Thromboxane A₂ modulates the fibrinolytic system in glomerular mesangial cells

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Coffman, Thomas M., Robert F. Spurney, Roslyn B. Mannon, and Richard Levenson. Thromboxane A₂ modulates the fibrinolytic system in glomerular mesangial cells. Am. J. Physiol. 275 (Renal Physiol. 44): F262–F269, 1998.—We examined the effects of thromboxane A₂ (TxA₂) on the activities of the plasminogen-plasmin system in glomerular mesangial cells. When mesangial cells are exposed to the TxA₂ agonist U-46619, a substantial increase in production of plasminogen activator inhibitor-1 (PAI-1) protein is observed that is significantly greater than that induced by 10% serum alone. This increase in PAI-1 protein production is accompanied by an increase in steady-state levels of PAI-1 mRNA. This stimulation is specifically mediated by TxA₂ (thromboxane prostanoid, TP) receptors, since U-46619 also stimulates PAI-1 expression in cells that are transfected with TP receptors, and this stimulation of PAI-1 production is completely blocked by the TxA₂ receptor antagonist, SQ-29,548. Despite the increase in PAI-1 production, there was net stimulation of plasmin activity in the medium of mesangial cells that had been exposed to U-46619. Furthermore, U-46619 also caused an increase in tissue plasminogen activator (tPA) mRNA levels. Thus TxA₂ stimulates the production of PAI-1 and plasminogen activators by mesangial cells through a receptor-dependent mechanism. In inflammatory renal diseases, the balance of these effects may modulate glomerular thrombosis and renal fibrosis.

Plasminogen activator inhibitor-1; tissue plasminogen activator; thromboxane receptor; glomerulonephritis

The labile arachidonic acid metabolite thromboxane A₂ (TxA₂) plays a key role in the pathogenesis of a diverse group of kidney diseases (6, 25, 30). Enhanced production of thromboxane in the kidney has been demonstrated in diseases such as lupus nephritis (17, 36), renal allograft rejection (35), ureteral obstruction (46), and nephrotic syndrome nephritis (19). In these settings, administration of thromboxane antagonists decreases the severity of kidney disease (19, 35, 36, 46). TxA₂ has a number of biological effects that could contribute to the development of kidney dysfunction and injury. For example, TxA₂ causes renal vasoconstriction (2) and mesangial cell contraction (22), promotes platelet aggregation (25, 30), and stimulates production of extracellular matrix proteins by mesangial cells (3). However, the relative contribution of these individual actions of TxA₂ in the pathogenesis of renal disease has not been completely defined.

In a murine model of lupus nephritis, we have shown that long-term treatment with a thromboxane receptor antagonist improves kidney function, reduces glomerular crescent formation, and decreases interstitial inflammatory cell infiltrates (36). One of the striking findings in this study was that intraglomerular thrombosis, a prominent abnormality in untreated animals, was completely absent in animals that received thromboxane receptor antagonist. Because thromboxane is a relatively weak stimulator of platelet aggregation in rodents (24), the ability of thromboxane receptor blockade to prevent glomerular capillary thrombosis suggested that thromboxane might have direct effects on the coagulation system. In view of recent reports demonstrating overexpression of the procoagulant protein plasminogen activator inhibitor-1 (PAI-1) in kidneys affected by glomerular disease (15, 16, 21, 28, 40, 44), we speculated that regulation of PAI-1 by TxA₂ might be a pathway through which TxA₂ may directly affect intravascular coagulation.

Plasminogen activators such as tissue plasminogen activator (tPA) convert plasminogen to active plasmin, which functions to degrade fibrin clots and also to break down extracellular matrix proteins during tissue remodeling. PAI-1 is a serine protease inhibitor that serves as the major physiological regulator of tPA (1, 7, 41). Alterations in plasmin activity in the glomerulus would lead to decreased clot lysis (procoagulation) and accumulation of extracellular connective tissue components (fibrosis) (8). In tissue culture, human mesangial cells (12, 26, 43) and glomerular epithelial cells (14) can synthesize PAI-1 and plasminogen activators. While very little PAI-1 is found in normal kidneys (16), enhanced renal PAI-1 production has been observed in several inflammatory kidney diseases including murine lupus nephritis (15, 16, 21, 28, 40, 44). However, the factors that regulate PAI-1 and plasminogen activator production in the kidney have not been characterized. As TxA₂ is one of the final common pathways for kidney injury in a number of inflammatory diseases, we examined the effects of TxA₂ on this system in glomerular mesangial cells.

