Selective estrogen receptor modulators suppress mesangial cell collagen synthesis

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Neugarten, Joel, Anjali Acharya, Jun Lei, and Sharon Silbiger. Selective estrogen receptor modulators suppress mesangial cell collagen synthesis. *Am J Physiol Renal Physiol* 279: F309–F318, 2000.—Estrogen receptor modulators (SERMs) are “designer drugs” that exert estrogen-like actions in some cells but not in others. We examined the effects of the SERMs LY-117018 (an analog of raloxifene) and tamoxifen on mesangial cells synthesis of type I and type IV collagen. We found that LY-117018 and tamoxifen suppressed mesangial cell type IV collagen gene transcription and type IV collagen protein synthesis in a dose-dependent manner, with a potency identical to that of estradiol. Type I collagen synthesis was also suppressed by LY-117018 in a dose-dependent manner with a potency identical to that of estradiol but greater than that of tamoxifen. Genistein, which selectively binds to estrogen receptor-β in nanomolar concentrations, suppressed type I and type IV collagen synthesis, suggesting that estrogen receptor-β mediates the effects of estrogen on collagen synthesis. Because matrix accumulation is central to the development of glomerulosclerosis, second-generation SERMs may prove clinically useful in ameliorating progressive renal disease without the adverse effects of estrogen on reproductive tissues.

estradiol; angiotensin II; transforming growth factor-β1; endothelin

Selective estrogen receptor modulators, SERMs, are compounds that bind to the estrogen receptor but have tissue-specific effects that may differ from those of estrogen itself (4, 12, 22). The promise of the SERMs lies in their ability to reproduce the beneficial effects of estrogen on bone, endothelium, and lipoprotein metabolism without reproducing the undesirable effects of estrogen on reproductive tissues (4, 12, 22). Raloxifene, a second-generation SERM, has a more favorable tissue selectivity profile than tamoxifen, a first-generation SERM (4, 12, 22). Although both compounds act as estrogen antagonists in mammary tissue, tamoxifen is a partial estrogen agonist in endometrial tissue whereas raloxifene is a complete antagonist in the uterus (4).

Renal disease progresses less rapidly in women than in men (24, 33). In the course of investigating the mechanisms responsible for this observation, we found that estradiol suppresses the synthesis of types I and IV collagen by mesangial cells grown in the presence of serum (18, 19, 25, 26, 31, 32). The ability of estradiol to suppress mesangial cell collagen synthesis may reduce matrix accumulation after glomerular injury and contribute to the beneficial effect of female gender on the progression of renal disease (33).

We reasoned that if second-generation selective estrogen receptor modulators reproduce estradiol’s suppressive effects on mesangial cell collagen synthesis, they might prove clinically useful in ameliorating progressive glomerulosclerosis in chronic renal disease without reproducing the adverse effects of estradiol on reproductive tissue. The potential clinical usefulness of second-generations SERMs in limiting progressive renal disease led us to explore in detail their antifibrotic actions. In the present study we examined the effects of a first-generation SERM (tamoxifen) and a second-generation SERM (LY-117018) on the synthesis of type I and type IV collagen by cultured mesangial cells. We also investigated the effects of the SERMs on the ability of fibrogenic agents such as transforming growth factor-β1 (TGF-β1), ANG II, and endothelin-1 (ET-1) to stimulate mesangial cell type α1(IV) gene (COL4A1) transcription and type IV collagen synthesis.

We found that LY-117018 and tamoxifen suppress COL4A1 gene transcription and type IV collagen protein synthesis in a dose-dependent manner, with a potency identical to that of estradiol. Type I collagen synthesis was also suppressed by LY-117018 in a dose-dependent manner with a potency identical to that of estradiol but greater than that of tamoxifen.

We also examined the effect of the SERMs on the ability of the fibrogenic agents TGF-β1, ANG II, and ET-1 to stimulate COL4A1 gene transcription and type IV collagen synthesis. Estradiol, in physiological concentrations, reversed the stimulatory effects of ANG II and ET-1 on COL4A1 gene transcription and type IV collagen synthesis by antagonizing the actions of TGF-β1. The SERMs reproduced the effects of estradiol, reversing the actions of ANG II and ET-1 on type IV collagen via antagonism of the autocrine/paracrine effects of TGF-β1.

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Last, we demonstrated that estrogen receptor-α (ER-α) and estrogen receptor-β (ER-β) are present in murine mesangial cells and that the effects of estrogen on collagen synthesis are mediated, at least in part, by ER-β.

METHODS

Isolation and characterization of murine mesangial cells. Mesangial cells were isolated from kidneys of 8- to 10-wk-old naive SJL/J (H-2d) mice by differential glomerular sieving. The present studies were performed in an immortalized, differentiated murine mesangial cell line transformed with nonreplicating, non-capsid-forming SV40 virus (strain Rh 911). The cells express receptors for ANG II and stain positive for Thy-1 antigen, desmin, vimentin, and types I and IV collagens but fail to bind antibody directed against a proximal tubular antigen (36). To exclude the possibility that our results were influenced by SV40 transformation per se, all experiments (except reporter gene studies) were reproduced in nontransformed male murine mesangial cells. Because identical results were obtained in all transformed and nontransformed cells, all data were combined.

