Estradiol suppresses mesangial cell type I collagen synthesis via activation of the MAP kinase cascade

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We have previously shown that estradiol suppresses type I collagen synthesis by murine mesangial cells grown in the presence of serum via activation of the transcription factor activator protein-1 (AP-1). We hypothesized that estradiol activates this pathway in several other cell types (7, 8, 21, 27, 32), we postulated that estradiol suppresses type I collagen synthesis by murine mesangial cells by enhancing AP-1 activity via activation of the MAP kinase pathway.

METHODS

Isolation and characterization of murine mesangial cells. Mesangial cells were isolated from kidneys of 8- to 10-wk-old naive male SJL/J (H-2b) mice by differential glomerular sieving. Studies were performed on an immortalized, differentiated murine mesangial cell line transformed with nonreplicating, noncapsid-forming SV40 virus (strain Rh 911). The cells express receptors for angiotensin II (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. To exclude the possibility that SV40 transformation per se might influence our results, replicate studies were performed with nontransformed mesangial cells, passages 5–7. Cells were grown in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) containing 10% fetal calf serum (FCS, GIBCO) and 1% penicillin-streptomycin (Sigma Chemical, St. Louis, MO). Results obtained with transformed and nontransformed cells were identical, and the data have been combined.

Western blotting. Mesangial cells were plated on six-well plates (Becton Dickinson, Franklin Lakes, NJ) and grown in phenol red-free DMEM medium supplemented with 10% FCS (GIBCO). Cells were grown to confluency and growth arrested before the experiments. The final estradiol concentration in the medium was <10⁻¹¹ M (manufacturer’s specifications). Cells were exposed to 17β-estradiol (10⁻¹⁰ to 10⁻⁷ M, Sigma), 17α-estradiol (10⁻⁷ M), a time-dependent mixed agonist-antagonist of 17β-estradiol, ICI-182780, a high-affinity estrogen receptor antagonist (Tocris Cookson, St. Louis, MO), PD-98059 (50 or 100 µM), a highly selective inhibitor of MAP kinase-extracellular signal-regulated protein kinase (ERK) 1 (MEK1) and MEK2, and genistein (18 µM), a nonselective tyrosine kinase inhibitor (Sigma). Cells were grown to confluency and growth arrested before the experiments. The final estradiol concentration in the medium was <10⁻¹¹ M (manufacturer’s specifications). Cells were exposed to 17b-estradiol (10⁻¹⁰ to 10⁻⁷ M, Sigma), 17α-estradiol (10⁻⁷ M), a time-dependent mixed agonist-antagonist of 17b-estradiol, ICI-182780, a high-affinity estrogen receptor antagonist (Tocris Cookson, St. Louis, MO), PD-98059 (50 or 100 µM), a highly selective inhibitor of MAP kinase-extracellular signal-regulated protein kinase (ERK) 1 (MEK1) and MEK2 (Calbiochem, La Jolla, CA), genistein (18 µM), a nonselective tyrosine kinase inhibitor (Sigma), sodium orthovanadate (1 mM), a tyrosine phosphatase inhibitor (Sigma), platelet-derived growth factor (PDGF)-AB (10 ng/ml; Upstate Biotechnology, Lake Placid, NY), ANG II (10⁻⁶ M, Sigma), or control medium for the indicated time periods. In studies in which we measured type I collagen secreted in the medium, cells were grown in the presence of β-aminopropionitrile (80 µg/ml) with or without ascorbic acid (50 µg/ml, Sigma). In addition, the medium contained a metalloproteinase inhibitor (1,10-phenanthroline (10 µM)) and a commercial protease inhibitor cocktail containing serine, cysteine, and aspartic protease inhibitors, and an aminopeptidase inhibitor (Sigma).

Medium was collected and concentrated using an Amicon Centricon-10 concentrator (Grace, Beverly, MA). Protein concentrations were measured by the Lowry method (1). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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content was measured in a 0.1-ml aliquot (Bio-Rad Protein Assay, Richmond, CA). To prepare the samples for SDS-PAGE electrophoresis, 1 ml of 10% trichloroacetic acid was added to 4 mg protein of concentrated medium, 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% bromphenol blue, 2% 5-mercaptoethanol, pH 7.6, all reagents from Sigma), boiled for 5 min, and then immediately placed on ice. The sample (50 μg) was loaded in each well, and SDS-PAGE electrophoresis was performed by standard techniques and proteins transferred to a polyvinylidene difluoride microporous membrane.

