Abstract. Platelet-activating factor (PAF) is a potent inflammatory mediator that participates in the pathogenesis of proteinuria and glomerular damage. However, the role of this lipid in glomerular sclerosis remains unknown. This study examines the effect of PAF on the regulation of extracellular matrix proteins by rat and human mesangial cells. PAF increased in a dose-dependent manner the gene expression of fibronectin and type IV collagen, but not type I collagen. Moreover, an increase in cell-associated and soluble fibronectin synthesis was also seen. These effects were abolished by BN52021 and WEB2086, two different PAF receptor antagonists. Because transforming growth factor (TGF)-β has been considered a profibrogenic cytokine, this study also evaluated whether PAF effects might be mediated by the production of endogenous TGF-β. PAF caused an increase in TGF-β1 mRNA expression (by a protein kinase C-dependent pathway) and TGF-β activity. Moreover, PAF-induced fibronectin synthesis was totally abolished when an anti-TGF-β-neutralizing antibody was added to the culture medium, suggesting that PAF stimulates fibronectin synthesis, at least in part, through the induction of TGF-β. Addition of cycloheximide, a protein synthesis inhibitor, upregulated PAF-induced fibronectin mRNA expression but downregulated PAF-induced TGF-β1 gene expression, suggesting the existence of different regulatory transcriptional factors of the two proteins. These results suggest that PAF may be implicated in matrix accumulation during renal injury and therefore contribute to the pathogenesis of glomerulosclerosis.

Glomerulosclerosis is the final common pathway of progressive proliferative or nonproliferative glomerular diseases. A central feature of glomerular obsolescence is the accumulation of extracellular matrix proteins within the glomerulus (1,2). Recent evidence suggests that arachidonic acid metabolites may participate directly in the stimulation of matrix protein synthesis, independent of their effects on renal hemodynamics and platelet aggregation. Thus, thromboxane and prostacyclin regulate extracellular matrix protein expression in mesangial cells (MC) (3) and in a murine cell line (4).

Platelet-activating factor (PAF) is a phospholipid mediator that exerts a variety of biological actions (5,6) and has been implicated in pathological conditions such as shock, ischemia, inflammation, and renal failure (7–10). The role of PAF in the pathogenesis of renal damage is supported by several facts. PAF infusion alters the glomerular permselectivity and induces proteinuria (11). An increase in glomerular PAF synthesis and urinary excretion was noted in experimental and human glomerular diseases (12–14). Furthermore, the administration of PAF receptor-specific antagonists reduced proteinuria and morphological lesions in several models of renal damage (15–18). However, the potential participation of PAF in the pathogenesis of tissue remodeling and sclerosis remains largely unknown. Recent data have shown that PAF induces proto-oncogenes and type I collagenase expression in corneal epithelial cells (19), suggesting its potential participation in the wound healing process.

Among the cytokines implicated in the pathogenesis of sclerosis, transforming growth factor (TGF)-β has emerged as one of the main regulators of the synthesis of glomerular extracellular matrix proteins (20) both in cultured MC (21) and in experimental models of glomerulosclerosis (22,23). TGF-β has been considered the mediator of the increase in MC matrix protein synthesis elicited by several agents such as thromboxane (24).

In the study presented here, we investigate the hypothesis that PAF may participate in the glomerular accumulation of matrix proteins, a common event in progressive renal diseases. Our data show that the exposure of cultured rat and human MC to PAF stimulated the gene expression and synthesis of matrix proteins through the induction of TGF-β. Although further studies are needed, our results suggest that PAF may participate in the pathogenesis of glomerulosclerosis.