Construction of reporter gene plasmids. A plasmid containing portions of the gene encoding murine α1(IV)-collagen was linked to luciferase coding sequences to form a reporter construct, HB35, which has previously been described (8). A 2.3-kb Hinf I fragment derived from p184 (gift of Dr. P. Killen, University of Michigan) (5, 13) spanning the first three exons, the first two introns, and a portion of the third intron of the α1(IV)-collagen gene, the intergenic bidirectional promoter region, and a portion of the α1(IV)-collagen transcription unit was cloned into pBlueScript (Stratagene, La Jolla, CA) and was truncated within the third intron of the α1(IV) gene by excising the appropriate fragment with Hinf III. The fragment was then ligated into the Hind III site of the luciferase expression plasmid, pSVAlucD5′ (7), and its orientation was determined by restriction mapping (17). An additional 3.2-kb fragment of the first intron of the α1(IV)-collagen gene, previously shown to modulate the activity of the α1(IV) promoter (5, 13), corresponding to a BamHI-linked 3.2-kb XbaI fragment of the first intron of α1(IV), was inserted into the BamHI site downstream from the 3′ end of the luciferase coding sequences following partial BamHI digestion (17). Intron fragment orientation and position were confirmed by restriction mapping (17).

Stable transfection. Murine mesangial cells were cotransfected with HB35 and a selection plasmid pSV2-Neo encoding neomycin-resistance at a molar ratio of 10:1 by using the CaPO4-DNA precipitation procedure (2). Cells were then grown in selection medium containing Geneticin (G-418). Cells surviving in medium with Geneticin were expanded for neomycin-resistance at a molar ratio of 10:1 by using the CaPO4-DNA precipitation procedure (2). Cells were then grown in selection medium containing Geneticin (G-418). Cells surviving in medium with Geneticin were expanded and selected with the neomycin analog. Stable transfectants exhibited patterns of growth behavior and protein synthesis similar to the parent cell line (8).

Luciferase assay. Cells were plated in six-well plates and grown in phenol-free, serum-free RPMI. In some experiments cells were exposed to 17β-estradiol (Sigma Chemical, St. Louis, MO); LY-117018 (a second-generation SERM analog of raloxifene; Lilly Research Laboratories, Indianapolis, IN); tamoxifen (a first-generation SERM; Sigma Chemical), and genistein (a phytoestrogen; Sigma Chemical); TGF-β1 (R & D Systems, Minneapolis, MN); ANG II (Sigma Chemical); ET-1 (Sigma Chemical); ANG II antipeptide (a nonselective ANG II receptor antagonist; Sigma Chemical); neutralizing antibody to TGF-β1 (R&D Systems); nonimmune IgG (Sigma Chemical); or PD 0156707 (a selective ETA receptor antagonist; Parke-Davis Pharmaceutical Research, Ann Arbor, MI), alone or in combination, for 24 h. Cells were washed twice with PBS, then lysed with 100 μl Reporter Lysis Buffer (Promega, Madison, WI) at room temperature for 15 min. Wells were then scraped, and the cell lysate was transferred to a microcentrifuge tube and placed on ice. Tubes were vortexed and microcentrifuged for 2 min at 4°C. The suspension was transferred to a new microcentrifuge tube and stored at -70°C until assayed. Twenty microliters of cell extract were mixed with 10 μl of assay reagent (20 mM tricine, 1.07 mM MgCO3, 1.07 mM Mg(OH)2 · 5H2O, 2.67 mM MgSO4, 0.1 mM EDTA, 33.3 mM dithiothreitol (DTT), 270 μM coenzyme A, 470 μM luciferin, 530 μM ATP, pH 7.8) at room temperature (all reagents were from Sigma Chemical, St. Louis, MO). Light emission was measured directly at room temperature over a 10-s period in a luminometer (Promega). Blank reactions were determined with equivalent volumes of lysis buffer substituted for cell lysates, and these values were subtracted from experimental values. Luciferase activity was expressed per milligram of cellular protein in the suspension as determined by the Bio-Rad protein assay (Bio-Rad, Richmond, CA). Relative luciferase units were calculated as percentages of control values, where 100% is the value obtained with control media (serum-free, no added agents).