After blotting, the membrane was immediately placed into blocking buffer [2% BSA 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 primary antibody. The membrane was incubated with the primary antibody solution on a shaking apparatus for 30 min at 37°C. The membrane was next washed for 30 min with agitation. The membrane was then placed in 5% nonfat milk in wash buffer containing secondary antibody conjugated to horseradish peroxidase (Sigma). 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 in our studies included goat anti-bovine type I collagen antibody (Southern Biotechnology, Birmingham, AL; 1:250 dilution). We determined that the amount of protein loaded into each lane was within the linear range of detection of type I collagen by Western blotting in our system. Human type I collagen (Sigma) was used as a positive control in Western blotting experiments. Appropriate negative controls using irrelevant antibodies were also performed. The anti-type I collagen antibody did not significantly cross-react with type IV collagen (Sigma).

We also utilized polyclonal rabbit anti-phospho-specific p44/p42 MAP kinase antibody, which detects ERK1 and ERK2 (p44 MAP kinase and p42 MAP kinase, respectively) only when activated by phosphorylation at Thr202 and Tyr204 by the upstream kinase 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 (28). The nuclei were resuspended in hypotonic buffer (in mM, 10 HEPES, pH 7.9, 1.5 MgCl2, 10 KCl, 0.2 phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol, Sigma). While being stirred gently, 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. Nuclear extracts (4 μg) 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 dithiothreitol, 4 mM MgCl2, 150 mM NaCl, 5% glycerol, 0.005% bromphenol blue, and 0.05% Nonidet P-40 (Sigma) (28). The mixture was incubated on ice for 15 min followed by addition of 10 fmol of 32P end-labeled AP-1 consensus binding sequence oligonucleotide (Promega, Madison, WI) (nucleotide sequence: 5′-CGTTGATGATCGAGCCGGAA-3′). 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 (Molecular Dynamics, model 3005). Competition experiments were performed with a 200-fold excess of unlabeled AP-1 consensus binding sequence oligonucleotide.

Messenger RNA for type I collagen. The steady-state message for the α1 chain of procollagen type I was evaluated by dot-blot analysis. Mesangial cells were grown as previously described and exposed to the indicated agents for 48 h. After extraction of total RNA (RNAid PLUS Kit, Bio 101, Vista, CA), 100-μg aliquots were dissolved in diethyl pyrocarbonate-treated water and a mixture of formaldehyde and 20× saline-sodium citrate (SSC) in equal volumes. This solution was heated for 15 min at 65°C to denature the RNA. The material was then dot blotted at various concentrations onto nitrocellulose membranes (Bio-Rad dot-blot apparatus). The blotted membranes were heated at 80°C for 1.5 h. The cDNA probe for the α1 chain of human type I collagen (ATCC 61322, Rockville, MD) (11) was prepared by the Rad-Prime Kit (Life Technologies, Gaithersburg, MD) using 25 ng of DNA. The probe was heated for 5 min at 100°C and kept on ice before use. Prehybridization and hybridization of the labeled probe with the RNA fixed to the nitrocellulose filter was accomplished in a solution of 5% dextran, Denhardt, 20× saline-sodium phosphate-EDTA (all from Sigma) and H2O at 42°C. Prehybridization was done for 1.5 h, and hybridization was accomplished overnight. After hybridization, the filters were washed twice in 2× SSC and 0.1% SDS at room temperature and then with 0.2% SSC and 0.1% SDS at 55°C for 45 min. Filters were then autoradiographed using an intensifying screen. Filters were stripped with a solution consisting of Tris·HCl (5 mM), EDTA (2 mM), and 0.1× Denhardt solution, and reprobed with glyceraldehyde-3-phosphate dehydrogenase (Oncogene Research Products, Cambridge, MA) to correct for variations in RNA loading.

Statistics. For each individual experiment, the mean of replicate determinations was calculated. Data are given as means ± SE. Comparisons among groups were performed with analysis of variance with Scheffé’s correction or Student’s two-tailed t-test for independent variables. Statistical significance was defined using an overall type I error of 0.05.

