Fasudil inhibits tissue factor and plasminogen activator Inhibitor-1 secretion by peripheral blood mononuclear cells in CAPD patients

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ABSTRACT
Disturbances in hemostasis are common complications of kidney diseases and correlate well with cardiovascular mortality. Little is known about the effects of fasudil on tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1) expression in peripheral blood mononuclear cells (PBMCs) in CAPD patients. PBMCs were isolated from 13 individuals with CAPD and 13 healthy subjects. After 4 h of incubation with or without LPS (10 ng/mL), TF and PAI-1 mRNA of PBMCs were detected by RT-PCR. The levels of TF and PAI-1 in culture supernatants of PBMCs were determined by ELISA. Compared with healthy controls, CAPD patients had increased TF, PAI-1 protein and mRNA expression by PBMCs at baseline and after stimulated by LPS (10 ng/mL) \( p < 0.001 \). The fasudil treatment resulted in a significant effect in decreasing TF and PAI-1 \( p < 0.05 \) synthesis in PBMCs. TF and PAI-1 mRNA expression and activities in PBMCs were increased in CAPD patients. Fasudil reduced LPS-mediated TF and PAI-1 expression and activity in PBMCs. These effects may partially be relevant to the clinical benefits of fasudil in the treatment of CAPD patients.

Abbreviations: CAPD: continuous ambulatory peritoneal dialysis; LPS: lipopolysaccharide; TF: tissue factor; PAI-1: plasminogen activator inhibitor-1; PBMC: peripheral blood mononuclear cells

Introduction
It is apparent that hemostatic abnormalities are closely associated with increased atherosclerosis risk and correlate well with cardiovascular mortality.\(^1\) Due to the significant increase in cardiovascular morbidity and mortality in dialyzed patients,\(^2,3\) more attention has been paid to the role of thrombogenic factors in the pathogenesis of cardiovascular events in uremia.\(^4\)

Disturbances in hemostasis are common complications of kidney diseases. Their occurrence and severity correlate with the progressive loss of renal function to end-stage renal disease. Uremic patients seem to have two opposite aspects in hemostatic status, which are bleeding diathesis and thromboembolism.\(^5\) Clinical statistics suggest that uremic patients have a high incidence of thrombotic events.\(^6,7\) A thrombotic tendency, caused by factors such as platelet hyperaggregability and hypercoagulability, has been described in dialysis patients.\(^8,9\) Moreover, the relationship between hypoalbuminemia and hemostatic factors has been suggested that the elevated fibrinogen levels in CAPD patients could be induced by protein loss, which in turn could stimulate hepatic synthesis of fibrinogen.\(^10\)

Tissue factor (TF), a transmembrane cell surface glycoprotein located on the surfaces of certain cell types, is generally held to be the physiological trigger of coagulation in normal hemostasis and ultimately leads to thrombin formation. It activates both intrinsic and extrinsic coagulation pathways. Excessive expression of TF occurs in prothrombotic conditions such as sepsis, endotoxemia, systemic lupus erythematosus, atherosclerosis, Crohn’s disease, transplant rejection reactions and hemolytic uremic syndrome (HUS).\(^11,12\)

In addition, TF-induced coagulation plays an important role in the pathophysiology of many diseases, including atherosclerosis, thrombosis, ischemia-reperfusion injury, sepsis, or glomerulonephritis.\(^13\) TF-dependent coagulation and the concentrations of TF are significantly higher in CAPD patients compared to the healthy volunteers.\(^14,15\)

Several longitudinal cohort studies have also provided evidence that impaired fibrinolysis due to increased PAI-I activity is implicated in the pathogenesis of atherosclerotic disease. Recently, an enhanced PAI-I expression was observed in atherosclerotic coronary arteries with acute coronary thrombosis, and it was
suggested that an increased PAI-1 level may precipitate thrombosis in cases of sudden cardiac death.\textsuperscript{16} CAPD patients with atherosclerosis had significantly higher PAI-1 levels than those without atherosclerosis and the normal controls.\textsuperscript{9}

The small GTPase, RhoA, belongs to the Rho subfamily and has been implicated in many cellular functions, such as cell adhesion, cell motility and migration, growth control, cell contraction, and cytokinesis. One of its main effectors, Rho-kinase (ROCK), has two known isoforms (ROCK1 and ROCK2) and regulates cytoskeletal reorganization by phosphorylating myosin phosphatase, which results in an increase in myosin light chain (MLC) phosphorylation.\textsuperscript{17,18}

The aims of this study were to investigate the effects of fasudil on TF and PAI-1 expression in peripheral blood mononuclear cells in CAPD patients.

