profile after 5 year past its onset.
Aging can be associated to a switch from a beneficial anti-inflammatory action by estrogen, at earlier stages of menopause, to a pro-inflammatory profile after 5 year past its onset.
Conclusions

 ✓ The complex regulation of cellular responses to estrogen ligands are specific to the ligand and dependent not only on the relative levels of ER in a given tissue, but also on how ligand-induced conformational changes in ERs leading to differential modulation of transcription depending upon the cell type.

 ✓ Furthermore, cardiovascular responses to estrogens can vary based on different physiological and pathophysiological situations, such as during aging, in part, due to alterations in the tissue methylation status of key regulators of cardiovascular function.
Conclusions

Gaining a detailed understanding of the cell- and tissue-specific signaling pathways induced by various ER ligands and the subsequent effects on gene regulation under physiological and pathophysiological circumstances, may ultimately lead to the development of new therapeutics for the treatment of cardiovascular disease in both men and women.
KEEPS
(Kronos Early Estrogen Prevention Study)

✓ Rationale: “earlier intervention than that performed in the WHI and HERS trials will provide cardiovascular benefit to women”
✓ Multicenter, randomized, double-blind, placebo-controlled 5-year clinical trial.
✓ Will evaluate the effectiveness of conjugated equine estrogens or transdermal 17β-estradiol, and placebo in preventing progression of cardiovascular disease in women aged 42-58 years who are within 36 months of their final menstrual period.
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Zuleica B Fortes, PhD
Graziela Ceravolo, PhD

Georgetown University Medical Center

Kathryn Sandberg, PhD

Carlos Hermenegildo, MD PhD
Susana Novella, PhD
Pascual Medina, PhD
Gloria Segarra, PhD

University of Wisconsin

Erin Séanle, PhD
Wei Xu, MD

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