ORIGINAL ARTICLES

Impact of native VLDL on tissue factor pathway inhibitor in endothelial cells and interactions between TFPI and lipoprotein lipase

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Tissue factor pathway inhibitor (TFPI) is a potent inhibitor of tissue factor (TF)-induced blood coagulation. A positive association between very low density lipoproteins (VLDLs) and TFPI has been reported *in vivo*. In contrast, one *in vitro* study indicates that TFPI may enhance lipoprotein lipase (LPL) activity, thereby increasing triglyceride hydrolysis. The current study was conducted to investigate how native VLDL influenced the synthesis and release of TFPI in endothelial cells, and how TFPI affected the LPL-induced hydrolysis of VLDL *in vitro* and at the endothelial surface. A spontaneously transformed immortal endothelial cell line (ECV304) and primary coronary artery cells (CoEc) were used, and VLDL was isolated from healthy volunteers by density gradient ultracentrifugation. Sequential free fatty acid (FFA) measurements were used to evaluate the kinetics of the LPL-induced hydrolysis. The levels of TFPI mRNAs in the stimulated cells were determined by quantitative real-time reverse transcription-ploymerase chain reaction (qPCR) using the ABI PRISM 7700 Sequence Detection System. Stimulation of ECV304 cells for 24 hours with native VLDL (0–100 μg/mL) caused a dose-dependent increase of TFPI in the medium (6.7–23.8 ng/10^6 cells, $P < 0.001$), without affecting the cellular content of TFPI. The expression of TFPI mRNA was significantly upregulated after 10 minutes of stimulation with n-VLDL. Both recombinant TFPI (r-TFPI) and LPL showed a dose-dependent binding to ECV 304 cells without saturation, and no competitive binding interactions between LPL and TFPI were observed at the endothelial surface. The addition of increasing concentrations of r-TFPI to ECV 304 cells, preincubated with LPL, did not affect the hydrolysis of VLDL triglycerides. The maximal reaction velocity (V_{max}) of LPL-induced hydrolysis of n-VLDL was not affected by the addition of increasing concentrations of r-TFPI to the reaction mixture *in vitro*. The current experimental study indicates an upregulation of TFPI synthesis and release by VLDL. LPL-induced hydrolysis of VLDL *in vitro* was not influenced by TFPI neither in suspension nor at the endothelial surface. (J Lab Clin Med 2006;147:167–173)

Abbreviations: CM = culture medium; FFA = free fatty acid; FVIIa = coagulation factor VIIa; GAG = glucosaminoglycan; GLM = general linear model; HAS = human serum albumin; LPL = lipoprotein lipase; NCS = newborn calf serum; PBS = phosphate-buffered solution; qPCR = reverse transcription-ploymerase chain reaction; r-TFPI = recombinant TFPI; TF = tissue factor; TFPI = tissue factor pathway inhibitor; VLDL = very low density lipoprotein

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Blood coagulation is triggered when blood is exposed to TF and forms a complex with FVIIa, by which the proteolytic TF-FVIIa complex initiates clotting through the cleavage and activation of both factors IX and X.[1] TFPI is a Kunitz-type serine protease inhibitor currently thought to be the principal regulator of TF-induced blood coagulation.[1] TFPI exerts its inhibitory function by neutralizing factor Xa directly, and by feedback inhibition of TF-FVIIa complexes in the presence of factor Xa.[2,3] The vascular endothelium is the primary site of TFPI synthesis,[4] and 50–80% of intravascular TFPI that is stored is associated with the endothelium under normal conditions.[5] Heparin administration causes mobilization of TFPI into the circulation, and heparin-releasable TFPI is thought to contribute significantly to the anticoagulant effect of heparins.[5–7]

LPL is the major lipase in lipoprotein metabolism.[8] The enzyme is synthesized by and released from parenchymal cells in many tissues, and it becomes anchored to GAGs at the endothelial surface.[8] LPL hydrolyses triglycerides in chylomicrons and VLDLs, providing fatty acids to the underlying tissues.[9] In addition, LPL has a non-enzymatic molecular "bridging" function, acting as a ligand in lipoprotein–cell surface interactions, mediating cellular binding and uptake of lipoproteins.[10] LPL may, therefore, have anti-atherogenic effects by clearing circulating lipoproteins and pro-atherogenic effects by promoting the uptake of potentially atherogenic lipids by the arterial wall.[8]

Patients with high serum triglycerides levels are at increased risk for coronary thrombosis.[11–14] A strong positive relationship between VLDL and TFPI has been reported and suggested as a compensatory mechanism for increased susceptibility to thrombosis in patients with hypertriglyceridemia.[13–16] In contrast, *in vitro* studies have shown that TFPI enhances LPL activity, thereby increasing triglyceride hydrolysis.[17] Thus, it is pertinent to investigate how native VLDL affects TFPI synthesis and release in endothelial cells and how TFPI affects LPL-induced triglyceride hydrolysis of native VLDL at the endothelial surface and *in vitro*.

