Expression and Regulation of Estrogen Receptors in Mesangial Cells: Influence on Matrix Metalloproteinase-9

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Abstract. Diabetic glomerulosclerosis is characterized by the accumulation of extracellular matrix (ECM) in the mesangium. Estrogens seem to retard whereas estrogen deficiency seems to accelerate progressive glomerulosclerosis. Thus, mesangial cells (MC) may be a target for estrogens. Estrogen action is mediated via estrogen receptor (ER) subtypes ERα and ERβ. Both ER subtypes were expressed in human and mouse MC. Using an estrogen-responsive reporter construct in transfection assays, it was also demonstrated that the nuclear ER were transcriptionally active. In the presence of 17β-estradiol (E2; 10⁻¹⁰ to 10⁻⁸ M), there was a progressive increase in the mRNA levels of both ERα (approximately 1.8-fold and approximately 2.7-fold after 24 and 72 h, respectively) and ERβ (approximately 1.3-fold and approximately 2.2-fold after 24 and 72 h, respectively). ERα protein levels increased approximately 2.5-fold after 24 h (10⁻¹⁰ M, E2) and up to approximately 5.4-fold after 72 h (10⁻⁹ M, E2). ERβ protein levels increased approximately 2.1-fold in the presence of E2 (10⁻⁹ M) after 24 h. Thus, estrogens positively regulate the expression of the ER subtypes, thereby maintaining or increasing MC responsiveness to estrogens. Because diabetic glomerulosclerosis may be due partly to a decrease in ECM degradation, the effects of estrogens on matrix metalloproteinases (MMP) were studied. It was found that E2 (10⁻¹⁰ to 10⁻⁸ M) increased both MMP-9 mRNA and MMP-9 activity in MC. This may be an important mechanism by which estrogens influence ECM turnover and protect against progression of diabetic glomerulosclerosis.

The accumulation of extracellular matrix (ECM) in the mesangium in diabetic glomerulosclerosis may result from an imbalance between ECM synthesis and ECM degradation (1). Mesangial cells (MC) play a central role in this process, because they synthesize and secrete both the structural components of the ECM and the enzymes that degrade them (2). Several lines of evidence suggest that there is a decrease in ECM degradation in diabetic nephropathy and that this may be due to decreased matrix metalloproteinase (MMP) synthesis and secretion by MC (3). Thus, MC provide a potential therapeutic target to modify progression in diabetic glomerulosclerosis.

The United States Renal Data System data reveal that women in the premenopausal age range have a lower incidence of end-stage renal disease (ESRD) caused by diabetic nephropathy than do men (4). After menopause, there is a sharp increase in the incidence of ESRD caused by diabetic nephropathy, especially in women from ethnic minority groups (4). For instance, the women:men ESRD incident ratio nearly doubles in African Americans (0.8 in the 35- to 39-yr age group compared with 1.5 in the 65- to 69-yr cohort). However, the female preponderance in ESRD caused by diabetic nephropathy after menopause cannot be explained by an increased incidence of diabetes in postmenopausal women compared with age-matched men. A large prospective study that examined the risk to develop type 2 diabetes mellitus in African American and Caucasian adults aged 45 to 64 yr over a 9-yr period showed a nearly equal incidence per 1000 person-years of 25.1 in African American women versus 23.4 in African American men (female:male ratio, 1.07) (5). In Caucasian study participants, there was even a male predominance in developing type 2 diabetes mellitus with a 0.67 female:male incident ratio. Thus, although other mechanisms may be invoked, estrogen status may play an important role in the protection or potentiation of progressive glomerulosclerosis. In fact, estrogens have been shown to inhibit transforming growth factor-β-mediated type IV collagen and to suppress type I collagen expression via activation of activator protein-1 (AP-1) in MC (6,7). Thus, estrogens could decrease the synthesis of ECM components, resulting in decreased accumulation of ECM in the glomerulus. Estrogens could also decrease ECM accumulation by increasing degradation of ECM by MMP. However, this has not been studied in MC.

