Rationale: Tissue factor pathway inhibitor (TFPI) is a potent regulator of the tissue factor pathway and is found in plasma in association with lipoproteins.

Objective: To determine the role of TFPI in the development of atherosclerosis, we bred mice which overexpress TFPI into the apolipoprotein E–deficient (apoE<sup>−/−</sup>) background.

Methods and Results: On a high-fat diet, smooth muscle 22α (SM22α)-TFPI/apoE<sup>−/−</sup> mice were shown to have less aortic plaque burden compared to apoE<sup>−/−</sup> mice. Unexpectedly, SM22α-TFPI/apoE<sup>−/−</sup> had lower plasma cholesterol levels compared to apoE<sup>−/−</sup> mice. Furthermore, SM22α-TFPI mice fed a high-fat diet had lower cholesterol levels than did wild-type mice. Because TFPI is associated with lipoproteins and its carboxyl terminus (TFPIct) has been shown to be a ligand for the very-low-density lipoprotein (VLDL) receptor, we hypothesized that TFPI overexpression may regulate lipoprotein distribution. We quantified VLDL binding and uptake in vitro in mouse aortic smooth muscle cells from SM22α-TFPI and wild-type mice. Mouse aortic smooth muscle cells from SM22α-TFPI mice demonstrated higher VLDL binding and internalization compared to those from wild-type mice. Because SM22α-TFPI mice have increased circulating levels of TFPI antigen, we examined whether TFPIct may act to alter lipoprotein distribution. In vitro, TFPIct increased VLDL binding, uptake, and degradation in murine embryonic fibroblasts. Furthermore, this effect was blocked by heparinase treatment. In vivo, systemic administration of TFPIct reduced plasma cholesterol levels in apoE<sup>−/−</sup> mice.

Conclusions: These studies suggest that overexpression of TFPI lowers plasma cholesterol through the interaction of its carboxyl terminus with lipoproteins and heparan sulfate proteoglycans. (Circ Res. 2009;105:713-720.)

Key Words: atherosclerosis  ■  coagulation  ■  murine models  ■  tissue factor  ■  lipoproteins

TFPI is cleared from the plasma by binding the low-density lipoprotein receptor–related protein and heparin sulfate proteoglycans.14,15 It has been previously shown that the carboxyl terminus of TFPI (TFPIct) can bind lipoproteins16–18 and is a ligand for cell surface receptors including the very-low-density lipoprotein (VLDL) receptor.19 Vascular overexpression of TFPI has been shown to reduce acute thrombosis and neointimal formation following vascular injury in murine models.20,21 Similarly, TFPI haploinsufficiency increases the atherosclerotic burden and thrombotic tendency in wild-type and apoE-deficient backgrounds.22,23 Taken together, these findings suggest a potentially important role for TFPI in the development and progression of acute and chronic vascular disease.

To further define the role of TFPI in vascular disease, we bred mice in which a murine TFPI transgene was expressed under the control of the SM22α promoter24,25 into the apoE<sup>−/−</sup> background. As expected, TFPI overexpression reduced plaque formation in this model. Unexpectedly, this
decrease was associated with a reduction in plasma cholesterol suggesting a novel role for TFPI in lipid metabolism.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Materials**

125I-Human VLDL, human VLDL, and lipoprotein-deficient bovine calf serum were purchased from Biomedical Technologies Inc (Stoughton, Mass). Culture medium Dulbecco’s modified PBS without Ca2+ or Mg2+, Hank’s balanced salt solution, and DMEM were purchased from Invitrogen (Carlsbad, Calif). Smooth muscle growth medium was from Lonza (Walkersville, Md). Soybean trypsin inhibitor, bovine serum albumin, heparin, heparinase I and III, collagenase type I, and elastase were purchased from Sigma-Aldrich Corp (St Louis, Mo). RAP (receptor related protein) was purchased from Innovative Research (Novi, Mich). Murine TFPI C-terminal 56mer peptide HRFNYTGCGGNNNNFTTRRRCLRS-CKITL1KNSKGVKRI-QRRKAPFVKVVYESIN (according to National Center for Biotechnology Information database accession no. NP-035706 from amino acids 251 to 306) was synthesized by Sigma-Aldrich Corp (St Louis, Mo). RAP (receptor related protein) was purchased from Innovative Research (Novi, Mich). Murine TFPI C-terminal 56mer peptide HRFNYTGCGGNNNNFTTRRRCLRS-CKITL1KNSKGVKRI-QRRKAPFVKVVYESIN (according to National Center for Biotechnology Information database accession no. NP-035706 from amino acids 251 to 306) was synthesized by Sigma-Aldrich Corp (St Louis, Mo). RAP (receptor related protein) was purchased from Innovative Research (Novi, Mich). Murine TFPI C-terminal 56mer peptide HRFNYTGCGGNNNNFTTRRRCLRS-CKITL1KNSKGVKRI-QRRKAPFVKVVYESIN (according to National Center for Biotechnology Information database accession no. NP-035706 from amino acids 251 to 306) was synthesized by Sigma-Aldrich Corp (St Louis, Mo). RAP (receptor related protein) was purchased from Innovative Research (Novi, Mich). Murine TFPI C-terminal 56mer peptide HRFNYTGCGGNNNNFTTRRRCLRS-CKITL1KNSKGVKRI-QRRKAPFVKVVYESIN (according to National Center for Biotechnology Information database accession no. NP-035706 from amino acids 251 to 306) was synthesized by Sigma-Aldrich Corp (St Louis, Mo). RAP (receptor related protein) was purchased from Innovative Research (Novi, Mich).

