Original Article

The Renin-Angiotensin System Is Involved in the Production of Plasminogen Activator Inhibitor Type 1 by Cultured Endothelial Cells in Response to Chylomicron Remnants

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Triglyceride-rich lipoproteins have been suggested to promote atherosclerosis. Plasminogen activator inhibitor type 1 (PAI-1) plays an important role in the events of cardiovascular pathophysiology. The renin-angiotensin system influences various vascular functions, including PAI-1 production. We examined whether or not chylomicron remnants increased PAI-1 mRNA and protein production in endothelial cells and whether or not an inhibition of the renin-angiotensin system interfered with this effect. Chylomicron remnants were isolated from functionally hepatectomized rats injected with chylomicrons. Human umbilical vein endothelial cell cultures (HUVECs) were incubated with chylomicron remnants with or without an angiotensin-converting enzyme inhibitor (temocaprilat), an angiotensin II receptor type 1 antagonist (RNH-6270), or an angiotensin II receptor type 2 antagonist (PD123319). Chylomicron remnants increased PAI-1 secretion in HUVECs (0.5 µg/ml; 128.3 ± 6.1%, the mean ± SEM) as well as angiotensin II (10 nmol/l; 130.7 ± 9.5%) in 18 h, as compared with the controls, as well as stimulated PAI-1 mRNA expression to a maximum level at 4 h. Temocaprilat and RNH-6270, but not PD123319, attenuated all of these effects. Chylomicron remnants enhanced nuclear extract binding to a very low-density lipoprotein response element in the PAI-1 promoter region and activated nuclear factor-κB. Extracellular signal-regulated kinase (ERK 1/2) was phosphorylated in response to chylomicron remnants. These effects were inhibited by temocaprilat or RNH-6270. In conclusion, chylomicron remnants increased protein secretion and mRNA expression of PAI-1 in HUVECs. Inhibition of the renin-angiotensin system reduced this stimulation. (Hypertens Res 2003; 26:315–323)

Key Words: chylomicron remnants, plasminogen activator inhibitor type 1, endothelial cells, renin-angiotensin system

Introduction

Thrombolytic potential is mainly under the control of plasminogen activator inhibitor type 1 (PAI-1), the modulation of which has been shown to influence fibrin and extracellular matrix accumulation (1, 2). Elevated PAI-1 concentration in plasma is considered as a risk factor for coronary artery events (2, 3). The endothelium is a likely major source of PAI-1, in addition to the liver, adipose tissue, and circulating platelets (2, 4). Several mechanisms regulating PAI-1 have been investigated. Angiotensin II increases PAI-1 production

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in several cell types, including endothelial cells (5), smooth muscle cells (6), and monocytes (7). Inhibition of angiotensin II by an angiotensin converting enzyme (ACE) inhibitor or an angiotensin II receptor type 1 (AT1) antagonist reduced PAI-1 production in vitro and in clinical studies (5–10). On the other hand, increased plasma PAI-1 has been demonstrated in patients with hypertriglycerideremia, hypertension, obesity, and type II diabetes mellitus (2, 11, 12). Recent reports indicate that triglyceride-rich liver-derived lipoproteins, very low-density lipoproteins (VLDL), can accelerate PAI-1 secretion by endothelial cells by increasing transcription of the PAI-1 gene (13, 14). Oxidized low-density lipoproteins (OxLDL) also increase PAI-1 production via oxidative stress (15).

Accumulating evidence suggests the significance of hypertriglycerideremia in the development of atherosclerosis (16). Not only elevated fasting concentrations but also persistent postprandial increases of plasma triglyceride have been associated with cardiovascular diseases (17, 18). Chylomicron remnants, which are intestine-derived lipoproteins, are known to be atherogenic (19). These remnants are formed after hydrolysis of triglycerides by lipoprotein lipase from intestine-derived chylomicrons, and they are rapidly removed from the circulation. Delayed clearance of these triglyceride-rich lipoprotein particles induced by genetic or metabolic abnormalities is often associated with accelerated atherosclerosis (18, 20, 21). However, no evidence has yet been found for a connection between chylomicron remnants and PAI-1 production.

