Smoking Induces Glomerulosclerosis in Aging Estrogen-Deficient Mice through Cross-Talk between TGF-β1 and IGF-I Signaling Pathways

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Smoking is a known risk factor for the progression of chronic kidney diseases. However, its independent contribution to the development of ESRD and the underlying molecular mechanism have not been well elucidated. Although the risk for ESRD is higher in postmenopausal women according to the US Renal Data System, the number of women who smoke is on the rise worldwide. Therefore, the effects of smoking and estrogen status on glomerular function and structure were studied in female B6 mice that were ovariectomized at 3 (young) and 15 mo (aged) of age. The mice received either 17β-estradiol (E2) replacement or placebo (Pla) and were divided further into groups that were exposed to cigarette smoke (S) and not exposed (NS). Six months of exposure to smoke had no effect on young mice, although aging S/Pla mice exhibited a phenotype of increased albumin excretion associated with a moderately increased glomerular collagen type IV deposition compared with NS/Pla mice. S/Pla mice also had a two-fold increase in glomerular TGF-β, Smad3, and IGF-I receptor mRNA expression compared with the NS group. Mesangial cells that were isolated from S/Pla mice had an increase of IGF-I receptor protein, and IGF-I stimulated a TGF-β reporter construct promoter three-fold. This was blocked by pretreatment with a neutralizing antibody to IGF-I, LY294002 (phosphatidylinositol-3 kinase inhibitor) or a dominant negative Smad construct. In addition, Smad3 activation was stimulated by IGF-I and blocked by LY294002, suggesting cross-talk between Smad and the phosphatidylinositol-3 kinase/AKT pathways. The smoking phenotype was reversed by E2 replacement. In conclusion, smoking induces a phenotype in E2-deficient mice that is characterized by activation and cross-talk between the TGF-β1 and IGF-I signaling pathways.


Smoking has been shown to be a significant risk factor in the aggravation of a variety of progressive renal diseases (1,2). Despite this, there have been few studies of the mechanisms that are involved in this important problem. It is interesting that the renal diseases that are involved have been found to be very diverse and include primary vascular diseases such as hypertension, metabolic diseases that affect glomeruli such as diabetic nephropathy, and genetic diseases that affect tubules such as polycystic kidney disease (3–6). In addition, a large body of evidence shows that smoking is associated with risk for cardiovascular disease and renal cell carcinoma (7,8). This suggests that smoking may alter fundamental biologic functions that are common to the pathogenesis of several different disease states.

Recent US Renal Data System data suggest that women after menopause have a higher risk than men for ESRD, but there is little information on the role that smoking may play in this increased risk (9). A study by Haroun et al. (10) showed that the risk that is associated with chronic kidney disease increases with smoking for both men and women. Other studies reported a higher risk for men compared with women, although no distinction was made between pre- and postmenopausal women in the data analysis (11–14).

We propose that tobacco and estrogen deficiency represent compounding risk factors for renal injury, especially in postmenopausal women. Because the number of aging women in the population is increasing and the number of women who are smokers also is increasing, the relationship between estrogen deficiency and smoking now can be considered to be a significant health care issue. We used C57BL/6 mice as a model, because they are resistant to glomerulosclerosis during the premenopausal period but progressively develop this lesion after cessation of estrogen synthesis, and investigated whether estrogen deficiency in young and aging mice coupled with exposure to smoke would increase their vulnerability to indices of renal damage such as albuminuria and collagen deposition. In addition, we explored the potential role of the IGF-I and TGF-β prosclerotic signaling pathways in these processes (15–17).

Materials and Methods

Animal Model

Female C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were ovariectomized (ovx) at either 3 (young)
or 15 mo (aged) of age using the previously described procedure that has been approved by the Committee for Animal Safety at the University of Miami School of Medicine. The mice received either placebo (Pla) or 17β-estradiol (E2; 0.025 mg) 90-d release pellets 2 wk after ovariectomy as described previously (18). The 3-mm pellets were implanted subcutaneously into the back of the mice using a sterile trochar and forceps. These pellets were replaced every 90 d during the course of the experiment. The groups were divided further into a group that was exposed to smoke (S) and a group of non–smoke-exposed controls (NS).