RESULTS

In control mesangial cells grown in the presence of 10% FCS, the ratio of phosphorylated p42 ERK to phosphorylated p44 ERK was 2.8 ± 0.2 to 1. Estradiol upregulated phosphorylated p44 ERK and p42 ERK in a dose-dependent manner (10-10 to 10-7 M, n = 10; Fig. 1). Cells exposed to physiological concentrations of
estradiol (10⁻¹⁰ to 10⁻⁷ M) for 5 min showed an increase in phosphorylated p44 ERK (270.3 ± 24.0%, expressed as percentage of control values, P < 0.001 vs. control). The maximum increase in p44 ERK was seen with 10⁻⁷ M estradiol (476.9 ± 42.4, P < 0.001). Physiological concentrations of estradiol (10⁻⁹ to 10⁻⁸ M) also increased phosphorylated p44 ERK (350.1 ± 61.3, P < 0.001). The maximum increase in phosphorylated p44 ERK was seen with 10⁻⁷ M estradiol (746.9 ± 42.2, P < 0.001). In contrast, estradiol (10⁻¹⁰ to 10⁻⁷ M) had no significant effect on total (phosphorylated + unphosphorylated) p44 ERK or p42 ERK (not shown).

ICI-182780 (10⁻⁶ M), a high-affinity estrogen receptor antagonist, reversed the activation of MAP kinase induced by estradiol (10⁻⁷ M) [p44 ERK 97.5 ± 9.3 vs. 476.9 ± 42.2, P not significant (NS) vs. control, P < 0.001 vs. estradiol; p42 ERK 91.8 ± 12.4 vs. 742.9 ± 118.0, P NS vs. control, P < 0.001 vs. estradiol], whereas ICI-182780 alone had no effect (p44 ERK 89.8 ± 7.9; p42 ERK 88.0 ± 8.7, P NS vs. control; Fig. 1). 17α-Estradiol (10⁻⁷ M), a time-dependent mixed agonist-antagonist of 17β-estradiol, had only a minor effect on MAP kinase, which did not achieve statistical significance (p44 ERK 126.5 ± 12.3, P NS vs. control; p42 ERK 159.2 ± 21.5, P NS vs. control; Fig. 1).

Activation of the MAP kinase pathway by estradiol (10⁻⁷ M) was already evident by 1 min (p44 ERK 208.9 ± 22.1%, expressed as percentage of control values, P < 0.001 vs. control; p42 ERK 200.6 ± 30.1, P < 0.003; n = 11; Fig. 2). The peak response was observed at 10 min (p44 ERK 1,670.2 ± 714.4, P < 0.0001; p42 ERK 1,243.6 ± 152.2, P < 0.001) but completely dissipated by 2 h (Fig. 2).

We also assessed the effects of estradiol (10⁻⁷ M, 10-min exposure) on mitogen-stimulated MAP kinase activity in serum-starved mesangial cells (n = 3; Fig. 3). In the absence of serum, estradiol had no effect on basal p44 or p42 ERK phosphorylation but reversed the stimulatory effects of PDGF-AB (10 ng/ml) and of ANG II (10⁻⁶ M) on ERK phosphorylation. Total (unphosphorylated + phosphorylated ERK) was not changed by any of these agents (data not shown).

We measured the effects of PD-98059 (50 µM), a highly selective inhibitor of MEK1 and MEK2, on estradiol-induced activation of p44 and p42 ERK (n = 3), upregulation of AP-1 activity (n = 3), and suppression of type I collagen synthesis (n = 6) in murine mesangial cells (Fig. 4, A, B, and C1). As expected, PD-98059 suppressed basal MAP kinase activity (p44 ERK 23.6 ± 9.4; p42 ERK 38.6 ± 10.8, P < 0.001 vs. 10.220.32.247 on October 20, 2017 http://ajprenal.physiology.org/ Downloaded from http://ajprenal.physiology.org/
control; Fig. 4A). PD-98059 returned estradiol (10^{-7} M)-stimulated MAP kinase activity to control levels (p44 ERK 86.0 ± 35.9 vs. 344.6 ± 56.0, P NS vs. control, P < 0.01 vs. estradiol; p42 ERK 111.4 ± 21.6 vs. 495.3 ± 14.8, P NS vs. control, P < 0.01 vs. estradiol).