Western blotting. In studies utilizing TGF-β1, ANG II, or ET-1, cells were grown in phenol-free, serum-free RPMI as described above. In all other studies, cells were grown in phenol-free RPMI supplemented with 20% FCS. Cells were not growth arrested before study. Media was collected and concentrated to an identical final volume by using an Amicon Centriprep-10 concentrator (Grace, Beverly, MA). Protein content was measured in a 0.1-ml aliquot of the concentrated media (Bio-Rad Protein Assay, Richmond, CA). To prepare the samples for SDS-PAGE, 1 ml of 10% trichloroacetic acid was added to 4 mg protein of concentrated media, and the final volume was brought to 2 ml with distilled water. The sample was vortexed and then centrifuged at 2,000 rpm for 10 min. The pellet was dissolved in 1 ml of loading buffer (2% SDS, 10% glycerol, 50 mM Tris, 3% bromophenol blue, 2% β-mercaptoethanol, pH 7.6), boiled for 5 min, then immediately placed on ice. Samples (25, 50, 75 or 100 μg) were loaded for electrophoretic separation of proteins. SDS-PAGE was performed by standard techniques, and proteins were transferred to a polyvinylidene difluoride microporous membrane.

After blotting, the membrane was immediately placed into blocking buffer [2% bovine serum albumin in wash buffer (10 mM Tris, 100 mM NaCl, 0.1% Tween-20, distilled H2O added to 1 liter)] on a shaking apparatus for 30 min at 37°C. The blocking buffer was discarded and replaced with new blocking buffer containing the indicated primary antibody. Membranes were incubated with the primary antibody on a shaker, washed, and reselected for secondary antibody conjugated to horseradish peroxidase (Sigma Chemical). The antibody conjugate was allowed to incubate for 30 min at 37°C with agitation. After washing, the membrane was treated with enhanced chemiluminescence reagent (Amersham Life Science, Arlington Heights, IL) according to the instructions of the manufacturer. Kodak X-OMAT 4R film was exposed to the blot for 10 min. Bands were quantitated by laser densitometry.

Primary antibodies utilized included monoclonal mouse anti-ER-α antibody (Affinity Bioreagents, Golden, CO), polyclonal rabbit anti-ER-β antibody (Affinity Bioreagents), goat
anti-bovine type I collagen (Southern Biotechnology, Birmingham, AL), or goat anti-bovine type IV collagen antibody (Southern Biotechnology; 1:250 dilution). Recombinant human ER-α and ER-β (Affinity Bioreagents) and human type I and type IV collagen (Sigma Chemical) were used as positive controls in Western blotting experiments. Appropriate negative controls using irrelevant antibodies were also performed.

We also utilized polyclonal rabbit anti-phospho-specific p44/p42 MAP kinase antibody, which detects extracellular signal-regulated protein kinase (ERK)1 and ERK2 (p44 MAP kinase and p42 MAP kinase, respectively) only when activated by phosphorylation at Thr 202 and Tyr 204 by the upstream kinase, MAP kinase-ERK (MEK; New England Biolabs, Beverly, MA). This antibody does not cross-react with unphosphorylated MAP kinase. Bacterially expressed unphosphorylated p42 MAP kinase served as a negative control (New England Biolabs). The same protein phosphorylated by MEK served as a positive control (New England Biolabs). In other blots, we utilized polyclonal rabbit anti-p44/p42 MAP kinase antibody, which detects total (phosphorylated and unphosphorylated) MAP kinase (p44 Erk and p42 Erk) (New England Biolabs). Bacterially expressed unphosphorylated p42 MAP kinase served as a positive control (New England Biolabs).

Preparation of nuclear extracts. Mesangial cells were scraped into PBS and pelleted by centrifuging at 3,000 g for 10 min at 4°C (30). The cells were resuspended in hypotonic buffer (in mM) 10 HEPES, pH 7.9, 1.5 MgCl2, 10 KCl, 0.2 phenylmethylsulfonyl fluoride (PMSF), 0.5 DTT (Sigma Chemical), centrifuged at 3,000 g for 5 min, resuspended in the hypotonic buffer, and allowed to swell for 10 min on ice. The cells were homogenized in a glass Dounce homogenizer. Nuclei were collected by centrifuging at 3,300 g for 15 min. The nuclear pellet was resuspended in low-salt buffer [(in mM) 20 HEPES, pH 7.5, 1.2 mM MgCl2, 10 KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT (Sigma Chemical)]. With gentle stirring, a volume of high-salt buffer (1.2 M KCl) equal to one-half the packed nuclear volume was added dropwise. The protein concentration was measured by colorimetric assay (Bio-Rad, Hercules, CA).

Electrophoretic mobility shift assay. Four micrograms of nuclear extracts were mixed with 2 μg of poly (dI:dC) in 20 μl of a reaction buffer consisting of 25 mM HEPES, pH 7.5, 1.2 mM DTT, 4 mM MgCl2, 150 mM NaCl, 5% glycerol, 0.005% bromphenol blue, and 0.05% Nonidet P-40 (Sigma Chemical) (30). The mixture was incubated on ice for 15 min, followed by addition of 10 fmol of 32P end-labeled activator protein-1 (AP-1) consensus binding sequence oligonucleotide (nucleotide sequence: 5'-GGTGTAGAGTCTAGCCGGA-3'; Promega). Incubation was continued for 30 min. The incubation mixture was subjected to electrophoresis on a 6% polyacrylamide gel in Tris-glycine buffer. The gels were dried, and autoradiography was performed at −70°C with an intensifying screen. Bands were quantitated by laser densitometry (model 300S, Molecular Dynamics). Competition experiments were performed with a 200-fold excess of unlabeled AP-1 consensus binding sequence oligonucleotide.