**Smoking Protocol**

The S group was exposed 5 d/wk, 3 h/d for 6 mo to mainstream and sidestream cigarette smoke in a TE-10 smoke machine (Teague Enterprises, Woodland, CA). Carbon monoxide was monitored. The cigarettes were University of Kentucky reference cigarettes (1R2F), manufactured by Tobacco and Health Research Institute, and were used as described previously (19). The cigarettes were conditioned in a humidifier for 48 h before use at 23°C, 60% relative humidity. To ensure that all mice received adequate smoke exposure, we measured urine levels of cotinine by ELISA (Ora Sure Technologies, Bethlehem, PA) according to the manufacturer’s directions and normalized to creatinine in each sample.

**Animal Death**

Mice were allowed free access to food and water and were killed at 9 mo for young mice and 21 mo for aged mice. As described previously, the left kidney was perfused with a buffer solution that contained collagenase and RNase inhibitors for microdissection of glomeruli (20). The right kidney was perfused in situ with 6 ml of PBS, followed by 3 ml of 4% paraformaldehyde. Thin cross-sections were postfixed in 4% paraformaldehyde solution for at least 12 h, embedded in methylacrylate, and sectioned at 4 µm, and the sections were stained with periodic acid-Schiff. Other kidney fragments were frozen immediately in OCT. The uterus was removed and weighed when the mice were killed as a measure of the efficacy of estrogen levels. Estrogen replacement was considered to be effective when uterine weight reached a value within 10% of the uterine weight of sham-operated mice.

**Measurements of Urinary Albumin and Creatinine**

Spot urine samples were collected at the same hour on a weekly basis and when the mice were killed. Albumin in the urine, measured by ELISA (Bethyl, Houston, TX), was corrected for the concentration of creatinine in the urine and expressed as the urinary albumin/creatinine excretion ratio (UAE).

**Morphometry**

A morphometric approach was used to quantify the degree of glomerulosclerosis (21). Fifty cortical glomeruli, randomly selected from each mouse, were recorded with an Olympus BH-2 microscope (Microptics, Fort Lauderdale, FL) and Micro Image A209RGB color video camera (Microptics). Glomerular volume (µm³) and mesangial area (µm²) were measured using MetaMorph 4.5.4 Imaging System computer program (Universal Imaging Corp, West Chester, PA).

**Immunohistochemistry**

Deparaffinized kidney sections (4 µm) were blocked for endogenous peroxidase. Sections were stained with either rabbit anti-mouse collagen type IV (Biodesign, Saco, ME) or rabbit anti-mouse laminin (Research Diagnostics, Flanders, NJ). After 1 h, the slides were washed and incubated for 30 min at room temperature with biotinylated-labeled goat anti-rabbit, followed by Vectastain ABC reagent (Vector Labs, Burlingame, CA) and 3,3’-diamino-benzidine chromogen solution (Sigma, St. Louis, MO). Finally, tissue sections were counterstained with hematoxylin and eosin, and coverslips were applied. The sections were examined and graded on a scale of 0 to 4 by a renal pathologist who was blinded to the treatment group.

**Isolation of RNA and Quantitative Analysis of RNA Expression by Real-time Reverse Transcriptase–PCR**

Total RNA was extracted from 100 glomeruli, microdissected from each mouse, using the guanidinium thiocyanate-phenol-chloroform method as described previously (20). Amplification and quantification of target RNA was performed on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The primers and probes for TGF-β, IGF-I receptor (IGFR); and Smad2, 3, and 7 were synthesized commercially (ABI Primer & Probes; Applied Biosystems). TaqMan ribosomal RNA control reagents that are designed to detect 18S ribosomal RNA, which served as an endogenous control to normalize for variations in the isolated RNA amount, were purchased from Applied Biosystems.

