Estradiol upregulates mesangial cell MMP-2 activity via the transcription factor AP-2

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METHODS

We have previously shown that gender exerts a major impact on the rate of progression of chronic renal disease (17, 28). We performed a meta-analysis examining the effect of gender on the rate of deterioration of renal function in non-diabetic renal disease using 68 studies that contained a total of 11,345 patients (17). The results indicated that men with chronic renal failure due to IgA nephropathy, membranous nephropathy, polycystic kidney disease, or unspecified chronic renal disease show a more rapid decline in renal function with time than do women (17). Sex hormones may directly influence many of the processes implicated in renal disease progression, including mesangial matrix synthesis and degradation and the synthesis and release of various cytokines, vasoactive agents, and growth factors (19, 28). In animal models of renal disease, manipulation of the hormonal milieu by gland ablation or by the administration of exogenous sex hormones replicates the effects of gender on the course of chronic renal disease (19, 28). These data suggest that the impact of gender on renal disease progression may reflect direct receptor-mediated effects of sex hormones (19, 28).

The accumulation of extracellular matrix in the glomerular mesangium reflects the net balance between the synthesis and degradation of matrix components (20). Mesangial cells secrete matrix metalloproteinases (MMPs) that degrade intact glomerular basement membrane, gelatin, and soluble type IV collagen at neutral pH (3, 11). Metalloproteinases and their specific inhibitors, tissue inhibitors of metalloproteinase (TIMPs), play an important role in regulating glomerular matrix remodeling (3, 11, 20). Thus disturbances in the balance between matrix synthesis and matrix degradation may contribute to the progression of glomerular sclerosis in chronic renal disease (3, 11, 20). Accordingly, in the present study, we sought to determine whether differing effects of sex hormones on MMP-2 and TIMP-2 activity might contribute to the sexual dimorphism that characterizes the progression of chronic renal disease.

Isolation and characterization of murine mesangial cells. Mesangial cells were isolated from kidneys of 8- to 10-wk-old naive SJL/J (H-2b) mice by differential glomerular sieving.

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The present studies were performed on a well-characterized, immortalized, differentiated murine mesangial cell line transformed with nonreplicating, non-capssid-forming SV40 virus (strain Rh 911) (10). To exclude the possibility that our results were influenced by SV40 transformation per se, all experiments were repeated in nontransformed male murine mesangial cells. Identical results were obtained in transformed and nontransformed cells. Data are reported for transformed cells only. Cells were not tested for mycoplasma contamination.

Collection of conditioned media. Cells were washed, and media were replaced with serum-free, phenol red-free RPMI media supplemented with lactalbumin hydrolysate (0.2%) with or without estradiol or testosterone for 72 h before collection of conditioned media. The cell layer was harvested for determination of total protein (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA). Conditioned media were concentrated by centrifugation (Centricon-10 microconcentrator, Grace, Beverly, MA), and the final volume was adjusted to 100 μl of a reaction buffer consisting of (in mM) 25 HEPES, pH 7.5, 1.2 dithiothreitol, 4 MgCl₂, and 150 NaCl, as well as 5% glycerol, 0.005% bromophenol blue, and 0.05% Nonidet P-40 (Sigma). The mixture was incubated on ice for 15 min followed by the addition of 10 fmol of [3P] end-labeled AP-2 consensus binding sequence oligonucleotide (nucleotide sequence: 5'-GAT CGA ACT GAC CGC CCG CCC GT-3'; Promega, Madison, WI). 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 for 30 min. The gels were then stained for 2 h with 0.1% Coomassie blue in distilled water. Removal of SDS allows the MMP proenzyme to renature in an active form, enabling detection byzymography. Gels were incubated overnight at 37°C in reaction buffer (0.05 M Tris·HCl, 0.2 M NaCl, 10 mM CaCl₂, pH 7.4). Gels were then stained for 2 h with 0.1% Coomassie blue in distilled H₂O, 50% methanol, and 10% acetic acid, followed by destaining for 5 min with 20% methanol and 10% acetic acid in distilled H₂O. Gelatinolytic activity was demonstrated as a clear band against a blue background of stained gelatin. Bands were quantitated by laser densitometry.