**Murine Models**

SM22α-TFPI transgenic mice with C57BL/6 background were generated as previously described.21 apoE-deficient mice on C57BL/6J background were reverse purchased from Jackson Laboratory (Bar Harbor, ME). SM22α-TFPI mice were cross bred into apoE-deficient mice to mice which were heterozygous (SM22α-TFPI+/apoE−/−) for each allele. The double homozygous (SM22α-TFPI+/apoE−/−) mice were produced by backcrossing the heterozygous mice, which were identified by polymerase chain reaction (PCR) (for lack of wild-type apoE allele) and real-time PCR analysis (for TFPI transgene) of blood DNA samples.21 Mice were maintained on standard chow unless noted. All procedures comply with the standards for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, Md) and were approved by Mayo Clinic Institutional Animal Care and Use Committee.

**Analysis of Atherosclerotic Lesions**

Mice used in these experiments were 6 to 8 weeks old and 15 to 18 g in weight. Groups of apoE−/− and SM22α -TFPI/apoE−/− mice were fed a high-fat Western diet (TD.88137, Harland, Madison, Wis). At the end of 5 weeks and 20 weeks of treatment, the mice were euthanized; aorta and blood samples were collected and analyzed. Additional methods are available in the Online Data Supplement.

**Plasma Lipid and Lipoprotein Analysis**

**Plasma Samples**

Blood samples from SM22α -TFPI/apoE−/− or apoE−/− mice (pooled groups of 6 to 8 mice/group) were obtained through retroorbital bleeding into EDTA-coated vials followed by centrifugation for 20 minutes at 3000 rpm at room temperature to obtain a final plasma volume of 1 mL. Plasma samples were kept frozen at −20°C until fast protein liquid chromatography (FPLC) was performed.

**Cholesterol Assay**

The WAKO Total Cholesterol E kit (Osaka, Japan) was used to determine the cholesterol concentration of each FPLC fraction. See the Online Data Supplement.

**FPLC Instrumentation**

FPLC was performed at 4°C using a Bio-Logic Pathfinder FPLC system interfaced with an automatic fraction collector (Bio-Rad, Hercules, Calif). See the Online Data Supplement.

**Cell Culture**

To generate murine aortic smooth muscle cells (mASMCs), aortas were removed from mice and cleaned of adventitia under a dissecting scope. Murine embryonic fibroblasts (MEFs) and PEA13 cells were purchased from American Type Culture Collection and cultured according to provided protocols. See the Online Data Supplement.

**Measurement of Murine TFPI in Plasma**

Murine TFPI levels in plasma were measured by using murine TFPI–specific rabbit antibody and Protein Detector ELISA Kit. See the Online Data Supplement.

**Small Interfering RNA Transfection**

MEFs were transfected with small interfering RNA targeted to the VLDL receptor. See the Online Data Supplement.

**Analysis of mRNA Expression of VLDL Receptor**

Cells were harvested directly for RNA extraction by using RNeasy Plus Mini Kit (Qiagen). RNA extractions were reverse transcribed using SuperScript III first-Strand synthesis system (Invitrogen). Two microliters of cDNA was used as template to amplify with primer pair (forward: 5'-TGACGCAGACTGTTCAGACC-3'; reverse: 5'-GCCGTGGGATACAGCTACCAT-3') by PCR. The products of PCR were analyzed using agarose gel electrophoresis. For normalization of RNA loading, β-actin housekeeping gene was used as control.