Materials and methods

Patients

The study protocol was approved by the Ethics Committee on Human Studies at Hainan General Hospital, Hainan, Haikou, China. Informed consent was obtained from each patient. Thirteen uremic patients undergoing CAPD with standard glucose solutions for more than 6 months were enrolled in this study. They were 10 men and 3 women with ages ranging from 25 to 71 years (49.92 ± 14 years). Duration of CAPD treatment ranged from 6 to 60 months (mean, 22.23 months). Causes of renal failure were chronic glomerulonephritis in 7 patients, hypertensive nephropathy in 1 patient, obstructive nephropathy in 1 patient, and Diabetic nephropathy in 4 patients. No patient had experienced peritonitis in the past 6 months and no liver dysfunction was observed (prothrombin time, alanine, and asparaginase aminotransferases within normal range). Patients with acute illnesses were excluded. All patients performed three 4-h 2-L exchanges of 1.5% glucose solution during the day and one 9-h 2-L exchange of 1.5% or 2.5% glucose solution while sleeping at night. They continued their regular medications, such as antihypertensives, erythropoietin, and phosphate binders, except for lipid lowering agents. Their current medications ranged from 6 to 60 months (mean, 22.23 months). Patients with atherosclerosis had significantly higher CRP (mg/dL) than those without atherosclerosis and the normal controls.\textsuperscript{9}

The TF and PAI-1 assay were quantified by enzyme-linked immunosorbent assay (ELISA) (Shanghai JingMa Biotechnology, China). TF and PAI-1 concentrations in culture supernatants of PBMCs were harvested and stored at −70 °C for cytokine analysis.

Activity of TF and PAI-1 in culture supernatants of PBMCs

The TF and PAI-1 assay were quantified by enzyme-linked immunosorbent assay (ELISA) (Shanghai JingMa Biotechnology, China). TF and PAI-1 concentrations were determined by measuring absorbance at 450 nm.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA of equivalence PBMCs was isolated by using TRIzol reagent (Gibco-BRL) according to the manufacturer’s instructions. Equal amounts of RNA were

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### Table 1. Characteristics of participants.

|                          | Control          | CAPD Patients   |
|--------------------------|------------------|-----------------|
| Men/women                | 10/3             | 10/3            |
| Age (years)              | 49.15 ± 12.71    | 49.92 ± 14.84   |
| Time on CAPD (months)    | N/A              | 22.23 ± 16.39   |
| Primary kidney disease   |                  |                 |
| Glomerulonephritis (n/%) | N/A              | 7 (53.8%)       |
| Diabetic nephropathy (n/%)| N/A              | 4 (30.7%)       |
| Hypertension (n/%)       | N/A              | 1 (7.69%)       |
| Obstructive nephropathy (n/%)| N/A              | 1 (7.69%)       |
| Systolic blood pressure (mm Hg) | 117.69 ± 7.53 | 136.15 ± 10.82 |
| Diastolic blood pressure (mm Hg) | 66.54 ± 4.27 | 82.15 ± 7.78   |
| Biochemical parameters   |                  |                 |
| Hemoglobin (g/dL)        | 139.85 ± 7.88    | 91.54 ± 14.17   |
| Urea nitrogen (mg/dL)    | 6.68 ± 1.09      | 19.18 ± 8.41    |
| Creatinine (mg/dL)       | 78.46 ± 10.64    | 1033.46 ± 353.48|
| Uric acid (mg/dL)        | 3.83 ± 48.42     | 394.31 ± 61.78  |
| Albumin (g/dL)           | 42.32 ± 2.50     | 31.76 ± 3.08    |
| Triglycerides (mg/dL)    | 1.11 ± 0.15      | 2.07 ± 1.39     |
| Cholesterol (mg/dL)      | 4.5 ± 0.52       | 5.71 ± 1.64     |
| Kt/V                     | N/A              | 1.93 ± 0.53     |
| Calcium (mg/dL)          | 2.36 ± 0.08      | 2.14 ± 0.37     |
| IP (mg/dL)               | 1.12 ± 0.07      | 1.51 ± 0.47     |
| Intact PTH (pg/ml)       | 61.11 ± 10.89    | 370.79 ± 266.70 |
| CRP (mg/dL)              | 4.80 ± 1.35      | 5.84 ± 6.37     |