Statistics. For each individual data point, the mean of at least three to seven experiments (using 3 replicate wells/experiment) was calculated. The data are means ± SE. Differences among groups were tested by analysis of variance with Scheffe's correction. P < 0.05 was considered a significant difference.

RESULTS

Identification of ERs in mesangial cells. We used a mouse monoclonal anti-ER-β antibody that did not cross-react with ER-α to demonstrate the presence of ER-β in murine mesangial cell extracts by Western blotting (Fig. 1A, top). We used a rabbit polyclonal anti-ER-α antibody that did not cross-react with ER-β to demonstrate the presence of ER-α in murine mesangial cell extracts by Western blotting (Fig. 1A, bottom).

SERMs suppress mesangial cell type IV collagen gene transcription and protein synthesis. The SERM LY-117018 suppressed COL4A1 gene transcription in a dose-dependent manner in murine mesangial cells grown in 20% FCS (Fig. 1B). At a LY-117018 concentration of 10−7 M, LY-117018 decreased COL4A1 gene transcription by 31.4% (P < 0.0001 vs. control). At a concentration of LY-117018 equivalent to physiological concentrations of estradiol in premenopausal women (10−9 M), COL4A1 gene transcription was reduced by 16.4% (P < 0.0001 vs. control). The potency of LY-117018 was similar to that of estradiol and tamoxifen at identical concentrations (10−9 M; LY-117018: 83.7 ± 1.7, expressed as a percentage of control values; estradiol: 82.3 ± 1.0; tamoxifen: 89.1 ± 1.8; not significant (NS) vs. LY-117018 and P NS vs. estradiol (Fig. 1B)). The suppressive effects of LY-117018, tamoxifen, and estradiol on COL4A1 gene transcription were not additive.

LY-117018 suppressed type IV collagen synthesis by murine mesangial cells in a dose-dependent manner (Fig. 1C). Type IV collagen synthesis was reduced by 74.3% at an LY-117018 concentration of 10−7 M (P < 0.0001 vs. control) and by 49.9% at a LY-117018 concentration of 10−9 M (P < 0.0001 vs. control). The potency of LY-117018 was similar to that of estradiol and tamoxifen at identical concentrations (10−9 M; LY-117018: 42.2 ± 7.5, expressed as a percentage of control values; estradiol: 40.0 ± 9.0; tamoxifen: 55.7 ± 8.3, P NS vs. LY-117018, P NS vs. estradiol, P < 0.0001 vs. control) (Fig. 1D). Suppression of type IV collagen synthesis was receptor mediated, as evidenced by the ability of a 10-fold excess of a pure ER antagonist (ICI-182780) to reverse the effect of the estrogenic compounds [LY-117018 (10−9 M)+ICI-182780 (10−8 M): 91.9 ± 13.4, P NS vs. control, P < 0.0001 vs. LY-117018; estradiol+ICI-182780: 97.0 ± 7.6, P NS vs. control, P < 0.0001 vs. estradiol; tamoxifen+ICI-182780: 92.8 ± 6.9, P NS vs. control, P < 0.0001 vs. tamoxifen; ICI-182780 alone: 101.3 ± 3.2, P NS vs. control] (Fig. 1D).

SERMs suppress mesangial cell type I collagen synthesis. LY-117018 suppressed type I collagen synthesis by murine mesangial cells in a dose-dependent manner (Fig. 2A). Type I collagen synthesis was reduced by 75.7% at an LY-117018 concentration of 10−7 M (P < 0.0001 vs. control) and by 47.8% at a LY-117018 concentration of 10−9 M (P < 0.0001 vs. control). The potency of LY-117018 was similar to that of estradiol but greater than that of tamoxifen at identical concentrations (10−9 M; estradiol: 37.3 ± 6.9, P < 0.002 vs.
control; LY-117018: 36.4 ± 8.3, expressed as a percentage of control values, *P < 0.009 vs. control, ¶ NS vs. estradiol; tamoxifen: 68.6 ± 10.7, ¶ NS vs. control, *P < 0.05 vs. LY-117018 (Fig. 2B).

Suppression of type I collagen synthesis was receptor mediated, as evidenced by the ability of a 10-fold excess of a pure ER antagonist (ICI-182780) to reverse the effect of the estrogenic compounds [estradiol (10^{-9} M) + ICI-182780 (10^{-8} M): 90.0 ± 5.5, ¶ NS vs. control, *P < 0.0001 vs. estradiol; LY-117018 + ICI-182780: 88.7 ± 8.4, NS vs. control, *P < 0.0001 vs. LY-117018; tamoxifen + ICI-182780: 89.8 ± 12.6, ¶ NS vs. control, *P < 0.0001 vs. tamoxifen; ICI-182780 alone (10^{-8} M): 100.2 ± 4.7, ¶ NS vs. control] (Fig. 2B).