In this study, we investigated whether chylomicron remnants can stimulate PAI-1 production in endothelial cells. Moreover, we examined whether inhibition of the renin-angiotensin system reduced this effect.

Methods

Materials

Male Sprague-Dawley rats were purchased from Japan SLC (Hamamatsu, Japan). M199 medium, fetal bovine serum (FBS), angiotensin II, and angiotensin II receptor type 2 (AT2) antagonist (PD123319) were obtained from Sigma (St. Louis, USA). A penicillin-streptomycin mixture was purchased from GIBCO BRL (Grand Island, USA). Endothelial cell growth supplement was obtained from BD Biosciences Clontech (Palo Alto, USA). Bio-Gel A-50m (100–200 mesh) was purchased from Bio-Rad (Richmond, USA). Angiotensin-converting enzyme inhibitor (temocaprilat) and AT1 antagonist (RNH-6270) were kindly supplied by Sankyo (Tokyo, Japan). PAI-1 antigen was assayed by enzyme-linked immunosorbent assay (ELISA) using kits (TimELISE PAI-1) from Biopool AB (Umeå, Sweden). [α-32P]dCTP and [γ-32P]dATP, and polyclonal primary antibodies against phosphorylated extracellular signal-regulated kinase (ERK 1/2) and anti-rabbit horseradish peroxidase-conjugated immunoglobulin G (IgG) were obtained from American Biosciences (Buckinghamshire, UK). Human PAI-1 cDNA was obtained from Oncogene (San Diego, USA). All other chemicals were obtained from Wako (Osaka, Japan).

Cells

Human umbilical vein endothelial cells (HUVECs) were prepared as previously described (22). Cells were cultured on 0.2% gelatin-coated dishes in M199 medium with 20% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 U/ml heparin, and 20 µg/ml endothelial cell growth supplement with incubation in 5% CO2 at 37°C. Cells were passaged by trypsinization and used between passages 3 and 7. In the experiments, cells were incubated in M199 medium with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (control medium) with 0.5–1 µg/ml chylomicron remnants for 5 min–18 h. In the experiments with antagonists, cells were preincubated with 100 nmol/l temocaprilat, 100 nmol/l RNH-6270, or 100 nmol/l PD123319 for 15 min. Cell viability was examined with a cell proliferation reagent, WST-1 (Boehringer Manhein, Wilmington, USA) according to the manufacturer’s protocol. Lactate dehydrogenase (LDH) release into the culture medium was also determined as previously described (23).

Lipoproteins

Rat mesenteric lymph chylomicrons and chylomicron remnants were prepared in vivo as previously described (19). All experiments were performed in accordance with a protocol approved by the Guidelines for Animal Experimentation at Hyogo College of Medicine. Briefly, chylomicrons were isolated by ultracentrifugation from lymph collected from gastrotomized male Sprague-Dawley rats fed with egg solution for 48 h. These functionally hepatectomized rats were injected with chylomicrons and then exsanguinated after 3 h. Finally, chylomicron remnants were isolated from the plasma of these hepatectomized rats by ultracentrifugation. To determine whether chylomicron remnants were successfully prepared, selected preparations of chylomicrons and chylomicron remnants were analyzed to their lipid compositions, as previously described (19). The ratios of triglyceride to total cholesterol (w/w) were 60.2 in chylomicrons and 5.8 in chylomicron remnants (n = 5). Moreover, lipids were extracted from chylomicrons and chylomicron remnants that contained equal amounts of phospholipids with mixtures of chloroform and methanol, as previously described by Bligh and Dyer (24). Extracted phospholipids were subjected to silica gel thin-layer chromatography, visualized by iodine, and analyzed by scanning photodensitometry, as described previously (25). The thin-layer chromatography showed an increase in the amount of lysophosphatidylcholine extracted from chylomicron remnants, compared with that of chylomicrons (7.7 ± 0.2% vs. 0.3 ± 0.2%, n = 5, the mean ± SEM). These
Data indicated that chylomicrons were successfully hydrolyzed (26).