Nuclear extracts isolated from mesangial cells exposed to estradiol (10^{-7} M) for 4 h showed increased binding to a consensus AP-1 binding sequence oligonucleotide in gel shift assays (180.1 ± 11.9%, expressed as percentage of control values, n = 3, P < 0.001 vs. control; Fig. 4B). In contrast, nuclear extracts exposed to PD-98059 (50 µM) for 4 h showed reduced binding to the AP-1 oligonucleotide (31.2 ± 5.0, P < 0.001 vs. control). PD-98059 returned estradiol-stimulated nuclear extract binding to control levels (102.1 ± 7.9 vs. 180.1 ± 11.9, P NS vs. control, P < 0.001 vs. estradiol, P < 0.001 vs. PD-98059).

Estradiol (10^{-7} M) suppressed mesangial cell type I collagen synthesis (23.9 ± 5.1%, expressed as percentage of control values, n = 6, P < 0.001 vs. control; Fig. 4C1). In contrast, PD-98059 (50 µM) increased mesangial cell type I collagen synthesis (195.4 ± 18.8, P < 0.001 vs. control). When cells were exposed to both estradiol and PD-98059, type I collagen synthesis re-
Estradiol suppressed type I collagen synthesis by inducing the expression of AP-1 (specifically c-Fos), which in turn binds to AP-1 regulatory motifs to suppress type I collagen gene transcription.

The MAP kinase signaling pathway is a cascade of serine-threonine and tyrosine kinases that convert extracellular stimuli into intracellular responses (3). The MAP kinases ERK1 and ERK2 (p44 MAP kinase and p42 MAP kinase, respectively) are activated by phosphorylation of threonine and tyrosine regulatory sites by MEK, a dual specificity kinase with serine-threonine and tyrosine kinase activity (3). ERK, a proline-directed serine-threonine kinase, translocates from the cytoplasm to the nucleus after activation to phosphorylate Elk-1, which in turn stimulates transcription of c-fos (3, 14). In addition, RSK2, a serine-threonine protein kinase in the p90S6K family, is another substrate for phosphorylation by ERK (26). Activated RSK2 in turn phosphorylates and activates the transcription factor cAMP response element-binding protein, which binds to the c-fos promoter to activate transcription (14).

Estradiol has been shown to activate the MAP kinase pathway in a wide variety of cell types from various species (5, 7, 8, 18, 21, 27, 32). In MCF7 human breast cancer cells, physiological concentrations of estradiol activate c-src, which in turn initiates a cascade of phosphorylation events leading to activation of ERK (21). ERK activation occurs within 2–5 min and completely dissipates within 15–60 min (21). The rapid onset of these effects suggests that they are nongenomic. Activation of MAP kinase by estradiol is not reversed by cycloheximide or actinomycin D and is also induced by a cell membrane-impermeable form of estradiol (8, 32). These effects are thought to be mediated by binding to cell membrane receptors and in most studies are reversed by a specific estrogen-receptor antagonist (7, 21).

Although estradiol upregulates MAP kinase in most cell types, including mesangial cells grown in serum-supplemented medium (described herein), several other patterns of response have been observed. In serum-starved human umbilical vein smooth muscle cells, estradiol has no effect on basal MAP kinase activity but reverses mitogen (endothelin-1 or serum)-stimulated MAP kinase activity (22). Similarly, estradiol inhibits ANG II-induced activation of MAP kinase in serum-starved bovine aortic endothelial cells without affecting basal activity (23). We observed a similar pattern in serum-starved mesangial cells stimulated with PDGF or ANG II but not in cells stimulated with serum itself. A third pattern is seen in human umbilical vein endothelial cells grown in 1% BSA (16). In these cells, estradiol induces a delayed, receptor-mediated increase in MAP kinase activity which peaks at 3 h and is mediated by autocrine production of basic fibroblast growth factor (16).

We hypothesized that estradiol suppresses mesangial cell type I collagen synthesis via activation of the MAP kinase cascade, which in turn upregulates AP-1 activity. This hypothesis was based on the following observations: 1) AP-1 suppresses type I collagen synthesis (29). These data led us to conclude that estradiol suppresses mesangial cell type I collagen synthesis by inducing the expression of AP-1 (specifically c-Fos), which in turn binds to AP-1 regulatory motifs to suppress type I collagen gene transcription.