ER-β mediates the suppressive effects of estrogen on collagen synthesis. Genistein, which selectively binds to ER-β in nanomolar concentrations, suppressed type I and type IV collagen synthesis in a dose-dependent manner (10^{-9} to 10^{-7} M) (Fig. 2C). The effects of genistein (10^{-9} M) were reversed by ICI-182780 (10^{-8} M) (Fig. 2C).

SERMs upregulate MAP kinase and AP-1 activity. We have previously shown that estradiol suppresses mesangial cell type I collagen synthesis by increasing the activity of AP-1 via upregulation of the mitogen-activated protein kinase pathway (25, 31). We examined the hypothesis that the SERMs suppress type I collagen synthesis via an identical mechanism. Phospho-MAP kinase was markedly increased by all three estrogenic compounds (10^{-9} M) after 10 min of exposure (Fig. 2D).

We performed electrophoretic mobility shift assays using an AP-1 consensus sequence oligonucleotide and nuclear extracts from mesangial cells treated with con-
trol media, estradiol, or the SERMs. All three estrogenic compounds increased the binding of nuclear extracts to the AP-1 oligonucleotide (Fig. 2E). The increase induced by estradiol and LY-117018 was greater than that induced by tamoxifen.

SERMs reverse TGF-β1-stimulated COL4A1 gene transcription. COL4A1 gene transcription was increased in mesangial cells incubated with TGF-β1 (2 ng/ml for 24 h; 137.7 ± 2.7, expressed as a percentage of control values, P < 0.0001) (Fig. 3A). Physiological concentrations of all three estrogenic compounds (10^{-9} M) reversed TGF-β1-stimulated COL4A1 gene transcription (TGF-β1 + estradiol: 96.0 ± 1.9, P < 0.0001 vs. TGF-β1, P NS vs. control; estradiol alone: 107.9 ± 1.8, P NS vs. control; TGF-β1 + LY-117018: 99.6 ± 2.7, P < 0.0001 vs. TGF-β1, P NS vs. control; LY-117018 alone: 105.6 ± 1.2, P NS vs. control; TGF-β1 + tamoxifen: 105.9 ± 0.7, P < 0.0001 vs. TGF-β1, P NS vs. control; tamoxifen alone: 108.9 ± 1.2, P NS vs. control) (Fig. 3A).

SERMs reverse ANG II-stimulated COL4A1 gene transcription. ANG II (10^{-8} to 10^{-6} M) stimulated COL4A1 gene transcription in a dose-dependent manner, achieving statistical significance at a concentration of 10^{-6} M (125.3 ± 2.1, expressed as a percentage of control values, P < 0.0001 vs. control). ANG II (10^{-6} M)-stimulated gene transcription was reversed by a nonselective ANG II receptor antagonist (ANG II antipeptide, 10^{-5} M; 100.3 ± 0.5, P NS vs. control, P < 0.0001 vs. ANG II; ANG II antipeptide alone: 100.2 ± 0.6, P NS vs. control) (Fig. 3B). A neutralizing antibody to TGF-β1 completely reversed the stimulatory effect of ANG II on COL4A1 gene transcription: (100.4 ± 0.8, NS vs. control, P < 0.0001 vs. ANG II; ANG II antipeptide alone: 100.7 ± 0.5, P NS vs. control). IgG lacking anti-TGF-β1 specificity had no effect on ANG
II-stimulated COL4A1 gene transcription (126.4 ± 3.1, P NS vs. ANG II alone, P < 0.0001 vs. control; IgG alone: 100.9 ± 0.4, P NS vs. control) (Fig. 3B)

All three estrogenic compounds (10^-9 M) completely reversed the stimulatory effects of ANG II on COL4A1 gene transcription (ANG II + estradiol: 100.1 ± 0.4, P NS vs. control, P < 0.0001 vs. ANG II; estradiol alone: 108.9 ± 0.3, P NS vs. control; ANG II + LY-117018: 100.7 ± 0.3, P NS vs. control, P < 0.0001 vs. ANG II; LY-117018 alone: 107.6 ± 1.8, P NS vs. control; ANG II + tamoxifen: 108.5 ± 0.6, P NS vs. control, P < 0.0001 vs. ANG II; tamoxifen alone: 109.0 ± 2.2, P NS vs. control) (Fig. 3C). The effects of the estrogenic compounds and of anti-TGF-β1 antibody were not additive (ANG II + estradiol + anti-TGF-β1: 99.3 ± 0.5, P NS vs. control, P NS vs. ANG II + estradiol, P NS vs. ANG II + anti-TGF-β1 antibody, P < 0.0001 vs. ANG II; ANG II + LY-117018 + anti-TGF-β1 antibody: 99.2 ± 1.1, P NS vs. ANG II + LY-117018, P NS vs. ANG II + anti-TGF-β1 antibody, P NS vs. control, P < 0.0001 vs. ANG II; ANG II + tamoxifen + anti-TGF-β1 antibody: 99.2 ± 0.9, P NS vs. ANG II + tamoxifen, P NS vs. ANG II + anti-TGF-β1 antibody, P NS vs. control, P < 0.0001 vs. ANG II) (Fig. 3C).