**VLDL–Peptide Complex Formation and Agarose Gel Electrophoresis**

VLDL–peptide complex formation was tested using an agarose gel electrophoresis method.26 Briefly, 10 μg of VLDL and 10 μg of VLDL plus various concentrations of TFPIct were incubated at room temperature for 1 hour. The samples of VLDL and VLDL-TFPIct mixtures were electrophoresed on a 0.7% agarose gel at a constant voltage of 100 V for 20 minutes. The gel was stained with Coomassie blue and photographed.
In Vivo Administration of TFPIct
Administration of TFPIct in mice was performed at concentrations previously described for apolipoprotein mimetics. ApoE<sup>−/−</sup> mice (8 to 12 weeks old) fed high-fat or normal chow were anesthetized, and blood samples were taken from the retroorbital sinus before the injection. In acute studies, 100 µL of peptide (1 µg/µL in saline) was injected via tail vein. Saline was injected into the control group of mice. Blood samples (100 µL) were taken from individual mice at 1 or 3 and 6 hours after injection, and the plasma was separated by centrifugation. Duplicate 10-µL aliquots of each plasma sample were used for cholesterol determination. For chronic administration, 6- to 8-month-old apoE<sup>−/−</sup> mice were used. 100 µL of peptide or saline was given intraperitoneally daily. Blood samples were taken at day 3 and 7 for cholesterol determination.

Statistical Analysis
All results are expressed as means±SEM. In all experiments, differences between control and treated groups were analyzed for statistical significance using a 1-way ANOVA, 2-way ANOVA, or an unpaired Student’s t-test (2-tailed). When applicable, a repeated measures analysis was performed. In the case of ANOVA, a post test comparison was used to compare all groups.

Results
To determine the effect of vascular overexpression of TFPI on the development of atherosclerosis, 6- to 8-week-old apoE<sup>−/−</sup> and SM22α-TFPI/apoE<sup>−/−</sup> mice (n>10 for each group) were fed with a Western diet. After 20 weeks, atherosclerotic lesions were quantified in the aorta (Figure 1A and 1B). Compared to apoE<sup>−/−</sup> mice, the extent of plaque surface area was significantly reduced in SM22α-TFPI/apoE<sup>−/−</sup> mice (Figure 1C). There were no differences in plaque area between sexes in this model (data not shown).

Plasma cholesterol in both groups was measured from mice fed a high-fat diet for 5 weeks and 20 weeks. Surprisingly, the total cholesterol level in SM22α-TFPI/apoE<sup>−/−</sup> mice was significantly lower at both time points than that in apoE<sup>−/−</sup> mice (Figure 2A; P<0.01). Triglyceride levels were significantly lower at 5 weeks in the SM22α-TFPI/apoE<sup>−/−</sup> mice compared with apoE<sup>−/−</sup> (165±31 mg/dL versus 269±28 mg/dL, P<0.014), but this difference was not significant at 20 weeks (185±40 versus 267±48 mg/dL, P=0.21) (Figure 2B). On normal chow, the cholesterol levels were also decreased after 20 weeks (P<0.01) (Figure 2A). As with plaque development, no difference was noted between sexes. FPLC analysis on pooled samples confirmed that the differences between the mice were predominantly in the VLDL and low-density lipoprotein (LDL) fractions (Figure 2C). Baseline plasma cholesterol and triglyceride levels did not differ between SM22α-TFPI and C57BL/6 mice (Figure 2D and 2E). However, when fed a high-fat diet for 20 weeks, plasma cholesterol levels in SM22α-TFPI mice were lower than C57BL/6 mice. These data suggest that vascular smooth muscle cell–directed overexpression of TFPI attenuates the hyperlipidemia induced by high-fat diet or apoE deletion.

The model system studied here allows for several potential mechanisms for the observed effects of TFPI overexpression on plasma cholesterol levels. These mechanisms might include differences in feeding habits or dietary absorption of lipid between mice. To that end, the SM22α-TFPI mice are developmentally normal and have normal body weight and lifespan, which would not be supportive of that mechanism.

