

Simvastatin inhibits tissue factor and plasminogen activator inhibitor-1 expression of glomerular mesangial cells in hypercholesterolemic rabbits

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ABSTRACT

Tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1) activity and/or expression are up-regulated in hypercholesterolemia. Despite extensive research on anti-thrombotic effect of statins, little is known about their effects on TF and PAI-1 expression in glomerular mesangial cells under hypercholesterolemic condition. Male rabbits were fed on either normal or high-cholesterol diet for 8 weeks. Then cholesterol-fed rabbits were randomly assigned to simvastatin or starch. At the end of 12 weeks, glomerular mesangial cells were collected. The concentrations of TF and PAI-1 mRNA were detected by RT-PCR. The plasma activities of TF and PAI-1 were determined with enzyme linked immunosorbent assay (ELISA) and chromogenic substrate method, respectively. The atherogenic diet caused a consistent increase in serum concentrations of total cholesterol (TC) and serum triglyceride (TG) ($p < 0.05$), increased TF and PAI-1 mRNA expression in glomerular mesangial cells and plasma activities as compared to the normal diet ($p < 0.01$). Four-week simvastatin treatment resulted in significant decrease of mesangial TF and PAI-1 mRNA ($p < 0.01$), and also of the plasma activities of TF ($p < 0.05$) and PAI-1 ($p < 0.01$). These results suggest that simvastatin might protect kidney from the formation of microthrombus under hypercholesterolemic condition and might be a possible pathogenesis of obesity-related glomerulopathy.

Lipid abnormalities often accompany and exacerbate renal disease (11, 16, 54). Hypercholesterolemia is considered as an independent risk factor for renal injury (45). Previous reports show that even a short exposure to diet-induced hypercholesterolemia is associated with an increase in oxidative stress, formation of oxidized low-density lipoprotein (ox-LDL) and renal inflammation (4, 8). There is a close relationship among hypercholesterolemia, coagulation, fibrinolysis and glomerular damage. Glomerular fi-

brin deposits and capillary microthrombi are histopathological hallmarks in many inflammatory kidney diseases (18). Fibrin may exert detrimental effects by direct cytotoxicity, by altering glomerular haemodynamics or by attracting leukocytes.

The coagulation and fibrinolytic systems are two separate but reciprocally linked enzyme cascades that regulate the formation and breakdown of fibrin. Tissue factor (TF), a membrane-anchored glycoprotein, plays an important role in promoting coagulation and thrombosis (42). TF initiates blood coagulation by forming a complex with circulating factors VII and VIIa (14). It is reported that the activation of TF is associated with renal insufficiency (37), experimental and human crescentic glomerulonephritis (10, 18), renal injury after ischemia and reperfusion (36, 53), interstitial fibrosis (56) and

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diabetic nephropathy (51). Plasminogen activator inhibitor-1 (PAI-1) is a 50 kDa glycoprotein member of the serine protease inhibitor (SERPIN) family and is the major physiological inhibitor of tissue plasminogen activator (tPA) and urokinase-like plasminogen activator (uPA). The protease-inhibitory actions of PAI-1 extend beyond fibrinolysis, and include modulation of extracellular matrix (ECM) turnover, cell migration and activation of several pro-enzymes and latent growth factors. Normal human kidneys do not express PAI-1 but PAI-1 is overexpressed in renal pathologic conditions including thrombotic microangiopathy, proliferative and crescentic glomerulonephritis, diabetic nephropathy, and chronic allograft nephropathy (13, 15, 28, 49, 50). PAI-1 gene deficiency attenuates transforming growth factor-beta1 (TGF-beta1)-induced kidney disease, decreasing both glomerular and interstitial ECM deposition (25).

