Effects of Estradiol and Its Metabolites on Glomerular Endothelial Nitric Oxide Synthesis and Mesangial Cell Growth

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Abstract—Reduced nitric oxide synthesis by glomerular endothelial cells and increased proliferation of glomerular mesangial cells is associated with glomerular remodeling that leads to accelerated glomerulosclerosis. Estradiol induces nitric oxide synthesis and slows the progression of renal disease. Because the estradiol metabolites 2-hydroxyestradiol and 2-methoxyestradiol are more potent than estradiol in inhibiting growth of vascular smooth muscle cells, which are phenotypically similar to mesangial cells, we compared the effects of estradiol, 2-hydroxyestradiol, and 2-methoxyestradiol on growth of glomerular mesangial cells and on basal nitric oxide synthesis by glomerular endothelial cells. In human glomerular mesangial cells, estradiol and its metabolites concentration-dependently (1 nmol/L to 10 μmol/L) inhibited serum (2.5%)-induced DNA synthesis, cell proliferation, and collagen synthesis with the order of potency being 2-methoxyestradiol > 2-hydroxyestradiol > estradiol. ICI182780 (100 μmol/L, an estrogen receptor antagonist) blocked the growth inhibitory effects of estradiol but not 2-hydroxyestradiol or 2-methoxyestradiol. Treatment with estradiol, but not 2-hydroxyestradiol and 2-methoxyestradiol, induced nitric oxide synthesis (P<0.05, assayed by the formation of [1H-3H]-citrulline from [1H-3H]-arginine) in human glomerular endothelial cells, and these effects were blocked by ICI182780 and L-NMA (a nitric oxide synthesis inhibitor). In conclusion, estradiol may attenuate glomerulosclerosis by inducing nitric oxide synthesis via an estrogen receptor–dependent mechanism and by conversion to 2-hydroxyestradiol and 2-methoxyestradiol, which inhibit glomerular mesangial cell proliferation independent of estrogen receptors. (Hypertension. 2001;37[part 2]:645-650.)

Key Words: 2-hydroxyestradiol ■ nitric oxide ■ 2-methoxyestradiol ■ glomerulosclerosis ■ renal disease, end-stage ■ postmenopause

Estradiol may induce protective effects on the kidney. Compared with age-matched men, the rate of progression of renal disease in premenopausal women is decreased.1,2 However, with the onset of menopause and decreased synthesis of 17β-estradiol (estradiol), the progression of renal diseases accelerates, and estradiol replacement therapy slows this process.3–5 Although estradiol induces protective effects on the kidney, the mechanisms via which it induces its effects remain undefined. Estradiol is known to protect the vasculature by inhibiting processes that initiate or mediate vascular remodeling associated with neointima formation and the vaso-occlusive process.1 In this regard, estradiol stimulates endothelial cell–derived nitric oxide (NO) synthesis and inhibits vascular smooth muscle cell growth. Analogous to the vasculature, decreased NO synthesis by damaged and dysfunctional glomerular endothelial cells (GECs)4 and abnormal growth of glomerular mesangial cells (GMCs), a cell phenotype typically similar to smooth muscle cells, is associated with the pathogenesis of renal diseases, such as glomerulosclerosis.4,5 It is feasible that, similar to the effects on the vasculature, estradiol may protect against glomerulosclerosis and reduce the rate of progression of renal disease by stimulating NO synthesis by GECs and inhibiting growth of GMCs.

In the present study, we investigated whether estradiol stimulates NO synthesis in human GECs and inhibits growth of GMCs. Although some of the biological effects of estradiol are estrogen receptor (ER) mediated,6 recent findings suggest that ER-independent effects may also play a role in mediating the protective effects of estradiol.7,8 Because 2-hydroxyestradiol and 2-methoxyestradiol, major endogenous metabolites of estradiol with no affinity for ERs, possess biological activity and are more potent than estradiol in inhibiting vascular smooth muscle cell growth,10 we also investigated the effects of estradiol metabolites on NO synthesis by human GECs and on growth of GMCs. Moreover, using ICI182780, an ER antagonist,1 we...
investigated the role of ERs in mediating the effects of estradiol and its metabolites. Because estradiol is used in combination with progesterin, we also evaluated how the effects of estradiol on NO synthesis and GMC growth are influenced by natural and synthetic progestins.