Materials and Methods
Reagents
PAF (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) was purchased from Calbiochem (San Diego, CA) and stored in a 1-mM stock solution in ethanol. PAF receptor antagonists BN52021 (a gift from...
Dr. Pierre Braquet, Institut Henri Beaufour, Les Plessis-Robinson, France) and WEB2086 (Boehringer, Ingelheim, Austria) were dissolved in dimethyl sulfoxide and added to the medium to reach a final concentration of <0.01%. [35S]Methionine (1000 Ci/mmol) and [α-32P]dCTP (3000 Ci/mmol) were purchased from Amersham (Buckinghamshire, United Kingdom). [3H]Thymidine (70 Ci/mmol) and [3H]leucine (25 Ci/mmol) were from New England Nuclear (Boston, MA). Polyclonal anti-fibronectin (FN) antibody was obtained in rabbits by immunization against human FN (25). Monoclonal mouse anti-TGF-β1 antibody was obtained from Immugenex (Los Angeles, CA), and normal control rabbit IgG was from Sigma Chemical (St. Louis, MO). Recombinant human TGF-β1 was purchased from Immugenex. The rest of the chemicals used were from Sigma.

MC Culture

MC were cultured from isolated glomeruli according to a method described previously (26). Briefly, glomeruli were obtained from the renal cortices of Sprague-Dawley rats (150 to 200 g) or from human cadaver kidneys unsuitable for transplantation by several sieving techniques and differential centrifugations. The final preparation was treated with 500 U/ml collagenase (Type Ia, Sigma), and glomeruli were resuspended in RPMI 1640 buffered with 25 mM Hepes (Gibco BRL, Paisley, Scotland) at pH 7.4 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine in the presence of 10% fetal calf serum (FCS) (Gibco) for rat MC, and in the presence of 20% FCS, 5 μg/ml human transferrin, 5 μg/ml bovine insulin, and 5 ng/ml sodium selenite (Sigma) for human MC, and plated on Petri dishes (Costar Corp., Cambridge, MA) and cultured at 37°C in 5% CO2 atmosphere. MC at confluence were used for RNA studies or trypsinized (0.05% trypsin-0.02% ethylenediamine tetra-acetic acid; Gibco) and subcultured for 3 to 6 d in 24-well plates (Costar) for protein synthesis studies. MC were morphologically characterized by phase-contrast microscopy. This technique showed stellate or fusiform cells, with no evidence of either glomerular endothelial or epithelial cells, which grow in monolayers of polygonal cells. Additionally, the cells showed positive staining for desmin, vimentin, and α-smooth muscle myosin and negative staining for keratin and factor VIII antigen, excluding epithelial and endothelial contamination, respectively (27). Furthermore, MC were characterized by their positive response to angiotensin II and the phagocytosis of immune complexes (26).

Biosynthetic Labeling and Immunoprecipitation

FN synthesis was measured by [35S]methionine incorporation and immunoprecipitation with anti-FN antibodies (28). MC grown in 24-well plates were made quiescent by incubation for 48 h in RPMI 1640 with 0.5% FCS and then incubated in methionine-free culture medium RPMI 1640 (Gibco) with 20 μCi/ml [35S]methionine, 0.5% FCS, and the appropriate stimuli. At the end of the incubation period, medium was collected and cells were lysed with extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 2 mM ethylenediamine tetra-acetic acid, and 5 mM N-ethyldiamine, pH 7.4). Aliquots of the supernatants (diluted 1:10 in extraction buffer) and cell lysates were immunoprecipitated with an excess of anti-FN antibody (50 μg) for 16 to 18 h at 4°C. After this incubation, immune complexes were recovered by addition of protein A-Sepharose beads (Pharmacia, Upssala, Sweden) and incubated for an additional hour. Beads were washed in extraction buffer. FN was released by heating at 100°C for 5 min in electrophoresis sample buffer and analyzed under denaturing conditions in 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels. The gels were handled by fluorography techniques, and autoradiography was performed by standard methods.

As a negative control for immunoprecipitation, isotopic normal rabbit IgG (30 μg/ml) was used, and recombinant human TGF-β1 (50 pM) was employed as a positive control for FN synthesis. For experiments with PAF antagonists, cells were preincubated with 10 μM BMS2021 and 10 μM WEB2086 for 1 h at 37°C before the addition of 10−8 M PAF. For experiments with anti-TGF-β antibody, MC were coincubated with 10 μg/ml antibody and 10−8 M PAF. To normalize FN production to cell number, sample aliquots (20 μl) of cell lysates were taken for determination of DNA content.