**DISCUSSION**

We have previously shown that estradiol suppresses type I and type IV collagen synthesis by cultured murine and rat mesangial cells grown in the presence of serum (17, 19, 25, 29–31). We found that estradiol suppresses mesangial cell type I collagen synthesis by activating the transcription factor AP-1 (29). Upregulation of AP-1 by phorbol esters suppressed mesangial cell type I collagen synthesis, whereas inhibition of AP-1 by curcumin enhanced type I collagen synthesis (29). Estradiol induced a rapid increase in the steady-state message for c-fos, which peaked at 30 min (29). Corresponding to this increase in c-fos mRNA, nuclear proteins from estradiol-treated mesangial cells showed increased binding to an AP-1 consensus sequence oligonucleotide (29). Estradiol-enhanced binding was reversed by cycloheximide and thus dependent on new protein synthesis (29). Curcumin, an inhibitor of AP-1, also reversed the enhanced binding observed in estradiol-treated cells (29). These data led us to conclude that estradiol suppresses mesangial cell type I collagen synthesis by inducing the expression of AP-1 (specifically c-Fos), which in turn binds to AP-1 regulatory motifs to suppress type I collagen gene transcription.

Because PD-98059 in a concentration of 50 µM did not depress estradiol (10⁻⁷ M)-stimulated MAP kinase activity below control levels (Fig. 4A), we repeated our studies using a higher concentration of PD-98059 (100 µM) and a lower concentration of estradiol (10⁻⁹ M). At these concentrations, both PD-98059 alone and PD-98059 plus estradiol raised type I collagen synthesis above control levels (PD-98059 344.6 ± 96.8, P < 0.01 vs. control; PD-98059 + estradiol 327.0 ± 68.6, P < 0.01 vs. control, P NS vs. PD-98059; estradiol 37.8 ± 2.4, P < 0.001 vs. control; Fig. 4C2). The effects of estradiol, PD-98059, and PD-98059 plus estradiol on collagen I protein synthesis were closely paralleled by their effects on steady-state levels of mRNA for the α₁ chain of type I collagen (Fig. 4C3).

Estradiol-induced suppression of type I collagen synthesis was completely reversed by ICI-182780 (97.9 ± 2.8, P NS vs. control), whereas ICI-182780 alone had no effect on type I collagen synthesis (96.2 ± 3.2, P NS vs. control). Estradiol suppressed type I collagen synthesis to the same degree in cells grown in the presence or absence of ascorbic acid (data not shown).

We also examined the effects of other inhibitors and activators of the MAP kinase cascade on type I collagen synthesis. Genistein is a tyrosine kinase inhibitor that nonselectively blocks MAP kinase activation, whereas sodium orthovanadate is a tyrosine phosphatase inhibitor that nonselectively upregulates MAP kinase. Genistein (18 µM) increased mesangial cell type I collagen synthesis (170.3 ± 23.0%, expressed as percentage of control values, n = 6, P < 0.001). The ability of genistein to stimulate type I collagen synthesis was not inhibited by a 10-fold excess of ICI-182780 (229.2 ± 28.9% vs. 229.2 ± 22.9%, n = 3, P NS). When cells were exposed to both genistein and estradiol, type I collagen synthesis returned to control levels (98.6 ± 10.6, P NS vs. control, P < 0.001 vs. genistein). Sodium orthovanadate (1 mM) suppressed mesangial cell type I collagen synthesis (63.0 ± 2.8, P < 0.006 vs. control).
sion in murine mesangial cells and mediates the suppressive effects of estradiol on type I collagen synthesis, 2) estradiol stimulates AP-1 activity by upregulating c-fos transcription, 3) c-fos transcription is stimulated by activation of the MAP kinase pathway, and 4) estradiol activates MAP kinase in many other cell types.

We found that physiological concentrations of estradiol activated p44/p42 ERK in murine mesangial cells grown in the presence of serum by specifically increasing the phosphorylated form. Associated with MAP kinase activation, AP-1 activity was increased as reflected by increased binding of nuclear extracts from estradiol-treated cells to an AP-1 oligonucleotide in gel shift assays. We used an inhibitor of the MAP kinase cascade to examine the relationship between this pathway and type I collagen synthesis. PD-98059 is a highly selective inhibitor of MEK1 and MEK2 activation, which interrupts the MAP kinase cascade. PD-98059 does not inhibit activation of other dual specificity protein kinases or other serine-threonine protein kinases (1). We found that blockade of the MAP kinase cascade with PD-98059 suppressed AP-1 activity in gel shift studies and increased type I collagen synthesis by murine mesangial cells. These data suggest that the MAP kinase pathway regulates type I collagen synthesis by modulating AP-1 activity. In addition, PD-98059 reversed the effects of estradiol on AP-1 activity and on collagen I synthesis. These observations suggest that estradiol suppresses type I collagen by activating MAP kinase, which in turn upregulates AP-1 activity.

