

Original Article

Aldosterone and TGF- β_1 synergistically increase PAI-1 expression in hepatic stellate cells of rats

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Abstract: Objective: Aldosterone is related to the fibrosis of several organs, but the specific mechanism underlying the aldosterone induced hepatic fibrosis is still unclear. Methods: Separation, culture and identification of primary hepatic stellate cells (HSCs): The fluids and digestives used in the present study were able to completely remove blood cells, digest hepatocytes and matrix, and effectively separate HSCs. The *in situ* perfusion was performed at 2 steps: *in situ* perfusion with pre-perfusion fluid and *ex vivo* perfusion with enzyme-containing perfusion fluid. Influence of Ald on PAI-1 and Smad expressions in HSCs: cells were divided into control group, Ald group (10^{-6} M), spironolactone (SPI) group and Ald+SPI group, and the mRNA and protein expressions of PAI-1 and Smad were detected. Ald induced type I collagen expression in HSCs: Immunohistochemistry was performed to detect type I collagen expression in the supernatant of control group, Ald group (10^{-6} M), TGF- β_1 group, and Ald+TGF- β_1 group. Influence of Ald and TGF- β_1 on PAI-1 expression in HSCs: cells were divided into control group, Ald group (10^{-6} M), TGF- β_1 group, and Ald+TGF- β_1 group, and the mRNA and protein expressions of PAI-1 were determined by RT-PCR and Western blot assay, respectively. Synergistic effect of Ald and TGF- β_1 on PAI-1 expression in HSCs: cells were divided into control group, Ald group (10^{-6}), TGF- β_1 group, Ald (10^{-6} M)+TGF- β_1 group, Ald (10^{-7} M)+TGF- β_1 group and Ald (10^{-8} M)+TGF- β_1 group, and the mRNA and protein expressions of PAI-1 were detected by RT-PCR and Western blot assay, respectively. Results: The survival rate, purity, markers and activation of HSCs were determined after separation. Influence of Ald on PAI-1 expression in HSCs: PAI-1 expression increased in HSCs of Ald group, SPI group and Ald+SPI group, and the PAI-1 expression in Ald group and Ald+SPI group was significantly higher than in control group ($P<0.01$). Influence of Ald on Smad expression in HSCs: Smad expression in Ald group, TGF- β_1 group and Ald+TGF- β_1 group was markedly higher than in control group ($P<0.05$). Smad expression in Ald+TGF- β_1 group increased significantly when compared with Ald group ($P<0.01$). Ald induced type I collagen expression in HSCs: type I collagen expression in Ald group, TGF- β_1 group and Ald+TGF- β_1 group was dramatically higher than in control group ($P<0.05$), and it in Ald+TGF- β_1 group was also significantly different from that in Ald group and TGF- β_1 group ($P<0.01$). Synergistic effects of Ald and TGF- β_1 on PAI expression in HSCs: PAI-1 expression in treated cells was markedly higher than in control group ($P<0.01$). PAI-1 expression in 10^{-6} M Ald+5 ng/ml TGF- β_1 group increased dramatically as compared to Ald group and TGF- β_1 group ($P<0.01$), but the increased PAI-1 expression reduced after SPI treatment. Ald at different concentrations exerts synergistic effect with TGF- β_1 to increase PAI-1 expression in HSCs: PAI-1 expression in HSCs after different treatments increased markedly as compared to control group ($P<0.01$). Significant difference in PAI-1 expression was observed in 10^{-6} M Ald+50 pg/ml TGF- β_1 group and 10^{-6} M Ald group ($P<0.01$), PAI-1 expression in 10^{-7} M Ald+50 pg/ml TGF- β_1 group was significantly higher than in 50 pg/ml TGF- β_1 group ($P<0.01$), but the PAI-1 expression in 10^{-7} M Ald+50 pg/ml TGF- β_1 group was similar to that in 10^{-6} M Ald group ($P>0.05$). Conclusion: Aldosterone is able to activate HSCs and increase PAI-1 expression during hepatic fibrosis, which may be inhibited by spironolactone. Aldosterone and TGF- β_1 may synergistically act on HSCs to increase PAI-1 expression as compared to treatment with aldosterone or TGF- β_1 alone. Aldosterone or TGF- β_1 alone may slightly increase PAI-1 expression in HSCs, which can be inhibited by spironolactone.

