Aldosterone and TGF-β₁ synergistically increase PAI-1 and decrease matrix degradation in rat renal mesangial and fibroblast cells

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Huang W, Xu C, Kahng KW, Noble NA, Border WA, Huang Y. Aldosterone and TGF-β₁ synergistically increase PAI-1 and decrease matrix degradation in rat renal mesangial and fibroblast cells. *Am J Physiol Renal Physiol* 294: F1287–F1295, 2008. First published March 26, 2008; doi:10.1152/ajprenal.00017.2008.—Aldosterone is thought to modulate renal fibrosis, in part, through increasing plasminogen activator inhibitor type 1 (PAI-1), a major inhibitor of ECM degradation. The present study investigated aldosterone effects on PAI-1 and transforming growth factor (TGF)-β₁ and asked whether PAI-1 effects were TGF-β₁ mediated and whether aldosterone and TGF-β₁ acted synergistically to increase PAI-1 and decrease ECM degradation. Rat mesangial cells (MCs) and fibroblast cells [normal rat kidney (NRK)-49F] were used. ³H-labeled ECM was produced by MCs. The effect of aldosterone and TGF-β on ECM degradation by newly plated MCs or NRK-49F was measured by the release of ³H into medium. Aldosterone markedly increased PAI-1 mRNA and protein in both cell types, increases completely blocked by spironolactone and partially blocked by TGF-β neutralizing antibody. Adding both aldosterone and TGF-β₁ produced PAI-1 mRNA and protein increases higher than the sum of increases seen with either compound alone. Aldosterone or TGF-β₁ alone inhibited matrix degradation by 39 and 49% in MCs and 21 and 23% in NRK-49F, respectively. When both compounds were added, matrix degradation was further decreased by 93% in MCs and 61% in NRK-49F. The results indicate that aldosterone-induced PAI-1 increases are partially mediated by TGF-β₁ and lead to decreased ECM degradation. While aldosterone alone induced TGF-β₁ weakly, aldosterone and TGF-β₁ added together produced dramatic synergistic effects on PAI-1 production and subsequent ECM accumulation. Thus the elevated aldosterone induced by renin-angiotensin-aldosterone system activation may amplify renin-angiotensin-aldosterone system profibrotic actions.

**ACTIVATION OF THE RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM (RAAS) and generation of angiotensin II (ANG II) play a crucial role in fibrotic renal disease through both pressure-dependent and pressure-independent mechanisms (29). ANG II blockade has been a great therapeutic breakthrough for a variety of renal diseases. However, accumulating evidence from both clinical and laboratory research has suggested that halting renal fibrosis cannot be achieved by angiotensin-converting enzyme inhibitors (ACEi) or ANG II receptor blockers alone or in combination, given at maximal dosages and correct frequency (14, 18, 37, 43). One reason could be the effects of aldosterone. Elevated plasma aldosterone levels are usually observed in patients chronically treated with ACEi by a phenomenon known as “aldosterone escape” (48). The elevated aldosterone level is seen not only in plasma, but also in tissue due to alternative pathways of ANG II generation, including chymase and chathespin G (34). Although it was once thought that aldosterone acts primarily as a circulating hormone involved in the regulation of sodium excretion through mineralocorticoid receptor (MR)-dependent mechanisms, evidence has been mounting to suggest that aldosterone also contributes to inflammatory and fibrotic effects that were previously attributed solely to ANG II (16, 53). In fact, aldosterone receptor blockers have been shown to decrease mortality in patients with congestive heart failure, to improve endothelial function, to reduce circulating markers of collagen turnover, and to decrease microalbuminuria (17, 44, 45, 50). Addition of spironolactone (SPR) to ACEi markedly reduced proteinuria in patients with renal failure and in patients with diabetes (11, 46). In rats, studies have consistently demonstrated that aldosterone causes glomerular injury, interstitial inflammation, and fibrosis in hypertensive rat kidneys (5). Aldosterone receptor blockers markedly ameliorated glomerular and/or tubulointerstitial injury in several models of nephropathy, including spontaneously hypertensive stroke-prone rats, diabetic rats, nephritic rats, and chronic cyclosporine-induced nephrotoxic rats (20–22, 39, 42, 47). Moreover, MR blockade not only reduced the development of glomerulosclerosis, but also induced regression of existing glomerulosclerosis in rats after 5/6 nephrectomy (1). These studies together not only emphasized the beneficial effect of MR antagonism in progressive renal diseases, but also suggested that the profibrotic effects of aldosterone might be independent of other components of the RAAS. Thus the aldosterone escape phenomenon occurring during long-term use of ANG II blocker may, in fact, contribute to the limited effectiveness of this therapy. Understanding the mechanisms underlying ANG II-independent actions of aldosterone may guide the use of combination therapy with ANG II and aldosterone blockers in these patients. One possible mechanism by which aldosterone may contribute to renal fibrosis is through induction of plasminogen activator inhibitor-1 (PAI-1) expression. Aldosterone was first observed to induce PAI-1 expression in vascular smooth muscle cells and then in endothelial cells, cardiomyocytes, and recently in mesangial cells (MCs) (8, 12, 56). Treatment with an MR antagonist decreased PAI-1 expression in the heart of
ANG II/N^G^-nitro-l-arginine methyl ester-treated mice (8) and in the rat kidney following radiation injury (9), streptozocin-induced diabetic nephropathy (20), and 5/6 nephrectomy (1). Moreover, PAI-1 deficiency protected against aldosterone/salt-induced glomerular hypertrophy and injury (35). Elevated PAI-1, the major inhibitor of plasminogen activators, is thought to inhibit plasmin generation and activation of matrix-degrading metalloproteinases, thereby decreasing ECM degradation and enhancing ECM accumulation in the kidney (26). It is likely that PAI-1 mediates aldosterone-induced profibrotic effects in renal disease.