MATERIAL AND METHODS

Materials. Medium 199 and L-glutamine was purchased from GIBCO Ltd (Middlesex, United Kingdom), and NCS was purchased from Biowhitaker (Wakersville, Md). Recombinant full-length TFPI (r-TFPI) was obtained from American Diagnostica Inc (Greenwich, Conn) and LPL from Sigma (L-9656; Sigma–Aldrich, St Louis, Miss). VLDL was isolated from human plasma obtained from fasting normolipemic subjects by density gradient ultracentrifugation,[18] using SW 40 swinging bucket rotors at 40,000 rpm for 22 h (Beckman). The VLDL solution (density, 0.94–1.006 g/mL) was collected at the top of the centrifuge tube.[20] VLDL solution was concentrated using Amicon Ultrafiltration cells (Milipore Corporation, Bedford, Mass). The protein concentrations were determined by a commercial available protein assay system, based on the method of Bradford et al[19] (Bio-Rad; Hercules, Calif). All VLDL preparations were filtered through a sterile 0.45-μm filter (Millex-GS; Millipore Corporation) and stored in darkness at 4°C until usage within 7 days.

Cell cultures. A spontaneously transformed immortal cell line (ECV 304) was obtained from American Type Culture Collection (ATCC CRL-1998, Rockville, Md) and served as the model system. In addition, primary coronary artery cells (CCC-2585) from Clonetics (Bio Whittager, MedProbe AS, Norway) were used. ECV 304 is a human EC line that like HUVECs, exhibits EC markers expected for endothelial cells such as factor VIII, Weibel–Palade bodies, and surface-specific molecules.[20] These cells are characterized by a cobblestone monolayer growth pattern and have been used to assess the anticoagulant role of TFPI in endothelial cells.[21–23] The cells were grown in plastic tissue flasks (Nunclon, Nalge Numc Int., Denmark) containing medium 199 supplemented with 10% heat inactivated NCS and 2 mM glutamine at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was changed every 2–3 days and always 24 h before an experiment.

Measurement of TFPI in cells and supernatants. The ECV 304 cells were seeded on 24-well polystyrene culture dishes (Falcon 3046, Becton Dickinson Labware, Lincoln Park, NJ) in medium 199 with 10% heat inactivated NCS and 2 mM glutamine at a density of 1.0×10^5 per dish. The cells were incubated for 24 h and washed twice in PBS and relayered with 1.0-mL cell medium containing 2% NCS and then exposed to different experimental procedures.

The ECV 304 cells were exposed to various concentrations of VLDL for 24 h. Supernatants were pipetted off and immediately frozen in two aliquots of 0.5 mL from each dish. Monolayers of ECV 304 cells were scraped from the plastic dish, transferred into a plastic tube with a volume of 1.0-mL cell medium, and subjected to three repeated freezing and thawing circles before measurement of TFPI. The number of ECV 304 cells under each experimental condition was counted. Signs of cell toxicity, determined by trypan blue exclusion, were not present in samples of endothelial cells stimulated with VLDL at the different concentrations. The TFPI antigen level in the endothelial cells and supernatant was then measured by a commercial available ELISA kit (IMUBIND Total TFPI; American Diagnostica Inc.), employing a rabbit antihuman TFPI polyclonal antibody as the capture antibody. The assay was performed as described by the manufacturer.

Assay of Cell Proliferation. Incorporation of ^{3}H-thymidine into DNA is proportional to *de novo* DNA synthesis and thereby reflects cell proliferation[24] A total of 2.5×10^3 ECV 304 cells were seeded on 96-well polystyrene culture dishes (Falcon 3072, Becton Dickinson Labware), grown for 24 h in CM with 10% NCS washed twice and relayered with CM containing 2% NCS with or without different concentrations