Keywords: Aldosterone, hepatic stellate cells, PAI-1, TGF- β_1 , spironolactone

Introduction

Aldosterone (Ald) is an important mineralocorticoid in humans and released after stimulation

of angiotensin II, potassium and ACTH. Classically, Ald mainly acts on mineralocorticoid receptor (MR) to induce the water reabsorption and potassium excretion, which are

crucial for the regulation of fluid and electrolytes. In addition, Ald has non-classic activities to function on epithelial tissues and non-epithelial tissues, inducing inflammation and organ fibrosis. Ald is one of important members of renin angiotensin-aldosterone system (RAAS), and its role in the liver diseases has been paid attention to. In hepatic cirrhosis, the RAAS is activated, and factors including angiotensin II (Ang II) may stimulate the synthesis and secretion of Ald, exerting multiple bioeffects. Ald is able to bind to and form a complex with MR on target cells, and this complex then translocates into nucleus to regulate the expression of some target genes including ENaC and Na⁺-K⁺-ATPase, modulate the metabolism of water and salt, and induce sodium and water retention, resulting in hypertension [1]. Moreover, Ald may also activate NADPH oxidase to increase reactive oxygen species (ROS), which promotes the inflammation [2, 3]. Ald may up-regulate the expressions of type I and IV collagens, transforming growth factor [3, 4], connective tissue growth factor (CTGF) [5, 6] and plasminogen activator inhibitor-1 (PAI-1) [7], leading to the aggregation of extracellular matrix (ECM).

Hepatic fibrosis is a shared pathophysiology in chronic liver injury and also a consequence of liver injury. Hepatic fibrosis has been found to be one of causes of portal hypertension and liver dysfunction and also a risk factor of liver cancer. Hepatic stellate cells (HSCs) are major cells producing collagens during the liver injury and have been found to be a key factor involved in the occurrence and development of fibrosis. Ald has been found to be a pivotal pathogenic factor in the fibrosis [8]. It is one of potent factors causing collagen synthesis and mitosis, and may promote the fibrosis of some organs including heart, lung and kidney [9]. In the presence of factors able to induce liver injury, the occurrence and development of fibrosis are as a result of interaction of these factors. However, little is known on the role of Ald as an independent stimulus in the hepatic fibrosis.

In recent years, studies on fibrotic diseases reveal that PAI-1 is a pro-fibrotic factor and plays important roles in the pathogenesis of fibrotic diseases. PAI-1 is an important member of fibrinolytic system and a major physiological inhibitor of tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). Under the pathological condition,

high PAI-1 expression may inhibit the plasminogen activator which decreases the fibrin degradation and increases the ECM deposition. Increased PAI-1 expression has been found to be associated with renal tubular sclerosis and renal interstitial fibrosis [10-12]. In some studies, increasing attention has been paid to PAI because TGF- β_1 as a crucial factor regulating fibrosis may induce PAI expression, and TGF- β_1 over-expressed mice with glomerulosclerosis have concomitant high PAI-1 expression [13]. It has been confirmed that TGF- β_1 may induce the PAI-1 synthesis, and there is a TGF- β_1 response element at the promoter of PAI-1 gene [14]. Ald may increase TGF- β_1 expression [15]. Available studies also demonstrate that Ald is able to increase the expression of pro-fibrotic factors, leading to pathological consequences. Ald may interact with Ang II to elevate PAI-1 expression, which promotes the deposition of ECM among vascular endothelial cells [16]. These findings have been found in Ald treated mesangial cells of rats [17]. In addition, Ald may also increase the PAI-1 mRNA expression in wide-type mice [18], and PAI-1 expression is closely associated with sclerosis in mice with radiation induced renal injury [12]. Finally, blood Ald concentration is related to PAI-1 expression [19], which further supports the regulatory role of Ald in PAI-1 expression. TGF- β_1 is a potent pro-fibrotic factors and able to increase the synthesis and secretion of ECM and block the degradation of ECM. As a downstream gene of TGF- β_1 , PAI-1 also involves in the fibrosis of tissues and organs and might play a special role in the pathogenesis of fibrotic diseases. Studies on the role of PAI-1 in fibrotic diseases may provide new targets for the therapy of these diseases. Thus, the present study was undertaken to investigate the effect of Ald on the PAI-1 expression in hepatic stellate cells (HSCs) in vitro, which may provide evidence for the Ald induced hepatic fibrosis and identify therapeutic targets for hepatic fibrosis.