Several other key modulators of renal fibrosis also induce PAI-1. Transforming growth factor-β (TGF-β) increases PAI-1 production by cultured glomeruli, and overexpression of TGF-β in disease is associated with increased PAI-1 expression (15, 43, 51). ANG II upregulates PAI-1 expression by mechanisms both independent of, and dependent on, TGF-β (30). Therapeutic strategies aimed to reduce ANG II or TGF-β also reduce PAI-1 overexpression (43, 55). Recently, renin was found to induce PAI-1 expression via a renin receptor-mediated, ANG II-independent mechanism partially mediated by TGF-β (27). The increasingly recognized connection between aldosterone and progressive kidney disease stimulated us to ask whether aldosterone, like ANG II and renin, induces TGF-β, whether aldosterone induces PAI-1 expression through a TGF-β-mediated pathway, and whether aldosterone exerts a synergistic effect with TGF-β on PAI-1 overexpression, thereby leading to decreased ECM degradation.

We chose to study the profibrotic action of aldosterone on MCs and renal fibroblasts, since abundant MRs are present, particularly in the cytoplasm of MCs (38, 40) and because they are key cell types involved in glomerular and interstitial fibrosis.

To study ECM degradation, we developed an in vitro system utilizing MCs cultured on extracellular matrices produced by MCs themselves, which contain similar matrix components to those seen in vivo (23). Utilizing this system, we confirmed that ECM degradation by cultured rat MC or normal rat kidney fibroblasts (NRK-49F) was dependent on added plasminogen and was mediated by plasmin (23, 54). The fact that addition of urokinase-type (uPA) or tissue-type plasminogen activator (tPA) was not required for matrix degradation confirmed other published studies showing that MCs or NRK-49F cells produce uPA and tPA. ECM degradation by cultured rat MCs or NRK-49F cells thus is dependent on the presence of a functional plasminogen activator/plasminogen/plasmin system (3, 4). As the primary in vivo inhibitor of the plasminogen activators tPA and uPA, PAI-1 tightly regulates this system (25). Increased PAI-1 levels can dramatically reduce plasmin generation, thereby decreasing plasmin-dependent extracellular matrix degradation and defining a mechanistic path for increasing matrix accumulation in progressive kidney disease.