The inhibitory profile of the gelatinases was assessed by addition of the following inhibitors to the reaction buffer (in mM): 10 EDTA, 10 EGTA, 2 phenanthroline, 200 leupeptin, and 1 phenylmethylsulfonyl fluoride, as well as 10 mg/ml pepstatin (all from Sigma).

Reverse zymography. To assess TIMP-2 activity in mesangial cell conditioned media, we performed reverse zymography utilizing reagents obtained from University Technologies International (Calgary, BC). The protocol employed was identical to that described for zymography except that matrix MMP-2 was incorporated into the acrylamide-gelatin gel matrix. Concurrent TIMP-1, -2, and -3 standards were electrophoresed as positive controls. Most of the resultant gel does not stain with Coomassie brilliant blue because the embedded gelatin was degraded by embedded MMP. Bands staining with Coomassie blue reflect inhibition of MMP activity by TIMPs within the conditioned media.

Western blotting. Media were collected and concentrated to an identical final volume using a Centriprep-10 microconcentrator (Grace). Protein content was measured in a 0.1 ml aliquot of the concentrated media (Bio-Rad Protein Assay). SDS-PAGE was performed, and proteins were transferred to a polyvinylidene difluoride microporous membrane as previously described (27). MMP-2 protein (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a positive control. Goat polyclonal antihuman MMP-2 (Santa Cruz Biotechnology) was used to detect MMP-2 protein, rabbit polyclonal anti-human AP-2α (Santa Cruz Biotechnology) was used to detected AP-2 protein, and goat polyclonal anti-TIMP-2 antibody (Santa Cruz Biotechnology) was used to detect TIMP-2 protein by Western blotting, as previously described (27). We determined that the amount of protein loaded into each lane was within the linear range of detection by Western blotting in our system. Negative controls using irrelevant antibodies and positive controls were also performed as indicated.

Electrophoretic mobility shift assay. Mesangial cell nuclear extracts were prepared as previously described (10). Four micrograms of nuclear extract were mixed with 20 μg of poly (dI:dC) in 20 μl of a reaction buffer consisting of (in mM) 25 HEPES, pH 7.5, 1.2 dithiothreitol, 4 MgCl₂, and 150 NaCl, as well as 5% glycerol, 0.005% bromophenol blue, and 0.05% Nonidet P-40 (Sigma). The mixture was incubated on ice for 15 min followed by the addition of 10 fmol of [3P] end-labeled AP-2 consensus binding sequence oligonucleotide (nucleotide sequence: 5'-GAT CGA ACT GAC CGC CCG CCC GT-3'; Promega, Madison, WI). 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-2 consensus binding sequence oligonucleotide.

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

RESULTS

Estradiol stimulated MMP-2 enzyme (gelatinase) activity, as assessed by zymography and increased MMP-2 protein levels, as assessed by Western blotting, in a dose-dependent manner (Fig. 1, A and B). The effect of estradiol on MMP-2 gelatinase activity and protein levels achieved statistical significance at a physiological concentration of estradiol (10⁻¹⁰ M). Gelatinase activity 194.6 ± 17.8, P < 0.05, was expressed as a percentage of values obtained in control mesangial cells grown in serum-free, phenol red-free RPMI media in the absence of estradiol (MMP-2 protein: 145.2 ± 2.5, P < 0.05).

The gelatinase activity identified by zymography was completely abolished by calcium chelation (EDTA or EGTA) and by zinc chelation (phenanthroline), whereas inhibitors of serine, cysteine, and aspartic acid proteases had no effect on gelatinase activity (data not shown).

