# Simvastatin Inhibits Tissue Factor and Plasminogen Activator Inhibitor-1 Secretion by Peripheral Blood Mononuclear Cells in Patients with Primary Nephrotic Syndrome

Jia-li Wei[1], Hui-ming Cui[2], Chun-yang Ma[3]

[1]Department of Nephrology, The Second Xiangya Hospital of Central South University, Changsha, Hunan, PR China;
[2]Department of Nephrology, Zhongshan city people's hospital, Zhongshan, Guangdong, PR China;
[3]Department of Neurology, The affiliated Hospital of Hainan medical college, Haikou, Hainan, PR China

## Abstract

*Background:* Tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1) activity and/or expression are upregulated in nephrotic syndrome. Despite extensive research on antithrombotic effect of statins, little is known about their effects on TF and PAI-1 expression in peripheral blood mononuclear cells in patients with primary nephrotic syndrome(PNS).

*Methods:* PBMCs were isolated by gradient centrifugation from 25 individuals with PNS and 25 healthy subjects. TF and PAI-1 mRNA were detected by RT-PCR. The activities of TF and PAI-1 were determined with ELISA and chromogenic substrate method, respectively. The patients with PNS were then treated with simvastatin 40mg/day for 2 weeks. The activities of TF, PAI-1 and TF, PAI-1 mRNA of PBMCs were also measured.

*Results:* Compared with controls, patients with PNS had increased TF, PAI-1 secretion by PBMCs at baseline [$70.4 \pm 15.6$ ng/l vs. $32.7 \pm 8.2$ ng/l; $15.9 \pm 2.4$ ($\times 10^3$AU/l) vs. $3.9 \pm 1.5$($\times 10^3$AU/l), $P<0.01$] and after stimulated by LPS (10 ng/mL) [ $89.2 \pm 13.4$ ng/l vs. $49.5 \pm 10.3$ ng/l; $23.8 \pm 3.3$ ($\times 10^3$AU/l) vs. $8.1 \pm 2.1$, $P<0.01$]. The simvastatin treatment resulted in a significant effect in decreasing TF and PAI-1 [$69.1 \pm 14.6$ ng/l vs. $89.2 \pm 13.4$ ng/l; $16.5 \pm 4.8$ ($\times 10^3$AU/l) vs. $23.8 \pm 3.3$ ($\times 10^3$AU/l), $P<0.05$] secretion in PBMCs. Increased TF and PAI-1 mRNA expression in PBMCs from PNS ($1.034 \pm 0.043$ and $0.982 \pm 0.056$, respectively) as compared to the control ($0.221 \pm 0.015$ and $0.221 \pm 0.015$, respectively) ($p<0.01$). two-week simvastatin treatment resulted in significant decrease of TF ($0.535 \pm 0.028$, $p<0.01$) and PAI-1 mRNA ($0.602 \pm 0.037$, $p<0.01$).

*Conclusion:* TF and PAI-1 mRNA expression and activities in PBMCs were increased in PNS. Simvastatin reduced TF and PAI-1 expression and activity in PBMCs. These effects may partially be relevant to the clinical benefits of statins in the treatment of PNS.

*Key words:* HMG-CoA reductase inhibitors; tissue factor; PAI-1; nephrotic syndrome

## 1. Introduction

The fibrinolytic activity is increased in patients with nephrotic syndrome [1]. Acquired deficiency of naturally occurring anticoagulant proteins, due to loss in the urine, has been proposed as one of the major thrombogenic alterations in nephrotic proteinuria[2]. Tissue factor (TF) is an integral membrane protein essential for the initiation of the extrinsic pathway of hemostasis. Concentrations of TF and TF pathway inhibitor (TFPI) were found to be significantly elevated in patients with nephrotic syndrome [3]. Nephrotic proteinuria is not associated with TFPI deficiency, but with a marked increase of this anticoagulant protein [2]. Expression of plasminogen activator inhibitor 1(PAI-1) is enhanced in patients with nephrotic syndrome as well [1, 4], an increased expression of PAI-1 may be involved in the intraglomerular fibrinogen/fibrin-related antigen deposition seen in nephrotic syndrome [1].

