Aldosterone Up-regulates Production of Plasminogen Activator Inhibitor-1 by Renal Mesangial Cells

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In vivo studies have demonstrated that aldosterone is an independent contributor to glomerulosclerosis. In the present study, we have investigated whether aldosterone itself mediated glomerulosclerosis, as angiotensin II (Ang II) did, by inducing cultured renal mesangial cells to produce plasminogen activator inhibitor-1 (PAI-1), and whether these effects were mediated by aldosterone-induced increase in transforming growth factor β1 (TGF-β1) expression and cellular reactive oxygen species (ROS) activity. Quiescent rat mesangial cells were treated by aldosterone alone or by combination of aldosterone and spironolactone, Ang II, neutralizing antibody to TGF-β1 or antioxidant N-acetylcysteine (NAC). This study indicate that aldosterone can increase PAI-1 mRNA and protein expression by cultured mesangial cells alone, which is independent of aldosterone-induced increases in TGF-β1 expression and cellular ROS. The effects on PAI-1, TGF-β1 and ROS generation were markedly attenuated by spironolactone, a mineralocorticoid receptor antagonist, which demonstrate that mineralocorticoid receptor (MR) may play a role in mediating these effects of aldosterone.

Keywords: Aldosterone, Mineralocorticoid receptor, Plasminogen activator Inhibitor-1, Reactive oxygen species, Transforming growth factor β1.

Introduction

Recently, extensive clinical and animal model studies indicate that aldosterone is an independent contributor to the progression of renal injury, and mineralocorticoid receptor (MR) antagonists can markedly ameliorate renal injury (Brown et al., 2000; Miñé et al., 2001; Fetiá et al., 2003; Sato et al., 2003; Fujisawa et al., 2004; Hollenberg, 2004; Nishiyama and Abe, 2004). Several studies have indicated that aldosterone can be synthesized by rat glomerular mesangial cells (Lai et al., 2003; Nishiyama et al., 2005; Nishiyama and Abe, 2006) and mineralocorticoid receptor are highly expressed in rat glomerular mesangial cells (Lai et al., 2003; Nishiyama et al., 2005; Nishiyama and Abe, 2006). These studies suggest that locally produced aldosterone can act on mesangial cells, the key producers of extracellular matrix (ECM) in progressive glomerulosclerosis.

The amount of ECM deposited in the kidney depends on the balance between the synthesis and degradation of ECM (Eddy, 2000). Although enhanced ECM synthesis has been demonstrated to contribute to ECM accumulation, decreased degradation of the ECM could also play an important role in matrix accumulation and glomerulosclerosis. Two major ECM protease systems, plasminogen activator (PA)/plasmin/plasminogen activator inhibitors (PAI) system and matrix metalloproteinases (MMP)/tissue inhibitors of matrix metalloproteinases (TIMP) system, are interrelated and involved in matrix degradation (Mignatti, 1995; Schmarer, 1995; Steetler-Stevenson, 1996). Plasminogen activator inhibitor-1 (PAI-1), a principal inhibitor of plasminogen activators (tissue-type plasminogen activator and urokinase-type plasminogen activator) (Vaughan, 2001; Eddy, 2002), can promote glomerulosclerosis by preventing the activation of matrix metalloproteinases (MMPs) and the degradation of ECM by plasminogen activators and plasmin (Vassalli et al., 1991; Eddy, 2002). Recently, several animal experiments have indicated that aldosterone contribution to glomerulosclerosis is partly due to up-regulation of PAI-1 expression, and spironolactone can ameliorate glomerulosclerosis via the inhibition of PAI-1 expression in streptozotocin-diabetic rats (Miñé et al., 2001; Fujisawa et al., 2004), 5/6 nephrectomy (Aligiet et al., 2005) and rat kidney radiation model (Brown et al., 2000). Furthermore, levels of PAI-1 correlate significantly with aldosterone concentration during periods of low salt intake (Brown et al., 1998). Despite the implications of the aforementioned studies, there are few studies on the effect of
aldosterone alone on PAI-1 expression by mesangial cells. Brown et al reported that in cultured human umbilical vein endothelial cells and rat aortic smooth muscle cells, aldosterone itself could not regulate PAI-1 expression, but modulated the effect of angiotensin II (Ang II) on PAI-1 expression (Brown et al., 2000). It is not clear presently whether aldosterone itself mediates glomerulosclerosis, as Ang II does (Kagami et al., 1997), by inducing cultured mesangial cells to produce PAI-1.