Materials and methods

Separation, culture and identification of primary HSCs

The liver of male SD rats was perfused with enzymes at 2 steps for the digestion, and HSCs were harvested after removal of confounding cells by density gradient centrifugation. HSCs were observed and counted under a micro-

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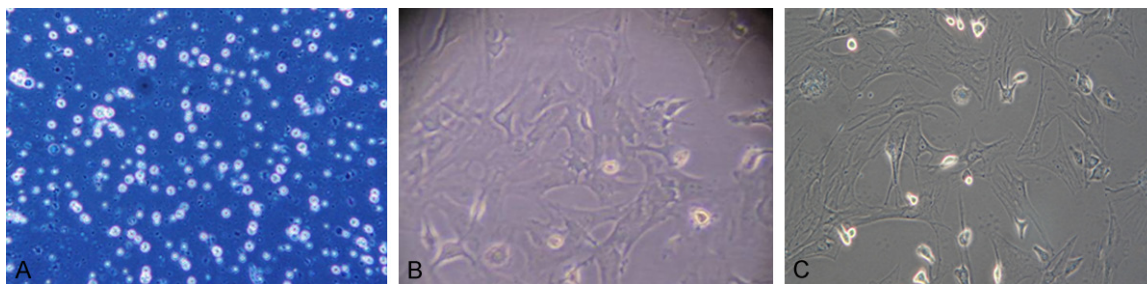


Figure 1. Survival rate, purity, marker and morphology of HSCs. A. HSCs after trypan blue staining ($\times 10$); B. HSCs after 3-day culture ($\times 20$); C. HSCs after 7-day culture ($\times 20$).

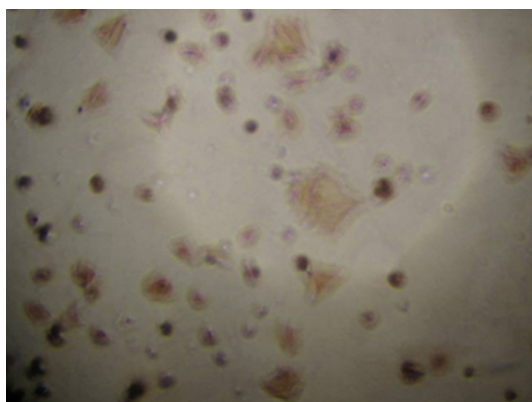


Figure 2. Immunocytochemistry for desmin ($\times 10$).

scope. The survival rate was determined after trypan blue staining. HSCs were identified by immunocytochemistry for desmin. After Ald treatment for 48 h, immunohistochemistry was performed to detect α -SMA expression in HSCs, aiming to evaluate the HSCs activation.

Detection of PAI-1 mRNA expression by RT-PCR

Monolayer cells were treated with TRIzol for lysis, followed by addition of chloroform and a 15-s shaking. The mixture was incubated at room temperature for 3 min and centrifuged at $12000\times g$ for 15 min at 4°C . PCR amplification was performed using Taq polymerase (Kataru) in a total volume of 50 μl . The upstream and downstream primers for rat PAI-1 mRNA were 5'-CCTCCTCATCCTGCCTAAGTT-3' and 5'-CTTGACCTTTGTAGTGCTTG-3' RNA in water was precipitated with isopropanol at room temperature for 10 min, followed by centrifugation at $12000\times g$ for 10 min at 4°C . The supernatant was removed, and RNA washed in 75% ethanol. Centrifugation was done at $12000\times g$ for 5 min at 4°C . The supernatant was removed, and RNA air-dried for 5-10 min at room tempera-

ture. The RNA purity was determined, and PAI-1 mRNA expression was detected by RT-PCR.

Detection of PAI-1 and Smad protein expression by Western blot assay

Total protein was extracted from HSCs and subjected to SDS-PAGE. Proteins were transferred onto PVDF membrane which was then blocked in 5% non-fat milk and treated with primary antibody at 4°C over night. After incubation with secondary antibody at room temperature for 2 h, visualization was done with chemiluminescence method.