**METHODS**

**Materials**

Aldosterone and SPR were supplied by Acros Organics. Recombinant human TGF-β1, 1D11 used as the pan-specific TGF-β neutralization antibody (Ab), and 13C4 used as control IgG were obtained from R&D Systems (Minneapolis, MN). Unless otherwise indicated, materials, chemicals, or culture media needed were purchased from Sigma Chemical (St. Louis, MO).

**Cell Culture**

Primary MCs were derived from intact rat glomeruli of 4- to 6-wk-old Wistar rats and characterized according to published methods (30). Cells were used between passages 4 and 7 and were maintained in RPMI-1640 medium supplemented with 20% FBS (HyClone Laboratory, Logan, UT), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.1 U/ml insulin, and 25 mM HEPES buffer at 37°C in a 5% CO₂ incubator. Subconfluent cells seeded on six-well plates were made quiescent in serum-free RPMI-1640 medium for 48 h before experimental studies.

NRK-49F rat fibroblast cells were obtained from ATCC (Rockville, MD) and were maintained in DMEM medium (ATCC) supplemented with 5% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Subconfluent NRK-49F cells seeded on six-well plates were made quiescent by DMEM medium with 0.2% FBS for 16 h before experimental studies.

Aldosterone and SPR were dissolved in alcohol first, and the final alcohol concentration was 0.1% with 0.5% DMSO in serum-free RPMI-1640 (used for MC treatment) or in DMEM medium with 0.2% FBS (used for NRK-49F treatment). Vehicle-treated control cells were incubated in the same media containing 0.1% alcohol and 0.5% DMSO. MCs and NRK-49F cells were preincubated with SPR or 1D11 or control IgG for 1 h before treatment with aldosterone or TGF-β1. In initial control experiments, no changes in cell viability were seen with solvent (0.1% alcohol with 0.5% DMSO), SPR, aldosterone, or 1D11 (data not shown).

Cells were harvested after 8- or 24-h incubation for determination of mRNA levels by Northern blot. Supernatant was collected after 36 or 48 h for analysis of PAI-1 protein expression by Western blot. Cell lysate was obtained 24–48 h after treatment for detection of TGF-β1 protein by ELISA.

**RNA Preparation and Northern Hybridization**

Total RNA of cultured cells was extracted by a guanidinium isothiocyanate method using TRIzol Reagent (GibcoBRL, Gaithersburg, MD), according to the manufacturer’s instructions. After being denatured and fractionated by electrophoresis through a 1.0% agarose gel (20 µg/lane), RNA was transferred to a nylon membrane (BrightStar Plus, Ambion, Austin, TX) overnight, and nucleic acids were immobilized by UV irradiation (Stratagene, La Jolla, CA). Membranes were prehybridized with ULTRAhyb buffer (Ambion) and hybridized with DNA probes labeled with 32P-DCTP by random oligonucleotide priming (Strip-EZ DNA, Ambion). The blots were washed in 2x SSC, 0.1% SDS at 42°C for 10 min, and in 0.1x SSC, 0.1% SDS at 42°C for 15 min twice. DNA probes used were 1) rat GAPDH cDNA, a gift from Dr. P. G. Kondaiah and M. B. Sporn (19); 2) PAI-1 cDNA, kindly provided by T. D. Gelehrter (10); and 3) TGF-β1 cDNA, kindly provided by Dr. H. L. Moses (13). Four blots per probe were performed. Autoradiographic signals obtained with GAPDH cDNA probe served as controls for equal loading of gels. Autoradiographs were scanned on a Bio-Rad GS-700 imaging densitometer. Changes in mRNA levels were determined by first correcting for the densitometric intensity of GAPDH for each sample. For comparison, this ratio was set at unity for normal control samples, and other lanes on the same gel were expressed as fold increases over this value.