An alternative mechanism would be that the transgene might be involved in affecting lipoprotein levels. To investigate the possibility that overexpression of TFPI alters local lipoprotein binding and internalization, we isolated mASMCs from SM22α-TFPI and wild-type mice to study the effect of TFPI overexpression on <sup>125</sup>I-VLDL binding and internalization in these cells. At concentrations of 2 µg and 10 µg/mL of <sup>125</sup>I-VLDL, the surface binding of <sup>125</sup>I-VLDL on mASMCs isolated from SM22α-TFPI mice was increased compared to that of cells isolated from wild-type mice (14±1 versus 11±0 for 2 µg/mL and 51±0 versus 41±1 for 10 µg/mL; Figure 3). Also, the internalization of <sup>125</sup>I-VLDL in mASMCs isolated from SM22α-TFPI mice was increased by approximately 50% compared with wild-type cells (93±11 versus 38±0 for 2 µg/mL and 272±9 versus 181±8 for 10 µg/mL). These data demonstrate that TFPI overexpression in mouse aortic smooth muscle cells (mASMCs) can regulate VLDL binding and internalization. However, as shown by Wamhoff et al.,<sup>30</sup> the SM22α promoter is not expressed in advanced atherosclerotic lesions in apoE-deficient mice; therefore, the effect on lipoprotein handling may be attributable to overexpression of TFPI outside of the plaque itself.

We have previously demonstrated that SM22α-TFPI mice have higher levels of vascular TFPI activity but similar levels...
of plasma TFPI activity.\textsuperscript{21} However, using a murine specific ELISA, it was demonstrated that SM22\textsubscript{a}-TFPI mice had increased levels of plasma TFPI antigen compared to wild-type mice (Figure 4). In addition, TFPI antigen was found in VLDL and LDL fractions (data not shown). The increased levels of TFPI in apoE-deficient mice parallel findings in humans with atherosclerosis and hyperlipidemia.\textsuperscript{12} Again, similar to humans, we were unable to detect TFPI protein in the livers of transgenic or wild-type mice (data not shown). Thus, the SM22\textsubscript{a}-directed overexpression of TFPI resulted in increased circulating TFPI antigen. Therefore, an endocrine/paracrine mechanism for the lipoprotein effect was considered.

Because TFPI circulates in lipoprotein fractions and the TFPIct is a known ligand of several cell surface receptors, we hypothesized that the TFPI, which is overexpressed in our model, may associate with VLDL and act to alter lipoprotein binding, uptake, and degradation. First, to determine whether TFPIct is capable of altering VLDL binding, uptake, and degradation, an in vitro model system was used. At a constant VLDL concentration, addition of TFPIct enhanced binding and uptake of VLDL in a concentration-dependent manner (Figure 5A and 5B) in MEFs. When 1 \(\mu\)g of TFPIct was added to VLDL, it resulted in a 15-fold increase in VLDL binding and a 20-fold increase in VLDL internalization. At the highest ratio we tested, 10 \(\mu\)g of TFPIct mixed with 10 \(\mu\)g of VLDL, the degradation rate increased by more than 100\% (Figure 5C). The rate of VLDL degradation accelerated as the TFPIct:V-LDL ratio increased.

To define the mechanism by which TFPIct alters VLDL internalization, known TFPI receptors were studied. Knockdown of VLDL receptor expression by small interfering RNA partially attenuated TFPIct mediated binding and uptake of VLDL in these cells (Figure 6). Furthermore, although RAP attenuated VLDL binding and uptake in the absence of TFPIct (Online Figure I, A), the same dose of RAP did not affect the increase observed with TFPIct (Online Figure I, B). Similarly the increase was only partially attenuated in cells deficient in lipoprotein receptor–related protein (PEA13) (Online Figure II). These results indicate that known receptors for VLDL are only partially responsible for TFPIct-mediated enhancement of VLDL binding and uptake in MEF.
Because TFPI is known to bind heparin sulfate proteoglycans (HSPGs), similar studies were performed following treatment of MEFs with heparinase I and III. Heparinase treatment did not affect baseline VLDL binding and uptake in MEF but significantly reduced VLDL binding and uptake when TFPIct was added (Figure 7). These results demonstrate that the HSPG pathway is the major route for the TFPI-mediated VLDL binding and subsequent uptake. Taken together, these data suggest that TFPIct enhances the binding and internalization of VLDL via the HSPG pathway in coordination with known receptors for VLDL.