Measurement of Cellular Proliferation

The effect of PAF on cell proliferation was determined by methylene blue assay. Subconfluent rat MC grown in 96-well plates were incubated in a serum-free medium for 48 h to make them quiescent. Then, cells were stimulated for 48 h in serum-free medium in the presence of appropriate stimuli. After the incubation period, cells were washed with PBS and fixed by adding 100% formal saline for 30 min. Cells were stained with methylene blue in 0.01 M borate buffer. The absorbance was measured at 650 nm in a microplate photometer (30).

Bioassay of TGF-β Activity

Previous studies have established that mink lung epithelial cells (CCL-64; American Type Culture Collection, Rockville, MD) can be used to quantify TGF-β, because they are very sensitive to the inhibitory growth effects of active TGF-β (31).

Confluent MC grown in 24-well plates were made quiescent as described above. Cells were incubated for 24 h with stimuli in the presence of 0.5% FCS. For the last 6 h of culture, 1 μCi of [3H]leucine was added to each well. The cells were washed three times in ice-cold phosphate-buffered saline (PBS), precipitated twice in ice-cold 10% trichloroacetic acid (TCA), redissolved in 0.5 M NaOH with 0.1% Triton X-100, and counted for β emissions.

Bioassay Biosynthetic Labeling and Immunoprecipitation

FN synthesis was measured by [35S]methionine incorporation and immunoprecipitation with anti-FN antibodies (28). MC grown in 24-well plates were made quiescent by incubation for 48 h in RPMI 1640 with 0.5% FCS and then incubated in methionine-free culture medium RPMI 1640 (Gibco) with 20 μCi/ml [35S]methionine, 0.5% FCS, and the appropriate stimuli. At the end of the incubation period, medium was collected and cells were lysed with extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 2 mM ethylenediamine tetra-acetic acid, and 5 mM N-ethyldiamine, pH 7.4). Aliquots of the supernatants (diluted 1:10 in extraction buffer) and cell lysates were immunoprecipitated with an excess of anti-FN antibody (50 μg) for 16 to 18 h at 4°C. After this incubation, immune complexes were recovered by addition of protein A-Sepharose beads (Pharmacia, Upssala, Sweden) and incubated for an additional hour. Beads were washed in extraction buffer. FN was released by heating at 100°C for 5 min in electrophoresis sample buffer and analyzed under denaturing conditions in 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels. The gels were handled by fluorography techniques, and autoradiography was performed by standard methods.

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Confluent MC grown in 24-well plates were made quiescent as described above. After washing twice with PBS, MC were incubated with 10−8 M PAF for 48 h in serum-free culture medium. As a positive control of TGF-β synthesis, phorbol myristate acetate (100 nM) was used (32). Supernatants were collected and stored at −20°C until analysis. Inactive TGF-β was converted to the active form by treating culture supernatants with 10N HCl (final pH, 1.5 to 2.0) for 30 min at room temperature, followed by neutralization with an equimolar amount of NaOH (33)
Specificity was proven by neutralization of the TGF-β activity with an anti-TGF-β antibody and an unrelated control IgG.

**RNA Isolation and Northern Blot Assay**

Quiescent MC were stimulated in RPMI 1640 with 0.5% FCS. After the incubation period, total RNA was extracted by the Chomczynski and Sacchi method (34) and quantitated by absorbance at 260 nm. Equal amounts of RNA (10 to 30 μg) were denatured, electrophoresed in a 1% agarose-formaldehyde gel, and transferred to nylon membranes (Genscreen, New England Nuclear). RNA was fixed to the nylon membrane by baking for 90 min at 80°C.