Aldosterone also increases transforming growth factor β1 (TGF-β1) (Jukenievius et al., 2004) and reactive oxygen species (ROS) (Miyata et al., 2005) production in kidney. Both TGF-β1 and ROS play an important role in renal injury (Lee et al., 2003). Both aldosterone and ROS have been shown to increase the expression of PAI-1 (Jiang et al., 2002; Lee et al., 2005). These reports prompted us to investigate whether the effect of aldosterone on PAI-1 induction is mediated by aldosterone-induced increases of TGF-β1 expression and cellular ROS.

Therefore, we plan to verify the hypothesis that aldosterone-induced PAI-1 expression may be independent of angiotensin II and act through the mediation of mineralocorticoid receptor, and to explore the role of ROS and TGF-β1 in aldosterone-induced PAI-1 expression.

Materials and Methods

Cell culture. The rat mesangial cells line (HBZY-1) purchased from China center for type culture collection (Wuhan, China) was plated in MEM containing 10% fetal bovine serum (FBS, Gibco), 15 mM N-2-hydroxy-ethylpiperazine-N-2-ethane-sulfonic acid (HEPES), 20 mM NaHCO3, 100 U/ml penicillin, 100 µg/ml streptomycin, and cultured in a humidified atmosphere of 95% air and 5% CO2 at 37°C. Cells were cultured in 100-mm culture dish for western blot measurement, on cover glass coated with polylysine for intracellular ROS measurement, and 6-well culture plate for western blot measurement, and 5% CO2 for 60 s and extending at 72°C for 60 s with initial heating at 94°C for 3 min and final extending at 72°C for 10 min. The PCR products were separated by 1.5% agarose electrophoresis and stained with ethidium bromide (EtBr) and P-AI-1-ELISA detection Kit (Sunbiotech) respectively.

Statistical analysis. All results are expressed as means ± standard deviation (SD). Analysis of variance was used to assess the differences between multiple groups. If the F statistics was significant, the mean values obtained from each group were then compared by Fisher’s least significant difference method. P value < 0.05 was used as the criterion for a statistically significant difference.

Results

Effect of aldosterone and angiotensin II on PAI-1 mRNA and protein expression by mesangial cells. Mesangial cells were exposed to aldosterone (100 nM), Ang II (100 nM) or
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Combination of aldosterone and Ang II for 24 h. The effects of aldosterone and Ang II on PAI-1 mRNA expression were analyzed by RT-PCR and shown in Fig. 1. Aldosterone at 100 nM up-regulated PAI-1 mRNA expression by 1.8-fold compared to the control at 24 h. Ang II also up-regulated PAI-1 mRNA expression by 2-fold that of control. Furthermore, exposure of the cells to combination of aldosterone and Ang II resulted a 2.3-fold increase in the expression of PAI-1 mRNA.

After 48-h incubation, in comparison to control group (120.9 ± 23.78 pg/ml) both aldosterone and Ang II increased PAI-1 protein secretion by mesangial cells into media to 241.2 ± 26.3 pg/ml and 251.7 ± 20.3 pg/ml respectively (Fig 2). Co-treatment of aldosterone with Ang II further increased PAI-1 protein secretion to 293.5 ± 44.7 pg/ml.