Methods

Endothelial and Mesangial Cell Culture

Human GECs and GMCs were obtained from Cell System Corporation (Kirkland, Wash). Cells were cultured in multiwell plates under standard-cell culture conditions. GECs (passage 4 to 7) were maintained in endothelium growth medium (EGM), and GMCs (passage 3) were grown in phenol red-free DMEM/F12 medium supplemented with 10% FCS (steroid free) and antibiotics.

Determination of Nitric Oxide Synthase Activity

Confluent monolayers of GECs were fed EGM supplemented with charcoal-stripped FCS (steroid free) that contained or lacked the following treatments and were dissolved in sterile DMSO or H2O: estradiol, 2-hydroxyestradiol, 2-methoxyestradiol, estradiol plus ICI182780, 2-hydroxyestradiol plus ICI182780, 2-methoxyestradiol plus ICI182780, estradiol plus L-NMA, estradiol plus EDTA, and estradiol plus progesterone or medroxyprogesterone. The controls were treated with an equal volume of vehicle (DMSO, 0.1% vol/vol).

After 1 hour or 24 hours, NOS activity was determined by measuring the conversion of L-[3H]arginine to L-[3H]citrulline according to the method of Davda et al.11 with minor modifications. Briefly, after 1 or 24 hours of treatment, the medium was removed and cells were incubated with 0.5 mL Krebs-HEPES buffer that contained (in mmol/L): 131 NaCl, 5.5 KCl, 2.5 CaCl2, 1.0 MgCl2, 25.0 NaHCO3, 1.0 NaHPO4, 5.5 D-glucose, 20.0 HEPES and 0.05 mM-arginine and 2 μCi L-[3H]arginine for 1 hour at 37°C. The experiment was terminated by removing the medium and rapidly washing the cells 3 times with ice-cold phosphate buffer solution (PBS) that contained 10 mM unlabeled L-arginine. Cells were solubilized in 0.5 mL 1% Triton X-100, and 50-μL aliquots were taken for determination of total uptake of L-[3H]arginine in a manner by ICI182780 (Figure 2, top). Moreover, the effects of estradiol (10−10 mol/L) and medroxyprogesterone (Figure 3).

Statistics

Results are expressed as mean±SEM. Statistical analysis was performed with ANOVA, Student's t test, or Fishers least significant difference test, as appropriate. Values of P<0.05 were considered to be significantly different.

Results

Treatment of GECs with estradiol induced NOS activity in a concentration-dependent fashion (Figure 1). Physiological concentrations (10−10 mol/L) of estradiol induced NOS activity in GECs from 16.8 to 24.6 pmol/min per milligram protein. The lowest concentration of estradiol that significantly induced NOS activity was 10−12 mol/L. In GECs treated with estradiol (10−10 mol/L) for 1 hour and 24 hours, the NOS activity was stimulated by 19% and 48%, respectively, indicating that the stimulatory effects of estradiol were dependent on the time of exposure. Basal as well as estradiol-stimulated NOS activity was blocked by L-NMA (Figure 1). Neither 2-hydroxyestradiol nor 2-methoxyestradiol altered NOS activity (Figure 1). The stimulatory effects of estradiol on NOS activity were blocked in a concentration-dependent manner by ICI182780 (Figure 2, top). Moreover, the effects of 2-hydroxyestradiol and 2-methoxyestradiol on NOS activity remained unaltered in the presence of ICI182780 (Figure 2, bottom). The stimulatory effects of estradiol on NOS activity were significantly abrogated by both progesterone and medroxyprogesterone (Figure 3).
In GMCs, FCS stimulated $^3$H-thymidine and $^3$H-proline incorporation and cell number by 5- to 7-fold ($P<0.05$). Estradiol inhibited FCS-induced $^3$H-thymidine incorporation in a concentration-dependent manner (Figure 4). In this regard, significant inhibition occurred even with physiological concentrations of estradiol (1 nmol/L), and a 50% decrease was observed at $\sim$3 to 4 nmol/L of estradiol (Figure 4). Estradiol also inhibited FCS-induced $^3$H-proline incorporation and cell number in a concentration-dependent fashion (Figures 5 and 6, respectively). The lowest concentration of estradiol that significantly inhibited FCS-induced increases in cell number and proline incorporation was 1 nmol/L, and this concentration inhibited cell number by 23$\pm$3% and proline incorporation by 12.6$\pm$2.6%. The inhibitory effects of estradiol on FCS-induced $^3$H-thymidine and $^3$H-proline incorporation and cell number were completely blocked by ICI182780 (100 nmol/L).