The cDNA probes used were (a)I (Hf677) and (a)IV (pCVIV-1-PE16) collagens (American Type Culture Collection). Probes were prepared as described previously (35) and radiolabeled by nick translation (Boehringer Mannheim, Mannheim, Germany) with [α-32P]dCTP. The cDNA probes of rat FN (SR270), used as a 270-bp EcoRI fragment (provided by R. O. Hynes, Massachusetts Institute of Technology) (36), human TGF-β1 (p03244), used as a 274-bp EcoRI fragment, and murine TGF-β1, used as a 279-bp EcoRI fragment (a gift from F. N. Ziyadeh, University of Pennsylvania) (37), were radiolabeled by random priming method (Boehringer Mannheim) with [α-32P]dCTP.

The membranes were prehybridized for 4 h at 42°C in hybridization solution (50% formamide, 1% SDS, 5× SSC, 1× Denhardt’s, 0.1 mg/ml denatured salmon sperm DNA, and 50 mM sodium phosphate buffer, pH 6.5), and hybridization was carried out at 42°C for 16 to 18 h in fresh hybridization solution with 20% dextran sulfate and [α-32P]dCTP-denatured probe. The membranes were washed using 2× SSC, 0.1% SDS, for 30 min at room temperature and then twice with 0.2× SSC, 0.1% SDS, at 55°C for 15 min. Autoradiographs were scanned by densitometry (Image Quant densitometer, Molecular Dynamics, Sunnyvale, CA). Ethidium bromide staining was used as RNA quality control. Autoradiographic signals obtained with the 28S cDNA probe served as a control for equal loading of the gel. The ratio of mRNA versus 28S was set at unity for basals, and other lanes on the same gel were expressed as n-fold increases over this value.

**Statistical Analysis**

Results are expressed as the mean ± SEM. Significance was established using the GraphPAD Instat (GraphPAD Software, San Diego, CA), and differences were considered significant if the P value was <0.05.

**Results**

**PAF Increases mRNA Levels of Extracellular Matrix Proteins in MC**

We first studied the effect of several concentrations of PAF (10-6, 10-8, and 10-10 M) on the gene expression of extracellular matrix proteins in rat MC. As shown in Figure 1, A and C, the maximal increase of FN transcript was observed with 10-8 M PAF after 18 h of incubation (1.9-fold versus control). A similar stimulatory effect was observed on type IV collagen gene expression. The maximal increase was observed at 24 h of incubation with 10-8 M PAF (2.5-fold). By contrast, type I collagen mRNA expression was not modified during the period of study (Figure 1A).

To better extrapolate what occurs in patients with glomerulosclerosis, parallel experiments were performed in human MC (Figure 1, B and D). Incubation of cells with 10-8 M PAF also induced an increase in gene expression of FN (maximal response at 18 h with a 2.8-fold increase versus control) and type IV collagen (maximal response at 24 h with a 2.6-fold increase), with no effect on type I collagen.

**PAF Triggers FN Synthesis in Cultured MC**

To investigate whether the induction of mRNA transcription was accompanied by an increase in the production of the protein, we chose FN as a representative extracellular matrix protein because it is synthesized early during renal damage (38) and participates in cell adhesion and migration (39,40). Rat MC were metabolically labeled with [35S]methionine and immunoprecipitated with an anti-FN antibody. As pointed out previously, extracellular matrix production depended on the degree of cell confluence (41). In suprapconfluent MC, no increase in FN production was observed after PAF stimulation (data not shown). By contrast, the exposure of confluent MC to PAF significantly increased FN production in a dose- and time-dependent manner, reaching the maximal response at 24 h of incubation (10-8 M PAF; 180 ± 45% increase versus control, P < 0.05) (Figure 2, A and B). FN was found both in cell fraction (40 ± 7% versus total FN) and in the conditioned media (60 ± 5%) (Figure 2C).

In parallel experiments, we compared the PAF stimulatory effect on FN synthesis with several mediators implicated in renal sclerosis, such as TGF-β (42), interleukin (IL)-6 (43), and angiotensin II (44) (Table I). Under the same experimental conditions, the FN synthesis induced by PAF was in a similar range to that of TGF-β and IL-6, but significantly higher than that of angiotensin II (P < 0.05). These data suggest that PAF can be considered a relatively strong mediator of FN synthesis.