Statins, reversible inhibitors of the microsomal enzyme hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase which converts HMG-CoA to mevalonate, an early rate-limiting step in cholesterol biosynthesis, include atorvastatin, cerivastatin, fluvastatin, lovastatin, pravastatin, simvastatin, pitavastatin and rosuvastatin. Treatment of hyperlipidaemia with statins has led to a significant reduction in major cardiovascular events in humans (5, 21). Recent evidences suggest that even in the absence of cholesterol lowering, therapeutic benefits can be achieved in hypercholesterolemia due to the direct tissue effects of statins on upregulation of endothelial nitric oxide (NO) synthase (eNOS) with increased bioavailability of NO, decreased cellular proliferation, and/or decreased oxidative stress (35, 58). Alterations in these pathways are often involved in renal disease progression, and the findings *in vitro* and *vivo* suggest that statins can provide protection against kidney diseases characterized by inflammation and/or enhanced proliferation of epithelial and mesangial cells (7). The renoprotective effects of statins have also been reported in ischemia-reperfusion injury (23), subtotal renal ablation (29), puromycin-induced nephrosis (19), and unilateral ureteral obstruction (40).

In addition, statins diminish procoagulant activity, which is observed at different stages of the coagulation cascade, including TF activity, conversion of prothrombin to thrombin and thrombin activity. Statins also reduce fibrinogen levels and stimulate fibrinolysis by altering the levels and activities of tPA and PAI-1 (26). Despite extensive research on antithrombotic effect of statins, little is known about

their effects on TF and PAI-1 expression in glomerular mesangial cells under hypercholesterolemic condition. In this study, we used simvastatin, the methylated form of lovastatin, which competes with HMG-CoA for HMG-CoA reductase after being hydrolyzed.

MATERIALS AND METHODS

Animal experimental protocol and the diet. All animal experiments were conducted in accordance with the guidelines of animal ethical committee for animal experimentation in China. Fifteen male New Zealand rabbits (2–3 months old, weight 2000–2300 g) were provided by a breeder (Hainan Medical College, China) and individually housed in air-conditioned room equipped with laminar flow. They were randomly divided into normal diet group (control group, $n = 5$) and high-cholesterol diet group (1% cholesterol, 8.5% coconut oil, 7.5% protein, $n = 10$) for 8 week. Then cholesterol-fed rabbits were randomly assigned to simvastatin (Merck & Co., Inc., USA) (simvastatin group, $n = 5$) or starch (starch group, $n = 5$) at 30 mg/kg/day. Starch was used as the vehicle control for simvastatin, and was administered as a diet admixture. Simvastatin was prepared by mixing the drug with plain chow. Chow (≈ 5 –10 g) containing the dose of the drug was administered daily by adding it to a small chow dispenser that made chow pellets available and ensured the ingestion of the entire dose. Afterwards, drug-free high-cholesterol chow was available to the animals. Feeding was restricted to 120 g/day. At the end of 12 weeks of the experiment, all rabbits were sacrificed by an overdose of pentobarbital (4th Pharmaceutical Factory, Shanghai, China). The kidneys were removed for the following experiments.

Isolation and identification of glomerular mesangial cells. Rabbit glomerular mesangial cells were prepared by a modification of the method of Lovett *et al.* (31). Glomerular cells collected as described above were incubated in phosphate buffered saline (pH 7.4) containing 0.1% gentamicin solution (Sigma), 1% antibiotic antimycotic (Invitrogen), and 0.5% collagenase (Sigma), at 37°C for half an hour to remove epithelial cells, and were vortexed every 10 min during the incubation, leaving the glomerular cores containing mesangial and endothelial cells. The cores were diluted in 1.5 mL RPMI 1640 medium (Gibco-BRL, USA) plus hydroxyethyl piperazineethanesulfonic acid (HEPES) (Sigma) per kidney, containing 0.1% gentamicin solution, 1%

antibiotic antimycotic, 0.5% insulin-transferring solution, and 20% fetal bovine serum (Gibco-BRL), conditions that favor growth of mesangial cells. Cells were incubated at 37°C in a humidified atmosphere of 95% air-5% CO₂. Cell viability was assessed by standard dye exclusion techniques, using 0.1% trypan blue. Mesangial cells were identified by the following criteria. Mesangial cells were stained positive for intracellular cytoskeletal fibrils of actin and smooth muscle cell-specific myosin (indicative of contractile cells), desmin, and vimentin and negative for cytokeratin and factor VIII antigens. Morphologically, the mesangial cells had an elongated and stellate or spindle-shaped morphology. Mesangial cells isolated by this procedure were homogeneous and were used in all studies between passages 3–8. Before experiment, mesangial cells were cultured in 6-well plates at a density of 1×10^6 cells/mL.