There are two estrogen receptor (ER) subtypes, ERα and ERβ, that belong to the superfamily of nuclear receptors (8–11), which mediate the effects of estrogens. Interestingly, both ER subtypes α and β, as well as ER splice variants, have been previously identified in vascular smooth muscle cells including those from the aorta and coronary vessels (12,13–18), where the direct actions of estrogens contribute substantially to their cardiovascular protective effects (19). ER subtypes and their
regulation and biologic effects have not been reported in MC. Thus, in this study, we investigated ER expression and regulation. We also studied the effect of estrogens on MMP expression and activity in MC. MC expressed both ER subtypes ERα and ERβ, and estrogens regulated their expression. Estrogens and anti-estrogens also regulated the expression of the MMP-9 in MC. These findings may have important pathophysiological implications for sclerosing glomerular diseases, such as diabetic nephropathy.

Materials and Methods

Materials

Culture media, supplements, and primers were obtained from GIBCO Life Technologies BRL (Grand Island, NY). Charcoal-stripped fetal bovine serum (FBS) was purchased from Hyclone (Pittsburgh, PA). 17β-estradiol (E2), tamoxifen, anti-α-smooth muscle actin, and β-galactosidase substrate were purchased from Sigma (St. Louis, MO). ICI 182,780 (ICI) was obtained from Tocris (Ballwin, MO). First Strand cDNA Synthesis Kit for reverse transcription-PCR (RT-PCR) (AMV) and Taq polymerase were purchased from Boehringer Mannheim (Indianapolis, IN). For Western analysis, protein concentrations were measured by using the Pierce BCA Assay from Bio-Rad Laboratories (Hercules, CA). Prestained molecular weight markers were purchased from Bio-Rad Laboratories. ER antibodies and their respective blocking peptides were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The ERα antibody MC20 is a rabbit polyclonal antibody raised against a peptide mapping at the carboxy terminus of the ERα of mouse origin. The ERα antibody H-184 is a rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acid 2 to 185 mapping at the amino terminus of ERα of human origin. The two ERα antibodies recognize both mouse and human ERα protein. N-19, the antibody against human ERα, is a goat polyclonal antibody raised against a peptide mapping at the amino terminus of the ERα of human origin. Y-19, the antibody against mouse ERα, is a goat polyclonal antibody raised against a peptide mapping at the amino terminus of the ERα of mouse origin. Protein A-Agarose and chemiluminescence detection system were purchased from Santa Cruz Biotechnology. ERα and ERβ human recombinant peptide were purchased from Panvera (Madison, WI). Nitrocellulose membranes (Hybond ECL) and films (Hyperfilm ECL) for chemiluminescence detection were purchased from Amersham Pharmacia Biotech (Buckinghamshire, England). For the transfection studies, TransFast and lysis buffer were purchased from Promega (Madison, WI). Zymography gels were purchased from NOVEX (San Diego, CA).

Cell Culture

Human MC were obtained from microdissected glomeruli of kidneys not suitable for transplantation. Mesangial-like glomerular outgrowths were patch-cloned, propagated, and grown in Waymouth’s medium supplemented with 20% FBS, 1 mM L-glutamine, 100 µg/ml penicillin/streptomycin, and 0.075% Na2HCO3. Human MC were characterized by their stellate morphology and staining pattern, including positivity for α-smooth muscle actin (20). Experiments were performed using cells between passages 3 and 6. MC from C57BL/6J female mice used for these experiments have been previously described (21). Cells were used between passages 19 and 26. Previous experiments have shown that the cells retain their phenotype at the passages studied. Murine MC were routinely cultured in Dulbecco’s modified Eagle’s medium/F12 (3/1 vol/vol), supplemented with 20% FBS.

Cell Culture Conditions

To characterize initially the presence of ER in MC, we performed experiments on human and murine MC maintained in culture medium supplemented with 20% FBS. For the purpose of examining ER regulation, cells were transferred into phenol-red free medium supplemented with charcoal-stripped FBS, because a lipophilic impurity contained in the phenol red has been described as a weak estrogen agonist (22).