PAF displays its effects through binding to specific receptors in target cells (5,6). Preincubation of MC with two structurally unrelated PAF receptor antagonists, BN52021 (10-5 M), a terpene derived from the Ginkgo biloba (45), and WEB2086 (10-5 M), a chemically derived PAF antagonist belonging to the triazobenzodiazepine group (46), substantially reduced the PAF-induced FN synthesis (65 and 79% inhibition, respectively). BN52021 or WEB2086 alone did not have any significant effect on FN production. These results indicate that the observed effects of PAF are mediated by its interaction with specific cell membrane receptors.

To assess the specificity of PAF effects on FN synthesis, studies on [3H]leucine incorporation and cell proliferation were conducted. Treatment of quiescent MC with PAF for 24 h had no significant effect on TCA-precipitable [3H]leucine incorporation, either on human or on rat MC (Table 2). These data suggest that PAF does not modify total cellular protein synthesis, and that the effect on FN synthesis is a specific phenomenon. Previous studies from our group have shown that PAF does not induce hyperplasia in glomerular and tubular epithelial cells (47). In the same manner, in our experimental culture conditions, PAF did not significantly affect MC proliferation (Table 3), further suggesting that changes in matrix synthesis were not due to modifications in cell number.
Figure 1. Effect of platelet-activating factor (PAF) on extracellular matrix expression in rat and human mesangial cells (MC). A representative Northern blot analysis of rat (A) and human (B) MC incubated with $10^{-8}$ M PAF for 18 and 24 h. The membranes were hybridized with cDNA probes of fibronectin (FN), type IV collagen ((α1)IV), type I collagen ((α1)I), and 28S. Lane 1, basal 18 h; lane 2, $10^{-8}$ M PAF 18 h; lane 3, basal 24 h; lane 4, $10^{-8}$ M PAF 24 h. Molecular size markers are shown on the left. These blots are representative of a total of three experiments. Densitometric analysis of the mRNA bands of rat (C) and human (D) MC. Values are expressed as $n$-fold of increase versus control, normalized to the respective 28S bands.

**TGF-β Is Responsible for the Matrix Synthesis Elicited by PAF**

Because TGF-β is the major cytokine involved in matrix regulation (20), we tested the hypothesis that endogenous TGF-β may be responsible for the matrix production observed upon PAF stimulation.

We first studied the effect of PAF on TGF-β1 mRNA expression and synthesis. In preliminary experiments, maximal increase of TGF-β1 mRNA expression was noted between 6 and 8 h of incubation (data not shown). Therefore, this timing was chosen for further studies. Incubation of quiescent rat and human MC with $10^{-8}$ M PAF for 6 h induced upregulation of TGF-β1 mRNA expression (Figure 3). In rat MC, we observed that this effect was maximal at $10^{-8}$ M PAF after 6 h (2.7-fold versus control).

To establish whether the rise in TGF-β1 mRNA expression was accompanied by the synthesis of biologically active or latent forms of protein, we measured TGF-β activity in the conditioned media by the CCL-64 cell growth-inhibition assay. After exposure to $10^{-8}$ M PAF for 48 h, rat MC secreted...
Table 1. Comparative effect of PAF on total FN synthesis in rat MC

<table>
<thead>
<tr>
<th>Category</th>
<th>% Increase</th>
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<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>PAF (10⁻⁸ M)</td>
<td>180 ± 45b</td>
</tr>
<tr>
<td>TGF-β (50 pM)</td>
<td>190 ± 17b</td>
</tr>
<tr>
<td>Ang II (10⁻⁷ M)</td>
<td>125 ± 10bc</td>
</tr>
<tr>
<td>IL-6 (10 U/ml)</td>
<td>196 ± 28b</td>
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</table>

* Quiescent cells were metabolically labeled and stimulated for 24 h. Immunoprecipitation of newly synthesized FN using polyclonal anti-FN antibody was performed. Results are expressed as percentage increase versus control of densitometric intensity of electrophoretic bands. Mean ± SD of 6 to 9 experiments. PAF, platelet-activating factor; FN, fibronectin; MC, mesangial cells; TGF, transforming growth factor; Ang, angiotensin; IL, interleukin.

b P < 0.05 versus control.

c P < 0.05 versus PAF-treated cells.