The ability of estradiol (10⁻⁹ M) to stimulate gelatinase activity and increase MMP-2 protein levels was receptor mediated, insofar as the increase was reversed by ICI-182,780 (10⁻⁸ M; Torcis Cookson, Ballwin, MO), a pure estrogen receptor antagonist (gelatinase activity: 192.5 ± 31.3 vs. 87.9 ± 15.6, P < 0.05; MMP-2 protein: 156.7 ± 12.2 vs. 107.9 ± 10.2, P < 0.001) (Fig. 2, A and B). The ability of estradiol (10⁻⁹ M) to stimulate gelatinase activity and increase MMP-2 protein levels was also reversed by PD-98059 (100 μM; Calbiochem, La Jolla, CA), an MEK 1,2 inhibitor that blocks the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK)
cascade (gelatinase activity: 195.5 ± 31.3 vs. 104.8 ± 8.5, P < 0.05; MMP-2 protein: 156.7 ± 12.2 vs. 110.4 ± 8.2, P < 0.001) (Fig. 2A and B). The effect of estradiol (10^{-9} M) on gelatinase activity and MMP-2 protein was also reversed by curcumin (1 μM; Sigma), an inhibitor of both the ERK and c-Jun NH2-terminal kinase (JNK) signaling cascades (gelatinase activity: 192.5 ± 31.3 vs. 103.8 ± 7.9, P < 0.001; MMP-2 protein: 156.7 ± 12.2 vs. 97.9 ± 6.4, P < 0.001) (Fig. 2A and B). ICI-182,780, PD-98059, or curcumin alone had no effect on gelatinase activity or MMP-2 protein levels (gelatinase activity: 101.2 ± 5.8, 115.9 ± 12.2, 108.5 ± 7.6, not significant (NS) vs. control; MMP-2 protein: 106 ± 7, 97.8 ± 5.4, 101.4 ± 8.1, NS).

The ability of estradiol (10^{-9} M) to stimulate gelatinase activity was dependent on new protein synthesis insofar as cycloheximide pretreatment (10 μg/ml, 4 h) reversed this effect [201.9 ± 3.8 (estradiol) vs. 118.9 ± 10.4 (estradiol+ cycloheximide), P < 0.004] (Fig. 2C). Cycloheximide alone had no effect on MMP-2 activity (104.4 ± 9.5, NS vs. control).

AP-2 protein levels were markedly increased by estradiol (10^{-9} M) (see Fig. 4A). The stimulatory effect of estradiol on AP-2 protein levels was evident at 1 h (358.7 ± 10.2, expressed as a percentage of control values, arbitrarily set at 100%, P < 0.001), but levels returned to baseline by 4 h (101.1 ± 6.2, NS vs. control) (Fig. 3A). The ability of estradiol to increase AP-2 protein levels at 2 h was reversed by PD-98059 (100 μM; 328.3 ± 10.3 vs. 96.5 ± 2.1, P < 0.001) and by curcumin (1 μM; 103.2 ± 6.3, P < 0.001 vs. estradiol).
alone) (Fig. 3B). The effect of estradiol was receptor mediated, as evidenced by its reversal by ICI 182,780 (10^{-8} M; 98.5 ± 3.4, P < 0.001 vs. estradiol alone). PD-98059, curcumin, or ICI-182,780 alone had no effect on AP-2 protein levels (97.9 ± 15.6, 102.4 ± 7.2, NS vs. control) (not shown).

Nuclear extracts from mesangial cells exposed to estradiol (10^{-9} M) for 4 h showed markedly increased binding to an AP-2 consensus binding sequence oligonucleotide (358.8 ± 10.3, P < 0.001, expressed as a percentage of values obtained in control serum-free, phenol red-free RPMI media) (Fig. 3C). This effect was no longer present after 6 h of exposure (98.2 ± 5.2, NS vs. control). No band was observed in the absence of nuclear extracts, and binding was competitively inhibited by a 200-fold excess of unlabeled AP-2 consensus binding sequence oligonucleotide. The ability of estradiol to increase the binding of mesangial cell nuclear extracts to the AP-2 consensus binding sequence oligonucleotide was completely reversed by PD-98059 (100 μM; 102.9 ± 8.9, P < 0.001) and by curcumin (1 μM; 109.8 ± 10.4, P < 0.001).

Estradiol had no effect on TIMP-2 protein levels as assessed by Western blotting (Fig. 4A) and reverse zymography (Fig. 4B).