METHODS

Rat glomerular mesangial cell cultures. Primary cultures of glomerular mesangial cells were established from glomeruli isolated from 150- to 200-g PVG strain rats as described (37). In previous studies, we have extensively characterized thromboxane receptor binding and signaling mechanisms in these cells (32, 37). Experiments were carried out on confluent cells from passages 3 to 24. For 36 h prior to study, confluent cells were incubated in medium containing insulin, antibiotics, and 0.1% heat-inactivated fetal calf serum (serum deprivation). We have found that serum deprivation enhances both TxA₂ binding and physiological responses to TxA₂ agonists (37).
mesangial cell TxA₂ receptor responsiveness have been reported by other investigators (23).

Analysis of PAI-1 protein by Western blot. In some experiments, PAI-1 protein in cell culture supernatants was measured by Western blotting. A quantity of 5 µl of conditioned medium was combined with 5 µl of 2× Laemmli buffer and heated for 2 min at 95°C, and proteins were separated on 11% Bio-Rad SDS-PAGE minigels. The proteins were transferred to nitrocellulose, and PAI-1 was detected using rabbit polyclonal anti-PAI-1 antibody (10) and biotinylated goat anti-rabbit IgG and visualized using chemiluminescence detection system and Kodak X-AR film.

Analysis of PAI-1 protein synthesis by immunoprecipitation: time course and dose-response studies of the U-46619 effect. After mesangial cells were grown to confluence in 6-well tissue culture dishes, the cells were serum deprived for 36 h. In the dose-response studies, vehicle or doses of 0.5, 1, 2, 5, or 10 µg/ml of U-46619 were then added to the medium; in the time course studies, 1 µg/ml U-46619 was used. In the dose-response studies, cells were harvested 3 h after the addition of U-46619 (n = 3 for each group). In the time course experiments, cells were harvested at 1, 2, 3, 4, 6, 8, and 20 h after U-46619 exposure (n = 3 for each group). One hour before harvest, the medium was replaced with 800 µl of methionine-free RPMI containing 25 mM HEPES plus 10 µl of [³⁵S]methionine (specific activity, 1 mCi/83 µl). After 1 h, the medium was harvested, and 2 µl of affinity-purified rabbit anti-PAI-1 antibody was added to 100 µl of medium from each well. After incubation for 1 h at 4°C, 100 µl of a 10% (vol/vol) suspension of protein-A-agarose beads was added, and the samples were incubated with tumbling for an additional 90 min. The beads were then washed twice with RIPA buffer, and the bound proteins were eluted with 20 µl of Laemmli sample buffer at 100°C for 2 min. Volumes of 10 µl of each sample were then applied to wells in a Bio-Rad 11% SDS-PAGE minigel and electrophoresed at 200 V for 45 min. The gel was dried and exposed to Kodak X-AR film overnight.

RNA isolation and Northern blot analysis. Total cellular RNA was isolated from cultures of confluent mesangial cells using TRIzol reagent (GIBCO/BRL, Gaithersburg, MD), and RNA was size-fractionated on agarose gels after denaturation in glyoxal and DMSO (29). Briefly, RNA samples were dried, resuspended in 10 µl of a solution of 1.2 M denitrate glyoxal/20 mM sodium phosphate/50% DMSO, and incubated at 50°C for 1 h. The samples were placed immediately on ice, 2.5 µl of loading buffer was added, and the samples were separated on 1.2% agarose gels in recirculating 10 mM sodium phosphate buffer. The RNA was then transferred to nylon membranes that were subsequently exposed to ultraviolet irradiation and baked at 80°C for 2 h.