**PAI-1 Western Blot**

After treatment, the cultured cell supernatant was harvested and centrifuged immediately at 2,000 rpm for 5 min to remove any floating cells or fragments. The equal volume of supernatant (40 µl) without concentration mixed with 13.3 µl of 4X loading buffer was then separated by 10% Tris-glycine gel electrophoresis (Novex Tris-Glycine Gels, Invitrogen Life Technologies, Carlsbad, CA) and transferred to a 0.45-µm Immobilon-P transfer membrane (Millipore,
Nonspecific binding was blocked with 5% nonfat milk powder in Tris-buffered saline (TBS) for 1 h at room temperature followed by a 4°C overnight incubation with primary Ab (rabbit-anti-rat PAI-1 IgG, stock solution: 250 μg/ml; American Diagnostica, Greenwich, CT; diluted 1:200 in 5% BSA in TBS/0.1% Tween 20 with 0.02% NaN3). The blots were washed three times in TBS/0.1% Tween 20 for 10 min each. The second Ab (anti-rabbit horseradish peroxidase, stock solution: 400 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA) was incubated at a dilution of 1:2,000 for an additional 1 h at room temperature followed by three washes as described above. Bound Abs were detected by developing the blots in ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 min. Quantitation of the bands on autoradiograms was performed using a Bio-Rad GS-700 imaging densitometer.

**ELISA Analysis for TGF-β1 in Cell Lysate**

NRK-49F and MCs were exposed to 10⁻⁶ or 10⁻⁷ M aldosterone for 24, 36, and 48 h for measurements of TGF-β1 production. After culture supernatant was removed, cells were lysed with cell lysis buffer (Cell Signaling Technology, Beverly, MA). Protein concentration was determined using bicinchoninic acid protein assay kit (Pierce, Rockford, IL). The samples were acid activated, and TGF-β1 concentration was determined using a commercially available sandwich ELISA system (Quantikine, R&D Systems, Minneapolis, MN), according to the manufacturer’s instructions.

**Measurement of ECM Degradation**

Production of radioactive matrices. Rat MC matrices were obtained as described previously (23, 33). Briefly, MCs were seeded into six-well plates at 2 × 10⁵ cells/well. Ascorbic acid (25 μg/ml) was added to the culture on the second day and daily thereafter. The samples were acid activated, and TGF-β1 expression was determined using bicinchoninic acid protein assay kit (Pierce, Rockford, IL). The samples were acid activated, and TGF-β1 was measured using a commercially available sandwich ELISA system (Quantikine, R&D Systems, Minneapolis, MN), according to the manufacturer’s instructions.

Degradation of the matrices by MC and NRK-49F cells. Labeled matrices were washed twice with 2 ml of serum-free RPMI-1640 medium before addition of MCs at 2 × 10⁵ cells/well in 15% FBS RPMI-1640 medium. Medium was removed after 24-h incubation, allowing MCs to attach and to recover from plating. Cells were washed three times with 2 ml of serum-free RPMI-1640 medium to remove proteolytic enzyme inhibitors potentially present in the serum and then incubated for 24 h in 2 ml of serum-free RPMI-1640 containing 0.2% lactalbumin hydrolyzate and 4 μg/ml plasminogen. Exogenously added agents were dissolved in RPMI-0.2% lactalbumin hydrolyzate at the concentrations indicated below. At the end of the experiment, supernatants containing digested matrix were collected. Matrix remaining on the plates was also collected after being digested with 2 ml of 2 N NaOH at 37°C for 18 h. All samples were measured in a scintillation counter. Background values obtained with medium in the absence of cells were subtracted from these values. The sum of counts in the supernatant and residual undigested matrix were considered 100%. The percentage of ECM degradation was expressed as the value of supernatent counts divided by that of the sum of counts.

Degradation of the matrices by NRK-49F cells was measured by a similar procedure, except DMEM with 5% FBS was used for culture and DMEM with 0.2% lactalbumin hydrolyzate was used for treatment instead of RPMI-1640.

**Statistical Analysis**

Values are expressed as means ± SE. Statistical analyses of differences between the groups were performed by ANOVA and subsequent Student-Newman-Keuls or Dunnett testing for multiple comparisons. Comparisons with a P value < 0.05 were considered significantly different. Triplicate wells were analyzed for each experiment, and each experiment was performed independently a minimum of three times.

