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## Vascular-directed TFPI overexpression regulates plasma cholesterol and reduces atherosclerotic plaque development

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### Abstract

**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<sup>-/-</sup> (apoE) background.

**Methods and Results**—On a high fat diet, SM22 $\alpha$ -TFPI/apoE<sup>-/-</sup> mice were shown to have less aortic plaque burden compared to apoE<sup>-/-</sup> mice. Unexpectedly, SM22 $\alpha$ -TFPI/apoE<sup>-/-</sup> had lower plasma cholesterol levels compared to apoE<sup>-/-</sup> mice. Furthermore, SM22 $\alpha$ -TFPI mice fed a high fat diet had lower cholesterol levels than did wild type mice. Since TFPI is associated with lipoproteins and its carboxy terminus (TFPIct) has been shown to be a ligand for the 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 (mASMCs) from SM22 $\alpha$ -TFPI and wild type mice. mASMCs from SM22 $\alpha$ -TFPI mice demonstrated higher VLDL binding and internalization compared to those from wild type mice. Since SM22 $\alpha$ -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 carboxy terminus with lipoproteins and heparan sulfate proteoglycans.

### Keywords

atherosclerosis; coagulation; murine models; tissue factor; lipoproteins

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**Disclosures:** None

## INTRODUCTION

Tissue factor pathway inhibitor (TFPI), a Kunitz-type serine protease inhibitor, is an endogenous inhibitor of TF-mediated coagulation. TFPI suppresses factor Xa generation by binding via its Kunitz 1 domain to the TF:FVIIa catalytic complex and via its Kunitz 2 domain to factor Xa.<sup>1</sup> The formation of a quaternary TF:VIIa:TFPI:Xa complex dampens ongoing FXa generation. Additionally, TFPI also has been shown to regulate coagulation by inducing the internalization and degradation of TF:VIIa complex on cell surfaces.<sup>2-4</sup>

TFPI is expressed in many cells relevant to atherosclerosis including platelets, endothelial cells, vascular smooth muscle cells, and monocyte/macrophages.<sup>5-9</sup> In human carotid plaques, the level of TFPI expression is inversely associated with TF activity suggesting a local regulatory role.<sup>10</sup> In plasma, TFPI exists in small quantities (<5%) as a free full length protein but is predominantly associated with lipoproteins.<sup>11-13</sup> Lipoprotein-associated TFPI has been shown to have less anticoagulant activity than free TFPI.<sup>13</sup> TFPI is cleared from the plasma by binding the low density lipoprotein receptor related protein (LRP) and heparin sulfate proteoglycans.<sup>14, 15</sup> It has been previously shown that the carboxy terminus of TFPI (TFPIct) can bind lipoproteins<sup>16-18</sup> and is a ligand for cell surface receptors including the VLDL receptor.<sup>19</sup>

Vascular overexpression of TFPI has been shown to reduce acute thrombosis and neointimal formation following vascular injury in murine models.<sup>20, 21</sup> Similarly, TFPI haploinsufficiency increases the atherosclerotic burden and thrombotic tendency in wild type and apoE deficient backgrounds.<sup>22, 23</sup> 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 SM22 $\alpha$  promoter<sup>24, 25</sup> 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.

## MATERIAL AND METHODS

Please see Supplemental Methods for additional methods.

### Material

<sup>125</sup>I-hVLDL (human very low density lipoprotein), hVLDL, and LPDS (lipoprotein deficient bovine calf serum) were purchased from Biomedical Technologies Inc. (Stoughton, MA). Culture medium Dulbecco's modified PBS without Ca<sup>2+</sup> or Mg<sup>2+</sup> (D-PBS), Hank's Balanced Salt solution (HBSS), and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Invitrogen (Carlsbad, CA). Smooth muscle growth medium (SmGM) 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, Michigan). Murine TFPI C-terminal 56mer peptide HRFNYTGCGGNNNFTTRRRCLRSCKTGLIKNKSKGVVVKI-QRRKAPFVKVYESIN (according to NCBI database accession number NP-035706 from amino acid 251 to 306) was synthesized by Mayo Clinic protein core facility. The peptides were purified on a C-4 reversed-phase HPLC column, and the purity was determined by HPLC and confirmed by mass spectral analysis.

## Murine models

SM22 $\alpha$ -TFPI transgenic<sup>+/+</sup> mice with C57BL/6 background were generated as previously described.<sup>21</sup> apoE deficient mice on C57BL/6J background were purchased from Jackson Laboratory (Bar Harbor, MI). SM22 $\alpha$ -TFPI mice were cross bred into apoE-deficient mice to mice which were heterozygous (SM22 $\alpha$ -TFPI<sup>±</sup>/apoE<sup>±</sup>) for each allele. The double homozygous (SM22 $\alpha$ -TFPI<sup>+/+</sup>/apoE<sup>-/-</sup>) 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.<sup>21</sup> 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 6–8 weeks old and 15–18g in weight. Groups of apoE<sup>-/-</sup> and SM22 $\alpha$ -TFPI/apoE<sup>-/-</sup> mice were fed a high fat Western diet (TD.88137, Harland, Madison, WI). At the end of 5 weeks and 20 weeks of treatment, the mice were sacrificed; aorta and blood samples were collected and analyzed. Additional methods are available in Supplemental Methods.

## Plasma lipid and lipoprotein analysis

**Plasma samples**—Blood samples from SM22 $\alpha$ -TFPI/apoE<sup>-/-</sup> or apoE<sup>-/-</sup> mice (pooled groups of 6–8 mice/group) were obtained through retro-orbital bleeding into EDTA-coated vials followed by centrifugation for 20 minutes at 3000rpm at room temperature to obtain a final plasma volume of 1mL. Plasma samples were kept frozen at –20°C until 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 Supplemental Methods

## FPLC instrumentation

Fast Protein Liquid Chromatography (FPLC) was performed at 4°C using a Bio-Logic Pathfinder FPLC system interfaced with an automatic fraction collector (Bio-Rad, Hercules, CA).<sup>26</sup> See Supplemental Methods.