Detection of type I collagen expression by immunohistochemistry

Cells were seeded into 96-well plates at a density of 1×10^6 cells/L. Samples were diluted and added to plates. Deparaffinization and dehydration were done in xylene and ethanol at different concentrations, respectively. Antigen retrieval was performed in citrate buffer. Sections were then treated with 5% H_2O_2 to inactivate endogenous peroxidase. After blocking in goat serum for 20 min at 37°C , sections were incubated with primary antibody (1:100) at 4°C over night and then with biotin conjugated secondary antibody (1:100) at 37°C for 30 min. Visualization was done with DAB, and counterstaining with hematoxylin, followed by fixation.

Results

Survival rate, purity, marker and morphology of HSCs

Cells were counted as follow: cell count = (total cells in 4 big grids/4) $\times 10^4 \times$ volume (mL), cell counting was done twice for each sample, and an average was obtained. Survival rate was determined after trypan blue staining. Results

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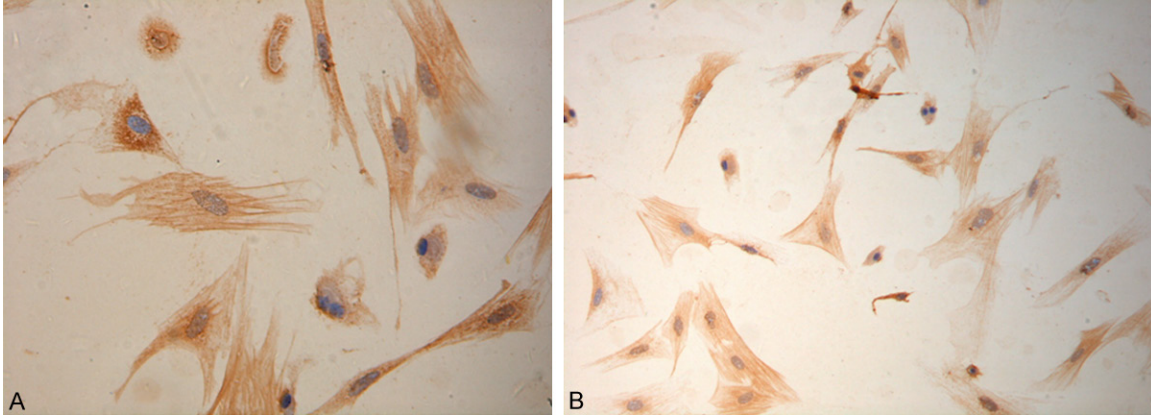


Figure 3. Immunohistochemistry for α -SMA in HSCs. A. ($\times 40$). B. ($\times 10$).