Table 2. Effect of PAF on [³H]leucine incorporation in MC

<table>
<thead>
<tr>
<th>Category</th>
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</tr>
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<tbody>
<tr>
<td>Rat MC</td>
<td>Human MC</td>
</tr>
<tr>
<td></td>
<td>Rat MC</td>
</tr>
<tr>
<td>Control</td>
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<tr>
<td>10⁻⁸M PAF</td>
<td>97 ± 5b</td>
</tr>
<tr>
<td>10⁻⁷M PAF</td>
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<tr>
<td>10⁻¹⁰M PAF</td>
<td>97 ± 5b</td>
</tr>
<tr>
<td>20% FCS</td>
<td>211 ± 58c</td>
</tr>
</tbody>
</table>

* Quiescent cells were stimulated with PAF for 24 h, and total protein synthesis was determined by trichloroacetic acid-precipitable [³H]leucine incorporation. Results are expressed as percentage increase versus control. Mean ± SEM of three experiments made in duplicate. ND, not determined; FCS, fetal calf serum.

b P = not significative.

c P < 0.05 versus control.

Figure 2. Effect of PAF on FN synthesis in rat MC. MC were metabolically labeled with [³⁵S]methionine, stimulated with PAF, and the newly synthesized FN was determined by immunoprecipitation, using polyclonal anti-FN antibody. (A) Dose-response. Cells were incubated for 24 h in medium alone or in the presence of PAF (10⁻⁶ to 10⁻¹⁰ M). The data are expressed as percentage increase versus unstimulated cells (control). (B) Time-course evolution. Cells were incubated for the indicated times in medium alone (open bars) or in the presence of 10 M PAF (filled bars). Results are calculated as percentage increase versus unstimulated cells at 6 h (control). Mean ± SEM of four to six experiments. *P < 0.05 versus control. (C) Representative autoradiography of SDS-PAGE analysis. Lanes 1 through 3, soluble FN; lanes 4 through 6, cell-associated FN. Lanes 1 and 4, medium alone; lanes 2 and 5, 10⁻⁸ M PAF; lanes 3 and 6, 10⁻¹⁰ M PAF. Molecular weight markers are shown on the left.

Biologically active TGF-β (Figure 4). Acid treatment of the conditioned media resulted in a significant increase in active TGF-β both in control and in PAF-treated cultures (P < 0.05). In control cultures, only 26% of the total TGF-β was active, whereas in PAF-treated cultures approximately 50% was active (P < 0.05). The TGF-β activity was completely abolished by the anti-TGF-β antibody (not shown). These results indicate that PAF not only increased the production of both latent and active TGF-β, but also stimulated the conversion of latent TGF-β into active TGF-β.

Furthermore, we investigated the possible role of endogenous TGF-β in PAF-stimulated FN production. In the presence of neutralizing anti-TGF-β antibody (10 µg/ml), the PAF-induced increase in FN production and the release by rat MC were totally abolished (Figure 5). The response appears to be specific because normal rabbit IgG failed to inhibit the PAF-stimulated FN synthesis (not shown). Neither anti-TGF-β antibody alone nor control IgG had any effect on FN synthesis. Similar results were observed in human MC. The FN release
Table 3. Effect of PAF on rat MC proliferation

<table>
<thead>
<tr>
<th>Category</th>
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<tbody>
<tr>
<td>Control</td>
<td>100 ± 10</td>
</tr>
<tr>
<td>10^{-6} M PAF</td>
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<td>10^{-8} M PAF</td>
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<td>10^{-10} M PAF</td>
<td>101 ± 4b</td>
</tr>
<tr>
<td>10% FCS</td>
<td>255 ± 28c</td>
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* Quiescent cells were stimulated with PAF for 48 h, and cell proliferation was determined by methylene blue assay. Results are expressed as percentage increase versus control. Mean ± SEM of four experiments. Abbreviations as in Tables 1 and 2.

b $P < 0.05$ versus control.

c $P = 0$ not significative.

induced by PAF was abolished in the presence of neutralizing anti-TGF-β antibody. These results suggest that autocrine TGF-β secretion mediates PAF-induced increase of FN synthesis in rat and human MC.