## Cell Culture

To generate murine aortic smooth muscle cells (mASMC), aortas were removed from mice and cleaned of adventitia under a dissecting scope. murine embryonic fibroblasts (MEF), and PEA13 cells were purchased from ATCC and cultured according to provided protocols. See Supplemental Methods.

## <sup>125</sup>I-VLDL binding, uptake, and degradation by mASMC, MEF and PEA13 cells

Studies of binding and uptake of <sup>125</sup>I-VLDL in mASMC, MEF and PEA13 cells were performed using modified method of Datta et al.<sup>27</sup> See Supplemental Methods.

## Measurement of mTFPI in plasma

mTFPI levels in plasma were measured by using mTFPI specific rabbit antibody and Protein Detector ELISA Kit. See Supplemental Methods.

### siRNA transfection

MEFs were transfected with siRNA targeted to the VLDL receptor. See Supplemental Methods.

### Analysis of mRNA expression of VLDL receptor

Cells were harvested directly for RNA extraction by using RNeasy Plus mini kit (Qiagen, MD). RNA extractions were reverse transcribed using SuperScript III first-Strand synthesis system (Invitrogen, CA). Two microliters of cDNA was used as template to amplify with primer pair (forward 5'-TGACGCAGACTGTTTCAGACC-3', reverse 5'-GCCGTGGATACAGCTACCAT-3') by polymerase chain reaction (PCR). The products of PCR were analyzed using agarose gel electrophoresis. For normalization of RNA loading,  $\beta$ -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.<sup>28</sup> Briefly 10 $\mu$ g VLDL and 10 $\mu$ g VLDL plus varying 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 100V 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.<sup>29</sup> ApoE<sup>-/-</sup> mice (8–12 weeks old) fed high fat or normal chow were anaesthetized, and blood samples were taken from the retro-orbital sinus prior to the injection. In acute studies, 100 $\mu$ l of peptide (1 $\mu$ g/ $\mu$ l in saline) was injected via tail vein. Saline was injected into the control group of mice. Blood samples (100 $\mu$ l) were taken from individual mice at 1 or 3 and 6 hours after injection, and the plasma was separated by centrifugation. Duplicate 10 $\mu$ l aliquots of each plasma sample were used for cholesterol determination. For chronic administration, 6–8 month old apoE<sup>-/-</sup> mice were used, 100 $\mu$ l 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  $\pm$  SEM. In all experiments, differences between control and treated groups were analyzed for statistical significance using a one-way ANOVA, two-way ANOVA or an unpaired Student's t test (two-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, 6–8 week-old apoE<sup>-/-</sup> and SM22 $\alpha$ -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 B). Compared to apoE<sup>-/-</sup> mice, the extent of plaque surface area was significantly reduced in SM22 $\alpha$ -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 with a high fat diet for 5 weeks and 20 weeks. Surprisingly, the total cholesterol level in SM22 $\alpha$ -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 SM22 $\alpha$ -TFPI/apoE<sup>-/-</sup> mice

compared with apoE<sup>-/-</sup> (165 ± 31mg/dl vs 269 ± 28 mg/dl, p<0.014), but this difference was not significant at 20 weeks (185 ± 40 vs 267 ± 48mg/dl, p=0.21) (Figure 2B). On normal chow, the cholesterol levels were also decreased after 20 weeks (p<.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 LDL fractions (Figure 2C). Baseline plasma cholesterol and triglyceride levels did not differ between SM22 $\alpha$ -TFPI and C57BL/6 mice (Figure 2D and E). However when fed a high fat diet for 20 weeks, plasma cholesterol levels in SM22 $\alpha$ -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 SM22 $\alpha$ -TFPI mice are developmentally normal and have normal body weight and life span 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 mASMC from SM22 $\alpha$ -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 2ug and 10ug/ml of <sup>125</sup>I-VLDL, the surface binding of <sup>125</sup>I-VLDL on mASMC isolated from SM22 $\alpha$ -TFPI mice was increased compared to that of cells isolated from wild type mice (14 ± 1 vs 11 ± 0 for 2ug/ml and 51 ± 0 vs 41 ± 1 for 10ug/ml, Figure 3). Also, the internalization of <sup>125</sup>I-VLDL in mASMC isolated from SM22 $\alpha$ -TFPI mice was increased by approximately 50% compared with wild type cells (93 ± 11 vs 58 ± 0 for 2ug/ml and 272 ± 9 vs 181 ± 8 for 10ug/ml). These data demonstrate that TFPI overexpression in mASMCs can regulate VLDL binding and internalization. However, as shown by Wamhoff,<sup>30</sup> the SM22 $\alpha$  promoter is not expressed in advanced atherosclerotic lesions in apoE deficient mice; therefore, the effect on lipoprotein handling may be due to overexpression of TFPI outside of the plaque itself.

We have previously demonstrated that SM22 $\alpha$ -TFPI mice have higher levels of vascular TFPI activity but similar levels of plasma TFPI activity.<sup>21</sup> However, using a murine specific ELISA, it was demonstrated that SM22 $\alpha$ -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.<sup>12</sup> 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 $\alpha$  directed overexpression of TFPI resulted in increased circulating TFPI antigen. Therefore, an endocrine/paracrine mechanism for the lipoprotein effect was considered.

Since 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 B) in MEFs. When 1ug of TFPIct was added to VLDL, it resulted in a 15-fold increase in VLDL binding and 20-fold increase in VLDL internalization. At the highest 16:10 (w/w) ratio of TFPIct:VLDL, a 55-fold increase of VLDL binding and a 40-fold increase in internalization was observed. The effect of TFPIct on degradation of VLDL was also measured after incubation of the cells with or without TFPIct for 6 hours at 37°C. At the highest ratio we

tested, 10ug TFPIct mixed with 10ug VLDL, the degradation rate increased by over 100% (Figure 5C). The rate of VLDL degradation accelerated as the TFPIct:VLDL ratio increased.

To define the mechanism by which TFPIct alters VLDL internalization, known TFPI receptors were studied. Knockdown of VLDLr expression by siRNA 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 (Supplemental Figure1A). The same dose of RAP did not affect the increase observed with TFPIct (Supplemental Figure1B). Similarly the increase was only partially attenuated in cells deficient in LRP (PEA13) (Supplemental Figure 2). These results indicate that known receptors for VLDL are only partially responsible for TFPIct-mediated enhancement of VLDL binding and uptake in MEF.