TFPIct has a net positive charge, a feature shared with peptides that have been shown to be apolipoprotein E mimetics.27 To determine whether TFPIct may associate and change the electrophoretic mobility of VLDL, VLDL was incubated with TFPIct at different weight ratios at room temperature for 1 hour and then analyzed by agarose gel electrophoresis (Online Figure III). VLDL alone migrated toward the anode as a single band. The migration of mixtures of VLDL and TFPIct was impeded, and the degree depended on the ratio of VLDL to TFPIct. Mobility was impeded at molar ratios as low as 1:15. These results indicated that TFPIct associates with VLDL and alters its electrophoretic properties.

Finally, to determine whether the TFPIct is sufficient to reduce the cholesterol level in vivo, TFPIct was administrated intravenously into apoE-deficient mice fed high-fat or normal diet using concentrations of peptide previously used in studies of apolipoprotein mimetics.29 In apoE-deficient mice after intravenous delivery of TFPIct, the cholesterol level was reduced significant after 6 hours compared with baseline in both fed Western diet and normal chow mice (Figure 8A and 8B). Reductions were also observed more than 7 days time with daily IP injections in these older apoE-deficient mice fed normal chow (Figure 8C). Thus, systemic delivery of TFPIct is sufficient to reduce plasma cholesterol levels.

Discussion
This is the first report that suggests that overexpression of TFPI may regulate atherosclerotic plaque formation. The potential known mechanisms for such effects might include the antithrombotic effects of TFPI in this model21 and its ability to block signaling via TF.31 Unexpectedly, we identified that overexpression of TFPI reduced plasma cholesterol. It was this unexpected finding which became the focus of this study. TFPI was originally referred to as lipoprotein-associated coagulation inhibitor because of its association with lipoproteins.32 Because TFPI is cleared from the blood through receptor-dependent and -independent mechanisms, a potential role in regulating lipoprotein clearance may not have been unexpected. Given the important roles of the TF pathway and lipoprotein metabolism in vascular disease, a biologically important association between the 2 remains intriguing. We have previously shown that the atherogenic lipoprotein Lp(a) can bind and inhibit TFPI activity through the binding of apo(a) to the TFPIct.16

Overexpression of TFPI resulted in reductions in elevated plasma cholesterol levels in mice from apoE-deficient and

Figure 5. Effect of TFPIct peptides on binding, internalization, and degradation of $^{125I}$-VLDL in MEFs. Cells were treated with $^{125I}$-VLDL complex containing different concentrations of TFPIct. TFPIct increased $^{125I}$-VLDL binding to MEF cell surface (A), internalization of $^{125I}$-VLDL into the cells (B), and degradation of $^{125I}$-VLDL (C) in presence of TFPIct in MEFs. $^{*}P<0.01$.

Figure 6. Knockdown of VLDL receptor (VLDLr) inhibits $^{125I}$-VLDL binding to MEF cell surface (A) and internalization (B) of $^{125I}$-VLDL into the cells when exposed to TFPIct. $^{*}P<0.01$ vs untreated cells. C, Results of RT-PCR demonstrating specificity of VLDL receptor knockdown.
wild-type backgrounds. Because TFPI has been shown to be a ligand of multiple cell surface receptors which also mediate lipoprotein clearance, we further demonstrated that the carboxyl terminus of murine TFPI can associate with and alter the distribution and degradation of VLDL in vitro and lower plasma cholesterol acutely in vivo following systemic injection. Taken together, we suggest that overexpression of TFPI regulates atherosclerotic lesion formation and does so in part through lowering cholesterol through the association of TFPI with atherogenic lipoproteins. These findings provide insights into a novel noncoagulant function for TFPI.

The present data suggest that smooth muscle cell–directed overexpression of TFPI can alter cholesterol levels through autocrine or endocrine effects. As a secreted peptide, in vitro and in vivo models of overexpression of TFPIct cannot distinguish the two. Analysis of mASMCs which express TFPIct from the SM22α promoter demonstrated increased binding, uptake and degradation of VLDL. Our subsequent focus on the TFPIct was based on its known functionality. TFPIct was shown to associate with VLDL and change its electrophoretic properties, and administration of TFPIct increased cell binding, uptake, and degradation of VLDL in vitro. This effect was mediated through enhanced binding of VLDL to HSPGs in a coordinated fashion with members of the LDL receptor family. In addition, acute or daily systemic delivery of TFPIct is sufficient to lower plasma cholesterol levels.