Regarding the inhibition of FCS-induced DNA synthesis (Figure 4), collagen synthesis (Figure 4), and cell proliferation (Figure 5), 2-hydroxyestradiol and 2-methoxyestradiol were more potent than estradiol. The order of potency was 2-methoxyestradiol > 2-hydroxyestradiol > estradiol. At physiological concentrations (1 nmol/L), estradiol, 2-hydroxyestradiol, and 2-methoxyestradiol inhibited cell proliferation by 23%, 31%, and 37%, respectively. A 50% decrease in proline incorporation in GMCs by estradiol, 2-hydroxyestradiol, and 2-methoxyestradiol was observed at 8 nmol/L, 0.15 nmol/L, and 0.03 nmol/L, respectively. The inhibitory effects of estradiol (1 nmol/L) on thymidine incorporation (Figure 6A), proline incorporation (Figure 6B), and cell proliferation (Figure 6C) were completely reversed by ICI182780 (100 nmol/L; Figure 6 A through C). In contrast, the inhibitory effects of 2-hydroxyestradiol (1 nmol/L) and 2-methoxyestradiol (1 nmol/L) on FCS-induced thymidine incorporation, proline incorporation, and cell pro-

Figure 1. Top, NOS activity in cytosolic extracts of GECs treated for 24 hours with estradiol ($\beta$-Est), 2-methoxyestradiol (2-MeOE), or 2-hydroxyestradiol. Bottom, Effects of L-NMA (5 nmol/L) on basal and estradiol-induced NOS activity in GECs treated for 24 hours. *$P<0.05$ vs basal (in presence of vehicle, 0.1% DMSO); §$P<0.05$ vs $\beta$-NMA.

Figure 2. Top, NOS activity in GECs treated for 24 hours with 0.1 nmol/L estradiol in the presence and absence of ICI182780 (ICI). Bottom, NOS activity in GECs treated for 24 hours with 0.1 nmol/L 2-hydroxyestradiol (2-OHE) or 2-methoxyestradiol (2-MeOE) in the presence and absence of 10 nmol/l ICI. *$P<0.05$ vs basal (in presence of vehicle, 0.1% DMSO); §$P<0.05$ vs estradiol.

Figure 3. Effects of progesterone (1 to 100 nmol/L) and medroxyprogesterone (MPA; 1 to 100 nmol/L) on the stimulatory effects of estradiol ($\beta$-Estr; 0.1 nmol/L) on NOS activity. *$P<0.05$ vs vehicle-treated control (0.1% DMSO); §$P<0.05$ vs estradiol.

Figure 4. Top, Inhibitory effects of estradiol, 2-hydroxyestradiol, and 2-methoxyestradiol on FCS-induced $^3$H-thymidine incorporation in GMCs. *$P<0.05$ vs vehicle-treated control (0.1% DMSO). Bottom, Inhibitory effects of estradiol, 2-hydroxyestradiol, and 2-methoxyestradiol on FCS-induced $^3$H-proline incorporation in GMCs. *$P<0.05$ vs vehicle-treated control (0.1% DMSO).
liferation were not blocked by ICI182780 (100 μmol/L; Figure 6A through C).

Treatment of GMCs for 4 days with 100 nmol/L progesterone alone inhibited 2.5% FCS-induced cell proliferation by 16%, and similar inhibitory effects were observed in GMCs treated with 100 nmol/L medroxyprogesterone (Figure 6D, right panel). The inhibitory effects of 1 nmol/L estradiol on GMC growth were reduced from 24% to 19% by progesterone and to 18% by MPA; however, these decreases did not attain statistical significance (Figure 6D, left panel).