**RESULTS**

**Effect of Aldosterone on PAI-1 and TGF-β1 mRNA Expression and Protein Production by Renal MCs**

When subconfluent, quiescent renal MCs were exposed to 10⁻¹¹ to 10⁻⁷ M aldosterone, a dose-dependent, significant
increase in PAI-1 mRNA expression was seen after 8 h, with a 2.35-fold increase at the highest dose (10^{-7} M) (P < 0.001, Fig. 1A). That mRNA increases resulted in protein increases is shown in Fig. 1C, where PAI-1 protein production increased by 2.75-fold after 36 h. Treatment of cells with 10^{-5} M SPR, an aldosterone receptor antagonist, completely blocked the stimulation of PAI-1 mRNA synthesis and protein production (P < 0.001, Fig. 1, B and D). As shown in Fig. 2A, a marked increase in TGF-β1 mRNA expression was seen only after 10^{-5} M aldosterone administration for 3 h (Fig. 2A). Aldosterone (10^{-6} M) resulted in 25 and 75% increases in TGF-β1 protein after 24- and 48-h treatment, respectively (P < 0.05) (Fig. 2B). Treatment of cells with SPR for 48 h completely blocked the stimulation of TGF-β1 protein synthesis (Fig. 2C). These results indicate that aldosterone markedly stimulates MC PAI-1 mRNA and protein through a classic MR-mediated pathway. Aldosterone stimulates TGF-β1 significantly but weakly compared with the expression of PAI-1.

**Effect of Aldosterone on PAI-1 and TGF-β1 mRNA Expression and Protein Production by Renal NRK-49F Fibroblast Cells**

Similarly, when subconfluent quiescent fibroblasts were exposed to 10^{-11} to 10^{-6} M aldosterone in 0.2% FBS DMEM medium, PAI-1 mRNA increased in a dose-dependent manner after 24 h, resulting in a 3.33-fold increase in protein levels after 48 h (P < 0.001, Fig. 3, A and C). Addition of 10^{-5} M SPR completely blocked aldosterone-induced increase of PAI-1 mRNA expression and protein production (Fig. 3, B and D). Aldosterone (10^{-6} M) also stimulated TGF-β1 mRNA expression by NRK-49F fibroblast cells slightly but signifi-

Fig. 2. Effect of Aldo dose on transforming growth factor (TGF)-β1 mRNA and protein production in renal mesangial cells. A: quiescent rat mesangial cells were incubated for 3 h in the presence of various concentrations of Aldo, and Northern blotting was performed. Results of the densitometric analyses of Northern blots after correction for GAPDH mRNA are shown graphically below the blots. Values are expressed relative to vehicle-treated control, which was set at unity. B: quiescent rat mesangial cells were incubated in the absence or presence of 10^{-6} M Aldo for the indicated times, and ELISA was performed. Graph shows the relative levels of PAI-1 protein comparison with that of control at each time point. *P < 0.05 compared with the increase at 24-h incubation. C: quiescent rat mesangial cells were incubated for 48 h in the presence of 10^{-6} M Aldo, with or without 10^{-3} M Aldo, and ELISA was performed. Values are expressed relative to vehicle-treated control, which was set at unity. *P < 0.05 compared with vehicle-treated control (A and C). #P < 0.05 compared with 10^{-6} M Aldo alone-treated cells (C).
Synergistic Effect of TGF-β1 and Aldosterone on the Expression of PAI-1 mRNA and Protein by Renal MCs and NRK-49F Cells