Cells were cultured in phenol red-free medium supplemented with 20% charcoal-stripped FBS in the presence of increasing concentrations of E2 (10^-10 M to 10^-8 M) for 5 d. Cell number was evaluated every 2 d with a Coulter cell counter (Hialeah, FL) and was not affected by the presence of E2 (data not shown).

To examine the regulation of ER mRNA and protein in the absence of estrogens and growth factors, we plated cells in six-well plates and maintained them in phenol red-free medium supplemented with 20% charcoal-stripped FBS. When cells were plated, cell number was adjusted according to the different conditions to obtain a similar high cell density at collection (day 1 and day 3).

To assess the functionality of endogenous ER, we performed transfection experiments. For this purpose, cells were plated, as described previously, and medium was replaced by phenol red-free medium supplemented with 0.1% charcoal-stripped FBS.

To assess the regulation of ER mRNA and protein expression, as well as the regulation of MMP-9 expression and activity by E2, we grew mouse MC initially for 3 d in phenol red-free medium supplemented with 20% charcoal-stripped FBS in six-well plates. The medium was replaced with phenol red-free medium supplemented with 0.1% charcoal-stripped FBS and increasing concentrations of E2 (10^-10 M to 10^-8 M) for 24 and 72 h. Confluent cells were harvested for RNA and/or protein collection while the supernatants were used to measure MMP-9 activity. All experiments were performed in triplicate.

Isolation of mRNA and RT-PCR

Total RNA was extracted from confluent cell cultures using the guanidium thiocyanate-phenol-chloroform method (Tri-Reagent) (23). RT was performed on 2 µg of total RNA in a total volume of 20 µl. After the total volume was adjusted to 100 µl with diethyl pyrocarbonate water, 2 µl of the cDNA solution was used as a template for the PCR. PCR amplifications were performed in a total volume of 50 µl with 1.5 U of Taq polymerase. The specificity of each reaction was monitored in control reactions, where amplifications were carried out on samples after omission of RT. Amplifications of human ER subtypes α and β in human MC were performed using specific primer pairs previously described by Enmark et al. (10), which resulted in amplicons of 344 bp and 392 bp length, respectively.

To investigate whether murine MC express ERα variants, we used a series of primers to amplify several overlapping segments spanning the entire coding regions of the murine wild-type ERα cDNA, as described previously (24). The forward primers were located in exons 1, 2, 4, and 6, whereas the reverse primers were located in exons 5, 7, and 8.

To assess ERα mRNA expression, we amplified a 408-bp cDNA fragment of the mouse ERα using forward primer TCTAACATT-GCTCTGGACAG located at nucleotides 1417 to 1438 and reverse primer CAGGACAGGTCTAGAGGG at nucleotides 1825 to 1805 (nucleotides are numbered according to the sequence published
by White et al. (9)). Restriction enzyme analysis was used to confirm the correct sequence of the amplicons (data not shown). To amplify a specific 409-bp amplicon of mouse ERβ, we used primers located at nucleotide positions 142 to 163 and 551 to 529. Nested PCR was used to confirm the correct sequence (25,26). MMP-9 and glyceraldehyde phosphate dehydrogenase (GAPDH) primer pairs were used as described previously (3), which resulted in PCR products of 414 bp and 561 bp length, respectively. PCR products were separated on 2% agarose gels containing 0.05% ethidium bromide gels and were photographed using an Alpha Innotech Digital Imaging System (San Leandro, CA). Analysis was performed using computer-aided densitometry (NIH image, NCBI, NIH, Bethesda, MD). To determine the PCR assay range, we plotted the number of PCR cycles against the integrated density obtained from the densitometry analysis. GAPDH was used as an internal standard and housekeeping gene. PCR data obtained for ERα, ERβ, and MMP-9 were normalized to GAPDH signals as described previously (3). The samples from at least three different experiments were run in duplicate.