Concentration dependence of effect of aldosterone on induction of PAI-1 mRNA. Aldosterone of the concentrations of 10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹, 10⁻¹⁰, 10⁻¹¹ mol/l was added in mesangial cells, and incubation time was 24 h. Aldosterone caused a concentration-dependent increase in PAI-1 mRNA expression, as demonstrated in Fig. 3. Maximal induction of PAI-1 mRNA was observed at the highest dose of 10⁻⁵ M aldosterone.

Time-course of PAI-1 mRNA expression induced by aldosterone. In this experiment, the aldosterone concentration was 100 nmol/l. After being deprived of serum for 24 h, mesangial cells were incubated with aldosterone (100 nM) for 0, 0.5, 1, 2, 4, 6, 8 and 24 h respectively. Total RNA was extracted from cell lysate and subjected to RT-PCR analysis. Enhancement of PAI-1 mRNA expression was seen after 4 h of exposure to aldosterone but not after shorter exposure (Fig. 4).
Effects of aldosterone in the absence or presence of spironolactone on cellular ROS and Expression of TGF-\(\beta\), in mesangial cells. Intracellular DCF-sensitive ROS was visualized by a confocal microscopy, and the results were analyzed by Leica confocal software program. TGF-\(\beta\) in conditioned media was quantitatively measured by ELISA method. The basal cellular ROS level was 3.59. After mesangial cells were incubated with 100 nM or 1 \(\mu\)M aldosterone for 0, 0.5, 1, 2, 4, 6, 8, 24 h respectively. (B) Relative mRNA level of PAI-1, which are normalized with GAPDH levels. *\(p<0.05\) versus control; **\(p<0.01\) versus control.

**Fig. 4.** Time-course of PAI-1 mRNA expression induced by aldosterone. PAI-1 mRNA was analyzed by RT-PCR as described in Methods. Time point 0 represents the control. (A) Typical RT-PCR results. Lane 1: Marker; lanes 2, 3, 4, 5, 6, 7, 8, 9 showed the results after treatment with 100 nM aldosterone for 0, 0.5, 1, 2, 4, 6, 8, 24 h respectively. (B) Relative mRNA level of PAI-1, which are normalized with GAPDH levels. *\(p<0.05\) versus control; **\(p<0.01\) versus control.

Effects of aldosterone in the absence or presence of spironolactone on aldosterone-induced PAI-1 mRNA and protein expression. This study examined whether TGF-\(\beta\) neutralizing antibody or NAC inhibited aldosterone-induced PAI-1 mRNA and protein expression. Neither 100 \(\mu\)g/ml TGF-\(\beta\) neutralizing antibody nor 5 mM NAC alone increased PAI-1 mRNA (Fig. 7) and protein expression (Fig. 8) by mesangial cells compared to the control value. But TGF-\(\beta\) neutralizing antibody or NAC effectively decreased aldosterone-induced PAI-1 mRNA expression 30% and 32% respectively (Fig. 7).

TGF-\(\beta\) neutralizing antibody or NAC also decreased aldosterone-induced PAI-1 protein expression to 190.7 ± 29.2 pg/ml and 184.1 ± 33.4 pg/ml respectively (Fig. 8). However, neither TGF-\(\beta\) neutralizing antibody nor NAC alone could inhibit aldosterone-induced PAI-1 mRNA (Fig. 7) and protein expression (Fig. 8) to normal level at 24 h.

**Effects of spironolactone on aldosterone-induced PAI-1 mRNA and protein expression by mesangial cells.** Effects of spironolactone on aldosterone-induced PAI-1 mRNA expression were analyzed by semi-quantitative RT-PCR. 1 nM Spironolactone efficiently inhibited induction of PAI-1 mRNA by 100 nM or 1 \(\mu\)M aldosterone (Fig. 9).

1 nM Spironolactone also markedly reduced synthesis of PAI-1 protein stimulated by aldosterone of 100 nM or 1 \(\mu\)M to control levels. Spironolactone did not affect basal PAI-1 protein synthesis (Fig. 10).