TG: triglyceride; TC: total cholesterol; Alb: albumin; Cr: creatinine; SBP: systolic blood pressure; DBP: diastolic blood pressure.
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 anti-sense primers used for amplification were as follows: TF 5’-AAGCAGTGATTCCCTCTCG-3’ and 5’-AACACAGCATTGCAGCAG-3’; PAI-1 5’-ATTGCTGCCCCTTATGAAAA-3’ and 5’-G CCAAGGTCTTGGAGACAGA-3’, GAPDH (internal control) 5’-GGAGGC CAAAAGGGTCA TC-3’ and 5’- CCA GT GAGTTTCCCGTTC-3’.19,20 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 39 cycles. PCR cycle for PAI-1 (596 bp) 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.

**Statistical analysis**

Results were expressed as mean ± SD. 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**

**Characteristics of the subjects**

The clinical and biochemical characteristics of CAPD patients and healthy individuals are shown in **Table 1**. Table 2 summarizes the medications of 13 CAPD patients.

**Secretion of TF and PAI-1 into culture supernatants by PBMCs**

At baseline, spontaneous secretion of TF and PAI-1 into culture supernatants by PBMCs differed significantly between CAPD patients and healthy controls \( (p < 0.001) \). TF and PAI-1 secretion stimulated by LPS were significantly higher in patients with CAPD vs. spontaneous secretion of healthy individuals \( (p < 0.001) \) (**Table 3**).

The changes of TF and PAI-1 concentrations stimulated by LPS after treatment with fasudil are shown in **Table 3**. It showed that Fasudil treatment resulted in a significant effect in decreasing TF and PAI-1 secretion in PBMCs \( (p < 0.05) \).

**TF and PAI-1 mRNA concentrations in PBMCs**

Experiments were performed to compare the concentration of gene expression in PBMCs between control and CAPD. TF mRNA concentrations in PBMCs from LPS-treated group were significantly increased when compared with the control, LPS and fasudil-treated group \( (p < 0.05, \text{Figure 1}) \). PAI-1 mRNA concentrations in PBMCs from LPS-treated group were significantly increased when compared with the control, LPS and fasudil-treated group \( (p < 0.05, \text{Figure 1}) \).

**Discussion**

Disturbances in hemostasis are common findings in renal abnormalities patients. Both bleeding diathesis and hypercoagulable state are observed. The principal cause of these abnormalities is the uremic state.15 Our present study provides evidence that these abnormalities might be partly due to the increased TF and PAI-1 synthesis by PBMCs.

Patients on CAPD showed evidence of a higher degree of hypercoagulation.15 Significant protein losses through the peritoneum, including fibrinolytic
activators, may be counterbalanced by the possible increase in protein synthesis in CAPD. The intensity of hypercoagulability is thought to be related to the degree of hypoalbuminemia. Koyama et al. reported that plasma TF concentrations were increased in uremic patients on chronic dialysis. PAI-1 levels in HD and CAPD patients are similar to healthy controls, and this supports many earlier studies. In contrast, Goedde et al. reported high blood levels of PAI-1 during CAPD. Our study showed that TF and PAI-1 mRNA expression and activities in PBMCs were increased in CAPD patients when compared with the healthy subjects. LPS-mediated TF and PAI-1 mRNA expression and activities in PBMCs were further upregulated.

In the present study, we first demonstrated that fasudil inhibited LPS-mediated TF and PAI-1 secretion by PBMCs in CAPD patients. The Rho/ROCK-mediated pathway plays a role in infiltration of Z cells both in vitro and in vivo. It is reported that Rho-kinase signaling plays a central role in LPS-mediated leukocyte-endothelial cell interactions. Thus, Rho-kinase inhibition might be useful in the prevention or treatment of pathological inflammation and endotoxin-mediated hypercoagulation. Meanwhile, fasudil inhibited PAI-1 mRNA and protein expression in bleomycin-induced pulmonary fibrosis.

Fasudil reduced LPS-mediated TF and PAI-1 expression and activity in PBMCs. These effects may partially be relevant to the clinical benefits of fasudil in the treatment of CAPD patients.

Disclosure statement
The authors declare that they have no competing interests.

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