The membranes were then hybridized at high stringency with 1) a full-length cDNA probe for rat PAI-1 (47) that had been labeled with ³²P by random priming or 2) with antisense riboprobes prepared from cDNA template for mouse tPA (27) or GAPDH using standard techniques. After hybridization and washing, the filters were exposed to Kodak X-AR film at −80°C for up to 1 wk with the cDNA probes and for 24 h with the riboprobe. The intensity of the autoradiographic signal was quantitated by laser densitometry (Molecular Dynamics, Sunnydale, CA), and the results are expressed in units that correspond to the area under the densitometric peak. To normalize for the amount of RNA in each sample, the autoradiographic signals for PAI-1 and tPA were factored by the corresponding GAPDH signal.

Transfection of a mouse mesangial cell line with a genomic clone containing the entire coding region of the TP receptor was isolated and cloned into the mammalian expression vector pcDNA 3 (Invitrogen, San Diego, CA) as described (34). This vector, which contains a neomycin resistance element, was transfected into a mouse mesangial cell line from SV40 transgenic mice (20) using the calcium-phosphate method (5). Cells were exposed overnight with serum, and the transfection efficiency was ~40%. To isolate permanent transfectants, cells were grown in complete medium containing 500 µg/ml G-418 and individual G-418-resistant clones were screened for expression of TP receptors by radioligand binding as described (34).

Assay of plasmin activity in mesangial cell cultures. Plasmin activity in medium obtained from cultured mesangial cells was determined as described (45) using the synthetic fluorometric plasmin substrate methoxy-succinyl-L-Ala-L-Phe-L-Lys-7-amido-4-methylcoumarin. A 100-µl volume of sample was mixed with 450 µl of 0.2 M Tris·HCl, pH 7.4, containing 0.2 M NaCl and 0.05% NaN₃ and 125 µl of water. The reactions were started by the addition of the substrate followed by incubation at 37°C for 40 min. The reaction was then stopped by adding 100 µl of soybean trypsin inhibitor (0.25 mg/ml) followed by vigorous mixing. The fluorescence of each tube was determined using a fluorometer equipped with appropriate filters for aminomethylcoumarin fluorescence (excitation at 360 nm, emission at 450 nm). Plasmin standards were also included in each assay, and the plasmin content of each sample was determined by a standard curve that was constructed based on the purified plasmin standards. Results are expressed as the means ± SE, corrected for background based on the appropriate blanks (n = 6 for each group).

Statistical analysis. Data are expressed as the mean ± SE where appropriate. The statistical significance of differences between groups was assessed by unpaired t-test.

RESULTS

In initial experiments, we examined PAI-1 production by glomerular mesangial cells in culture by Western blot. As shown in Fig. 1, quiescent mesangial cells incubated in 0.1% serum synthesize very little PAI-1 protein. Upon exposure to 10% serum, a modest increase in PAI-1 production was detected. To determine whether TxA₂ affected PAI-1 synthesis, mesangial cells
were incubated with 1 µg/ml of the TxA2 agonist U-46619. As can be seen in Fig. 1, within 3 h after exposure to thromboxane agonist, a substantial stimulation of PAI-1 production is present in the agonist-stimulated cells compared with either the quiescent cells or cells in 10% serum.

To determine whether this increase in PAI-1 protein production was associated with alterations in PAI-1 mRNA levels, we isolated RNA from mesangial cells and measured steady-state mRNA levels for PAI-1 by Northern blot. As seen in Fig. 2, PAI-1 mRNA was not detected in quiescent mesangial cells in 0.1% serum. When the cells were exposed to 10% serum, modest but detectable levels of PAI-1 mRNA were seen. Exposure to 1 µg/ml U-46619 resulted in a significant stimulation of PAI-1 mRNA that mirrored the increase in new PAI-1 protein synthesis demonstrated in Fig. 1.

In a separate series of experiments, we examined the time course for thromboxane stimulation of PAI-1. Aliquots of mesangial cells were harvested for measurement of PAI-1 protein production at intervals between 1 and 20 h following U-46619 exposure. As shown in Fig. 3, A and B, thromboxane caused a rapid induction of PAI-1 protein that peaked at 3 h following U-46619 exposure. This was a persistent effect and stimulation of PAI-1 production above baseline could be detected up to 20 h after exposure to thromboxane agonist.
To determine the dose-response relationship between thromboxane agonist and PAI-1 production, we incubated separate aliquots of mesangial cells with increasing concentrations of U-46619 up to 10 µg/ml and measured PAI-1 protein production 3 h later. The results of these studies are depicted in Fig. 4. As can be seen in Fig. 4, maximal stimulation was observed at a concentration of 1 µg/ml of U-46619. Within the dose range tested, increasing the concentration of U-46619 beyond 1 µg/ml caused little further stimulation of PAI-1 production.