Taken together, these findings define potential mechanisms for the observed effects of TFPI overexpression on lowering cholesterol in the setting of hyperlipidemia attributable to apoE deletion or hyperlipidemic feeding. TFPI, either on the cell surface or following secretion, binds lipoproteins and increases binding and clearance of these lipoproteins through receptor-dependent and HSPG-dependent mechanisms. This HSPG-dependent mechanism has been demonstrated to be important in MEFs33–35 and has been shown to be the mechanism by which apoE mimetics act to lower cholesterol.27

Previous studies suggest that TFPI plays an important role in pathogenesis of vascular thrombosis and atherosclerosis.9,10,21–23,40 Westrick et al have shown that systemic reduc-

![Figure 7. Effect of heparinases on the binding and uptake of 125I-VLDL or 125I-VLDL:TFPIct complex. MEF cells were pre-treated with heparinase I and III (each 3 U/mL) for 2 hours at 37°C and then incubated with 125I-VLDL (10 μg) or 125I-VLDL (10 μg):TFPIct (10 μg) complex. Binding and uptake were attenuated by heparinases only in the presence of TFPIct. *P<0.02.](http://circres.ahajournals.org/)

![Figure 8. Reduction of plasma cholesterol level in apoE-deficient mice after acute or chronic administration with TFPIct peptide. A, Cholesterol levels in high-fat-fed apoE-deficient mice after acute peptide injection are shown. *P<0.05 vs baseline. B, Cholesterol level in normal chow fed apoE-deficient mice after acute peptide injection. *P<0.01 vs baseline. C, Cholesterol levels in normal chow fed apoE−/− mice after daily peptide injections (IP). *P<0.01 vs baseline.](http://circres.ahajournals.org/)
carboxyl terminus, have not been described. In relatively small human studies, there is a positive correlation between circulating TFPI and VLDL levels.\textsuperscript{12,41–43} In this study, apoE–/– mice had higher levels of circulating TFPI antigen than did wild-type mice, suggesting a regulatory role of hyperlipidemia on TFPI expression. These data suggest a potentially important and complex interaction between TFPI and lipoproteins.

Human TFPIct has been shown to be important for its coagulant and noncoagulant properties. It is required for optimal inhibition of FXa.\textsuperscript{44,45} It is also required for TFPI binding to cells and cell surface receptors including the VLDL receptor\textsuperscript{19} and heparin sulfate proteoglycans.\textsuperscript{46} The binding to cells and cell surface receptors including the TFPIct is reminiscent of the multifunctional peptides in apoE.\textsuperscript{16–18} The broad functionality of the TFPIct dimension in which to consider the antiatherosclerotic effects of TFPI based on the unique multifunctional properties of its carboxyl terminus.\textsuperscript{16–18} The broad functionality of the TFPIct is reminiscent of the multifunctional peptides intended to serve as apolipoprotein mimetics.\textsuperscript{27,47} These mimetics contain moieties that bind lipoproteins and residues that mimic apoE bind the VLDL receptor. Systemic infusion of these peptides lowers cholesterol acutely in a similar manner to the TFPIct. In addition to the functional similarities, both are positively charged, which may play a role in their functionality. In fact, the mechanism for the effect of these peptides has been determined to be through dual pathways: HSPG-mediated and receptor-mediated (similar to our findings).

Although we cannot exclude the known anticoagulant functions of TFPI in the attenuation of atherosclerosis in this model, our data support a novel noncoagulant function of TFPI based on the unique multifunctional properties of its carboxyl terminus. This functionality adds another potent dimension in which to consider the antiatherosclerotic effects of TFPI. As such, the opportunities to use this functionality to understand its role in pathophysiology and treatment of human disease remain.

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Disclosures
None.

References


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Vascular-Directed Tissue Factor Pathway Inhibitor Overexpression Regulates Plasma Cholesterol and Reduces Atherosclerotic Plaque Development
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Supplemental Methods

Analysis of atherosclerotic lesions
To quantify the surface atherosclerotic area, mice were euthanized, aortas were dissected, and adventitial tissues were carefully removed. The tissue was immersed in 4% formalin and stained with Sudan IV for detection of atherosclerotic plaques. Computer-aided morphometric analysis (Image Pro Plus) was performed on the Sudan IV-positive areas.\(^1\) The percentage of atherosclerotic surface compared with total aortic surface was calculated.

Cholesterol assay
Briefly 10\(\mu\)l of each FPLC fraction or mouse plasma sample was mixed with 1ml of reagent from kit, incubated for 10 minutes at 37\(^\circ\)C, and spectrophotometric absorbance was measured at 600nm. The cholesterol concentration of each sample was calculated according to the manufacturer’s protocol. This kit was also used for cholesterol measurements of individual mouse blood samples in experiments including those examining the effects of \textit{in vivo} administration of TFPIct.