Testosterone (10^{-11} to 10^{-6} M) had no effect on MMP-2 activity as assessed by zymography (Fig. 4B) or TIMP-2 activity as assessed by reverse zymography (data not shown).

**DISCUSSION**

Mesangial cells in culture secrete a latent 72-kDa proenzym metalloproteinase (3, 11). Activation of this proenzyme occurs via an activation cascade that is initiated when an activator proteinase disrupts interactions between a cysteine residue within the cysteine switch sequence and the catalytic zinc atom (15). This cleavage leads to a conformational change that renders the intermediate molecule subject to a second proteolysis, which generates the active enzyme (15). Active MMP exhibits calcium and zinc dependence and is inhibited by chelators of these metals (3, 11).

The proenzyme is secreted complexed to a 21-kDa tissue inhibitor of metalloproteinase (TIMP-2) (3, 11). The binding of TIMP-2 to the activation site of MMP-2 (Fig. 4B).
inhibits catalytic activity (5, 29). Although synthesis of MMP-2 and TIMP-2 are independently regulated, potential metalloproteinase activity is determined by the level of MMP-2 relative to that of TIMP-2 (5, 29). Thus, a reduction in TIMP-2 levels facilitates MMP activation (5, 29).

It has been suggested that increased metalloproteinase activity may contribute to inflammatory glomerular injury by facilitating proteolysis of glomerular basement membrane and non-basement membrane proteins (1, 2, 7, 11, 20). On the other hand, decreased net activity of MMP may contribute to progressive glomerular sclerosis in noninflammatory renal diseases by promoting extracellular matrix accumulation (11, 20). Reduced MMP-2 activity contributes to the glomerular sclerosis that accompanies aging or after puromycin administration (8). In puromycin nephrosis, reduced net MMP activity is a consequence of increased synthesis of tissue inhibitors of metalloproteinase (8).

Male gender is associated with a more rapid rate of progression of chronic renal disease (17, 28). In this context, we have previously shown that estradiol suppresses mesangial cell type I and IV collagen synthesis (9, 10, 16, 18, 26, 27). However, the effects of sex hormones on MMP and TIMP activity and protein synthesis in the kidney have not been extensively studied. Estradiol has been shown to increase MMP-9 mRNA and MMP-9 activity in cultured human mesangial cells (22). In preliminary reports, Potier et al. (23, 24) showed that estradiol induced a dose-dependent increase in MMP-2 activity and mRNA in C57Bl/6J murine mesangial cells and increased MMP-2 activity in human mesangial cells. However, no effect was observed in mesangial cells from ROP mice (23). Several studies have examined the influence of gender on renal metalloproteinase activity in aging rats (4, 25). Reckelhoff and Baylis (25) found that glomerular MMP activity increased in aging intact or ovariec-tomized female rats and in castrated males but did not increase with age in intact males. Franki et al. (4) found increased expression of TIMP-2 with aging in the renal cortex from male but not female rats.

We measured gelatinase activity in media conditioned by mesangial cells exposed to estradiol or testosterone. We found that estradiol stimulates MMP-2 activity in a dose- and receptor-dependent manner. Increased MMP-2 activity reflected increased synthesis of MMP-2 protein. The ability of estradiol to increase MMP-2 protein was due to upregulation of AP-2 transcriptional activity, which in turn was mediated by increased AP-2 protein levels. The ability of estradiol to upregulate MMP-2 enzyme activity and protein levels was blocked by PD-98059, a selective inhibitor of the ERK signaling pathway at the level of MAP kinase 1,2. Because we have previously shown that estradiol rapidly activates the ERK/MAPK pathway (18), our studies with PD-98059 suggest that the effects of estradiol on MMP-2 are mediated through ERK signaling. Estradiol had no effect on TIMP-2 activity or protein levels. In contrast, testosterone had no effect on MMP-2 or TIMP-2.