Discussion

Glomerular remodeling associated with glomerulosclerosis and glomerular injury is a complex process that involves dysfunction/damage to GECs and abnormal growth of GMCs. The abnormal growth processes that lead to the dynamic changes in glomerular structure involve hypertrophic/hyperplastic growth of GMCs and modulation of the amount of extracellular matrix proteins, such as collagen.

Under normal conditions, GECs play a critical role in maintaining homeostasis by generating a battery of both growth-inhibitory and growth-stimulatory factors, as well as relaxing and contracting factors. Moreover, endothelial damage or dysfunction triggers a cascade of events that often lead to increased GMC proliferation and extracellular matrix synthesis. In this regard, decreased synthesis of nitric oxide by damaged/dysfunctional GECs as well as abnormal growth of GMCs is associated with glomerulosclerosis. Estradiol stimulates recovery and NO synthesis in damaged/dysfunctional vascular endothelial cells and inhibits mitogen as well as injury-induced growth of vascular smooth muscle cells. Because GECs and GMCs are phenotypically similar to vascular endothelial and smooth muscle cells, we hypothesize that the protective effects of estradiol against the progression of renal disease in females may in part be mediated by its stimulatory effects on NO synthesis by GECs and its inhibitory effects on GMC growth. Moreover, because endogenous metabolites of estradiol (methoxyestradiols) with no binding affinity for ERs inhibit growth of vascular smooth muscle cells, a cell phenotypically similar to GMCs, we hypothesize that estradiol metabolites may prevent glomerulosclerosis by inhibiting abnormal growth of GMCs.

The findings of the present study demonstrate that estradiol upregulates the synthesis of NO by GECs and that the stimulatory effects of estradiol on NO synthesis are blocked by ICI182780, an ER antagonist, suggesting that the stimulatory effects of estradiol on NO synthesis are ER mediated. In contrast to estradiol, the major active endogenous metabolites of estradiol (2-hydroxyestradiol and 2-methoxyestradiol) do not influence basal NO synthesis by GECs. Moreover, progesterone and medroxyprogesterone (a synthetic progestin used in combination with estradiol for hormone replacement therapy) abrogated the stimulatory effects of estradiol on NO synthesis.

We also demonstrate that estradiol inhibits GMC DNA and collagen synthesis and GMC proliferation. In contrast to the effects on NO synthesis, the inhibitory effects of estradiol on GMCs are mimicked by 2-hydroxyestradiol and 2-methoxyestradiol. The relative potencies of estradiol and its metabolites to inhibit GMC growth are 2-methoxyestradiol > 2-hydroxyestradiol > estradiol, and this relative potency does not match their relative affinities for ERs. Progesterone also inhibited GMC growth and attenuated the inhibitory effects of estradiol. The inhibitory effects of estradiol, but not 2-hydroxyestradiol and 2-methoxyestradiol, on GMC growth are blocked by ICI182780, a specific ER antagonist. Taken together, our findings provide the first evidence that estradiol induces NO synthesis in GECs via an ER-dependent mechanism. Moreover, estradiol and its endogenous metabolites inhibit GMC growth, and these antimitogenic effects are mediated via ER-dependent and ER-independent mechanisms, respectively.

The finding that physiological concentrations of estradiol induce NOS activity in GECs by almost 50% suggests that estradiol may protect against glomerular remodeling processes associated with glomerulosclerosis. This contention is supported by the fact that inhibition of NO leads to glomerular injury and glomerulosclerosis. Indeed, in the kidney, NO generated by endothelial constitutive NOS participates in physiological regulation of glomerular hemodynamics by modifying the tone of afferent arterioles and mesangial cells and maintaining glomerular capillary blood pressure, glomerular plasma flow, and the glomerular ultrafiltration. NO
synthesized under basal conditions has multiple antiglomeru-
loulosclerotic effects; for example, NO contributes to the anti-
thrombogenic properties of the endothelium by preventing
platelet aggregation and adhesion; improves the barrier func-
tion of endothelial cells within capillaries, thereby preventing
glomerular monocyte/macrophage infiltration; and inhibits
proliferation and extracellular matrix synthesis in GMCs.4,5
The fact that estradiol reduces the rate of progression of
end-stage renal disease15 and the finding that NO synthesis is
reduced in patients with end-stage renal disease16 suggests
that estradiol may induce its protective effects by upregulat-
ing NOS activity.