**Discussion**

Aldosterone plays a pivotal role in electrolyte and fluid homeostasis and thus control of blood pressure (Booth et al., 2002). If we investigated the effects of aldosterone on production of PAI-1 by aldosterone-infused rats, we could not avoid hypertension or other disorder induced by aldosterone. These factors maybe influence PAI-1 mRNA expression and protein synthesis (Fogari and Zoppi, 2005; Kuriyama et al., 2005). The in vitro study do not affect blood press and can exclude the possibility that hemodynamic changes influence the non-hemodynamic effect of aldosterone on production of PAI-1.

In agreement with previous study (Motojima et al., 2000), Ang II induced an increase in the expression of PAI-1 mRNA and protein by cultured mesangial cells in our study. The upregulation of PAI-1 co-treated by aldosterone with Ang II was more greater than that treated by Ang II alone, which suggested aldosterone together with Ang II may produce profound pro-fibrotic effects. But we also demonstrate that aldosterone can up-regulate PAI-1 mRNA and protein expression by renal mesangial cells, which occurred in absence of Ang II. Furthermore, aldosterone increased PAI-1 mRNA expression in a concentration-dependent manner. In this report, we provide direct evidence that aldosterone could stimulate production of PAI-1 by renal mesangial cells independently of Ang II, which has previously been shown to mediate the effect of aldosterone.

Aldosterone significantly increased DCF-sensitive cellular ROS in mesangial cells after mesangial cells were incubated with aldosterone for 24 h. Our results also are supported by the report that other kinds of cells, such as peripheral blood...
monocytes (Fiebeler and Luft, 2005) and vascular smooth muscle cells (Fiebeler and Luft, 2005), can also be influenced by aldosterone to produce ROS. It has long been recognized that ROS are harmful for cells, mainly because they injure lipids, proteins, and nucleic acids, which leads to structural and functional impairments (Rodrigo and Bosco, 2006). The glomerulus is considerably more sensitive to oxidative injuries than other nephron segments. Oxidative stress may alter the structure and function of the glomerulus because of the effect of ROS on mesangial cells (Rodrigo and Bosco, 2006). In a

Fig. 5. Effects of aldosterone in the presence or absence of spironolactone on reactive oxygen species in mesangial cells. (A) Representative image of fluorescent signals generated from composite images obtained by confocal microscopy of mesangial cells pretreated with the radical probe CM-H2DCFDA for 45 min. (B) Results of cellular ROS analyzed by Leica Confocal Software program are graphically shown. Lane 1: Control; lane 2: Mesangial cells + aldosterone (1 µM); lane 3: Mesangial cells + aldosterone (1 µM) + spironolactone (1 nM); lane 4: Mesangial cells + aldosterone (100 nM); lane 5: Mesangial cells + aldosterone (100 nM) + spironolactone (1 nM); lane 6: Mesangial cells + spironolactone (1 nM). *p < 0.05 versus control; **p < 0.01 versus control.
The present study also shows that aldosterone significantly up-regulates expression of TGF-β by Mesangial cells. Lane 1: Control; lane 2: Mesangial cells + aldosterone (1 µM); lane 3: Mesangial cells + aldosterone (1 µM) + spironolactone (1 nM); lane 4: Mesangial cells + aldosterone (100 nM); lane 5: Mesangial cells + aldosterone (100 nM) + spironolactone (1 nM); lane 6: Mesangial + spironolactone (1 nM). *p < 0.05 versus control; **p < 0.01 versus control.

Substantial clinical and experimental evidences suggest that TGF-β is the key mediator of glomerular fibrosis (Ziyadeh et al., 1998; Peters et al., 1999). In vivo studies have demonstrated that aldosterone could evoke TGF-β in the kidney, and this action contributed to aldosterone-dependent renal injury (Fena et al., 2003; Fujisawa et al., 2004; Juknevicius et al., 2004). The present study also shows that aldosterone significantly up-regulates expression of TGF-β by mesangial cells.