Statins, a structurally related group of hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are widely used in primary cardiovascular prevention, which cannot be explained only through their hypolipemic effect. As far as kidney diseases are concerned, statins therapy has been shown to prevent creatinine clearance decline and to slow renal function loss, particularly in case of proteinuria, and its favorable effect may depend only partially on the attenuation of hyperlipidemia, which can be triggered by other mediators, such as angiotensin receptor blockers. Possible pathways for the protective action of statins, other than any hypocholesterolemic effect, are as followed: cellular apoptosis/proliferation balance, inflammatory cytokines production, and signal transduction regulation. Statins also play a role in the regulation of inflammatory and immune response, coagulation process, bone turnover, neovascularization, vascular tone, and arterial pressure [5]. The renoprotective effects of statins have also been reported in nephrotic syndrome[11], ischemia-reperfusion injury [6], subtotal renal ablation [7], puromycin-induced nephrosis [8], and unilateral ureteral obstruction [9].

Nevertheless so far, TF and PAI-1 secretion by peripheral blood mononuclear cells (PBMCs) in patients with primary nephrotic syndrome (PNS) and the effects of statin have never been reported yet.

## 2. MATERIALS AND METHODS

### 2.1. SUBJECTS

We recruited 25 patients with PNS (17 males and 8 females) before steroid therapy at screening and 25 healthy age and sex-matched controls who attended the outpatient department for routine health checks with no evidence of renal diseases or other diseases that affect TF and PAI-1 levels.

All PNS patients were recruited consecutively from the Nephrology Unit, Zhongshan city people's hospital, Zhongshan, Guangdong, China from 2003-2005. The protocol for the present study was approved by the regional committee of medical ethics. After informed consent, kidney biopsy was performed in all PNS patients. Adequacy of biopsy was defined by the presence of at least 5 glomeruli in the specimen on light microscopy. All the biopsies were interpreted by the same pathologist and pathological changes included minimal glomerular abornormolities (10 patients), mesangial proliferative glomerulonephritis (8 patients), focal glomerular sclerosis (3 patients) and membranous glomerulonephritis(4 patients).None of these patients received any other medications or concomitant therapy before and during this study. None of the participants had hypertension, diabetes mellitus, creatinemia, hypothyroidism, abnormal liver and muscle enzymes, acute and/or chronic infections, autoimmune diseases or neoplastic diseases. None were taking medication or other agents known to affect coagulation.

### 2.2. CROSS-SECTIONAL STUDY AND INTERVENTION

At baseline, PBMCs were collected from all participants after an overnight fast (14h). Patients with PNS then took simvastatin 40 mg/day. After treatment for 2 weeks, PBMCs were also collected from all patients after an overnight fast.

### 2.3. ISOLATION OF PBMCS

Five milliliters of peripheral blood were drawn into sterile 15ml tubes containing 30μl of sodium heparin, layered on to an equal volume of Ficoll and centrifuged at 1500×g for 20 min. Cells were harvested from the Ficoll-plasma interface and washed three times in RPMI1640 medium (Gibco-BRL) containing 2 mmol/l glutamine (Sigma) and 10% heat-inactivated fetal calf serum (Gibco-BRL). Cells were suspended at $1 \times 10^6$/ml in RPMI1640 supplemented as above. Cell viability was always >95%, as estimated by trypan blue exclusion. The cell suspension was plated at 1 ml per well in 24-well flat-bottomed tissue culture plates (Costar). After 6h of incubation with or without LPS (10ng/mL) (Sigma) [10] at 37°C in 95% humidified air and 5% $CO_2$, cell supernatants were harvested and stored at -70 °C for cytokine analysis.