Plasma activity of TF and PAI-1. Blood was collected from heart at the end of 12 weeks of the experiment. The plasma TF antigen assay was quantified by enzyme-linked immunosorbent assay (ELISA) (American Diagnostic) as described by Almus *et al.* (2). TF concentrations were determined by measuring absorbance at 450 nm. The plasma PAI-1 activities were performed by chromogenic activity kit (Shanghai TaiYang Biotechnology, China). PAI-1 activity was assayed spectrophotometrically (44). One arbitrary unit (AU) was defined as the amount of PAI-1 activity that inhibited 1 IU of tPA.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA of equivalence glomerular mesangial cells 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'-AACA CAGCATTGGCAGCAG-3' (1); PAI-1 5'-ATTGCT GCCCCTTATGAAA-3' and 5'-GCCAAGGTCTT GGAGACAGA-3', GAPDH (internal control) 5'-GGAGCCAAAAGGGTTCATC-3' and 5'-CCAGT GAGTTTCCCGTTC-3' (1). PCR cycle for TF (254 bp) and GAPDH (346 bp) consisted of denaturing at 94°C for 50 s, annealing at 58°C for 50 s, and elongation at 72°C for 60 s, conducted for 40 cycles. PCR cycle for PAI-1 (596 bp) and GAPDH (346 bp) 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 37 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.

Statistical analysis. Results are expressed as mean \pm 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 $p \leq 0.05$.

RESULTS

Serum TC and TG concentrations

At the beginning of the experiment, the mean TC and TG concentrations (mmol/L) in the three groups had no statistical differences. Compared with the control group, the TC and TG concentrations in the starch and simvastatin group both increased significantly at the 8th and 12th weeks ($p < 0.05$). Simvastatin added to the cholesterol-rich diet decreased serum TC ($p < 0.01$ vs. starch group or pretreatment), and did not influence serum TG (Table 1).

Table 1 Serum TC and TG concentrations at the baseline, 8th week and 12th week (mmol/L)

Groups	n	TC			TG		
		Baseline	8th	12th	Baseline	8th	12th
Control	5	1.02 \pm 0.39	1.07 \pm 0.42	1.21 \pm 0.54	0.92 \pm 0.53	0.91 \pm 0.41	1.08 \pm 0.32
Starch	5	1.12 \pm 0.53	22.15 \pm 0.21*	25.43 \pm 1.59*	0.78 \pm 0.36	2.08 \pm 0.38*	2.01 \pm 0.25*
Simvastatin	5	0.98 \pm 0.38	23.27 \pm 0.18*	17.25 \pm 1.63 [#]	0.89 \pm 0.34	2.11 \pm 0.31*	1.92 \pm 0.69*

n indicates number of animals; values are mean \pm S.D.

* $p < 0.05$ vs. control group or baseline.

[#] $p < 0.01$ vs. starch group or pretreatment.

Table 2 Plasma activity of TF and PAI-1

Groups	n	TF antigen (ng/L)	PAI-1 activity ($\times 10^3$ AU/L)
Control	5	48.1 \pm 9.2	8.5 \pm 1.2
Starch	5	84.7 \pm 21.5*	17.9 \pm 2.3**
Simvastatin	5	58.6 \pm 10.9	10.6 \pm 1.5

n indicates number of animals; values are mean \pm S.D.

* $p < 0.05$ vs. control group or simvastatin group.