Western Blots

Confluent cell layers were washed once in phosphate-buffered saline (PBS) and collected in the presence of lysis buffer. Cell homogenates were centrifuged 30 min at 15,000 × g at 4°C. Supernatants were collected and protein concentrations were measured. For ERβ protein analysis, samples were immunoprecipitated using the human or mouse ERβ antisera, respectively. Briefly, 150 μg of protein was incubated overnight with the antisera and protein A-agarose. The immunoprecipitates were washed four times in PBS and resuspended in 40 μl of PBS. To analyze ERα protein, we processed protein homogenates without previous immunoprecipitation. All samples were then diluted in Laemmli buffer and boiled. Equal amounts of protein or immunoprecipitates from each experimental condition were run on a 10% polyacrylamide gel. Prestained markers were electrophoresed in parallel to estimate molecular weight. Electrotransfer of proteins from the gel to the nitrocellulose was performed by electroelution (27). Immunoblotting was performed with either human or mouse ERα and ERβ antisera, and immunoreactive bands were determined by exposing the nitrocellulose blots to a chemiluminescence solution followed by exposure to a Hyperfilm ECL film. Control experiments were performed in the presence of ERα and ERβ human recombinant peptides as positive controls, whereas specificity of the signal was demonstrated by incubating blots with an excess of the corresponding specific immunizing peptide.

Transfection and Luciferase Assays

Before transfection, mouse MC were transfected into 24-well plates and cultured 4 d in phenol-red free medium supplemented with 20% charcoal-stripped FBS. Subsequently, MC were transfected with the reporter construct, pVitA2-ERE-TKLuc (0.25 μg/well) using TransFast, according to the manufacturer’s recommendations. VitA2-ERE-TKLuc contains one copy of the Xenopus vitellogenin estrogen responsive element (ERE) proximal to the thymidine kinase promoter, which drives the expression of the luciferase reporter gene in an estrogen-dependent manner. The vector pTKLuc, which does not contain an ERE, served as a control. To adjust for transfection efficiency, MC were cotransfected with pRSV-βgal (0.25 μg/well), a vector that constitutively expresses the β-galactosidase gene. The constructs were a generous gift from Drs. G. Tremblay and V. Giguere (11). One h later, phenol-red free medium supplemented with 10% charcoal-stripped FBS was added to the transfected cells. Cells were incubated for an additional 24 h in the presence of 10−8 M E2 or vehicle (ethanol). The final ethanol concentration was 0.001% in both conditions. For luciferase and galactosidase assays, cells were lysed in 100 μl of Reporter Lysis buffer at room temperature. Light emission was detected using a luminometer (AutoLumat, Wallac, Gaithersburg, MD) after addition of luciferin to 40 μl of cell lysate. Values were expressed as arbitrary light units normalized to the β-galactosidase activity of each sample.

MMP-9 Activity

The cell supernatants were collected 24 and 72 h after treatment. At the time of medium collection, the cells were counted for the purpose of adjusting the volume of the medium to the cell number. MMP-9 activity was assessed using 10% zymogram gels as described previously (28). Briefly, the medium was diluted to normalize for cell number (approximately 25,000 cells/ml) before the addition of 5X Laemmli buffer under nonreducing conditions. After electrophoresis, gels were washed for 1 h in 2.5% Triton X-100 and incubated 40 h in 50 mM Tris buffer. The gels were stained with Coomassie Blue and air-dried. Densitometry, using NIH image 1.6, was used to analyze relative MMP-9 activity.

Statistical Analyses

All experiments were performed at least in triplicate. Data are expressed as percentage of control. Shown are means ± SEM of three or four independent experiments. One-way ANOVA and Dunnett’s multiple comparison post hoc test were performed. For transfection experiments, values are expressed as arbitrary light units, normalized to the β-galactosidase activity of each sample.