**Cell Culture**

When the mice were killed, glomeruli were microdissected and mesangial cells (MC) were isolated and propagated as described previously (22). All experiments were performed on MC between passages 6 and 15.

**Western Analysis**

MC were plated in phenol red–free medium that contained 20% charcoal-stripped serum. Twenty-four hours before collection, cells were treated as described for transfection studies. Confluent cell layers were washed once in 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. Samples were resolved by electrophoresis on 6% (IGFR) or 10% (Smad 2/3, pSmad 2/3, extracellular signal–regulated kinase [ERK], phosphorylated ERK1/2 [pERK1/2], AKT, and phosphorylated AKT [pAKT]) polyacrylamide gels as described previously (23). Electrophoretic transfer of proteins from the gel to the nitrocellulose was performed by electroelution and immunoblotting as described previously (24). Immunoreactive bands were determined by exposure of the nitrocellulose blots to a chemiluminescence solution (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 min, followed by exposure to X-OMAT AR film (Eastman Kodak Co., Rochester, NY). The film was scanned and saved on computer disks for densitometric analysis using Image J software (National Institutes of Health, Bethesda, MD).

**Transfections**

S and NS MC were transfected in phenol red–free medium that contained 10% charcoal-stripped serum with a luciferase-based Smad-responsive p3TP-Lux reporter reporter construct (0.3 µg/well; a gift from J. Massague, Memorial Sloan Kettering, New York, NY) as described previously (18). Twenty-four hours after transfections, cells were treated with TGF-β (0 to 1000 pg/ml); IGF-I (0, 10, and 50 ng/ml); and, in some experiments, PD98059, LY294002, or a neutralizing antibody to IGF-I (12.5 µg). To block Smad activation in some experiments, we used a dominant negative Smad3 construct (gift of Dr. Li, University of Colorado, Boulder, CO).
ELISA for TGF-β

TGF-β1 protein was measured in the supernatants of MC with a TGF-β1 ELISA kit (R & D, Minneapolis, MN). MC were plated in 24-well plates (35,000 cells/well) and maintained in phenol red–free medium that contained 2% estrogen-free serum, 3 d before the start of the experiment. On day 0, cells were placed in phenol red–free medium that contained 0.1% BSA and collected after 48 h. On the day of collection, supernatants were centrifuged to remove cell debris. MC in the cell culture media were incubated with 0.1% BSA and collected after 48 h. On the day of collection, supernatants were centrifuged to remove cell debris. MC in the cell layers were counted with a Z cell counter, and data were normalized to cell number.

Statistical Analysis

Statistical differences between experimental groups were determined by ANOVA and Tukey multiple comparison post hoc test. Each experiment was performed at least three times, and duplicate wells were collected. In the case of transfections, triplicate wells were collected for each treatment and transfections were repeated at least three times.

Results

Body, Kidney, and Heart Weight

Body weight was increased in young NS/Pla versus NS/E2 (P < 0.05) and NS/Pla versus S/Pla (P < 0.005) mice (Table 1). There was also an increase in body weight in aged NS/E2 compared with S/E2 mice. In addition, the kidney weight/body weight ratio was increased in E2 mice in both young and aged mice compared with the Pla mice, and there was no difference between the S and NS groups. Heart weight did not differ between the groups (data not shown). Uterine weight was increased in all mice that received E2 (Table 1). Cotinine, a metabolite of nicotine, was measured in the urine that was collected when the mice were killed and normalized to urine creatinine values. There was a significant difference between S and NS mice within groups (Table 1). Estrogen treatment reduced cotinine levels in all S mice. In addition, urine cotinine levels were lower in NS/E2 than in NS/Pla mice. This is not surprising, because a recent study showed that women metabolize nicotine and cotinine faster than men (25).