FPLC instrumentation
Fast Protein Liquid Chromatography was performed at 4\(^\circ\)C using a Bio-Logic Pathfinder FPLC system interfaced with an automatic fraction collector (Bio-Rad, Hercules, CA).\(^2\) Crude mouse plasma was first filtered using a 0.22\(\mu\)m Ultrafree-MC centrifuge filter (Millipore, Boston, MA) and then concentrated using 6ml 5 MWCO concentrator (Vivascience, Hannover, Germany). Once a loading volume of 250\(\mu\)l was obtained, the crude plasma was directly injected onto Pharmacia Superose columns in series, Superose 6 and Superose 12, with a total bed volume of 30ml. The flow rate was 0.2ml/min at 260psi for a total run time of 7 hours. The flow rate was selected through several attempts to obtain a clear profile and lipoprotein fractions separation. A BioLogic QuadTec UV-Vis detector (Bio-Rad) monitored absorbance of the eluted samples at a wavelength of 280nm, and 1ml fractions were collected.

Cell culture
To generate mouse aortic smooth muscle cells (mASMC), aortas were removed from mice and cleaned of adventitia under a dissecting scope. Aortas from 3 mice were pooled and minced in ice cold Dubelco’s modified PBS without Ca2+ or Mg2+ (D-PBS) and subsequently transferred to a sterile 10ml glass tube. Pieces of aorta were allowed to settle, and the D-PBS was carefully removed and replaced with 5ml of Hank’s Balanced Salt Solution buffered with 20mM HEPES (HBSS, pH 7.4) containing 0.25mg/ml soybean trypsin inhibitor, 2mg/ml bovine serum albumin, and 2mg/ml collagenase type I. The tube was placed on a rocking platform at 37\(^\circ\)C for 45 minutes. The tissue was triturated vigorously to disperse endothelial as well as other loosely associated cells. The tissue pieces were allowed to settle, and the supernatant containing dissociated cells was discarded and replaced with 5ml of HBSS containing 0.25mg/ml soybean trypsin inhibitor, 2mg/ml bovine serum albumin, 0.625U/ml elastase, and 10mg/ml collagenase type I. The tube was again placed on a rocking platform at 37\(^\circ\)C for 40 minutes. The tissue was triturated vigorously to disperse smooth muscle cells. Undigested pieces were allowed to settle, and the cell suspension was filtered through a 100\(\mu\)m cell strainer into a 50ml conical tube. The strainer was rinsed with 10ml smooth muscle growth medium (SmGM). The cells were pelleted by centrifugation at 300xg for 5 minutes and resuspended in 10ml SmGM, and this centrifugation and resuspension was repeated an additional 2 times. The final cell pellet was resuspended in SmGM and then seeded to a 75cm\(^2\) flask. The cells were then maintained in an incubator at 37\(^\circ\)C with 5% CO\(_2\).
Mouse embryonic fibroblasts (MEF) and LRP deficient cells (PEA13) (ATCC Manassas, VA) were cultured in DMEM supplemented with 4.5g/L glucose, 1.5g/L sodium bicarbonate, and 15% Fetal Bovine Serum at 37ºC with 5% CO₂.

**siRNA transfection**
MEFs were seeded in 6-well plates at 3 x 10⁴ cells/cm² with growth medium and incubated at 37ºC overnight. At 70% confluence, cells were rinsed with PBS and 2ml of Opti-MEM/well were added. siRNA of mouse VLDL receptor was purchased from Integrated DNA Technologies (IDT, Coralville, IA). 1.25ul of siRNA NM_013703 duplex 2 (20uM) or scrambled oligomers (20uM) were incubated at room temperature in Opti-MEM (250µl) for 5 minutes, and 5µl of the transfection agent, Lipofectamine 2000 Reagent (Invitrogen, CA) was then added and incubated with 250µl Opti-MEM in a separate tube for 5 minutes. The two mixtures were combined and mixed gently with agitation and incubated at room temperature for 30 minutes. The oligomer-Lipofectamine complexes (500ul) were added to each well and incubated at 37ºC for >6 hours. MEFs were washed with sterile PBS, and 2ml/well growth medium was added. Cultures were grown for 24 hours and then used for analysis of mRNA expression of VLDLr or for the VLDL binding and uptake experiments.