** $p < 0.01$ vs. control group or simvastatin group.

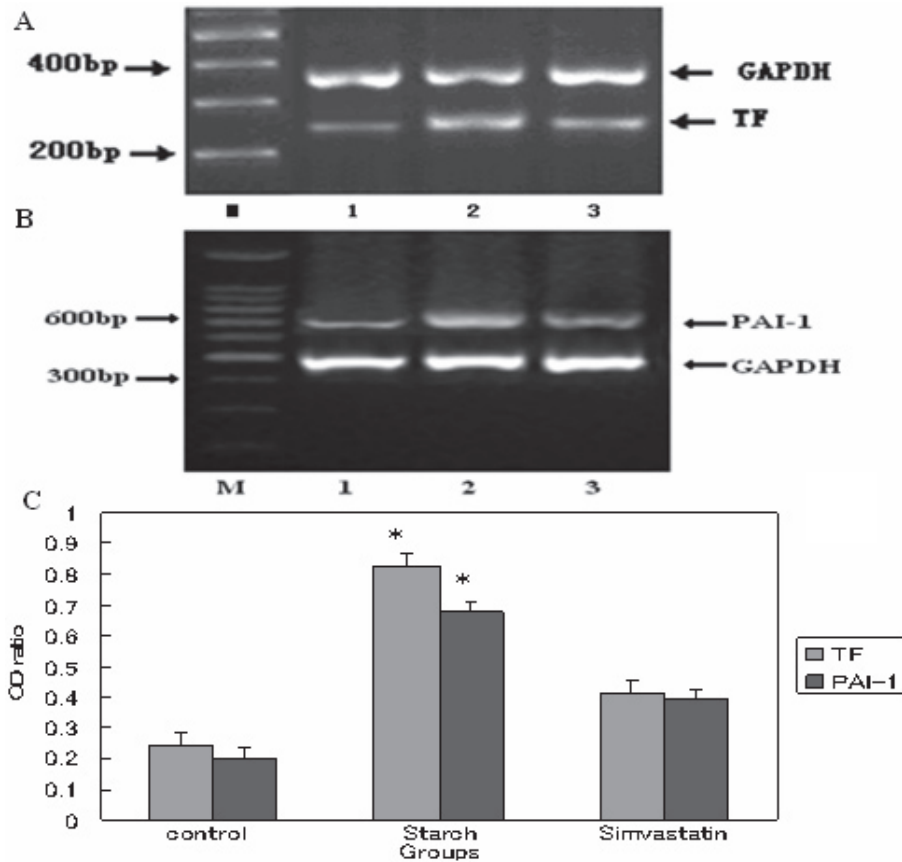


Fig. 1 mRNA expression of TF and PAI-1 in glomerular mesangial cells from control, starch, and simvastatin groups. Control indicates normal diet group; starch and simvastatin indicates hypercholesterolemic starch- or simvastatin-treated groups, respectively. *A* RT-PCR analysis of TF mRNA expression (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 diet group, lane 2: hypercholesterolemic starch-treated group, lane 3: hypercholesterolemic 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 \pm SD. * $p < 0.01$ vs. control group or simvastatin group.

Plasma activities of TF and PAI-1

The plasma activities of TF (84.7 \pm 21.5 ng/L) and PAI-1 (17.9 \pm 2.3 $\times 10^3$ AU/L) in high-cholesterol diet group were higher than those of normal diet group (48.1 \pm 9.2 ng/L, $p < 0.05$; and 8.5 \pm 1.2 $\times 10^3$ AU/L, $p < 0.01$). Four-week simvastatin treatment resulted in significant decrease of the plasma

activity of TF (58.6 \pm 10.9 ng/L, $p < 0.05$) and PAI-1 (10.6 \pm 1.5) $\times 10^3$ AU/L, $p < 0.01$) compared with the high-cholesterol diet group (Table 2).

TF and PAI-1 mRNA concentrations in glomerular mesangial cells

Experiments were performed to compare the con-

centration of gene expression in glomerular mesangial cells between control and starch rabbits. TF and PAI-1 mRNA concentrations in glomerular mesangial cells from starch group were 0.826 ± 0.043 and 0.667 ± 0.032 , respectively, which significantly increased when compared with their normal counterparts (0.241 ± 0.011 and 0.203 ± 0.021 , respectively) ($p < 0.01$). Four-week simvastatin treatment resulted in significant decrease of TF (0.412 ± 0.025 , $p < 0.01$) and PAI-1 mRNA (0.395 ± 0.018 , $p < 0.01$) (Fig. 1).