SERMs reverse ANG II-stimulated type IV collagen synthesis. We next examined the effect of the estrogenic compounds on ANG II-stimulated collagen IV protein synthesis as assessed by Western blotting (Fig. 3D). ANG II (10^-6 M) increased type IV collagen synthesis (191.4 ± 18.9, P < 0.0001 vs. control, n = 7). ANG II-stimulated type IV collagen synthesis was reversed by anti-TGF-β1 antibody (106.8 ± 8.9, P NS vs. control, P < 0.002 vs. ANG II; anti-TGF-β1 antibody alone: 106.0 ± 6.6, P NS vs. control). All three estrogenic compounds also completely reversed the stimulatory effects of ANG II on collagen IV synthesis (ANG II + estradiol: 100.1 ± 7.9, P NS vs. control, P < 0.0001 vs. ANG II alone; estradiol alone: 102.3 ± 6.3, P NS vs.

**Fig. 3.** A: estrogenic compounds reverse transforming growth factor-β1 (TGF-β1)-stimulated transcription of the gene encoding the α1-chain of type IV collagen (COL4A1) in mesangial cells grown in serum-free media as assessed by the activity of a COL4A1 reporter gene construct. B: TGF-β1 mediates the effects of ANG II (AII) on transcription of the gene encoding COL4A1 in mesangial cells grown in serum-free media as assessed by the activity of a COL4A1 reporter gene construct. ARA, ANG II receptor agonist; αTGF-β1, neutralizing antibody directed against TGF-β1. C: estrogenic compounds reverse angiotensin II-stimulated transcription of the gene encoding COL4A1 in mesangial cells grown in serum-free media by antagonizing the actions of autocrine TGF-β1. COL4A1 gene transcription was assessed by the activity of a COL4A1 reporter gene construct. D: estrogenic compounds reverse ANG II-stimulated type IV collagen synthesis in mesangial cells grown in serum-free media by antagonizing the actions of autocrine TGF-β1. Top: a representative Western blot for type IV collagen synthesis by mesangial cells grown in serum-free media in the presence of the indicated agents. Bottom: densitometry readings. *P < 0.001 vs. Con.
control; ANG II+LY-117018: 101.2 ± 5.8, P NS vs. control, P < 0.001 vs. ANG II alone; LY-117018 alone: 99.8 ± 3.8, P NS vs. control; ANG II+tamoxifen: 109.8 ± 6.6, P NS vs. control, P < 0.0001 vs. ANG II; tamoxifen alone: 106.7 ± 4.9, P NS vs. control) (Fig. 3D). The suppressive effects of the estrogenic compounds and anti-TGF-β1 antibody were not additive (ANG II+estradiol+anti-TGF-β1: 106.8 ± 8.9, P NS vs. ANG II+estradiol, P NS vs. ANG II+anti-TGF-β1 antibody, P NS vs. control, P < 0.002 vs. ANG II; ANG II+LY-117018+anti-TGF-β1 antibody: 102.2 ± 1.1, P NS vs. ANG II+LY-117018, P NS vs. ANG II+anti-TGF-β1 antibody, P NS vs. control, P < 0.0001 vs. ANG II; ANG II+tamoxifen+anti-TGF-β1 antibody: 104.9 ± 0.9, P NS vs. ANG II+tamoxifen, P NS vs. ANG II+anti-TGF-β1 antibody, P NS vs. control, P < 0.0001 vs. ANG II) (Fig. 3D).

SERMs reverse ET-1-stimulated COL4A1 gene transcription. ET-1 (10−9 to 10−6 M) stimulated COL4A1 gene transcription in a dose-dependent manner, achieving statistical significance at a concentration of 10−7 M (121.3 ± 1.3, P < 0.0001 vs. control). PD 0156707, an ETA-selective receptor antagonist, completely reversed the stimulatory effect of ET-1 (100.4 ± 2.9, P NS vs. control, P < 0.0001 vs. ET-1; ETA receptor antagonist alone: 100.1 ± 1.2, P NS vs. control) (Fig. 4A). A neutralizing antibody to TGF-β1 also completely reversed the stimulatory effect of ET-1 on COL4A1 gene transcription (99.1 ± 1.8, P NS vs. control, P < 0.0001 vs. ET-1; anti-TGF-β1 antibody alone: 101.3 ± 1.0, P NS vs. control) (Fig. 4A). IgG lacking anti-TGF-β1 specificity had no effect on ET-1-stimulated COL4A1 gene transcription (124.6 ± 2.6, P NS vs. ET-1 alone, P < 0.0001 vs. control; IgG alone: 100.9 ± 0.4, P NS vs. control).