TGF-β promotes glomerular fibrosis not only by enhancing ECM synthesis, but also by decreasing ECM degradation. TGF-β induces ECM synthesis, but also by decreasing ECM degradation.

Fig. 6. Effects of aldosterone in the presence or absence of spironolactone on expression of TGF-β by Mesangial Cells. Lane 1: Control; lane 2: Mesangial cells + aldosterone (1 µM); lane 3: Mesangial cells + aldosterone (1 µM) + spironolactone (1 nM); lane 4: Mesangial cells + aldosterone (100 nM); lane 5: Mesangial cells + aldosterone (100 nM) + spironolactone (1 nM); lane 6: Mesangial + spironolactone (1 nM). *p < 0.05 versus control; **p < 0.01 versus control.

Aldosterone has two modes of actions: the genomic actions of aldosterone and the nongenomic action of aldosterone. The classical actions of aldosterone are mediated by intracellular aldosterone-stimulated PAI-1 expression in mesangial cells just as NAC did. This finding is also in good agreement with results from animal experiments (Ma et al., 2003). Therefore, ROS or TGF-β induced by incubation of mesangial cells with aldosterone play an important role in aldosterone-induced PAI-1 expression, but aldosterone can induce PAI-1 expression through a TGF-β-dependent or ROS-independent pathway.

Besides the increase of expression of TGF-β, and cellular ROS, aldosterone also has been shown to increase tissue ACE activity, a further increase in angiotensin II (Krag et al., 2002). Furthermore, Ang II can up-regulate PAI-1 expression in mesangial cells as well (Kagami et al., 1997). Perhaps, there are several cell factors or pathways involved in aldosterone-induced PAI-1 expression. However, TGF-β neutralizing antibody treatment of mesangial cells. However, TGF-β neutralizing antibody only partially decreased this interrelationship of cell factors induced by aldosterone in upregulating PAI-1 expression are still to be studied.

Aldosterone has two modes of actions: the genomic actions of aldosterone and the nongenomic action of aldosterone. The classical actions of aldosterone are mediated by intracellular...
receptors that translocate to the nucleus upon ligand binding. Aldosterone binds to intracellular receptors and subsequently modulates transcription and protein synthesis, thus triggering genomic events finally responsible for delayed effects. The genomic actions of aldosterone mandate changes in gene expression, which results in a substantial latent period (from 0.5 to 1.0 h) prior to overt changes in cellular activity. The genomic actions of aldosterone can be blocked by MR antagonist. The nongenomic action of aldosterone is considered to be independent of direct effects on gene expression which results in rapid responses (<15 min). These rapid responses are not sensitive to mineralocorticoid receptor antagonist (Booth et al., 2002). Time-course studies indicated that Enhancement of PAI-1 mRNA expression was seen 4 h later when mesangial cells exposed to aldosterone but not after shorter exposure, and sensitivity to spironolactone of the PAI-1 induction are entirely consistent with being mineralocorticoid receptor-mediated events. Spironolactone, a mineralocorticoid receptor antagonist, also effectively inhibited TGF-$\beta_1$ expression and cellular ROS induced by aldosterone to normal level at the 24th hour. Taken together, these data clearly demonstrate that aldosterone increases generation of PAI-1, TGF-$\beta_1$ and cellular ROS via activation of mineralocorticoid receptor, which may contribute the pathophysiology of aldosterone-induced glomerulosclerosis. Our study also implies that therapy with mineralocorticoid receptor antagonists may have a therapeutic potential for glomerulosclerosis.

In conclusion, our present data show that aldosterone can increase PAI-1 mRNA and protein expression by cultured mesangial cells which have been shown to be a key regulator of fibrinolysis and ECM. That induction of PAI-1 expression by aldosterone is independent of aldosterone-induced increase in expression of TGF-$\beta_1$ or cellular ROS. The effects on PAI-1, TGF-$\beta_1$ and cellular ROS generation were markedly attenuated by spironolactone, which demonstrate that mineralocorticoid receptor may play a role in mediating these effects of aldosterone.
References