To determine whether stimulation of PAI-1 production by U-46619 was specifically mediated by the thromboxane receptor, we examined the effect of the thromboxane agonist on PAI-1 mRNA expression in mesangial cell lines that were transfected with a genomic clone containing the entire coding region for the mouse TP receptor. We have previously found that wild-type cells express negligible levels of thromboxane receptor and respond minimally to thromboxane agonists, whereas this transfected cell line expresses ~500 fmol thromboxane binding sites per milligram of protein (31). As seen in Fig. 5A, PAI-1 mRNA is not detected in unstimulated cells. However, marked stimulation of PAI-1 mRNA levels is observed following the addition of U-46619 (1,362 ± 599 vs. 5,100 ± 576 normalized densitometry units; P = 0.011). Stimulation of PAI-1 expression by U-46619 is completely blocked when the cells are pretreated with the specific thromboxane receptor antagonist SQ-29,548. Similarly, as shown in Fig. 5B, the stimulation of PAI-1 protein production by U-46619 is also blocked by SQ-29,548.

Since corticosteroids may induce PAI-1 synthesis in some systems (4, 9) and these agents are commonly used in the treatment of inflammatory renal diseases, we examined the effect of dexamethasone on the stimulation of PAI-1 production by mesangial cells. Dexamethasone exposure for 6–48 h caused a marked suppression of basal PAI-1 production as seen in Fig. 6. Moreover, pretreatment of mesangial cells with dexamethasone markedly blunted the stimulation of PAI-1 production by thromboxane agonist. This effect was most marked in cells exposed to dexamethasone for 6 h but was also seen following 24 or 48 h of dexamethasone pretreatment.

Although our studies clearly identified a direct stimulation of PAI-1 production mediated by stimulation of the thromboxane receptor, fibrinolytic activity is determined by the relative activities of PAI-1 and plasminogen activators. To determine the net effect of TXA2 agonists on this balance, we measured plasmin activity...
in the supernatants of mesangial cells that had been exposed to U-46619 for 3 h. As shown in Fig. 7, U-46619 caused a significant enhancement of plasmin production from $0.120 \pm 0.016$ to $0.220 \pm 0.020$ mU ($P < 0.01$). This increase in plasmin generation following U-46619 suggested that, in addition to stimulating PAI-1 production, the thromboxane agonist was affecting production of plasminogen activators. To examine this possibility, we exposed mesangial cells to 1 µM U-46619 and measured mRNA levels for tPA by Northern analysis. The results of these studies are shown in Fig. 8.

**DISCUSSION**

Glomerular capillary thrombosis and fibrin deposition in the glomerulus and within epithelial crescents are common features of glomerulonephritis (11, 18, 42). The presence of these abnormalities and the beneficial effects of anticoagulant therapies in certain glomerulopathies have suggested a role for the coagulation and fibrinolytic systems in the development and progression of renal diseases (18, 39, 48). Recent studies have demonstrated upregulation of renal PAI-1 production in disease states (15, 16, 21, 28, 40, 44), suggesting that PAI-1 might be one factor that promotes coagulation and fibrin deposition in glomerulonephritis. Although primary cultures of human mesangial cells and glomeru-
lar epithelial cells are capable of synthesizing components of the fibrinolytic system (12, 14, 26, 43), little is known about factors that regulate fibrinolysis in the diseased kidney. In this study, we have demonstrated a direct stimulatory effect of the arachidonic acid metabolite, TXA2, upon the production of PAI-1 and plasminogen activators by mesangial cells.