Since 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, 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.<sup>27</sup> 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, then analyzed by agarose gel electrophoresis (Supplemental Figure 3). VLDL alone migrated toward the anode as a single band. The migration of mixtures of VLDL and TFPIct was impeded, and the degree depended upon 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 utilizing concentrations of peptide previously utilized in studies of apolipoprotein mimetics.<sup>29</sup> 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 B). Reductions were also observed over seven 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 model<sup>31</sup> and its ability to block signaling via TF32. 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 (LACI) due to its association with lipoproteins.<sup>33</sup> As 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 tissue factor pathway and lipoprotein metabolism in vascular disease, a biologically important association between the two 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.<sup>16</sup>

Overexpression of TFPI resulted in reductions in elevated plasma cholesterol levels in mice from apoE deficient and wild type backgrounds. As TFPI has been shown to be a ligand of multiple cell surface receptors which also mediate lipoprotein clearance, we further demonstrated that the carboxy 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 non-coagulant function for TFPI.

The current 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 TFPI cannot distinguish the two. Analysis of mASMCs which express TFPI from the SM22 $\alpha$  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 due 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 MEFs<sup>34–36</sup> and has been shown to be the mechanism by which apoE mimetics act to lower cholesterol<sup>27</sup>.

Previous studies suggest that TFPI plays an important role in pathogenesis of vascular thrombosis and atherosclerosis.<sup>10, 37–41</sup> Westrick and colleagues have shown that systemic reduction of TFPI Kunitz 1 expression in mice increased atherosclerotic lesion formation and shortened the occlusion time following ferric chloride exposure in the carotid arteries of TFPI<sup>+/-</sup>/apoE<sup>-/-</sup> mice.<sup>22</sup> In that study, plasma lipid levels were not reported. It should be noted that the deletion in those mice is specific for the Kunitz 1 domain which leaves the carboxy terminus intact. Indeed, cholesterol levels in heterozygotic mice (K1 deletion) fed normal chow does not differ from wild type mice (data not shown).

Plasma TFPI found in lipoprotein fractions in humans has less anti-FXa and TF-FVIIa activity compared with free TFPI.<sup>11</sup> In our animal model, in spite of detecting increased plasma TFPI antigen and increased vascular TFPI activity, we are unable to detect increased circulating TFPI activity.<sup>21</sup> Human models of TFPI deficiency, particularly defects in the carboxy terminus, have not been described. In relatively small human studies, there is a positive correlation between circulating TFPI and VLDL levels.<sup>12, 42–44</sup> In this study, apoE<sup>-/-</sup> 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.<sup>45, 46</sup> It is also required for TFPI binding to cells and cell surface receptors including the VLDL receptor<sup>47</sup> and heparin sulfate proteoglycans.<sup>48</sup> The carboxy terminus has also been shown to mediate its interaction with lipoproteins.<sup>16–18</sup> The broad functionality of the TFPIct is reminiscent of the multifunctional peptides intended

to serve as apolipoprotein mimetics.<sup>27, 49</sup> These mimetics contain moieties that bind lipoproteins and residues which 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 can not 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 carboxy terminus. This functionality adds another potent dimension in which to consider the anti-atherosclerotic effects of TFPI. As such, the opportunities to utilize this functionality to understand its role in pathophysiology and treatment of human disease remain.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Non-standard Abbreviations and Acronyms

TFPI	Tissue factor pathway inhibitor
TFPIct	TFPI carboxy terminus
apo E	apolipoprotein E
FPLC	Fast Protein Liquid Chromatography
mASMCs	murine aortic smooth muscle cells
LRP	lipoprotein receptor related protein
hVLDL	human very low density lipoprotein
LPDS	lipoprotein deficient bovine calf serum
D-PBS	Dulbecco's modified PBS
HBSS	Hank's Balanced Salt solution
DMEM	Dulbecco's Modified Eagle's Medium
SmGM	Smooth muscle growth medium
MEF	murine embryonic fibroblasts
HSPGs	heparin sulfate proteoglycans
LACI	lipoprotein associated coagulation inhibitor