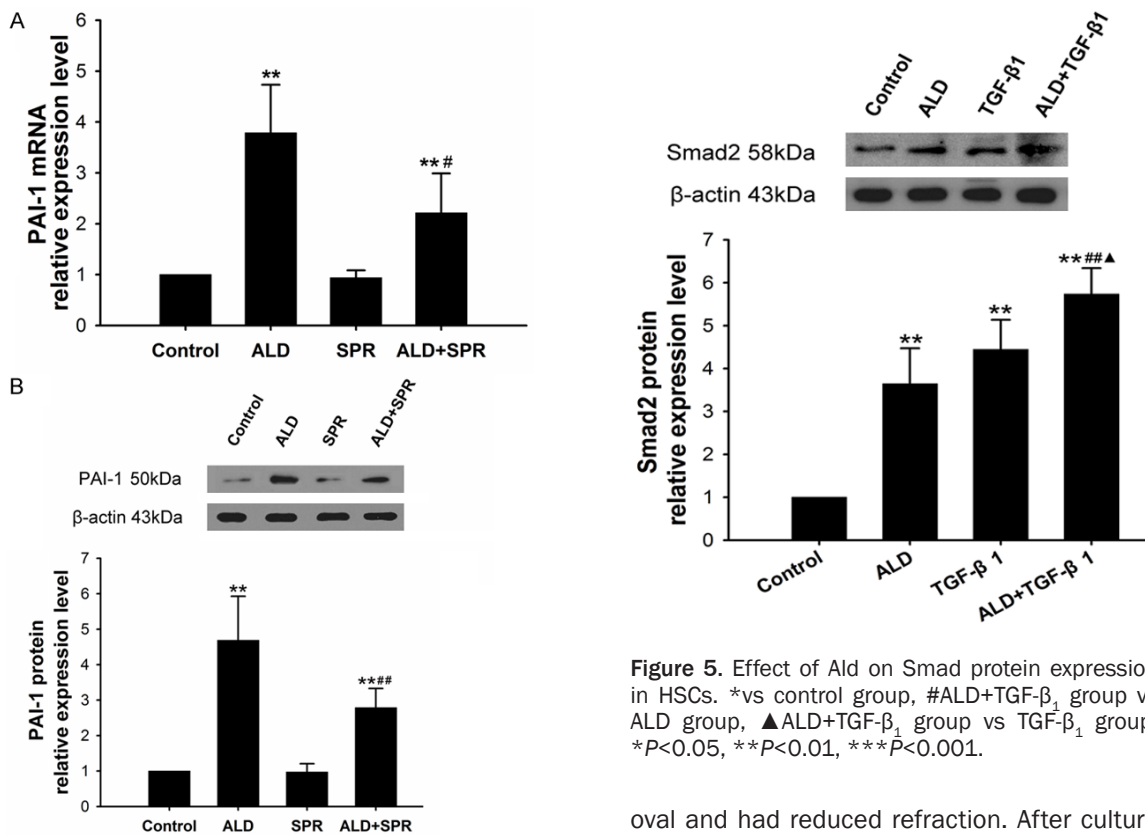


Figure 4. A. Effect of Ald on PAI-1 mRNA expression in HSCs. B. Effect of Ald on PAI-1 protein expression in HSCs. *vs control group; #Ald+SPI group vs Ald group; ** $P < 0.01$.

showed the survival rate of HSCs was $95.0 \pm 1.2\%$ after separation. Under an inverted phase contrast microscope, the newly separated HSCs were round, had small cell volume and lipid droplets in the cytoplasm, and presented high refraction. After culture for 24 h, cells were adherent to the wall, began to spread, became

Figure 5. Effect of Ald on Smad2 protein expression in HSCs. *vs control group, #ALD+TGF- β_1 group vs ALD group, \blacktriangle ALD+TGF- β_1 group vs TGF- β_1 group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

oval and had reduced refraction. After culture for 48 h, a majority of cells were adherent and extended pseudopods. At 7 days, these cells enlarged, became stellate and irregular, and presented focal growth (**Figure 1**).

Identification of HSCs by immunohistochemistry

Immunocytochemistry was performed to detect desmin expression, aiming to identify HSCs. Positive cells had brown granules in the cytoplasm and their nuclei were blue. The cell purity was higher than 90% (**Figure 2**).

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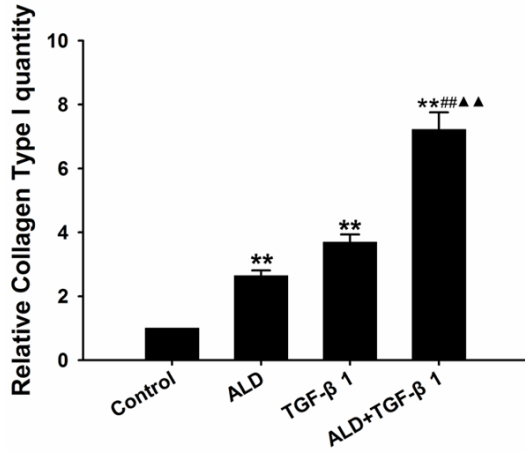


Figure 6. Ald induced type I collagen expression in HSCs. *vs control group, #ALD+TGF- β_1 group vs ALD group, ▲ALD+TGF- β_1 group vs TGF- β_1 group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