The stimulatory effects of estradiol on NOS activity are
blocked by ICI182780, which suggests that GECs possess
functional ERs. Although human kidneys express both ERα
and ERβ,1 whether these receptors are expressed in the GECs
is not known. Therefore, the present findings do not allow
deductions in regard to the relative importance of ERα versus
ERβ in mediating the stimulatory effects of estradiol on NOS
activity. Additionally, recent findings that estradiol induces
NO synthesis acutely in vascular endothelial cells via a
nongenomic mechanism that involves ERα localized within
the cell membrane17 suggest that estradiol may similarly
induce NOS activity in GECs.

Analogous to the vascular remodeling process in ather-
sclerosis, abnormal growth of GMCs after capillary or GEC
damage/dysfunction contributes to the glomerular remodeling
process associated with glomerulosclerosis.3,5 The abnormal
growth process involves increased proliferation of GMCs and
increased production and deposition of extracellular matrix
proteins, such as collagen. Moreover, multiple autocrine/
paracrine/endocrine factors are involved in inducing the
structural changes in the glomeruli.4,5 Because FCS contains
a battery of growth factors that may contribute to the
remodeling process, we thought it important to evaluate the
effects of estradiol and its metabolites on FCS-induced
growth of GMCs to elucidate the growth regulatory effects of
estrogens under more physiological conditions. The finding
that estradiol, its metabolites, and progesterone inhibit FCS-
induced GMC growth provides evidence that these hormones
are important modulators of GMC growth.

Physiological concentrations (1 nmol/L) of estradiol inhibit
cell proliferation and collagen synthesis by 13% and 11%,
respectively. Moreover, 1 nmol/L of 2-hydroxyestradiol and
2-methoxyestradiol inhibit cell proliferation and collagen
synthesis by 19% and 17%, respectively, and by 32% and
25%, respectively. This suggests that physiological concen-
trations of estradiol inhibit GMC growth and collagen
synthesis and that the inhibitory effects of estradiol in vivo may
be considerably higher owing to the presence of estradiol
metabolites. In addition, our findings suggest that the protec-
tive effects of estradiol on the kidney may vary and be
dependent on the metabolic capability of the individual.

The stimulatory effects of estradiol on NOS activity in
GECs are inhibited by progesterone and medroxyprogester-
one, a synthetic progestin.14 This finding is consistent with
our previous observation that in postmenopausal women
receiving hormone replacement therapy, the circulating NO
levels are increased during treatment with estradiol alone but
not during treatment with estradiol plus medroxyprogester-
one.18 In contrast, the inhibitory effects of estradiol on GMC
growth are not significantly influenced by progesterone and
medroxyprogesterone. Thus, progestins may reduce the ben-
eficial effects of estradiol mediated by NO generated by
GECs, but progestins may not attenuate the beneficial effects
of estradiol mediated by inhibition of GMC growth. Because
combined administration of a progestin with an estrogen is
currently the preferred method of hormone replacement
therapy in nonhysterectomized postmenopausal women, our
findings suggest that progestins may reduce the full benefits
of estradiol within the glomerulus.

In summary, our findings indicate that estradiol, but not its
metabolites, induce NOS activity in GECs and that these
effects are ER-mediated. The effects of estradiol on NOS
activity are abrogated by progesterone (natural progestin) and
medroxyprogesterone (synthetic progestin). The inhibitory
effects of estradiol on GMC growth and collagen synthesis
are mimicked by 2-hydroxyestradiol and 2-methoxyestradiol,
endogenous metabolites of estradiol with no binding affinity
to ERs. The antimitogenic effects of estradiol, but not
2-hydroxyestradiol and 2-methoxyestradiol, are blocked by
ICI182780. Together our findings indicate that estradiol may
protect against the progression of renal disease by inducing
NO synthesis in GECs and inhibiting GMC growth and that
both ER-dependent and ER-independent pathways may par-
ticipate in mediating these effects.

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