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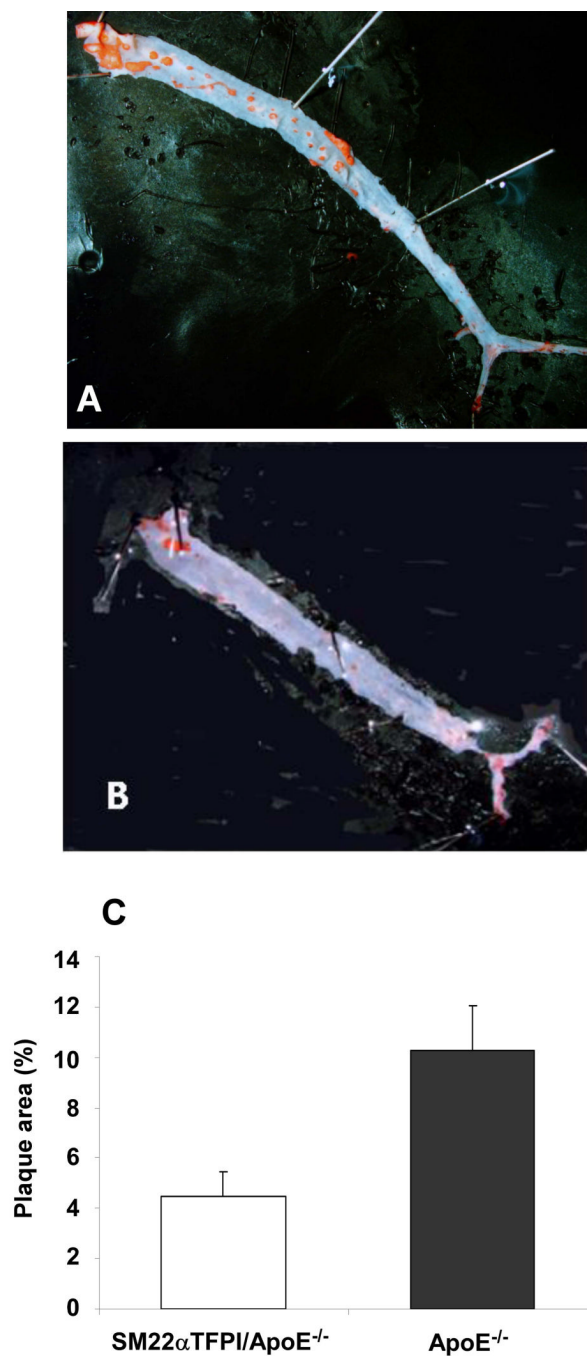
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