All three estrogenic compounds also completely reversed the effects of ET-1 on COL4A1 gene transcription (ET-1+estradiol: 98.9 ± 0.9, P < 0.0001 vs. ET-1, P NS vs. control; estradiol alone: 107.9 ± 1.8, P NS vs. control; ET-1+LY-117018: 98.6 ± 0.8, P NS vs. control P < 0.0001 vs. ET-1; LY-117018 alone: 105.6 ± 1.2, P NS vs. control; ET-1+tamoxifen: 105.6 ± 0.5, P NS vs. control, P < 0.0001 vs. ET-1; tamoxifen alone: 108.9 ± 1.2, P NS vs. control) (Fig. 4B). The suppressive effects of the estrogenic compounds and of anti-TGF-β1 antibody were not additive (ET-1+estradiol+anti-TGF-β1: 98.8 ± 1.2, P NS vs. ET-1+estradiol, P NS vs. ET-1+anti-TGF-β1 antibody, P NS vs. control, P < 0.0001 vs. ET-1; ET-1+LY-117018+anti-TGF-β1 antibody: 99.5 ± 1.0, P NS vs. ET-1+LY-117018, P NS vs. ET-1+anti-TGF-β1 antibody, P NS vs. control, P < 0.0001 vs. ET-1; ET-1+tamoxifen+anti-TGF-β1 antibody: 99.5 ± 1.0, P NS vs. ET-1+tamoxifen, P NS vs. ET-1+anti-TGF-β1 antibody, P NS vs. control, P < 0.0001 vs. ET-1) (Fig. 4B).

![Fig. 4](http://ajprenal.physiology.org/)

**Fig. 4.** A: TGF-β1 mediates the effects of endothelin-1 (ET-1; ET) on transcription of COL4A1 in mesangial cells grown in serum-free media as assessed by the activity of a COL4A1 reporter gene construct. B: estrogenic compounds reverse ET-1-stimulated transcription of COL4A1 in mesangial cells grown in serum-free media by antagonizing the actions of autocrine TGF-β1. COL4A1 gene transcription was assessed by the activity of a collagen IV/luciferase minigene construct. C: estrogenic compounds reverse ET-1-stimulated type IV collagen synthesis in mesangial cells grown in serum-free media by antagonizing the actions of TGF-β1. A representative Western blot for type IV collagen synthesis by mesangial cells grown in serum-free media in the presence of the indicated agents is shown. ERA, endothelin receptor antagonist, *P < 0.001 vs. Con.
SERMs reverse ET-1-stimulated type IV collagen synthesis. Last, we examined the effect of the estrogenic compounds on ET-1-stimulated collagen IV protein synthesis as assessed by Western blotting (Fig. 4C). The ability of ET-1 to stimulate type IV collagen synthesis was completely reversed by each of the following: anti-TGF-β1 neutralizing antibody, estradiol (10^-9 M), LY-117018 (10^-9 M), and tamoxifen (10^-9 M). The suppressive effects of anti-TGF-β1 antibody were not additive to those of estradiol, LY-117018, or tamoxifen.

DISCUSSION

SERMs are compounds that bind to the ER but have tissue-specific effects that may differ from those of estrogen itself (4, 12, 22). The promise of the SERMs lies in their ability to reproduce the beneficial effects of estrogen on bone, endothelium, and lipoprotein metabolism without reproducing the undesirable effects of estrogen on reproductive tissues (4, 12, 22). Raloxifene, a second-generation SERM, has a more favorable tissue-selectivity profile than does tamoxifen, a first-generation SERM (4, 12, 22). Although both compounds act as estrogen antagonists in mammary tissue, tamoxifen is a partial estrogen agonist in endometrial tissue whereas raloxifene is a complete estrogen antagonist in the uterus (4).

If these agents were to reproduce estradiol’s suppressive effects on mesangial cell collagen synthesis, they might prove clinically useful in ameliorating progressive extracellular matrix accumulation and resulting glomerulosclerosis in chronic renal disease without reproducing the adverse effects of estradiol on reproductive tissues. The potential clinical usefulness of second-generation SERMs in limiting progressive renal disease led us to explore in detail their antifibrotic actions. In the present study we examined the effects of a first-generation SERM (tamoxifen) and a second-generation SERM (LY-117018) on the synthesis of type I and type IV collagen by cultured mesangial cells. We also investigated the effects of the SERMs on the ability of fibrogenic agents such as TGF-β1, ANG II, and ET-1 to stimulate mesangial cell COL4A1 gene transcription and type IV collagen synthesis. We also examined the effect of the SERMs on the ability of the fibrogenic agents TGF-β1, ANG II, and ET-1 to stimulate COL4A1 gene transcription and type IV collagen synthesis. In a manner identical to estradiol, the SERMs also reversed the actions of ANG II and ET-1 on type IV collagen via antagonism of the autocrine/paracrine effects of TGF-β1.