Results

Expression of ERα and ERβ in Human MC

Total RNA and protein were extracted from MC between passages 3 and 6. Using RT-PCR, we detected both ERα and ERβ transcripts (shown are representative 344-bp and 392-bp amplicons of human ERα and ERβ mRNA; Figure 1A). The expression of ERα and ERβ protein was studied by Western blot analysis. Using ERα and ERβ antisera, we detected signals at approximately 66 and approximately 53 kD, respectively (Figure 1B). The estimated molecular weight of these bands corresponds to the size predicted for the wild-type human ERα and ERβ (8,10). Recombinant human ERα and ERβ peptides served as positive controls (lanes 1 and 4). Preincubation of the ER antisera with their respective immunizing peptides completely abrogated these signals, confirming that the detected bands were ERα and ERβ, respectively (data not shown). Thus, human MC express both ER subtypes ERα and ERβ.

Expression of ERα and ERβ in Mouse MC

The expression of ER was examined by RT-PCR and Western blot analysis in MC isolated from the glomeruli of C57BL/6J female mice. Representative amplicons of 408 bp and 409 bp length corresponding to mouse ERα and ERβ transcripts (9,11), respectively, are shown (Figure 2A). To exclude the presence of ERα variants, which originate from alternatively spliced ER transcripts, we amplified the entire coding region of ERα using specific primer pairs. Because all of the PCR products were of the predicted sizes, we concluded that there was no evidence of the expression of alternatively
spliced mouse ERα mRNA transcripts (data not shown). The expression of mouse ERα (approximately 67 kD) and ERβ (approximately 55 kD) protein was confirmed by Western blot analysis (Figure 2B). The corresponding signals were abrogated by preincubation with the respective immunizing peptide (data not shown). Human recombinant ERα (66 kD) and ERβ (53 kD) peptides served as positive controls. In summary, human and mouse MC expressed both ER subtypes at the mRNA and protein level.

**Transcriptional Activity of the ER in Mouse MC**

To test the ability of endogenous ER to modulate the transcriptional activity of an ERE-containing promoter, we transfected mouse MC with the reporter construct pVitA2-ERE-TKLuc (a generous gift from Drs. Tremblay and Giguere, Montreal, Canada) (11). In the transfected MC, E2 (10^{-8} M) induced approximately a twofold increase in luciferase activity (Figure 3). This demonstrates that the endogenous ER maintain their function as ligand-regulated transcription factors in MC.
Regulation of ERα and ERβ mRNA Expression by Culture Conditions in Mouse MC

Estrogens have been shown to regulate the levels of both ER subtype mRNA in reproductive tissues (29–31); however, little is known about the regulation of ER in vascular smooth muscle cells. We investigated whether E2 modulates ERα and ERβ mRNA in MC. Mouse MC were transferred into phenol red-free medium supplemented with 20% charcoal-stripped FBS to minimize the concentrations of compounds that may activate ER (32). After 1 or 3 d in this medium, levels of both ERα and ERβ mRNA were normalized to GAPDH mRNA levels. Shown is a representative graph of two independent experiments performed in triplicate.

Figure 3. Transcriptional activity of MC ER. Mouse MC were grown in phenol red-free DMEM/F12 supplemented with 20% charcoal-stripped FBS for 4 d. Transfection was performed as described in the Materials and Methods section. After transfection, cells were treated with vehicle or 10^{-8} M 17β-estradiol (E2) for 24 h in phenol red-free medium containing 20% charcoal stripped FBS. Data are expressed in relative luciferase units. Shown is a representative graph of two independent experiments performed in triplicate.

Regulation of ERα and ERβ Protein Expression by Culture Conditions in Mouse MC

Estrogens have been shown to accelerate ER protein turnover in a pituitary lactotrope cell line by a proteasome-mediated process, which in turn affects ER protein levels (33). There was approximately a 1.6-fold increase in ERα protein (Figure 5A) and approximately a 3.5-fold increase in ERβ protein levels (Figure 5B) in MC after 3 d of culture in phenol red-free medium supplemented with 20% charcoal-stripped FBS. Thus, the levels of ERα protein and the levels of ERα mRNA are discordant in MC under these culture conditions. Namely, ERα mRNA decreased and ERα protein increased, whereas both ERβ mRNA and ERβ protein increased.