Table 1. Body, kidney, uterine weight, and urine cotinine levels

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Body Weight (g)</th>
<th>Kidney Weight (g)</th>
<th>Kidney Weight/Body Weight Ratio (g)</th>
<th>Uterus Weight (g)</th>
<th>Cotinine Levels/Creatinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young (N)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>NS/Pla (4)</td>
<td>28.73 ± 0.87</td>
<td>0.20 ± 0.01</td>
<td>0.007 ± 0.0001</td>
<td>0.02 ± 0.001</td>
<td>6.95 ± 1.58</td>
</tr>
<tr>
<td>NS/E2 (10)</td>
<td>26.34 ± 0.41</td>
<td>0.23 ± 0.02</td>
<td>0.008 ± 0.0009</td>
<td>0.07 ± 0.02</td>
<td>2.77 ± 0.37</td>
</tr>
<tr>
<td>S/Pla (5)</td>
<td>24.90 ± 0.43</td>
<td>0.17 ± 0.006</td>
<td>0.007 ± 0.0002β</td>
<td>0.01 ± 0.0099</td>
<td>256.0 ± 49.8</td>
</tr>
<tr>
<td>S/E2 (8)</td>
<td>24.71 ± 0.74</td>
<td>0.22 ± 0.02</td>
<td>0.009 ± 0.0008</td>
<td>0.08 ± 0.02</td>
<td>99.8 ± 31.6</td>
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<tr>
<td>Aged (N)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NS/Pla (4)</td>
<td>28.8 ± 4.8</td>
<td>0.22 ± 0.06</td>
<td>0.007 ± 0.0008β</td>
<td>0.02 ± 0.003</td>
<td>18.8 ± 4.8</td>
</tr>
<tr>
<td>NS/E2 (5)</td>
<td>28.96 ± 1.20</td>
<td>0.27 ± 0.035</td>
<td>0.009 ± 0.001</td>
<td>0.10 ± 0.07</td>
<td>0.58 ± 0.12</td>
</tr>
<tr>
<td>S/Pla (6)</td>
<td>27.1 ± 1.1</td>
<td>0.22 ± 0.02</td>
<td>0.008 ± 0.0009β</td>
<td>0.03 ± 0.002</td>
<td>151 ± 21</td>
</tr>
<tr>
<td>S/E2 (7)</td>
<td>25.45 ± 1.6</td>
<td>0.24 ± 0.025</td>
<td>0.009 ± 0.001</td>
<td>0.08 ± 0.02</td>
<td>82.0 ± 21.6</td>
</tr>
</tbody>
</table>

Albumin/Creatinine Ratio

The UAE of all groups of mice was determined once monthly throughout the study and when the mice were killed. Ovx B6 mice that were 9 mo of age when they were killed had a normal UAE irrespective of smoke exposure (Figure 1) or estrogen status (data not shown). E2 treatment had no effect on UAE in 9-mo-old mice (data not shown). However, 21-mo-old S/Pla mice had a four-fold increased UAE (0.03 ± 0.004) compared with 9-mo-old S/Pla mice (0.015 ± 0.002; P < 0.05). There also was a significant increase in albumin excretion in aged S/Pla mice compared with young S/Pla mice (0.03 ± 0.004 versus 0.012 ± 0.003; P < 0.05). In addition, there was a significant increase in albumin excretion in aged S/Pla mice ameliorated the albumin excretion increase compared with Pla/ovx mice of the same age (0.03 ± 0.004 versus 0.015 ± 0.003; P < 0.05). Importantly, the UAE in the aged/S/E2 treatment group was reduced to levels that were found in 9-mo-old mice.

Histology

There were no noticeable pathologic glomerular or tubular changes in any of the groups examined.

Morphometry of the Glomeruli of Ovx and Estradiol-Treated Mice

There was no difference in glomerular area and volume between mice in any of the age groups. As described previously, there was an increase in glomerular volume between young and aged mice (young/NS/P versus aged/NS/P 1.609e + 0.006 μM³ [P < 0.05] and young/S/P versus aged/S/P 1.2068e + 0.006 μM³ [P < 0.05]).