### 2.3. ACTIVITY OF TF AND PAI-1 IN CULTURE SUPERNATANTS OF PBMCS

The TF antigen assay was quantified by enzyme-linked immunosorbent assay (ELISA) (American Diagnostic) as described by Almus et al. [11]. TF concentrations were determined by measuring absorbance at 450 nm. The PAI-1 activities were performed by chromogenic activity kit (Shanghai TaiYang Biotechnology, China). PAI-1 activity was assayed spectrophotometrically [12]. One arbitrary unit (AU) was defined as the amount of PAI-1 activity that inhibited 1 IU of t-PA.

### 2.4. RNA EXTRACTION AND REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA of equivalence PBMCs from three groups was isolated by using TRIzol reagent (Gibco-BRL) according to the manufacturer's instructions. RNA samples were dissolved in DEPC-treated water and the RNA concentration in each sample was determined spectrophotometrically. Equal amounts of RNA were analyzed for TF, PAI-1, glyceraldehydes -3-phosphate dehydrogenase (GAPDH) mRNA concentrations by quantitative reverse transcription-polymerase chain reaction (RT-PCR). The sequences of the sense and antisense primers used for amplification were as follows: TF 5'-AAGCAGTGATTCCCTCTCG-3 and 5'-AACACAGCATTGGCAGCAG-3' [13]; PAI-15'-ATTGCTGCCCCTTATGAAAA-3' and 5'-G CCAAGGTCTTGGAGACAGA-3',GAPDH (internal control) 5'-GGAGC CAAAAGGGTCA TC-3' and 5'-CCAGTGAGTTTCCCGTTC-3' [13]. PCR cycle for TF (254bp) and GAPDH (346bp) consisted of denaturing at 94 °C for 50 s, annealing at 58 °C for 50s, and elongation at 72 °C for 60 s, conducted for 39 cycles. PCR cycle for PAI-1 (596bp) and GAPDH consisted of denaturing at 94 °C for 50 s, annealing at 55 °C for 50 s, and elongation at 72 °C for 60 s, conducted for 38 cycles. Those PCR products were electrophoresed on 1.5% agarose gel. Densitometric measurements were made, and the relative density (normalized by the amount of internal control) was given.

### 2.5. STATISTICAL ANALYSIS

Results were expressed as mean ± S.D. Differences between groups were evaluated by the Student's unpaired/ paired two-tailed t-test. Statistically significant differences between groups were reported when $p \le 0.05$.

## 3. RESULTS

### 3.1. CHARACTERISTICS OF THE SUBJECTS

The clinical and biochemical characteristics of the PNS and healthy individuals were shown in Table 1. Age, blood pressure and serum Cr were not significantly different between the groups (P>0.05). PNS group had a significantly different in plasma cholesterol, triglycerides, Alb and proteinuria compared with the healthy controls(P<0.05).

*Table 1.* Characteristics of participants.

| | Contro (n = 25) | Pre-treatment (n = 25) | Post-treatment (n = 25) |
|---|---|---|---|
| Men/women | 14/11 | 17/8 | 17/8 |
| Age(years) | 30.3 ± 12 | 31.1 ± 14 | 31.1 ± 14 |
| Proteinuria(g/d) | 0.08 ± 0.06 | 7.7 ± 3.4* | 7.5 ± 4.6* |
| TG(mmol/L) | 1.39 ± 0.76 | 1.96 ± 0.56* | 1.83 ± 0.81* |
| TC(mmol/L) | 4.52 ± 0.93 | 8.47 ± 0.52* | 8.21 ± 0.67* |
| Alb(g/L) | 44.3 ± 2.1 | 26.21 ± 1.18* | 25.98 ± 1.05 |
| Serum Cr(umol/L) | 67.32 ± 7.21 | 65.34 ± 10.71 | 66.02 ± 9.28 |
| SBP (mmHg) | 123.6 ± 11.1 | 126.3 ± 9.5 | 125.1 ± 8.7 |
| DBP (mmHg) | 73.2 ± 7.2 | 72.6 ± 9.9 | 73.8 ± 10.9 |