Regulation of ERα and ERβ mRNA Expression by Estrogens in Mouse MC

After 3 d in culture, E2 (10^{-10} to 10^{-8} M) was added. There was a progressive increase in both ERα and ERβ mRNA levels. ERα mRNA levels were approximately 1.8-fold higher (183.8 ± 27.0% compared with baseline conditions after 24 h and increased to approximately 2.7-fold (276.4 ± 43.0%; P < 0.01) after 72 h in the presence of 10^{-9} M E2 (Figure 6A).

The increase in ERβ mRNA levels peaked at 10^{-9} M E2. The ERβ mRNA levels were approximately 1.3-fold higher (127.4 ± 14.5%) at 24 h, and there was approximately a 2.1-fold (211.8 ± 50.0%; P < 0.05) increase at 72 h (Figure 6B).

In summary, in the presence of physiologic concentrations of E2, the mRNA levels of both ER subtypes increased in MC cultured in phenol red-free medium supplemented with charcoal-stripped serum.

Regulation of ERα and ERβ Protein Expression by Estrogens in Mouse MC

E2 (10^{-10} to 10^{-8} M) was added to MC after 3 d of culture in phenol red-free medium supplemented with 20% charcoal-stripped FBS. There was an increase in the protein level of both ER subtypes. The maximal increase in ERα protein level (approximately 5.4-fold) was seen at 10^{-7} M E2 (P < 0.05) at 72 h (Figure 7A). ERβ protein levels increased approximately 2.1-fold in the presence of 10^{-9} M of E2 (P < 0.05) after 24 h (Figure 7B). It is interesting to note that the ERβ protein levels after 72 h were 2.3-fold higher than in the cells at 24 h.

In summary, this demonstrates that estrogens affect the ER protein levels in MC and may maintain or even increase estrogen responsiveness in this cell type.

MMP-9 mRNA Expression and MMP-9 Activity by Estrogens and Antiestrogens in Mouse MC

The levels of MMP-9 mRNA increased after 24 h in the presence of E2 (10^{-10} to 10^{-8} M) in cells that had been cultured 3 d in phenol red-free medium supplemented with 20% charcoal-stripped FBS. The maximal MMP-9 mRNA increase was approximately 1.7-fold (175.4 ± 25.0% of control; P < 0.01) in the presence of 10^{-8} M E2 after 24 h (MMP-9 mRNA was normalized to GAPDH mRNA). No changes in MMP-9 mRNA levels were observed after 72 h (Figure 8A). MMP-9 activity also changed markedly after treatment with E2. There was approximately a 3.3-fold increase (328.4 ± 73.4% of control; P < 0.05) in MMP-9 activity after 24 h of treatment with 10^{-8} M E2 (Figure 8B). After 72 h, there was approximately a 2.4-fold increase (238.3 ± 37.5% of control; P < 0.05) in MMP-9 activity in the presence of 10^{-8} M E2 (data not shown). These changes were abolished in the presence of the selective estrogen receptor modulator tamoxifen or the ER antagonist ICI 182,780 (Figure 9). Tamoxifen (10^{-6} M) and ICI (10^{-8} M) blocked the E2-induced increase in MMP-9 activity in the mouse MC (60.1 ± 15.4% and 59.9 ±
10.4% of control, respectively) during a 24-h incubation period. Neither of these inhibitors significantly affected baseline MMP-9 activity (126.7 ± 27.45% and 75.7 ± 26.7%, respectively). These studies demonstrate that E2 upregulates MMP-9 expression in mouse MC.