Immunohistochemistry

There were moderate increases in type IV collagen (3 and 4+ staining) and laminin (3 and 4+ staining) in the mesangial areas of aged/S/Pla-treated mice compared with aged/NS/Pla mice (1+ staining; Figure 2). Collagen deposition was decreased in...
Figure 1. Urinary albumin excretion increases in aged mice that were exposed to cigarette smoke. C57BL6 mice were made estrogen deficient by ovariectomy at either 3 (young [Y]) or 15 (aged [A]) mo of age. The groups were divided further into those that received placebo (Pla) or 17β-estradiol (E2). Mice from each group were either used as controls (NS) or exposed to smoke (S) for 6 mo. Data are mean ± SEM. *P < 0.05 versus A/S/Pla; #P < 0.05 A/S/Pla versus A/S/E2; n = 4 mice in Y/NS/Pla group, n = 5 mice for all other groups.

**Glomerular mRNA Expression**

mRNA expression of TGF-β1 and IGFR both were increased in 21-mo-old ovx S/Pla mice compared with age-matched NS/Pla mice (P < 0.05; Figure 3). E2 treatment resulted in decreased IGFR mRNA expression, but TGF-β1 mRNA expression was unaffected, suggesting that these two had a different sensitivity to E2. There also was a significant increase in Smad3 expression in MC from S mice aged 21-mo-old (1000 pg/ml). Treatment with TGF-β1 (1000 pg/ml) led to significantly increased transcription in MC that were isolated from Pla-treated (P < 0.05; Figure 6) and E2-treated mice (data not shown).

Because TGF-β1 and IGFR mRNA expression was increased in glomeruli and IGFR protein was increased in MC from S mice, we investigated potential cross-talk between these signaling pathways. MC that were isolated from S/Pla and NS/Pla mice were transfected with the reporter construct described in the previous paragraph and treated with IGF-1 (0, 10, and 50 ng/ml). IGF-I increased luciferase activity of a Smad-responsive reporter construct two- to three-fold only in MC that were isolated from S mice (Figure 7). Pretreatment with an IGF-I-neutralizing antibody abrogated the IGF-I-mediated increase in luciferase activity (273.0 ± 77.6 luciferase units after IGF-1 treatment versus 66.4 ± 29.6 after treatment with IGF-I and an IGF-I-neutralizing antibody; Figure 7C). MC that were isolated from E2-treated mice did not have an IGF-I-mediated increase in luciferase activity (data not shown).

**Cross-Talk Experiments**

MC were transfected with a TGF-β1 luciferase reporter construct and stimulated with increasing amounts of TGF-β1 (0 to 1000 pg/ml). Treatment with TGF-β1 (1000 pg/ml) led to significantly increased transcription in MC that were isolated from Pla-treated (P < 0.05; Figure 6) and E2-treated mice (data not shown).

MC were pretreated with either PD98059 (a Mek inhibitor) or LY294002 (a PI3K inhibitor) to determine which component of the IGF-I signaling pathway was responsible for the cross-talk. The IGF-I-mediated increase in luciferase was blocked by LY294002 (P < 0.005; Figure 7B) but not by PD98059 (data not shown). This suggested that there was cross-talk between the TGF-β and the PI3K/AKT signaling pathways after IGF-I stimulation but that the ERK/mitogen-activated protein kinase (MAPK) pathway was not involved.

Smad3 phosphorylation was increased by IGF-I, although Smad2 was not (P < 0.05; Figure 8, A and B). This activation was blocked by LY preincubation. E2 treatment reduced the activation of Smad3 (data not shown). To confirm the cross-talk between IGF-I signaling and Smad3, we transfected MC with a dominant negative Smad3 construct. The presence of the dominant negative construct blocked the IGF-I-mediated increase in the TGF-β responsive reporter (P < 0.05; Figure 8C).

**Discussion**

There is general agreement that smoking plays a role in development and progression of diabetic and nondiabetic kidney and cardiovascular disease (4,5,26,27). Although the risk for kidney disease increases for both men and women who smoke, to date, no data have addressed the effect of estrogen...
deficiency (menopause) on the development and progression of kidney disease in smokers.