In our study, the level of stimulation of mesangial cell PAI-1 production by thromboxane was proportional to the dose of TXA2 agonist and was associated with enhanced PAI-1 mRNA levels. Furthermore, the dose-response curve corresponds well with the Kd for binding of U-46619 in mesangial cells (37), consistent with a receptor-mediated action. The effect of thromboxane on PAI-1 synthesis was rapid; it was observed within 1 h after exposure to the thromboxane agonist, with maximal stimulation occurring at 3 h. The TXA2-stimulated production of PAI-1 was long lasting and could be detected for at least 20 h following thromboxane administration. Although the effect of the thromboxane agonist is specifically mediated through stimulation of the thromboxane receptor, the intracellular signaling pathways that mediate PAI-1 stimulation cannot be determined from our experiments. Studies by our group (32, 37) and others (23, 38) have demonstrated that thromboxane receptors in mesangial cells are coupled to protein kinase C activation. Since Peraldi and associates (26) have found that activation of protein kinase C increases PAI-1 release by human mesangial cells, we speculate that thromboxane stimulation of PAI-1 production in mesangial cells might also be mediated through pathways involving protein kinase C activation.

In some experimental systems, corticosteroids induce PAI-1 expression (4, 9), causing overall inhibition of tPA activity, despite concomitant induction of tPA synthesis (13). In glomerular mesangial cells, we found that the corticosteroid dexamethasone reduces basal PAI-1 synthesis and prevents stimulation of PAI-1 expression by thromboxane. This inhibitory effect was apparent for up to 48 h after dexamethasone exposure. Corticosteroids have been a cornerstone of therapy of glomerulonephritis, and these agents are believed to exert their beneficial effects through their potent anti-inflammatory and immunomodulatory properties. Preventing the induction of PAI-1 may represent another potential mechanism of action of corticosteroids in ameliorating kidney injury associated with inflammatory renal diseases.

The activity of the fibrinolytic system depends on the conversion of plasminogen to plasmin. The level of plasmin is regulated by the balance of the actions of plasminogen activators, such as tPA, and plasminogen activator inhibitors, such as PAI-1. Since protein kinase C activation stimulates tPA production in a number of cell types, including human renal mesangial cells (26), we considered the possibility that TXA2 might also stimulate tPA production by mesangial cells. Similar to its effects on PAI-1, we found that U-46619 caused a brisk upregulation of tPA expression that was mediated by TP receptors. Moreover, the overall effect of the thromboxane agonist in this system was to increase plasmin activation, suggesting that the net stimulation of plasminogen activators by U-46619 was greater than its effect on PAI-1. Stimulation of fibrinolytic activity by TXA2 was not predicted by our previous studies, which suggested that thromboxane promotes glomerular thrombosis in lupus nephritis (36). However, in the current in vitro studies, we have isolated only two components of the microenvironment of the inflamed glomerulus. In a complex milieu in vivo that includes platelets, endothelial cells, other circulating mediators, and immune complexes, the net effects of thromboxane on the fibrinolytic system might be quite different. Nonetheless, we have clearly demonstrated modulation of the plasminogen-plasmin system by TP receptors that was previously unrecognized. The specific role for these actions in the pathogenesis of glomerular disease remains to be determined.

TXA2, through its physiological and cellular effects, causes kidney dysfunction and injury in a number of renal diseases. TXA2 is a potent renal vasoconstrictor (2), and it mediates reversible vasoconstriction in several experimental and human diseases (17, 19, 35, 36, 46). In addition, Bruggeman and associates (3) have shown that thromboxane agonists directly stimulate production of extracellular matrix proteins such as laminin and type IV collagen. As accumulation of matrix proteins in the glomerulus and renal interstitium is the hallmark of chronic, irreversible kidney injury, this suggested a direct mechanism by which thromboxane could promote chronic renal injury. The effects of TXA2 on fibrinolysis may also contribute to these effects. While the most widely recognized activity of plasmin is its ability to degrade fibrin, plasmin is also capable of degrading extracellular matrix and probably plays an important role in the proteolysis that accompanies tissue repair (1, 7, 8, 41). Thus alterations in plasmin activity by thromboxane would tend to modulate its effects to promote coagulation and to stimulate matrix protein synthesis.

In summary, the arachidonic acid metabolite TXA2 regulates the fibrinolytic system in glomerular mesangial cells. The effects of thromboxane on PAI-1 and plasminogen activator production are receptor-mediated and may occur through signaling pathways involving protein kinase C. In kidney diseases, the balance of the effects of thromboxane in regulating fibrinolysis may impact on the development of pathological changes such as glomerular capillary thrombosis and intrarenal fibrosis.
REFERENCES