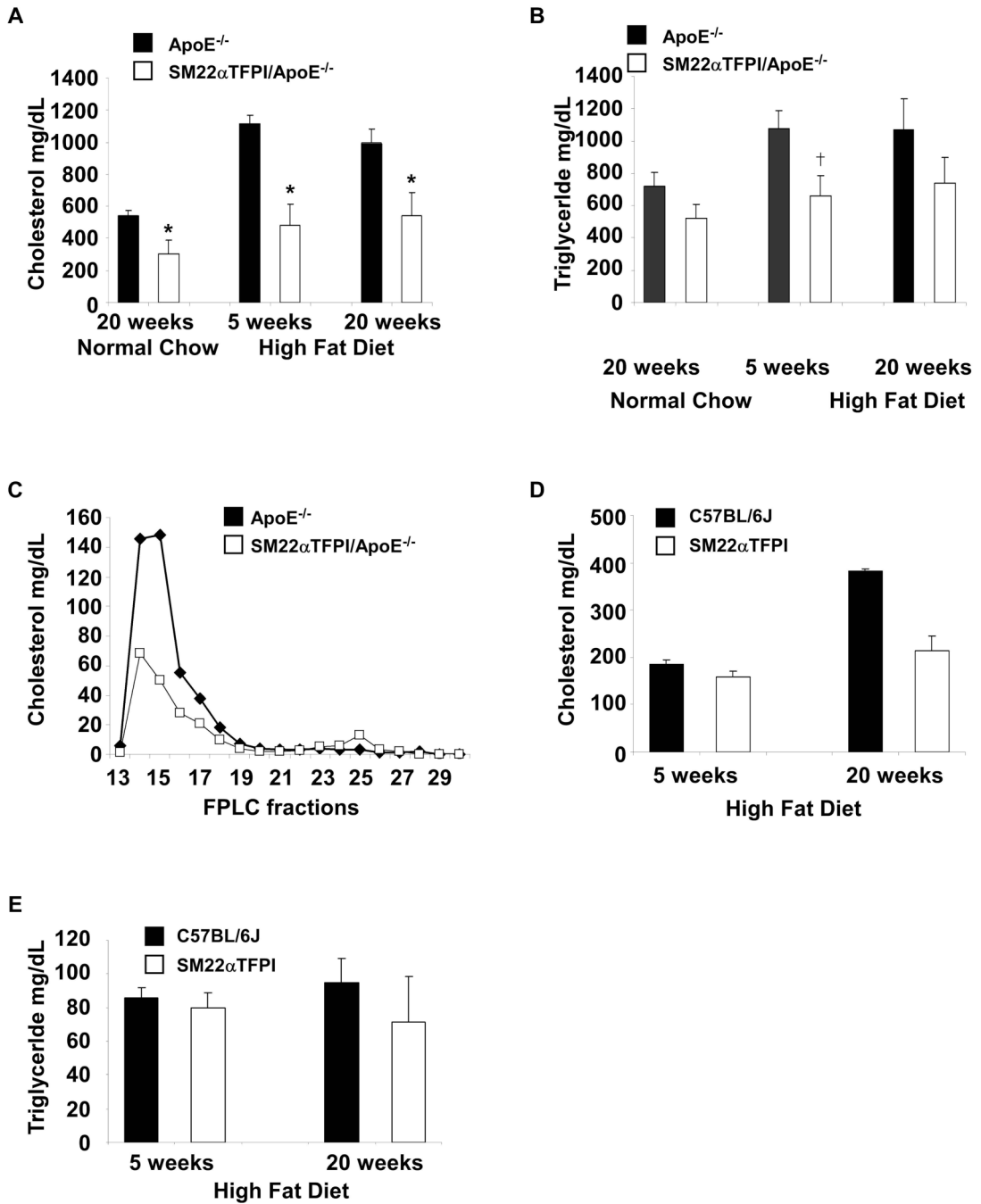
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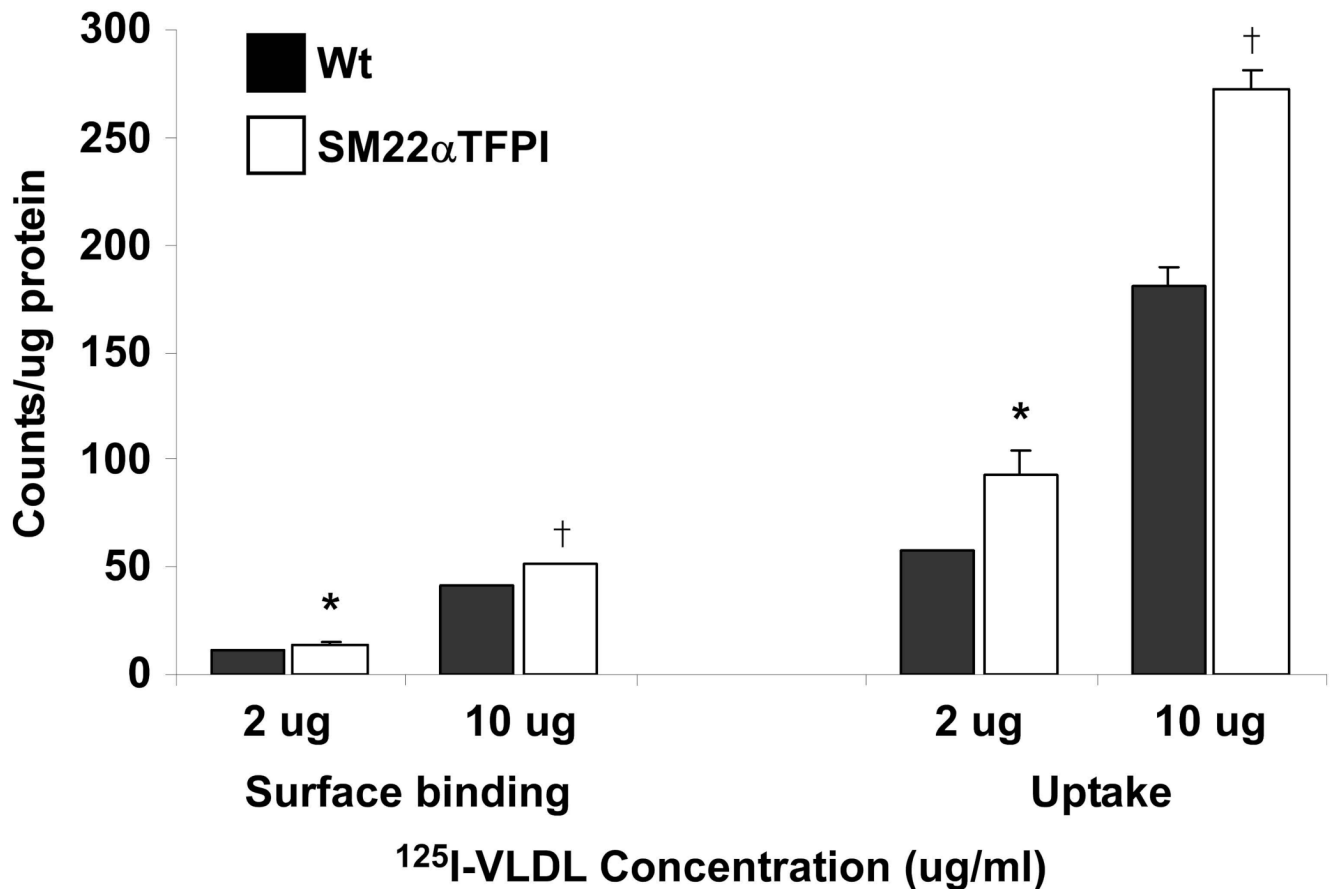