TG: triglyceride; TC: total cholesterol; Alb: albumin; Cr: creatinine; SBP: systolic blood pressure; DBP: diastolic blood pressure. NS: not significant. *:P<0.01 vs. control

*Table 2.* Secretion of TF and PAI-1 by PBMCs at baseline and stimulated by LPS.

| Groups | n | TF antigen (ng/l) | | PAI-1 activity (×$10^3$AU/l) | |
|---|---|---|---|---|---|
| | | Spontaneous | +LPS | Spontaneous | +LPS |
| Control | 5 | 32.7 ± 8.2 | 49.5 ± 10.3 | 3.9 ± 1.5 | 8.1 ± 2.1 |
| Pretreatment | 5 | 70.4 ± 15.6* | 89.2 ± 13.4[#] | 15.9 ± 2.4* | 23.8 ± 3.3[#] |
| Posttreatment | 5 | 58.5 ± 13.1 | 69.1 ± 14.6[&] | 11.2 ± 2.9 | 16.5 ± 4.8[&] |

n indicates number of animals; values are mean±S.D.
+LPS: stimulated by LPS(10ng/mL).
* p <0.01 vs. spontaneous control group.
[#]p < 0.01 vs. control group stimulated by LPS.

[&]<0.01 vs. pre-treatment stimulated by LPS.

### 3.2. Secretion of TF and PAI-1 into Culture Supernatants by PBMCs

#### 3.2.1

At baseline, spontaneous secretion of TF and PAI-1 into culture supernatants by PBMCs differed significantly between nephrotic syndrome patients and healthy controls. TF and PAI-1 secretion stimulated by LPS were significantly higher in patients with PNS vs. healthy individuals (Table 2).

#### 3.2.2

The changes of TF and PAI-1 concentrations stimulated by LPS after treatment with simvastatin were shown in Table 2. It showed that simvastatin treatment resulted in a significant effect in decreasing TF and PAI-1 secretion in PBMCs.

### 3.3. TF and PAI-1mRNA Concentrations in PBMCs

Experiments were performed to compare the concentration of gene expression in PBMCs between control and PNS. TF mRNA concentrations in PBMCs from pretreated group were 1.034 ± 0.043, which significantly increased when compared with the control and post-treated group (0.221 ± 0.015 and 0.535 ± 0.028, respectively) (p<0.01, Fig. 1). PAI-1 mRNA concentrations in PBMCs from pretreated group were 0.982 ± 0.056, which significantly increased when compared with the control and post-treated group (0.221 ± 0.015 and 0.602 ± 0.037, respectively) (p<0.01, Fig 1).

### Discussion

Disequilibrium in the coagulolytic system, platelet hyperactivity, hyperfibrinogenemia, disturbances in peripheral serotonergic system together with lipid abnormalities may contribute to the progression and development of atherosclerosis and an enhanced risk of thromboembolic complications in nephrotic syndrome [4].