**PAF Triggers TGF-β Expression in MC by a Protein Kinase C Pathway**

PAF stimulates the hydrolysis of phosphatidylinositol in a wide variety of cells (5,6), including glomerular MC (48), yielding second messengers that activate protein kinase C (PKC). We evaluated the possible involvement of PKC activation as a mediator of the PAF effect. In particular, we studied PAF-induced TGF-β1 mRNA expression in rat MC. For these experiments, cells were preincubated for 1 h with staurosporine (10^{-5} M), an inhibitor of PKC. The increase in TGF-β1 induced by PAF was markedly diminished (77% inhibition versus stimulated cells), suggesting a PKC-dependent pathway of TGF-β1 expression (Figure 6). Treatment with staurosporine (10^{-5} M) did not affect cell viability.

**PAF-Induced Gene Expression of FN and TGF-β Is Regulated by Cycloheximide in a Divergent Manner**

We studied the role of the de novo protein synthesis in PAF-induced matrix protein expression in MC. In rat MC, the inhibition of protein synthesis by 1 μg/ml cycloheximide (CHX), preincubated for 1 h before the addition of 10^{-8} M PAF, strongly potentiated the PAF-triggered FN mRNA expression (Table 4). In contrast, collagen I and IV mRNA were not overexpressed in CHX-pretreated, PAF-stimulated cells (Table 4). CHX also increased basal FN and collagen I mRNA. Previous studies have demonstrated that concomitant treatment with CHX resulted in superinduction of FN gene, showing an effect similar to that observed after PAF stimulation (49).

Pretreatment of cells with 1 μg/ml actinomycin D, an inhibitor of RNA synthesis, markedly reduced the amount of FN and type IV collagen mRNA induced by PAF (>95% inhibition versus stimulated cells). These data suggest that the increased matrix protein gene expression in PAF-treated cells was due, at least in part, to newly synthesized mRNA. Inhibition of the PAF-induced FN mRNA levels by actinomycin D and the increase of FN mRNA levels by cycloheximide suggest that PAF regulates FN mRNA synthesis at the transcriptional level. Although the mechanism of this effect is unknown, the gene superinduction could be due to the inhibition of the synthesis of a protein necessary for RNA degradation or a labile protein that repressed transcription (50).

In contrast, the inhibition of protein synthesis by CHX suppressed the stimulatory effect of PAF on TGF-β1 mRNA expression (88% inhibition versus stimulated cells) (Table 4). However, the addition of CHX 4 h after PAF stimulation had no effect on the PAF-induced increase in steady-state TGF-β1 mRNA levels. These data suggest that either a protein synthesized within the first few hours of agonist exposure or a short-lived constitutive protein mediates the increase in
TGF-β1 mRNA levels induced by PAF. All of these results indicate that PAF-induced TGF-β1 mRNA expression is regulated in a manner different from FN mRNA expression. Similar results were observed in human MC (not shown).

**Discussion**

In this work, we have observed that the exposure of quiescent rat and human MC to PAF increased FN and type IV collagen mRNA expression, two of the main components of mesangial matrix that are overexpressed during glomerular injury (51). PAF also increased FN synthesis, both cell-associated and released to the medium. The effect of PAF on FN production was in the same range of profibrogenic cytokines, such as TGF-β and IL-6, and greater than angiotensin II, under similar culture conditions.