The transcription factor AP-2 plays an important role in the regulation of MMP-2 gene transcription (12, 13). In the rat MMP-2 gene, Mertens et al. (13) have described a 40-base pair (bp) cis-acting enhancer sequence located -1,322 to -1,282 bp relative to the transcription start site. This sequence contains overlapping binding sites for the transcription factors AP-2 and YB-1 (12). AP-2 facilitates the binding of YB-1 to this sequence (12). This interaction is responsible for the synergistic activation of MMP-2 gene transcription by AP-2 and YB-1 (12). The human MMP-2 gene also contains a potent enhancer element in its 5′-flanking region, which contains an AP-2 binding sequence (12). Although the human enhancer does not contain a YB-1 binding sequence, both YB-1 and AP-2 are components of the complex that binds to and transactivates the enhancer element (12). Thus in both rats and humans, interactions between AP-2 and YB-1 regulate MMP-2 gene expression at the level of potent enhancer elements (12).

Activation of the ERK/MAPK cascade has previously been shown to upregulate AP-2/DNA binding activity (6, 14). In hamster fibroblasts, activation of the ERK/MAPK cascade increases binding of AP-2 to the vascular endothelial growth factor promoter (14). Similarly, activation of ERK/MAPK by phorbol-myristate acetate increases binding of AP-2 to the PAC-1 promoter to induce gene transcription (6). The mechanism whereby ERK/MAPK upregulates AP-2 binding activity, whether by increased binding affinity, posttranslational phosphorylation, or increased protein levels, has not been established.

Because the transcription factor AP-2 has been implicated as an important regulator of MMP-2 gene transcription in mesangial cells and because ERK/MAPK is known to regulate AP-2 activity, we examined the role of AP-2 in mediating the effects of estradiol on MMP-2. We found that physiological concentrations of estradiol markedly increased AP-2 protein levels in a receptor-dependent manner. This increase was mediated through the ERK/MAPK pathway insofar as the ability of estradiol to raise AP-2 levels was reversed by PD-98059, an inhibitor of the ERK/MAPK cascade and by curcumin, which down-regulates ERK and JNK pathway signaling (21). Increased AP-2 levels induced by estradiol were associated with an increase in the binding of mesangial cell nuclear extracts to an AP-2 consensus binding sequence oligonucleotide. Enhanced AP-2/DNA binding activity was reversed by PD-98059 and curcumin. In turn, PD-98059 and curcumin blocked the ability of estradiol to increase MMP-2 protein levels and gelatinase activity. These data suggest that estradiol enhances MMP-2 activity by upregulating AP-2 transcription of the MMP-2 promoter and that estradiol-induced AP-2 transcriptional activation is mediated by MAPK signaling. Contrary to these findings, estradiol has been shown to downregulate expression of AP-2 protein in human breast cancer cells without affecting AP-2/DNA binding activity (21).
Although curcumin has been widely utilized as a tool to selectively block the JNK pathway, it also inhibits ERK signaling, albeit at a higher 50% inhibition concentration (21). In murine mesangial cells, ERK/MAPK signaling is strongly inhibited by 1 μM curcumin, a concentration that is without effect in Jurkat T cells (18). Thus, although we have clearly established a role for ERK signaling in mediating estradiol-stimulated AP-2 levels, the data do not necessarily demonstrate a role for the JNK pathway.

Wingrove et al. (30) found that estradiol upregulates MMP-2 protein in human coronary artery and umbilical artery vascular smooth muscle cells in a receptor-dependent manner. It was hypothesized that estrogen directly activates MMP-2 gene transcription because the MMP-2 gene promoter contains three half-palindromic estrogen response elements (30). However, our data do not support direct transcriptional activation of MMP-2 by estradiol in murine mesangial cells because activation of MAPK signaling is required for estradiol to exert its stimulatory effect.

Our earlier demonstration that estradiol suppresses type I and IV collagen synthesis and our present finding that estradiol stimulates MMP-2 activity in mesangial cells suggest that estradiol shifts the balance of matrix metabolism away from matrix accumulation and glomerulosclerosis. These effects of estradiol on collagen metabolism may contribute to the protective effect of female gender on renal disease progression. However, because our studies were performed under traditional culture conditions, which increase MMP synthesis compared with cells cultured in a three-dimensional matrix, our conclusions are subject to this limitation.

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REFERENCES