TGF-β overexpression is a ubiquitous feature of renal fibrosis, both in animal models and in humans (7, 24). We asked whether elevated aldosterone and TGF-β, when present together, might contribute to a deteriorating cycle of amplified fibrogenic signals. Renal MCs and NRK-49F cells were treated with aldosterone (10^{-7} M in MCs and 10^{-8} to 10^{-6} M in NRK-49F) and TGF-β1 (5 ng/ml in MCs and 20 pg/ml in NRK-49F) alone and together. The results are shown in Fig. 7, A and B. TGF-β1 and aldosterone together dramatically increased PAI-1 mRNA and protein by 27.7- and 18.9-fold, respectively, in MCs (Fig. 7, A and B). The synergistic effect of these two factors was then revealed by the fact that increases in PAI-1 mRNA and protein were 137.8 and 79.9% greater than the sum increases seen with aldosterone or TGF-β alone. Coincubation with 10^{-5} M SPR diminished the synergistic effect of aldosterone and TGF-β1 on PAI-1 expression, where both levels of PAI-1 mRNA and protein were near to those of PAI-1 induced by TGF-β1 alone in MCs (Fig. 7). In fibroblasts, coincubation of 20 pg/ml TGF-β1 and 10^{-6} M aldosterone at the optimal concentrations induced 3.79- and 17.59-fold increases in PAI-1 mRNA and protein, respectively. When both compounds were present, a synergistic effect was detected where PAI-1 mRNA and protein were 75.4 and 43.4% higher, respectively, than the sum of increases seen with either compound alone (Fig. 8; P < 0.001). Importantly, as shown in Fig. 8, when the suboptimal concentration of aldosterone at 10^{-8} or 10^{-7} M was cotreated with 20 pg/ml TGF-β1, they yielded an even greater effect on PAI-1 mRNA and protein expression than either compound alone. These results suggest that aldosterone and TGF-β1 added together produce synergistic effects on PAI-1 production.

Synergistic Effect of TGF-β1 and Aldosterone on ECM Degradation by Renal MCs and NRK-49F Cells

As was expected, aldosterone or TGF-β1 alone inhibited MC matrix degradation by 39 and 49% and NRK-49F cell matrix degradation by 21 and 23%, respectively, in the presence of 4 μg/ml exogenous plasminogen (P < 0.001) (Fig. 9). Strik-
ingly, matrix degradation was further decreased to 93% by MCs and 61% by NRK-49F cells when both aldosterone and TGF-β1 were added, as shown in Fig. 9. These results support the synergistic effect of aldosterone and TGF-β1 on the downstream action of PAI-1 on ECM degradation.

**DISCUSSION**

This study clearly shows that addition of aldosterone to renal MCs and fibroblasts, the two major types of renal cells, produces similar fibrogenic effects on matrix protein metabolism. Although there are differences in the magnitude of response between the cell types, aldosterone does directly induce overexpression of PAI-1 and reduce matrix degradation through activation of a MR. SPR completely blocks the effects similarly in these two cell types. The findings on induction of PAI-1 in cultured renal MCs are consistent with those of Yuan et al. (56). Since PAI-1 is thought to be a key factor of fibrinolysis and ECM accumulation, aldosterone-induced decreases in matrix degradation are likely mediated by the induction of PAI-1. This is the first direct demonstration that aldosterone contributes to decreased matrix degradation by renal cells in addition to its physiological role in maintaining extracellular salt, potassium, and water homeostasis.

Recent data (35) showed that the glomerular area, mesangial area, glomerular collagen IV positive area, renal collagen content, and PAI-1 mRNA were significantly increased in the kidneys of aldosterone/salt-treated mice. However, when PAI-1 was deficient, all glomerular hypertrophy phenomena were significantly ameliorated. Although there is no direct evidence to link aldosterone-induced glomerular hypertrophy to the decreased matrix degradation in vivo, elevated PAI-1 may contribute to aldosterone-induced glomerular injury. Consistently, the increased glomerular PAI-1 expression and sclerosis observed in rats with diabetes or nephritis were reduced...
by blockade of MR with SPR (20, 21). Therefore, these findings, together with our results in MCs, suggest that aldosterone-induced increases in PAI-1 expression and subsequent ECM accumulation play a role in the development of glomerular fibrosis.