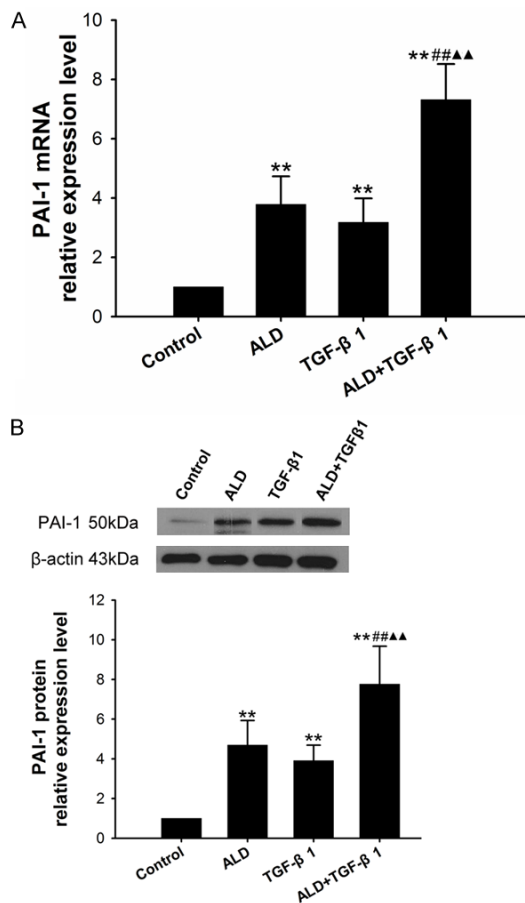


Figure 7. A. Effect of Ald and TGF- β_1 on the PAI-1 mRNA expression in HSCs. B. Effect of Ald and TGF- β_1 on the PAI-1 protein expression in HSCs. *vs control group, # 10^{-6} M Ald+TGF- β_1 group vs TGF- β_1 group, ▲ 10^{-6} M Ald+TGF- β_1 group vs ALD group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

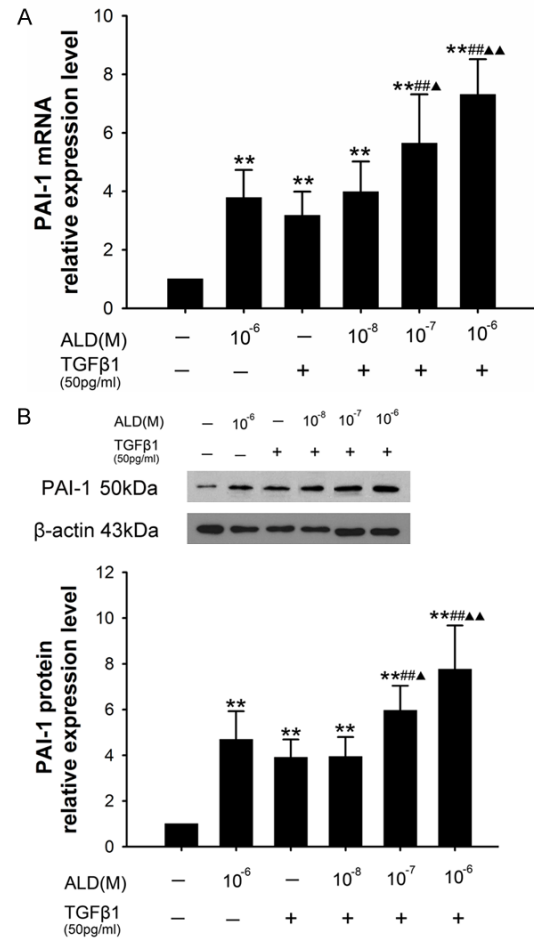


Figure 8. A. Effect of Ald and TGF- β_1 on the PAI-1 mRNA expression in HSCs. B. Effect of Ald and TGF- β_1 on the PAI-1 protein expression in HSCs. *vs control group, # 10^{-7} M Ald+TGF- β_1 group and 10^{-6} M Ald+TGF- β_1 group vs TGF- β_1 group, ▲ 10^{-7} M Ald+TGF- β_1 group and 10^{-6} M Ald+TGF- β_1 group vs ALD group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

HSCs activation after Ald treatment

Immunohistochemistry was done to detect α -SMA expression in HSCs. Results showed α -SMA expression increased significantly in HSCs after Ald treatment for 24 h, and HSCs had filamentous structures (Figure 3).

Effect of Ald and spironolactone (SPI) on the PAI-1 expression in HSCs

Cells were divided into control group, 10^{-6} M Ald group, 10^{-5} M spironolactone (SPI) group and Ald+SPI group. Results showed PAI-1 expression in Ald group, SPI group and Ald+SPI group was markedly different from that in control group ($P < 0.01$). In addition, significant differ-

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ences were also observed in PAI-1 mRNA and protein expressions between Ald group and Ald+SPI group ($P<0.05$). (**Figure 4**).