Discussion

MC play a central role in the progression of diabetic nephropathy (1,2). Estrogen deficiency in postmenopausal women is associated with a relative increase in the female: male ratio of ESRD caused by diabetic nephropathy (4). This may be due to the effects of estrogens on ECM turnover in the glomerulus, because MC synthesize both ECM components and MMP (2). Thus, estrogen replacement may halt the progression of diabetic glomerulosclerosis in states of estrogen deficiency, i.e., postmenopausal women. The addition of estrogens led to decreased type I collagen synthesis and reduced transforming growth factor-β1–mediated type IV collagen synthesis in mouse MC isolated from male mice (6,7). However, the expression of ER and their subtypes in MC, and their regulation by estrogens, remained to be elucidated. Furthermore, the effects of estrogens on MMP, which play a crucial role in ECM degradation, had not been previously investigated.

In this study, we identified the two known nuclear ER subtypes, ERα and ERβ, in both human and mouse MC (8–11). We detected the expression of the two ER subtypes at the mRNA level by RT-PCR and confirmed translation into their respective proteins by Western blot analysis. Using an estrogen-responsive reporter construct, we demonstrated further that the nuclear ER were transcriptionally active in MC. Furthermore, MC, similar to the findings in arterial vascular smooth muscle cells, expressed both ER subtypes ERα and ERβ (12–18). It is interesting to note that in experiments with ERα or ERβ knockout mice, the expression of one of the ER subtypes provided protection in a model of arterial vascular injury (34,35). This suggested some degree of redundancy in ERα and ERβ function in arterial vascular smooth muscle cells.
Nonetheless, this functional redundancy may be restricted to specific vascular sites and may not apply to MC.

The modulation of ER by estrogens has been studied extensively in reproductive organs and breast cancer cells. Estrogens either up- or downregulated ER expression, depending on the cell type or cell line (29,31,36). There have been no reports on the regulation of ER subtype expression in MC, and little is known about ER regulation in vascular smooth muscle cells from other vascular beds.

We found that cell culture conditions have a substantial impact on ER levels. The culture conditions that we chose consisted of phenol red-free medium supplemented with charcoal-stripped FBS, a condition generally accepted for studying steroid hormone effects. Phenol red-free medium was selected because phenol red supplements may contain lipophilic impurities, which have weak estrogen agonist activity (22). Charcoal treatment removes steroid hormones and numerous other substances, including growth factors (32,37). After transfer of MC into phenol red-free medium supplemented with charcoal-stripped FBS, there were changes in the ER subtype mRNA levels. ERα mRNA levels decreased after 1 d, and this decrease persisted until day 3, whereas ERβ mRNA increased and reached a maximum after 72 h. Thus, charcoal treatment had discordant effects on the regulation of ER subtype transcription. This observation also suggested that the regulatory regions of the genes for the two ER subtypes may differ in their organization, given that the transcription of the ER subtypes was either decreased (ERα) or increased (ERβ) when phenol red was removed from the basal medium in the presence of charcoal-stripped serum. After incubation in E2-containing medium for 24 or 72 h, we observed an increase in mRNA and protein levels of both ER subtypes at physiologic estrogen concentrations. Thus, the increase in ER protein synthesis seems to be only partially offset by accelerated ER protein turnover, a proteolytic process that occurs rapidly in the presence of E2 (33). These findings provide evidence that the levels of ER protein are sustained or increased in MC in the presence of estrogens. Thus, lack of estrogen for an extended time period, as in menopause, may decrease the capacity to mount an estrogen response. It should be noted that the MC used in these experiments were isolated from young, thus “premenopausal,” female C57BL/6J mice (21).