The poor solubility of PD-98059 in aqueous medium precludes the use of high concentrations in cell culture experiments (1). In the concentrations achievable, PD-98059 is only partially effective in suppressing MAP kinase activation in cells that are exposed to high concentrations of potent agonists (1). This limited activity explains why PD-98059 at a concentration of 50 µM merely returned estradiol (10⁻⁷ M)-suppressed type I collagen synthesis to control levels, whereas a higher concentration of PD-98059 was able to raise type I collagen synthesis above control levels in the presence of a lower concentration of estradiol (10⁻⁹ M).

Consistent with the results we obtained with PD-98059, genistein, a tyrosine kinase inhibitor that nonselectively blocks MAP kinase activation, increased mesangial cell type I collagen synthesis, whereas sodium orthovanadate, a tyrosine phosphatase inhibitor that nonselectively upregulates MAP kinase, suppressed mesangial cell type I collagen synthesis. Thus, 17β-estradiol activates MAP kinase activity and suppresses type I collagen synthesis, whereas, genistein, a plant-derived phytoestrogen, has opposite effects. These differential actions result from the opposite effects these agents have on tyrosine kinase activity. Genistein inhibits tyrosine kinase activity by competitive inhibition of ATP binding to tyrosine kinase via an estrogen receptor-independent mechanism (2, 20). Our observation that ICI-182780 reverses the effects of estradiol on type I collagen synthesis but fails to reverse those of genistein are consistent with the concept that genistein acts independently of the estrogen receptor. Other investigators have reported identical effects of genistein and sodium orthovanadate on type I collagen synthesis in primary human fetal skin fibroblasts. However, these authors invoked an entirely different mechanism of action (9).

We also assessed the effects of estradiol on mitogen (PDGF or ANG II)-stimulated MAP kinase activity in serum-starved mesangial cells. We found that in serum-starved cells, estradiol had no effect on basal MAP kinase activity, but reversed the stimulatory effects of PDGF and of ANG II. These data are consistent with our previous observation that estradiol suppresses type I collagen synthesis only in the presence of serum (17), i.e., only in situations where estradiol upregulates MAP kinase activity, and lend further support to our conclusion that estradiol suppresses collagen I synthesis in mesangial cells via activation of MAP kinase.

We are unable to explain why serum modulates the response of MAP kinase to estradiol in murine mesangial cells. The patterns of cellular MAP kinase responses to estradiol described in the literature do not segregate according to whether cells were serum starved or grown in the presence of serum. Some of the studies in which estradiol rapidly stimulated MAP kinase activity were performed in the presence of serum, whereas others were performed in serum-starved cells (5, 7, 8, 18, 21, 32). Studies that found that estradiol inhibited mitogen (ANG II or endothelin 1)-stimulated MAP kinase activity without affecting basal activity were performed using serum-starved cells (22, 23). However, in serum-starved human umbilical vein smooth muscle cells, estradiol antagonized the activation of MAP kinase induced by the addition of serum itself (22).

We postulate that PD-98059 reverses the suppressive effect of estradiol on type I collagen synthesis by blocking estradiol-induced upregulation of the MAP kinase cascade. However, an alternative explanation must be addressed. Growth factor-stimulated phosphorylation of the estrogen receptor at ser 118 is required for receptor activation and is mediated by the MAP kinase pathway in some cell types (4, 15). Thus MEK inhibition might reduce ser 118 phosphorylation and downregulate estrogen receptor activity. However, in MCF7 cells and in vascular smooth muscle cells, the cell type most closely related to the mesangial cell in phenotype and function, growth factor-stimulated phosphorylation of ser 118 is independent of the MAP kinase pathway (12, 13).

Interactions between type I collagen synthesis and the MAP kinase pathway are cell type- and extracellular stimulus-specific (6, 9, 10, 24). In some cell types, activation of the MAP kinase cascade stimulates type I collagen transcription (6). In addition, several studies suggest that ERK plays a role in mediating the transcriptional activation of type I collagen by transforming growth factor-β1 in NIH 3T3 cells and in human mesangial cells (10, 24).