**Figure 1.** Vascular directed overexpression of TFPI reduces aortic plaque burden in apoE deficient mice. Atherosclerotic plaques in apoE<sup>-/-</sup> (A) and SM22 $\alpha$ TFPI/apoE<sup>-/-</sup> (B) mice stained with Sudan IV for en face analysis. C. The comparison of plaque areas in aorta from apoE<sup>-/-</sup> (n=13) and SM22 $\alpha$ TFPI/apoE<sup>-/-</sup> mice (n=12), \* p < 0.02.

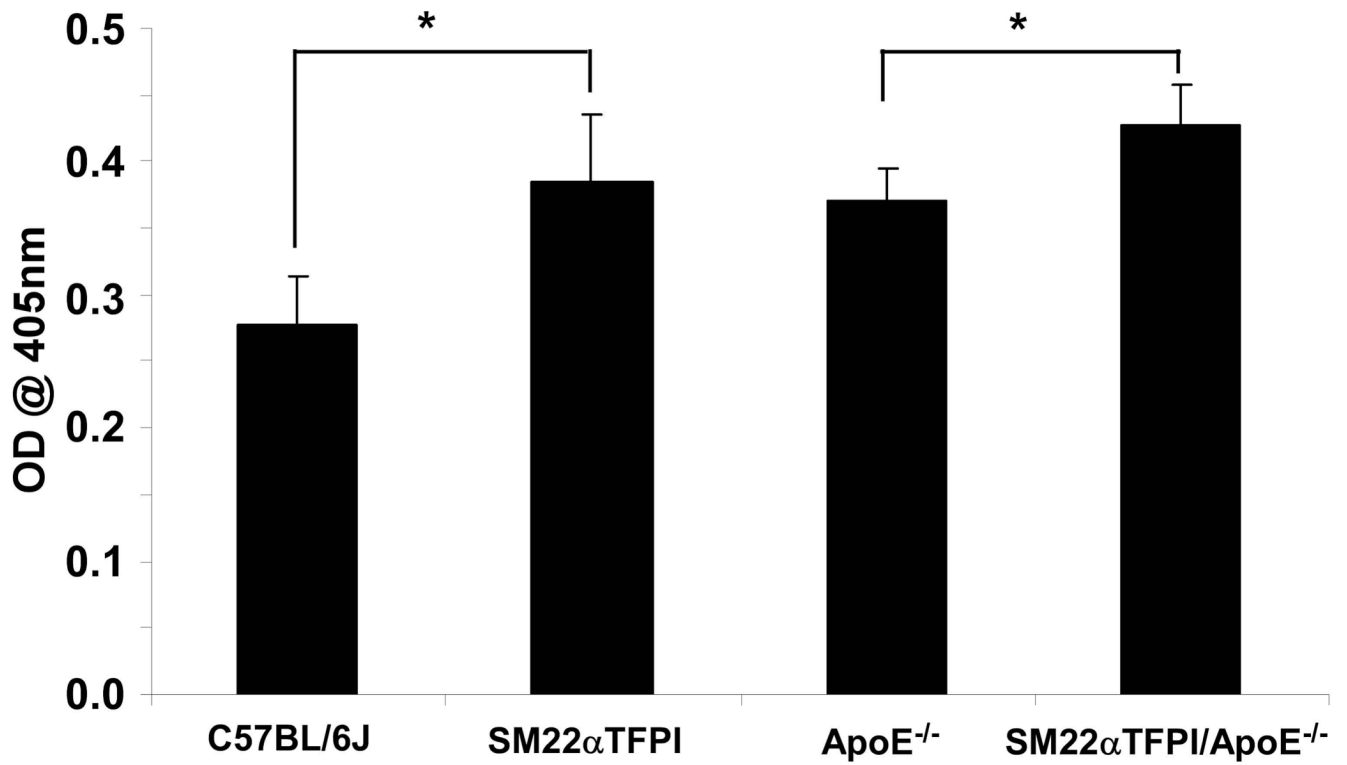


**Figure 2.**

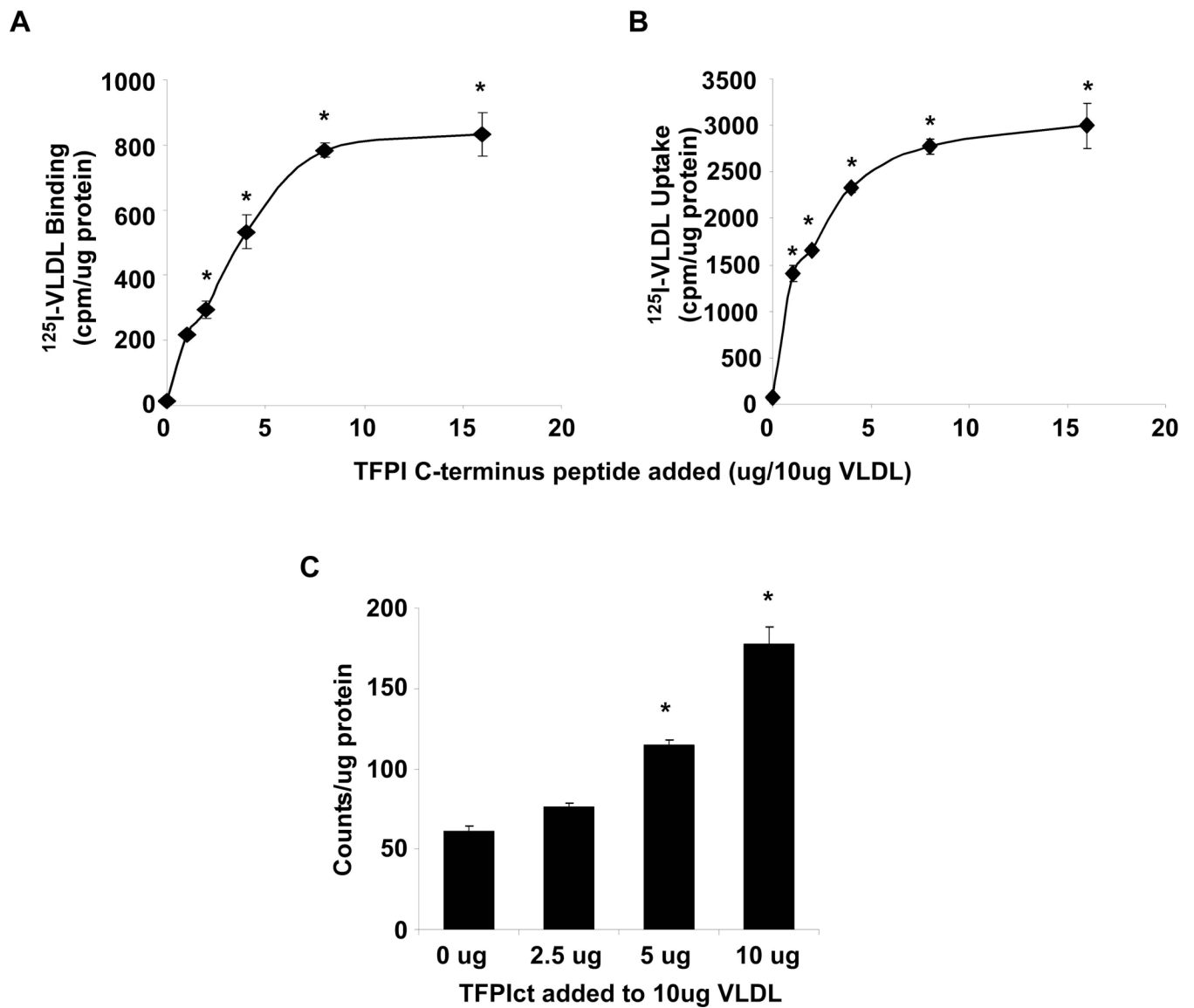
Vascular directed overexpression of TFPI reduces cholesterol levels in wild type and apoE deficient mice. Plasma cholesterol (A) and triglyceride (B) levels from apoE<sup>-/-</sup> and SM22 $\alpha$ TFPI/apoE<sup>-/-</sup> mice fed a high fat diet for 5 and 20 weeks were analyzed. Plasma from mice fed a high fat diet for 5 weeks were pooled and analyzed by FPLC (C). The fractions 14, 16, and 25 represent VLDL, LDL, and HDL respectively (confirmed by western blot). Plasma cholesterol (D) and triglyceride (E) levels from C57BL/6 and SM22 $\alpha$ TFPI mice fed a high fat diet for 5 and 20 weeks. \*  $p < 0.01$ , †  $p < 0.015$



**Figure 3.** Binding and internalization of  $^{125}\text{I}$ -VLDL in vascular smooth muscle cells. mASMCs from SM22 $\alpha$ TFPI transgenic and wild type mice were isolated, grown, and treated with  $^{125}\text{I}$ -VLDL at different concentrations. Significant differences between two groups are indicated as \* (for 2ug/ml of  $^{125}\text{I}$ -VLDL) and † (for 10ug/ml of  $^{125}\text{I}$ -VLDL). In binding assay \*  $p < 0.05$  and † $p < 0.01$  vs C57B/6. In the uptake assay \*  $p < 0.05$  and † $p < 0.001$  vs wild type.