It is known that tubulointerstitial fibrosis is associated with higher rates of progression of renal disease. Chronic treatment with aldosterone and salt in rats resulted in severe tubulointerstitial fibrosis, with an increased renal collagen content (35, 52). These effects were prevented by a selective MR antagonist, eplerenone (52). Similarly, MR blockade with SPR prevented the progression of renal tubulointerstitial fibrosis seen in rats with chronic cyclosporine nephrotoxicity (42). However, the mechanism involved in the induction of tubulointerstitial fibrosis by aldosterone has not been delineated. The main cellular target of aldosterone in renal interstitium was considered to be distal convoluted tubule cells and principal cells of the collecting duct. Recently, aldosterone has been reported to target renal fibroblasts via binding to the MR, since renal fibroblasts also express MR mRNA and protein (38). Addition of aldosterone to cultured renal fibroblasts induced collagen gene expression and synthesis, effects blocked by eplerenone (38). In the present study, addition of aldosterone to renal fibroblasts significantly reduced ECM degradation. Moreover, decreased ECM degradation in aldosterone-treated cells was associated with increased expression of PAI-1, and these increases were prevented by SPR. Therefore, aldosterone appears to contribute to interstitial matrix accumulation by both increased matrix production and decreased degradation.

TGF-β has a well-established role in a wide range of renal injuries. Many molecules upregulate TGF-β expression, particularly in the RAAS (6, 27). Whether aldosterone also stimulates TGF-β is receiving attention. Aldosterone infusion for 3 days increased urinary TGF-β excretion (28). This effect is independent of the steroid’s effects on potassium and extracellular volume expansion, since hypertension and renal injury were not present in 3 days. A number of studies have shown that treatment of MCs in vitro with aldosterone significantly enhanced TGF-β1 secretion and activation via MR. No data on mRNA levels were given (22, 32, 56). SPR administration decreased elevated renal TGF-β1 mRNA in rats with chronic cyclosporine nephrotoxicity and renal TGF-β1 protein production in rats with Type 1 diabetes or experimental glomerulonephritis (20, 21, 42). In our in vitro work, we have been unable to show anything but small increases in TGF-β expression in response to aldosterone treatment. Aldosterone at high concentration (10^-6 M) increased fibroblast TGF-β1 mRNA < 100% after 48-h incubation (Fig. 4). Similarly, aldosterone concentrations < 10^-5 M only slightly increased TGF-β1 mRNA expression by 20% in MCs. TGF-β1 protein levels became significant only at 48 h after treatment (Fig. 2). Therefore, our data suggest that aldosterone upregulates PAI-1 expression much more than TGF-β in these cells. It is not surprising that aldosterone-induced PAI-1 expression is only
partially mediated by TGF-β. This result is compatible with prior data in integrin-β3-deficient mice, in which aldosterone induced renal PAI-1 expression and fibrosis through a TGF-β-independent pathway, because β3 deficiency impaired TGF-β activation and its subsequent cellular actions (36). Aldosterone was demonstrated to stimulate collagen gene expression and synthesis via activation of cellular ERK1/2 signaling in rat renal fibroblasts (38). Further studies are required to determine whether aldosterone-stimulated PAI-1 expression is mainly regulated by a mitogen-activated protein kinase in renal cells.

Of note is where there is fibrosis, there is overexpression of TGF-β in both animal models and humans (7, 24). It is relevant that we could not suppress elevated TGF-β production by more than ~50% in experimental nephritis, whether maximally effective doses of enalapril or losartan were used alone or in combination (43). The present data suggest that increased aldosterone due to “aldosterone escape” could be another important contributor to renal fibrosis, because aldosterone interacts synergistically with TGF-β to increase PAI-1 overexpression and subsequently inhibit ECM degradation. If it acts in vivo, this synergistic effect may explain the beneficial effects of combination therapy with an ANG II blocker and an aldosterone blocker that have been observed in patients with heart failure, diabetic nephropathy, and nephritic range albuminuria (11, 44, 46, 49), and in animal models with hypertension and glomerulonephritis (2, 31).

In conclusion, the present experiments show that aldosterone-induced PAI-1 overexpression by both MCs and NRK-49F is partially mediated by TGF-β1. Increased PAI-1 leads to decreased ECM degradation. While aldosterone alone induces TGF-β1 weakly, aldosterone and TGF-β1 added together produce dramatic synergistic effects on PAI-1 production and subsequent ECM accumulation. Thus the elevated aldosterone induced by RAAS activation may amplify its fibrotic actions through a synergistic interaction of aldosterone and aldosterone-induced or disease-induced TGF-β1. Therapeutic combinations of an aldosterone blocker and an ANG II antagonist may hold promise for a further reduction in renal fibrosis.

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