The increased incidence of ESRD caused by diabetic nephropathy in postmenopausal women from ethnic minority
groups suggests that the accumulation of ECM is accelerated in estrogen deficiency states (4). Because MMP play an important role in ECM turnover, they may be an important mesangial target gene for estrogens (3). Increased MMP levels contribute to ECM degradation, which could have a protective role in progressive glomerulosclerosis. MMP-2 and MMP-9 belong to a subgroup of matrix-degrading enzymes that exhibit high activity against gelatin and native type IV collagen (3,38). In the present study, we focused on MMP-9. We found that E2 induced MMP-9 mRNA expression and activity in a dose-dependent manner. Tamoxifen, a selective estrogen receptor modulator, and the anti-estrogen ICI 182,780 blocked estrogen-induced MMP-9 activity, suggesting that these effects were ER mediated. The molecular mechanisms by which estrogens regulate MMP-9 transcription in MC are unknown. The MMP-9 promoter does not have a consensus ERE but contains several other important regulatory elements, including
three GC boxes, four AP-1 like binding sites, an AP-2 site, and three PEA3 consensus sequences, as well as a nuclear factor-κB (NF-κB) binding site (39). Several of these transcription factors, including the AP-1 complex or NF-κB, have been shown to interact with ER (40). Importantly, at AP-1 binding sites, the two ER subtypes ERα and ERβ signal in opposite directions upon ligation with E2 in vitro (41). Whereas E2 stimulation of ERα increases the transcriptional process, activation of ERβ by its natural ligand downregulates gene transcription at an AP-1 site. The expression of both ER subtypes and the potential of ERα and ERβ to form heterodimers, combined with the presence of four AP-1 binding sites in the 5′-flanking region, add several layers of complexity to the regulation of MMP-9 expression (39,42). In addition, ER have also been shown to interact with NF-κB, a transcription factor that is important in inflammatory processes (43). The complexity of the MMP promoter region may allow precise regulation of MMP-9 expression.

The present data suggest that one mechanism, by which estrogens contribute to the protection from ESRD caused by diabetic nephropathy in premenopausal women, is increased MMP-9 expression in MC. In addition, the data may inject a note of caution in the use of tamoxifen in postmenopausal women with diabetic glomerulosclerosis, because tamoxifen blocked upregulation of MMP-9 expression by estrogens.

In summary, we found that both ER subtypes were expressed in MC and that estrogens positively regulated their transcription and translation. Thus, estrogens maintain or in-

Figure 8. E2 increases matrix metalloproteinase-9 (MMP-9) mRNA expression and activity. Mouse MC were grown for 3 d in phenol red-free DMEM/F12 supplemented with 20% charcoal-stripped FBS. Medium was replaced with phenol red-free DMEM/F12 medium supplemented with 0.1% charcoal-stripped FBS and E2 (10⁻¹⁰ to 10⁻⁸ M) for 24 and 72 h. (A) MMP-9 mRNA expression (normalized to GAPDH) in the presence of E2 for 24 h (●) and 72 h (□). (B) MMP-9 activity as assessed by zymography 24 h after E2 addition. Data are expressed as percentage of control (V = 0.001% EtOH) at 24 h. Shown are means ± SEM of three independent experiments. Statistical significance is indicated by ** (P < 0.01) and * (P < 0.05) for comparison with 24-h baseline (V, ●) and by ▼ (P < 0.05), for comparison with 72-h baseline (V, □).

Figure 9. E2 increases MMP-9 activity: An effect abolished by ER antagonists. Mouse MC were grown for 3 d in phenol red-free DMEM/F12 supplemented with 20% charcoal-stripped FBS. Medium was replaced with phenol red-free DMEM/F12 medium supplemented with 0.1% charcoal-stripped FBS with vehicle, 10⁻⁸ M E2, 10⁻⁶ M tamoxifen (T) alone or in combination with 10⁻⁸ M E2, 10⁻⁶ M ICI alone or in combination with 10⁻⁶ M E2 for 24 h. When T and ICI were used in combination with E2, cells were incubated for 1 h with the antagonists before the addition of E2. (A) A representative zymography assessing MMP-9 activity in the presence of E2 with or without ER antagonists for 24 h (M, markers). (B) Data are expressed as percentage of control (V = 0.001% EtOH). Shown are means ± SEM of three independent experiments. Statistical significance is indicated by ** (P < 0.01) for the comparison, E2 versus V and by ▼▼ (P < 0.01), for comparisons E2 versus T+E2 or ICI+E2.
crease the estrogen responsiveness in MC. We found that estrogens upregulated MMP-9 expression and activity in MC. This may be an important mechanism by which estrogens influence ECM turnover and exert their protective effect on the progression of diabetic glomerulosclerosis.

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