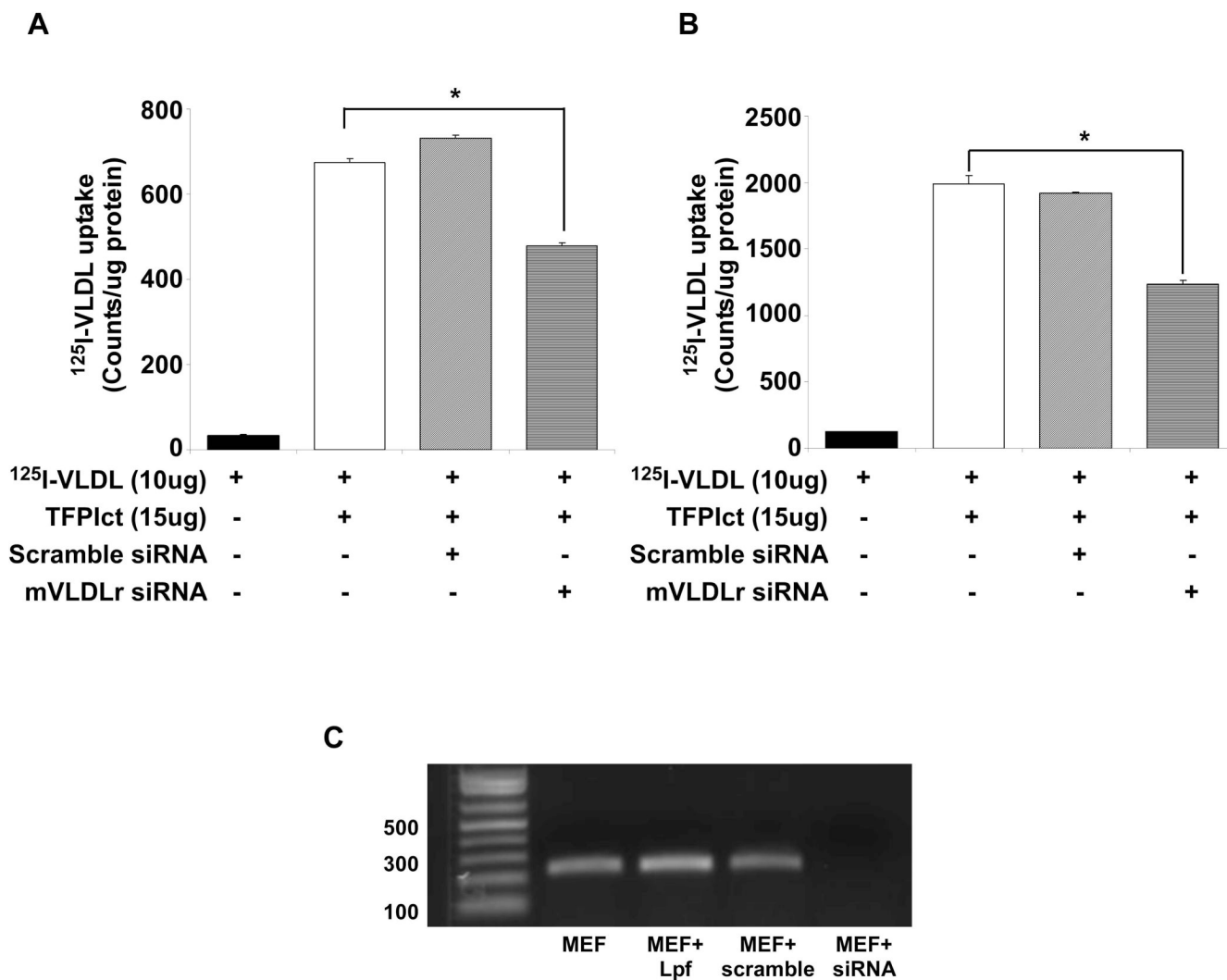


**Figure 4.** Vascular directed overexpression of TFPI increases circulating levels of TFPI antigen. Mice plasma samples were collected individually and tested by ELISA. (n>10/group) (\* p < 0.001)