Effect of Ald on Smad expression in HSCs

The Smad expression in 10^{-6} M Ald group, 5 ng/ml TGF- β_1 group and 10^{-6} M Ald+5 ng/ml TGF- β_1 group was significantly higher than in control group ($P<0.05$), and marked difference was also observed between Ald+TGF- β_1 group and Ald group ($P<0.01$) and between Ald+TGF- β_1 group and TGF- β_1 group ($P<0.05$) (**Figure 5**).

Effect of Ald on type I collagen expression in HSCs

The type I collagen expression in 10^{-6} M Ald group, 5 ng/ml TGF- β_1 group and 10^{-6} M Ald+5 ng/ml TGF- β_1 group was markedly higher than in control group ($P<0.01$), and type I collagen expression in Ald+TGF- β_1 group increased significantly as compared to Ald group and TGF- β_1 group ($P<0.01$) (**Figure 6**).

Effect of Ald and TGF- β_1 on PAI-1 expression in HSCs

PAI-1 expression in 10^{-6} M Ald group, 5 ng/ml TGF- β_1 group, and 10^{-6} M Ald+5 ng/ml TGF- β_1 group was markedly higher than in control group ($P<0.01$). Moreover, the PAI-1 expression in Ald+TGF- β_1 group increased dramatically as compared to Ald group and TGF- β_1 group ($P<0.01$) (**Figure 7**).

Effect of Ald at different concentration and TGF- β_1 on PAI expression in HSCs

PAI-1 expression in Ald group, 50 pg/ml TGF- β_1 group, 10^{-6} M Ald+TGF- β_1 group, 10^{-7} M Ald+TGF- β_1 group and 10^{-8} M Ald+TGF- β_1 group was significantly higher than that in control group ($P<0.01$). Moreover, significant difference was observed between 10^{-6} M Ald+TGF- β_1 group and Ald group ($P<0.01$), and between 10^{-7} M Ald+TGF- β_1 group and TGF- β_1 group ($P<0.01$), but the PAI-1 expression was comparable between 10^{-7} M Ald+TGF- β_1 group and Ald group ($P>0.05$) (**Figure 8**).

Statistical analysis

Statistical analysis was performed with SPSS version 17.0. Quantitative data are expressed as mean \pm standard deviation. Comparisons

were done with t test between two groups and analysis of variance among groups. A value of $P<0.05$ was considered statistically significant.

Discussion

HSCs are major cells regulating ECM deposition in the liver. HSCs may regulate the uPA and PAI expressions to modulate the activities of plasmin and MMP family members, and then to maintain the balance between ECM synthesis and degradation. This is one of important ways in which HSCs regulate the ECM metabolism. Our results showed Ald treated HSCs had significantly increased PAI-1 synthesis and markedly elevated PAI-1 expression as compared to control group ($P<0.01$). These results demonstrate that Ald may directly induced PAI-1 over-expression but SPI may partially inhibit the Ald-induced PAI-1 expression. Ald acts on HSCs to modulate the fibrotic matrix protein metabolism. PAI-1 is a key factor modulating the fibrolysis and ECM deposition, and the Ald induced matrix degradation might be PAI-1 dependent. Our results indicated that Ald could also reduce the ECM degradation to induce hepatic fibrosis except for it physiologically functioning to maintain the dynamic balance of extracellular salt, water and potassium. In addition, this study also revealed that, besides increasing PAI-1 expression, Ald was able to elevate the secretion of type I collagen in HSCs, and Ald and TGF- β_1 could synergistically promote the production of type I collagen in HSCs as compared to treatment with Ald alone, which however was blocked by SPI. There is evidence [18] showing that aldosterone/salt treated rats presented significantly increased expression of collagens in the kidney, accompanied by elevation of PAI-1 expression. Furthermore, the glomerular hypertrophy was significantly improved when there was PAI-1 deficiency. In addition, addition of MR blocker was able to reduce the PAI-1 expression and improve glomerulosclerosis in rats with diabetes or nephritis [20, 21]. Our findings were consistent with results in previous studies: Ald is able to induce PAI-1 expression in HSCs, and SPI inhibits the bioeffects of Ald on HSCs.