The upregulating effect of PAF on FN and type IV collagen gene expression in MC contrasts with its inhibition of proteoglycan synthesis in cultured glomerular epithelial cells, a process linked to the appearance of proteinuria (47). This phenomenon has also been observed with other arachidonic acid metabolites. Thus, in MC thromboxane increased the steady-state mRNA for genes encoding FN and type IV collagen, but decreased the level of mRNA for heparan sulfate proteoglycan (3,4). However, other eicosanoids, such as prostacyclin, exerted opposite actions. In a murine cell line, prostacyclin suppressed gene expression of type IV collagen and FN, but increased heparan sulfate proteoglycan (4). The fact that PAF
did not modify type I collagen mRNA expression in cultured MC, as shown in this article, indicates that its effect is gene-specific and suggests a differential regulation during tissue injury. PAF also induced collagenase gene expression in corneal epithelial cells (19). These results suggest that this lipid mediator, classically involved in the acute inflammatory response, could now be considered as an important mediator of matrix regulation.

Recent evidence has shown that TGF-β plays a pivotal role in the pathogenesis of fibrogenesis in several tissues. TGF-β increases matrix synthesis and degradation, and the expression of integrin receptors, which mediate matrix deposition (20). In cultured MC, TGF-β increases the production of FN, collagens, and the proteoglycans biglycan and decorin (21). In experimental models of renal injury, an overproduction of glomerular TGF-β has been linked to the development of glomerulosclerosis (22). In this article, we have identified TGF-β as an important cytokine mediating the stimulatory effect of PAF on matrix protein synthesis in cultured MC. In these cells, PAF increased TGF-β1 mRNA expression, the level of both latent and active TGF-β, and also the conversion of latent to active form. Moreover, when both PAF and an anti-TGF-β-neutralizing antibody were added simultaneously, a significant diminution in FN synthesis was seen. These results support the existence of a molecular cascade in which PAF triggers the production and secretion of TGF-β, and this TGF-β in turn stimulates the synthesis of matrix components.

To clarify the intracellular mechanism of PAF effects on matrix synthesis, we determined the role of some second messengers. When PAF binds to its receptor, it induces a variety of intracellular signals (5,6). In MC, PAF stimulates arachidonic acid synthesis, intracellular calcium concentration, protein phosphorylation, and PKC activation (48). The latter has been considered a signal needed for the increased TGF-β synthesis (31). Here, we show that PAF induces TGF-β1 gene expression in MC by a PKC-dependent pathway. These results are in accordance with that observed with thromboxane A₂, which also increased FN synthesis, involving both PKC activation (52) and TGF-β production (24).

The examination of the molecular mechanism of the PAF effect on gene expression of FN, type IV collagen, and TGF-β showed a different behavior. Cycloheximide experiments showed that the PAF-induced upregulation of the FN gene is (whereas that of type IV collagen is not) entirely dependent on protein synthesis, suggesting two different mechanisms for the enhancement of matrix protein gene expression. Moreover, the augmentation of TGF-β mRNA levels induced by PAF is dependent on either a short-lived constitutive protein or the synthesis of a protein within the first few hours of stimulation. Cycloheximide also abrogated the induction of TGF-β1 mRNA, which occurred after exposure of MC to PAF, suggesting the requirement of new protein synthesis.

During glomerular injury, PAF might be released by inflammatory cells (polymorphonuclear leukocytes and monocytes-macrophages) (53) and by resident glomerular cells (endothelial, mesangial, and epithelial cells) (54,55). PAF may also induce the synthesis of cytokines and growth factors, such as TNF-α, IL-1β, IL-6 (56,12), and TGF-β. Therefore, PAF can be considered a ubiquitous mediator of glomerular injury. Acting on cells in a direct manner, or through its interaction with a large array of proinflammatory and profibrogenic agents, PAF might be implicated in the remodeling and sclerotic processes.

In summary, our data provide evidence that in cultured rat and human MC, PAF induces the expression and synthesis of extracellular matrix proteins, at least in part, by autocrine TGF-β secretion. Although future studies are needed, our results suggest that PAF could participate in the process of extracellular matrix accumulation during glomerular injury.

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