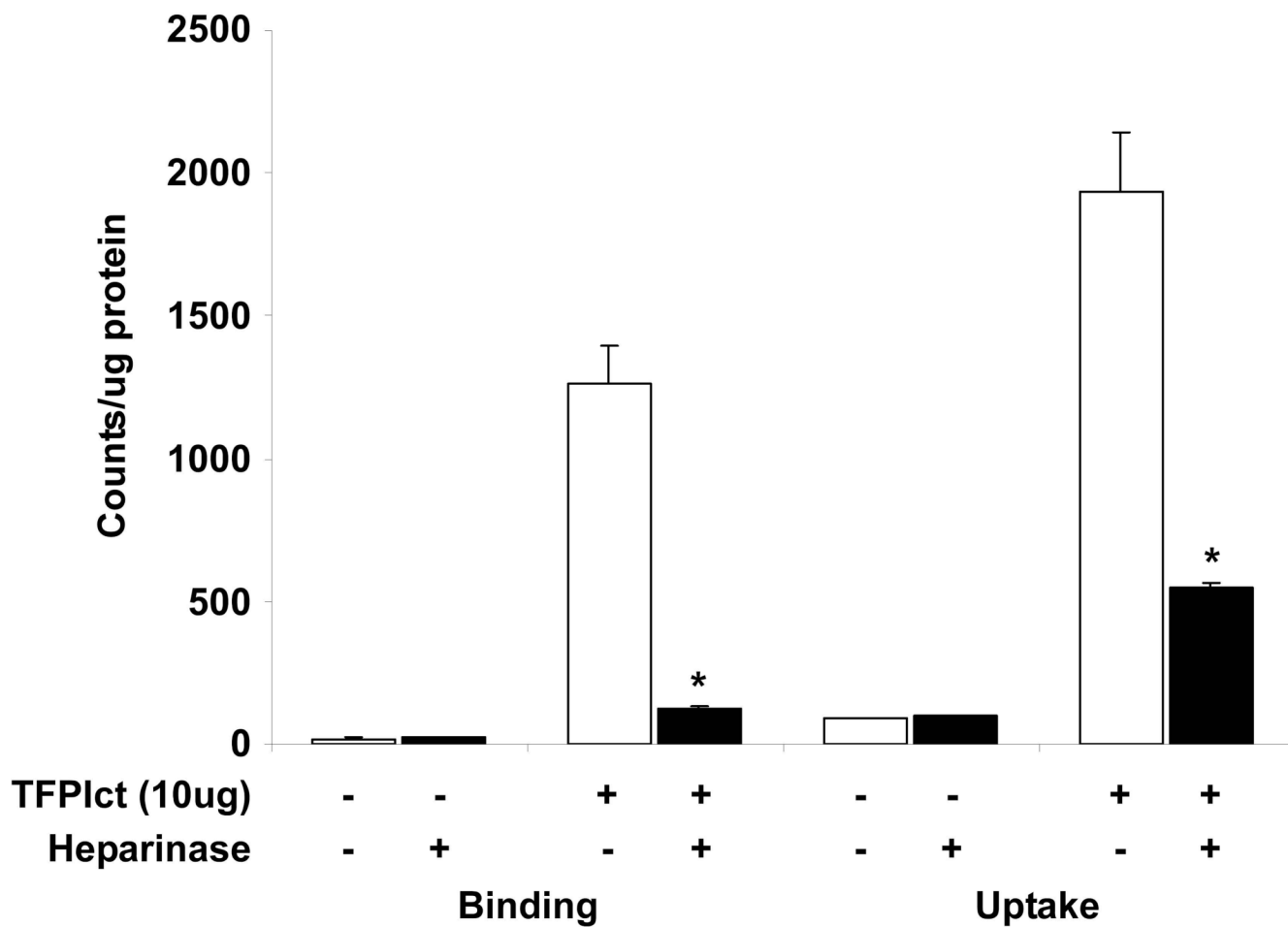


**Figure 5.** Effect of TFPIct peptides on binding, internalization and degradation of <sup>125</sup>I-VLDL in MEF. Cells were treated with <sup>125</sup>I-VLDL complex containing different concentrations of TFPIct. (A) TFPIct increased <sup>125</sup>I-VLDL binding to MEF cell surface, (B) internalization of <sup>125</sup>I-VLDL into the cells and (C) degradation of <sup>125</sup>I-VLDL in presence of TFPIct in MEF. \* p < 0.01.



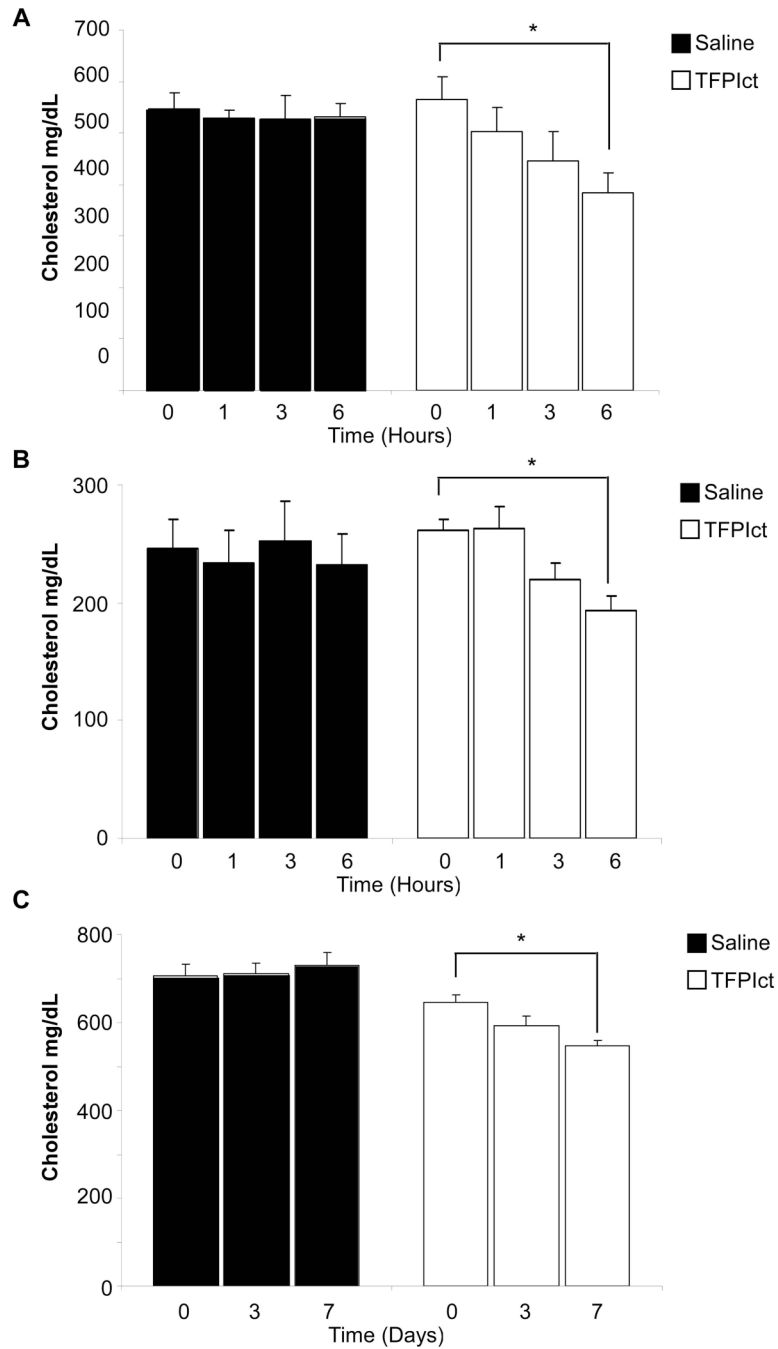


**Figure 6.** Knockdown of VLDLr inhibits  $^{125}\text{I}$ -VLDL binding to MEF cell surface (A) and internalization (B) of  $^{125}\text{I}$ -VLDL into the cells when exposed to TFPIct. (\*  $p < 0.01$  vs untreated cells). (C) Results of RT-PCR demonstrating specificity of VLDLr knockdown.



**Figure 7.**

Effect of heparinases on the binding and uptake of  $^{125}\text{I}$ -VLDL or  $^{125}\text{I}$ -VLDL:TFPIct complex. MEF cells were pretreated with heparinase I and III (each 3 units/ml) for 2 h at 37 °C and then incubated with  $^{125}\text{I}$ -VLDL (10ug) or  $^{125}\text{I}$ -VLDL(10ug):TFPIct (10ug) complex. Binding and uptake were attenuated by heparinases only in the presence of TFPIct. \*  $p < 0.02$



**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<sup>-/-</sup> mice after daily peptide injections (IP) (\*  $p < 0.01$  vs baseline).