**125I-VLDL binding, uptake, and degradation by mASMC, MEF or PEA13 cells**
Studies of binding and uptake of ¹²⁵I-VLDL in mASMC, mouse embryonic fibroblasts (MEF) and PEA13 cells were performed using modified method of Datta et al.⁴ For VSMC, cells were grown in SmGm in 6-well plates until reaching ~85% confluence. The culture media were replaced with SmGM containing 10% lipoprotein depleted serum 24 hours prior to beginning the experiments in order to upregulate lipoprotein receptors. In binding experiments, cells were incubated with ¹²⁵I-VLDL at 4ºC for 2 hours in presence or absence of TFPIcct. Nonspecific binding was assessed in the presence of a 50-fold excess of unlabeled VLDL with or without peptides. The cells were washed to remove unbound ¹²⁵I-VLDL, then incubated with heparin (10mg/ml) for 1 hour on a rotary shaker to release specifically bound ¹²⁵I-VLDL. The heparin solution was collected and subjected to scintillation counting to determine the amount of ¹²⁵I-VLDL released from cells surface. For measurement of the uptake of ¹²⁵I-VLDL, cells were incubated at 37ºC with various concentrations of ¹²⁵I-VLDL for 3 hours, then washed with ice cold PBS, treated with heparin to remove bound forms, washed with PBS again and dissolved by incubation at room temperature in 1ml of 0.1N NaOH. An aliquot of the cell lysate was counted to determine the internalized ¹²⁵I-VLDL.

To measure degradation of VLDL by MEF or PEA13 cells, same protocol described above was followed except the incubation time was extended to 6 hours at 37ºC. The conditioned medium was collected, and 0.5ml of 50% TCA was added to precipitate ¹²⁵I-VLDL from the medium. After centrifugation, the precipitate was removed, and 10ul of 40% potassium iodide and 40ul of 30% hydrogen peroxide were added into supernatant. The free ¹²⁵I was extracted with 2ml chloroform, and then the upper aqueous layer was counted using scintillation counter. The upper aqueous layer represents the amount of ¹²⁵I mono-iodotyrosine produced by the degradation of ¹²⁵I VLDL.

**Measurement of murine TFPI in plasma**
To determine the level of TFPI in mouse plasma, the Protein Detector ELISA Kit (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was used with the anti-mouse TFPI polyclonal rabbit antibody³ as the detection antibody. Supernatant from 293 cells transfected with plasmid expressing mTFPI was used as a positive control to optimize
the ELISA conditions. Blood samples were collected into 3.8% trisodium citrate solution at 9:1 ratio. The samples were centrifuged at 6000rpm for 15 minutes to separate the plasma. The plasma was diluted with coating buffer at 1:600, and cell culture supernatant and blocking buffer served as positive and negative controls respectively. Purified rabbit anti-mTFPI polyclonal antibody (50ug/ml) was diluted at 1:50 and used as primary antibody. The remainder of the procedure was performed according to the manufacturer’s instructions. Results are presented semi-quantitatively as OD@450nm.

References:


Supplemental Figure legends:

Supplemental Figure 1: Comparison of the binding and uptake of $^{125}$I-VLDL by MEF cells with or without RAP treatment. Before addition of VLDL or VLDL-TFPIct complex, RAP was added into culture medium at concentration of 1uM and incubated at 37°C for 30 minutes. (A) In the absence of TFPIct, RAP (1uM) inhibits binding and uptake of VLDL. (B) RAP (1uM) fails to significantly inhibit binding and uptake of VLDL induced by TFPIct.

Supplemental Figure 2: Effect of LRP deletion on the effects of TFPIct on the binding and uptake of $^{125}$I-VLDL. In PEA13 cells (LRP deficient) binding and uptake of VLDL was partially attenuated compared with MEF cells. * p<0.05 PEA13 vs MEF cells

Supplemental Figure 3. Coomassie Blue stained electrophoresis of TFPI C terminal peptide and VLDL on agarose gel (0.7%). The migration of VLDL was retarded by addition of TFPIct peptides. Lane 1, VLDL (10ug); lane 2, VLDL and TFPIct (0.1ug) (about 1:15 molar ratio); lane 3, VLDL and TFPIct (0.25ug) (about 1:40 molar ratio); lane 4, VLDL and TFPIct (0.5ug) (about 1:80 molar ratio); lane 5, VLDL: TFPIct (1ug) (about 1:160 molar ratio); lane 6, TFPIct (1ug).
Supplemental Figures:

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