In the present study, we found that the release of TF and PAI-1 in PBMC from PNS patients was significantly higher than that from healthy controls. This might at least partially explain that in vivo TF and PAI-1 levels are higher in PNS patients vs. healthy subjects. Furthermore, TF and PAI-1 secretion in
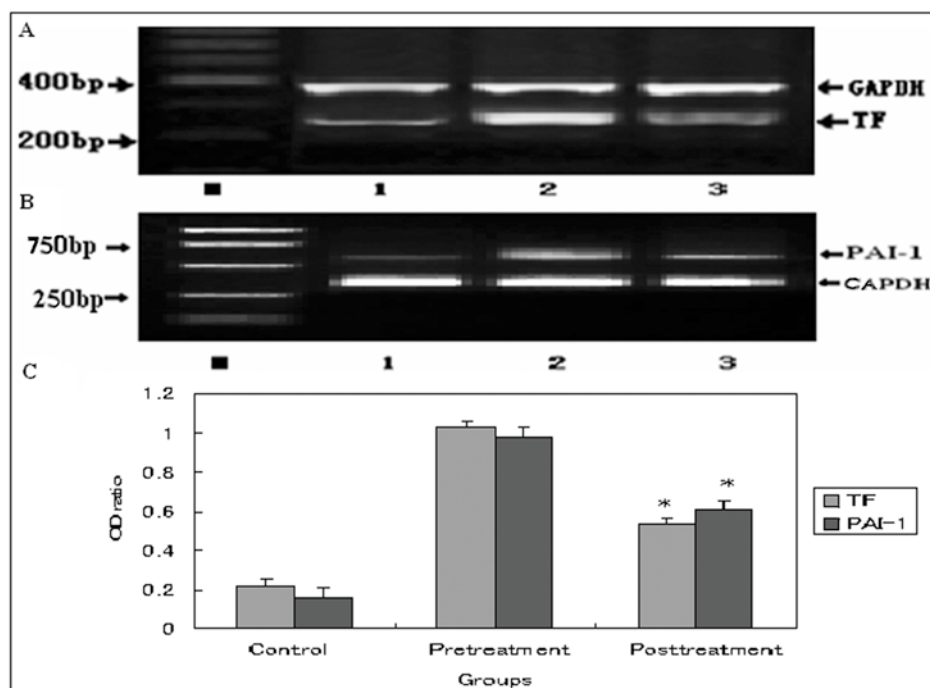
*Fig.1.* mRNA expression of TF and PAI-1 in HPMCs. Control indicates normal group; Pretreatment and postreatment indicates primary nephrotic syndrome pre- or post-simvastatin-treated groups, respectively. A: RT-PCR analysis of TF mRNA expression (upper bands: GAPDH mRNA,346 bp; lower bands: TF mRNA, 254 bp); B: RT-PCR analysis of PAI-1 mRNA expression (upper bands: PAI-1 mRNA, 596 bp; lower bands: GAPDH mRNA,346 bp); M: Marker; lane 1: normal group; lane 2: pre-simvastatin-treated group; lane 3: post-simvastatin-treated group. C: determination of relative signal intensity of TF and PAI-1 expression. Bar graph represents quantification of TF/GAPDH or PAI-1/ GAPDH mRNA signals of 3 to 4 reproductions of independent experiment. For each group, n = 5, mean ± SD. *P<0.01 vs. control group or pre-simvastatin-treated group.

PBMC stimulated by LPS were significantly higher in patients with PNS. TF is the primary cellular initiator of the blood clotting cascade. Recent evidence suggests that TF plays a role in renal fibrin formation and renal failure in experimental kidney disease. It is suggested that TF plays an important role in the development of renal injury after ischemia and reperfusion. The microcirculatory incompetence due to microthrombus might cause the formation and development of the necrosis [14]. Matsuyama et al. [15] demonstrated that antisense(AS) phosphorothioate oligodeoxynu cleotide (ODN) of TF protected the renal from ischemia-reperfusion injury. Mercier et al. [16] pointed out renal insufficiency is associated to the activation of the TF coagulation pathway. In ureteral ligation animal model, ureteral ligation leads to infiltration of inflammatory cells, increased AP-1 and NF-kappa B expression in the kidney, resulting in increased TF transcription and translation, and ultimately fibrin deposition increased [17]. The inhibition of TF activity can reduce fibrin deposition in the chronic stages of crescent formation [18]. PAI-1 is a multifunctional protein with actions that may be dependent on or independent of its protease inhibitory effects [9]. The protease-inhibitory actions of PAI-1 extend beyond fibrinolysis and include modulation of extracellular matrix turnover, cell migration and activation of several pro-enzymes and latent growth factors. PAI-1 is overexpressed in renal pathologic conditions including nephrotic syndrome, thrombotic microan-