We used female B6 mice, because they model kidney disease in women, namely, they are relatively resistant to the development of significant glomerular lesions at a young age, but this diminished response disappears after menopause (20,28,29). After ovariectomy, the mice were exposed to mainstream and sidestream cigarette smoke to mimic the noxious effects of active and passive smoking.

Cigarette smoke induces oxidative stress in the kidney and other organs (30). Gas-phase radicals and semiquinones that are produced by cigarette smoke lead to formation of superoxide and other oxidative molecules (31). The levels of these products have been found to correlate with an increased UAE in patients with and without diabetes (32–34). In our studies, despite a 6-mo regimen of smoking, UAE increased only in mice that were ovx at 15 mo of age. The moderate increase in UAE in 21-mo-old S mice correlated with a moderate increase in extracellular matrix accumulation, an indicator of glomerulosclerosis. However, UAE was not increased in any of the 9-mo-old mice. This is consistent with our previous data, showing that MC from 9-mo-old mice were normal but that those that were isolated from 28-mo-old female mice (aged) exhibited a proinflammatory phenotype (28). C57BL6 mice enter anestrous at approximately 18 mo of age (35), suggesting that prolonged estrogen deficiency may be partially responsible for the inflammatory phenotype. In our experiments, mice were ovx and then exposed to an injury. Importantly, 21-mo-old S mice with E2 supplementation had no increase in UAE and no change in collagen deposition compared with NS mice, suggesting that the MC phenotypic change that accompanies aging and smoke exposure may be prevented or slowed by E2 supplementation.

Although the increase in albumin excretion in S mice was double that of NS controls, the levels were relatively low compared with those previously obtained in sclerosis-prone animal models (ROPOS/+ and db/db) (20). We in fact would expect a more pronounced response to cigarette smoke in sclerosis-prone animal models (ROPOS/+ and db/db) (20).
prone mice or rats as shown by Odoni et al. (36). These data are similar to those in humans, which show that even in patients without kidney disease, a smoking history is associated with an elevation in UAE to a high normal level (2). This is especially important because increases in albumin excretion are predictive of cardiac and renal disease, which can occur even in the high normal range of albuminuria (37).

It is interesting that no pathologic changes were noted at the light microscopic level; this finding could be explained by the longer period of time required for the glomeruli and/or tubules to show damage at the morphology level after the derangement of proinflammatory and profibrotic molecules. It is possible that signs of glomerulosclerosis would appear if the mice had been exposed to smoke longer.

Cigarette smoke–induced oxidative injury was manifested in glomeruli that were isolated from 21-mo-old, S mice by increased IGFR and TGF-β1 mRNA expression. We and others previously showed that both of these pathways are prosclerotic and can induce increased extracellular matrix and decreased matrix metalloproteinase production. An increase of TGF-β1 in the kidney (38–41) was reported previously to occur with the oxidative injury of diabetes and cigarette smoking. We also found that Smad3 mRNA expression was increased in glomeruli of S mice. Smad3 plays an important role in cross-talk between steroid receptors and TGF-β. Estrogen-activated ERα has been shown to decrease Smad3 activity and may provide an explanation for the decrease that was seen in glomeruli that were isolated from E2-treated mice (42).

Reactive oxygen species stimulate the synthesis of IGF-I in vascular smooth muscle cells (43), and there is an upregulation of IGF binding protein 3 in the plasma of cigarette smoke–exposed ferrets (44). To our knowledge, this is the first report of an increase in IGFR and potentially signaling in glomeruli, as a result of smoke-induced oxidative injury.

After finding that both of these signaling pathways were increased at baseline only in the glomeruli of S E2-deficient mice, we investigated whether cross-talk between the two could be one of the potential mechanisms for smoke-induced phenotype changes. Furthermore, we speculated that E2 was modulating one or both pathways and thereby preventing the...
phenotype changes that are induced by aging and smoke exposure.