Several studies have shown that Ald is able to increase TGF- β_1 secretion *in vitro*, but little is known about TGF- β_1 mRNA expression after Ald treatment [7, 17, 22]. In recent years, investiga-

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tors focus on the effect of Ald on the TGF- β_1 in HSCs in the absence of RAAS. Our previous study [23] showed Ald could induce the TGF- β_1 expression in HSCs *in vitro* as compared to control group ($P < 0.05$), and the TGF- β_1 expression increased over time, but Ald in combination with SPI significantly reduced TGF- β_1 expression as compared to treatment with Ald alone ($P < 0.05$).

PAI-1 involves in some pathophysiological processes including fibrinolysis, recycling of ECM, fibrosis of tissues and organs and tissue repair after trauma. Thus, studies on the regulation of PAI-1 expression may provide new ways and methods for the therapy of related diseases. In recent years, there is evidence showing that PAI-1 plays multiple roles in the pathogenesis of chronic liver diseases. On one hand, PAI-1 induced inflammation and fibrosis mediate the destruction of tissues, and on the other hand PAI-1 is able to help the liver repair after liver injury [24]. Our results showed Ald treated HSCs had elevated PAI-1 expression, and Ald (10^{-6} M) could exert synergistical effect with TGF- β_1 to induce PAI-1 expression in HSCs ($P < 0.01$ vs 10^{-6} M Ald group). Although the PAI-1 expression increased in HSCs after treatment with Ald ($< 10^{-6}$ M) and TGF- β_1 , there was no marked difference between Ald ($< 10^{-6}$ M) and TGF- β_1 group and Ald (10^{-6} M) group. Thus, in our study, HSCs treated with Ald and TGF- β_1 showed significantly increased PAI-1 expression as compared to Ald group ($P < 0.01$). This suggests that Ald and TGF- β_1 may synergistically induce PAI-1 expression in HSCs. There is evidence showing that Smad signaling pathway is crucial for the TGF- β_1 mediated PAI-1 expression. Studies also reveal that TGF- β_1 is effective to increase the transcriptional activity of PAI-1, and TGF- β_1 may induce the formation of Smad/Spl complex to activate Sp1 and up-regulate PAI-1 expression [25, 26]. Our results also showed Ald significantly induced Smad in HSCs. Thus, we speculate that Ald induced PAI-1 expression is partially mediated by TGF- β_1 , which is consistent with findings from a study on pre-integrin β_6 deficient mice [27].

Of note, available studies confirm that TGF- β_1 over-expression is present in tissues with fibrosis [17, 28]. Ald may interact with TGF- β to induce PAI-1 over-expression and then inhibit ECM degradation. Ald binds to MR to induce

TGF- β_1 expression and activation, leading to the increase in PAI-1 expression, and TGF- β_1 neutralizing antibody may reduce the Ald induced PAI-1 expression, but it may not reduce to normal level. This indicates that Ald can stimulate PAI-1 expression in a TGF- β_1 dependent manner, but this is not a unique pathway [29]. This synergistic effect may be observed in patients with heart failure or diabetic nephropathy whom are treated with ang II receptor blockers and/or aldosterone blockers [28, 30-32] as well as animals with hypertension or glomerulonephritis [33-35]. *In vitro* experiments showed the TGF- β_1 and FN expressions increased in Ald treated mesangial cells, which was antagonized by SPI. Our *in vitro* experiments also indicated that Ald induced a slight increase in PAI-1 expression in HSCs, and Ald and TGF- β_1 could synergistically increase PAI-1 expression. These findings demonstrate that Ald may involve the fibrosis independent of RAAS, and can regulate TGF- β_1 signaling pathway to up-regulate collagen expression and reduce MMP release, leading to the proliferation of fibroblasts.

Taken together, our findings indicate that Ald is able to induce PAI-1 over-expression in HSCs, which is partially mediated by TGF- β_1 . Increased PAI-1 expression may reduce the ECM degradation. Although Ald alone only induces slight increase in PAI-1 expression, Ald may synergistically exert effect with TGF- β_1 to significantly induce PAI-1 expression, leading to the occurrence and development of fibrosis. SPI may partially inhibit the PAI-1 over-expression secondary to Ald treatment. Thus, PAI-1 may become a new target for the therapy of hepatic fibrosis. However, the specific signaling pathway involved in the Ald-induced hepatic fibrosis, the interaction among signal molecules and whether there is cross-talk among other signaling pathways are still poorly understood, and more studies are required to elucidate these issues.

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Disclosure of conflict of interest

None.

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