giopathy, proliferative and crescentic glomerulonephritis, diabetic nephropathy, and chronic allograft nephropathy [1, 19-23]. In addition, glomerular PAI-1 mRNA level correlate with level of proteinuria in patients with focal segmental glomerulosclerosis and membranous nephropathy [1]. It has been demonstrated that plasminogen activator(PA)/ plasmin/PA inhibitor (PAI) system is involved in ECM degradation and PAI-1 plays a critical role in ECM remodeling in the kidney. Further evidence showed that PAI-1 was directly involved in interstitial fibrosis and tubular damage via two primary overlapping mechanisms: early effects on interstitial cell recruitment and late effects associated with decreased urokinase activity [24]. Our results suggested that TF and PAI-1secretion by PBMCs might partly account for the progressive glomerulopathy in PNS and the increased secretion of TF and PAI-1 in circulation.

Hypertriglyceridemia and hypercholesterolemia in this case may depend on a reduction in triglyceride-rich lipoproteins catabolism and on an increase in hepatic synthesis of Apo B-containing lipoproteins. These alterations are the starting point of a self-maintaining mechanism, which can accelerate the progression of chronic renal failure. Indeed, hyperlipidemia can affect renal function, increase proteinuria and speed glomerulosclerosis, thus determining a higher risk of progression to dialysis. 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase is the rate-limiting enzyme in cholesterol synthesis from meval-

onate and its inhibitors, or statins, can therefore interfere with the above-mentioned consequences of hyperlipidemia. The beneficial effects of statins may extend to mechanisms beyond cholesterol reduction [25-28]. Statins not only decrease serum lipid levels, but also inhibit signaling molecules at several points in coagulation pathways. Potentially, reduced release of TF and PAI-1 from PBMC due to simvastatin, as found in the present study, may also account for part of such effects. Experimental and clinical studies have suggested statins might influence important intracellular pathways that were involved in the inflammatory and fibrogenic responses, which were common components of many forms of progressive renal injury [29]. Li et al. [30] confirmed pravastatin effectively abrogated the progression of tubulointerstitial inflammation and fibrosis in chronic CsA nephropathy.

The regulatory mechanism by which statins suppress TF and PAI-1 mRNA expression remains unclear. Cellular mechanisms, beyond reduced cholesterol synthesis, may underlie statin's vasculoprotective effect observed clinically. Statins reduce synthesis of cholesterol and metabolites of mevalonate used in posttranslational prenylation of proteins. Although the function of these prenyl modifications is not clearly understood, many prenylated proteins play important roles in the regulation of cell growth, cell secretion, and signal transduction. Recently Statins have been shown to inhibit PAI-1 expression by monocytes and smooth muscle cells and endothelial cells, an effect that is completely prevented by supplementation of the cells with mevalonate [31-32]. Lang D et al. [33]. revealed inflammatory mediators up-regulated TF expression in mesangial cell by a PKC dependent pathway whereas PKA can serve as a negative feedback link. Induction of monocytic TF expression by endotoxin is mediated by the activation of transcription factors such as AP-1 and NF-kappaB. Both these signaling pathways are modulated by peroxisome proliferator-activated receptor-alpha(PPAR-alpha) [34].

In summary, our study suggested that PBMCs in PNS were preactivated. Statins may partly attenuate the increased PBMCs activation, showing in the decreased levels of TF and PAI-1 excreted by PBMCs. These effects may partially be relevant to the clinical benefits of statins in the treatment of PNS. However, another prospective study of more patients should be performed to validate these results.

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*Address correspondence:*
Chunyang Ma, MD
Department of Neurology
The affiliated Hospital of Hainan medical college
Haikou, Hainan 510008
PR China
Tel.: +86-13322089516
E-mail: cyma323@yahoo.com.cn