We found that MC from S/Pla mice had higher baseline TGF-β1 levels than MC that were isolated from NS/Pla mice. This is not surprising, because rat MC that were exposed to cigarette smoke concentrates also were found to have an increased TGF-β1 production (40). Conversely, E2 treatment prevented the rise in TGF-β1 synthesis. This is consistent with data from our laboratory and others showing a decrease in TGF-β1 mRNA and/or synthesis after in vivo administration of E2 (18,45,46).

It is interesting that transfection studies on isolated MC revealed that sensitivity to TGF-β1 was not altered by smoke or estrogen status because cells that were isolated from the S mice were equally responsive to TGF-β1 compared with cells that were isolated from NS/Pla mice. These data suggested that the prosclerotic effects were driven by IGF-I signaling, not an increase in TGF-β1 signaling.

Physiologic concentrations of IGF-I also stimulated a TGF-β responsive reporter construct, an effect that was blocked by either an IGF-I-neutralizing antibody or a specific PI3K inhibitor. IGF-I treatment increased activation of Smad3, and the addition of a dominant negative Smad3 construct blocked the IGF-I–mediated luciferase increase of the Smad-responsive reporter construct. In total, these data suggest cross-talk between PI3K and Smad that is mediated at least in part by IGF-I. Cross-talk between ERK/MAPK and Smad-signaling pathways that lead to enhanced TGF-β–dependent responses also has been reported (47,48). We found, however, that incubation with PD, an inhibitor of MAPK did not block the IGF-I–mediated increase of the Smad-responsive reporter construct. Although we cannot exclude the possibility that there is a direct TGF-β

Figure 7. IGF-I stimulates a TGF-β1 responsive reporter construct through the phosphatidylinositol-3 kinase (PI3K) pathway. MC that were isolated from A/S/Pla mice were transfected with a TGF-β1 responsive reporter construct as described in Materials and Methods. (A) IGF-I at physiologic dosages was able to increase luciferase significantly as was TGF-β1. The two together did not magnify the response. Data are mean of triplicate experiments ± SEM. *P < 0.05, **P < 0.005 versus control (0 treatment). (B) Pretreatment of MC with LY294002 abrogated the IGF-I–mediated increase in the TGF-β1 responsive reporter (*P < 0.05, IGF-I treatment versus vehicle control; **P < 0.005, IGF-I versus LY + IGF-I). Data are the mean of triplicate experiments ± SEM. (C) IGF-I effects are decreased after incubation with an IGF-I-neutralizing antibody. *P < 0.05 versus vehicle control (0 ng/ml of IGF-I); n = 3.
response that is not IGF-I mediated, TGF-β1 synthesis did not increase after the addition of IGF-I. These data complement a recent study that described cross-talk between the PI3K pathway and Smad3, leading to the stimulation of human MC collagen type I expression (49). In contrast to our study, this group investigated the direct activation of PI3K by TGF-β1. However, in our studies, the addition of both TGF-β1 and IGF-I did not lead to a significant increase in luciferase activity of the TGF-β1 responsive reporter construct compared with either peptide alone.

Importantly, E2 replacement in the setting of cigarette smoke exposure was beneficial in that it reduced IGFR mRNA and protein expression and subsequent PI3K activation, lowered expression of Smad3 mRNA, and decreased Smad3 activation. Steroid receptor action has been shown to have either a positive or a negative effect on TGF-β signaling pathways (42,50). In fact, estrogen receptor can associate with Smad3 and act as a co-repressor in MC (42). In our study, however, the effect of E2 replacement seems to act by decreasing the IGF-I signaling pathway as previously shown in vascular smooth muscle cells (51).

**Conclusion**

We found that exposure to cigarette smoke in the setting of estrogen deficiency promoted a prosclerotic phenotype through cross-talk of TGF-β and IGF-I signaling pathways. Estrogen replacement partially protected against sclerosis by preventing this activation by its action on the IGF-I signaling pathway.

**Acknowledgments**

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**References**