DISCUSSION

Prothrombotic states occur in numerous human and experimental renal diseases and many evidences indicate that coagulation activation is linked to glomerular injury (24). Presence of glomerular fibrin deposits, capillary microthrombi and complete glomerular capillary occlusion are histological hallmarks of procoagulatory activity (18). Hypercholesterolemia is often accompanied by coagulation activation. Growing evidence indicate the role of hypercholesterolemia as an important risk factor for renal disease progression (9, 11, 16, 22, 54). Hypercholesterolemia can impair both the function and structure of many types of vascular beds, and even at early stage, hypercholesterolemia alters vasomotor regulation in both large vessels and microcirculation (41). In addition, hypercholesterolemia may induce renal cortical neovascularization (4) and may accelerate renal vascular, glomerular, and tubular damage (8).

Our study revealed that TF and PAI-1 mRNA in glomerular mesangial cells and their plasma activity were increased on hypercholesterolemic rabbits, indicating that TF and PAI-1 participate in the pathogenesis of early renal injury in hypercholesterolemia. It has been reported that the coagulation process proceeded on mesangial cells as the result of increased expression of endogenous factor V on its cell surface in cooperation with exogenous factor Xa (47). It is demonstrated that TF and PAI-1 gene transcriptions in mesangial cells are activated by inflammatory cytokines (12, 27, 38) and hypercholesterolemia is associated with inflammation (33), so the activation of TF and PAI-1 in glomerular mesangial cells of hypercholesterolemic rabbits might be related to the stimulation by inflammatory cytokines. Lang D *et al.* (27) revealed that inflammatory mediators up-regulate TF expression in mesangial cell by a protein kinase C (PKC)-dependent pathway whereas protein kinase A (PKA) can serve as a negative

feed-back link. Induction of monocytic TF expression by endotoxin is mediated by the activation of transcription factors such as activator protein-1 (AP-1) and NF-kappaB. Both these signaling pathways are modulated by peroxisome proliferator-activated receptor-alpha (PPAR-alpha) (43). Though, monocytes/macrophages are generally believed to be the major source of TF (3, 17, 39) and endothelial PAI-1 seems to be primarily responsible for PAI-1 levels in plasma (30), our results suggested that TF and PAI-1 secretion by glomerular mesangial cells might partly account for the obesity-related glomerulopathy and the increased secretion of TF and PAI-1 in circulation.

Statins are often used in the treatment of obesity/diabetes mellitus patients with dyslipidemia. Some of their observed benefits exceeded the degree of lipid lowering (6, 58), suggesting that statins have direct, lipid-lowering-independent cardiovascular effects (21, 55). Furthermore, previous studies have demonstrated that lipid-lowering doses of statins blunted renal insufficiency in a variety of experimental models of advanced renal disease (46), in association with a marked decrease in both blood pressure and plasma cholesterol (7, 48). Several studies have shown that statins exert a favorable influence on concentrations of TF and PAI-1 (34, 52). *In vitro*, pitavastatin, a novel, fully synthetic statin, markedly decreased the expression of TF in human monocytes, PAI-1 and tPA in human aortic smooth muscle cells and human umbilical vein endothelial cells (34). *In vivo*, simvastatin treatment significantly lowered plasma levels of matrix metalloproteinases (MMP-9), TF and PAI-1 in hypercholesterolemic and type 2 diabetes mellitus patients (32, 52). In our study, four-week simvastatin treatment resulted in significant decrease of TF and PAI-1 mRNA in glomerular mesangial cells. The plasma activity of TF and PAI-1 also decreased significantly. The reduction of TF and PAI-1 antigen levels in hypercholesterolemia by simvastatin may contribute to the protection of statins against thrombotic synthesis in circulation and renal glomeruli.

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, smooth muscle cells and endothelial cells, an effect that is completely prevented by supplementation of the cells with mevalonate (20, 57).

In summary, TF and PAI-1 mRNA expression and plasma activities increased in glomerular mesangial cells of hypercholesterolemic rabbits. Simvastatin reduced glomerular TF and PAI-1 expression and the plasma activities, suggesting that simvastatin treatment reduces thrombosis risk in the kidney, which may protect kidney from the formation of microthrombus and fibrosis under hypercholesterolemia. However, further study should be performed to validate the mechanism of these effects.

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