LY-117018 and tamoxifen, in concentrations identical to the physiological concentration of estradiol, reproduced the suppressive effects of estradiol on TGF-β1-stimulated COL4A1 gene transcription and type IV collagen synthesis. In addition, we found that LY-117018 and tamoxifen suppress COL4A1 gene transcription and type IV collagen protein synthesis in a dose-dependent manner, with a potency identical to that of estradiol. Type I collagen synthesis was also suppressed by LY-117018 in a dose-dependent manner with a potency identical to that of estradiol but greater than that of tamoxifen.

We have previously shown that estradiol suppresses mesangial cell type I collagen synthesis by increasing the activity of AP-1 via upregulation of the mitogen-activated protein kinase pathway (25, 31). Our demonstration that the SERMs upregulate MAP kinase and increase the binding of nuclear extracts to an AP-1 binding site supports the hypothesis that the SERMs suppress type I collagen synthesis via a mechanism identical to what we described for estradiol.

We also examined the effect of the SERMs on the ability of the fibrogenic agents TGF-β1, ANG II, and ET-1 to stimulate COL4A1 gene transcription and type IV collagen synthesis. We confirmed that the stimulatory effects of ANG II and ET-1 on mesangial cell COL4A1 gene transcription and type IV collagen synthesis are mediated by the autocrine/paracrine effects of TGF-β1 (9, 10). We showed that estradiol, in physiological concentrations, reverses the stimulatory effects of ANG II and ET-1 on COL4A1 gene transcription and type IV collagen synthesis by antagonizing the actions of TGF-β1.

Estrogens, SERMs, and ICI-182780 (a pure ER antagonist) all bind to the ER but elicit different transcriptional responses depending on the specific cell type and gene promoter (22). The diversity of responses may arise due to interactions with 1) different ER isoforms (1, 28), 2) hormone response elements other than the estrogen response element (37), or 3) different gene- or cell-specific effector molecules (coactivators, corepressors, transcription factors, chromatin remodeling proteins) (1, 4, 12, 22). Differences in the conformation of complexes formed between ER and specific ligands may contribute to tissue- and cell type-specific genomic responses by facilitating or interfering with the ability of the ER to recruit cell-specific coactivators or corepressors or by interfering with the ability of the ER to interact with DNA response elements (4, 12, 22). In addition, ligand/tissue selectivity may be conferred by differential ligand activation of the constitutive hormone-independent transcription activation function domain of the ER (AF-1) vs. the hormone-inducible AF-2 domain (35). In this regard, we found no ligand/tissue selectivity with respect to the effects of estradiol, tamoxifen, and LY-117018 on the synthesis of type I and type IV collagen by murine mesangial cells.

In addition to the classic estrogen receptor (ER-α), a second isotype (ER-β) has recently been identified (15). Vascular smooth muscle and vascular endothelial cells from a variety of species express both receptor subtypes (21). ER-β is the predominant isotype expressed in human fetal kidney, with minor expression of ER-α (3). Most studies have demonstrated moderate expression of ER-α in the absence of ER-β in adult renal tissue in humans, rats, and mice (14, 23, 34). However, other investigators have demonstrated ERβ in rat kidneys (27). The varying ratios of ER-α and ER-β in tissues that express both receptor isotypes may be involved in the tissue- and cell type-specific effects of estrogens vs. SERMs (29). ER-α and ER-β respond differently to ligands at AP-1 sites (28). In the presence of ER-α, estrogen and tamoxifen show agonist effects at AP-1 sites whereas raloxifene acts as a partial agonist
(28). In the presence of ER-β, tamoxifen, raloxifene, and ICI-182780 show agonist activity at AP-1 sites, whereas estrogen acts as an antagonist of raloxifene (28). In addition, at estrogen response elements, the transcriptional activity of estrogen is greater when bound to ER-α than ER-β (11, 23). In cells that express both receptor isotypes, heterodimer complexes may form that exhibit an intermediate DNA binding affinity and transcriptional activity (6, 29). In the present study we clearly demonstrate the presence of both ER-α and ER-β in male murine mesangial cells.

Genistein in micromolar concentrations inhibits tyrosine kinase activity. In these concentrations, genistein stimulates type I collagen synthesis in murine mesangial cells (25). In contrast, genistein in nanomolar concentrations lacks tyrosine kinase inhibitory activity but selectively binds to ER-β (16, 20). We found that genistein in nanomolar concentrations suppresses type I and type IV collagen synthesis by an ER-dependent mechanism. These data suggest that ER-β mediates, at least in part, the suppressive effects of estrogen on collagen synthesis. However, we cannot exclude the possibility that functional redundancy exists between ER-α and ER-β.

Because accumulation of glomerular extracellular matrix after renal injury is a precursor to the development of glomerular obsolescence and progressive loss of renal function, the ability of estrogens to inhibit fibrogenic cytokine-stimulated collagen IV synthesis may contribute to the protective effect of female gender on the progression of renal disease (33). Second-generation SERMs, such as LY-117018, may prove clinically useful in slowing the progression of chronic renal disease by reproducing the ability of estrogen to reduce glomerular matrix accumulation without reproducing its adverse effects on female reproductive tissues.

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