**Determination of PAI-1 Secretion**

Confluent 12-well plates of HUVECs were incubated in control medium in the presence or absence of the indicated concentrations of chylomicron remnants with or without antagonists. After incubation for 18 h, the conditioned medium was removed and stored at -20°C until analysis. Protein concentrations of PAI-1 in the medium were measured by ELISA, and were expressed as percentages relative to the control medium (0 µg/ml chylomicron remnants).

**Western Blot Analysis**

Confluent 6-cm dishes of HUVECs were incubated in control medium in the presence or absence of 0.5 µg/ml chylomicron remnants with or without antagonists for 5 min. Cells were disrupted in lysis buffer [phosphate-buffered saline (PBS), 1% Triton-X 100, 0.1% sodium dodecyl sulfate (SDS), and 0.5% deoxycholate]. Lysates were microcentrifuged for 10 min at 1,500 rpm and the supernatant was collected. Samples (30 µg/ml of total protein) were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then electroblotted onto a membrane as previously described (27). The membrane was incubated with polyclonal anti-phosphorylated ERK 1/2 primary antibody (1:500) for 1 h at room temperature, and then incubated with anti-rabbit horseradish peroxidase-conjugated IgG for 1 h at room temperature. Immune complexes were visualized by enhanced-chemiluminescence Western blotting detection regents (ECL; Amersham Biosciences).

**Northern Blot Analysis**

Confluent 6-cm dishes of HUVECs were incubated in control medium in the presence or absence of 0.5–3 µg/ml chylomicron remnants with or without antagonists for 4–18 h. Total RNA was extracted by use of TRIZol reagent (modified acid guanidium thiocyanate phenol/chloroform method; Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions. Northern blot analysis of PAI-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed as previously described (28). The relative intensities of the bands present were determined using a BAS 2000 phosphor imager (Fuji Photo Film, Tokyo, Japan).

**Electromobility Shift Assay**

To determine the effect of chylomicron remnant-induced transcription, nuclear extracts were prepared according to Dignam et al. (29) from 4 to 5 × 10⁶ HUVECs previously stimulated by chylomicron remnants for 2 h in the presence or absence of inhibitors. Nuclear extract binding to the VLDL response element was monitored by an electromobility shift assay (EMSA). Double-stranded oligonucleotides containing the VLDL-response element (5'-CGGGGAGTCAGCCGTGTATCATCGGAGGCGGC-3') of the human PAI-1 promoter were designed according to Eriksson et al. (14) as -672/-637 bp (upstream of the transcription start site in the human PAI-1 promoter). The probe was end-labeled with [α-32P]dCTP. All buffers were freshly supplemented with 10 µg/ml leupeptin, 0.023 TIU/ml aprotinin, 1 mmol/l phenylmethyl sulfonyl fluoride, 10 µg/ml antipain, and 1 mmol/l dithiothreitil. Samples were applied on a 4% (w/v) polyacrylamide/bisacrylamide gel (37.5:1) and electrophoresed in tris-borate ethylenediaminetetraacetic acid (EDTA) buffer for 1.5 h at 35 V. Dried gels were then exposed to an imaging plate for 30 min. The relative intensities of the bands present were determined using a BAS 2000 phosphor imager. For the detection of nuclear factor-κB (NF-κB) activation, we used a NUSHIFT KIT [NFκB p65 (HUMAN)] (Geneka Biotechnology, Montréal, Canada) according to the manufacture’s procedure. The specificity of the binding reaction was determined with a 100-fold excess of unlabeled oligonucleotide probe or with anti-NF-κB antibody (anti-p65).

**Other Assays**

Protein was measured by the method of Lowry et al. (30) or by the Pierce BCA protein assay method (Pierce; Rockford, USA); using bovine serum albumin (BSA) was used as the standard.

**Statistical Analysis**

All data were expressed as percentages relative to the control data. Statistical analysis was performed using an analysis of variance (ANOVA) with Scheffé’s post hoc test. P < 0.05 was considered to indicate statistical significance. Data are expressed as the mean ± SEM.

**Results**

**WST-1 Assay and LDH Release**

We completed all experiments within 18 h because we found that incubation of HUVECs with chylomicron remnants at a concentration of 0.5 µg/ml for longer than 24 h induced detachment of the cells. Cell viability estimated by the results of WST-1 assays and LDH release did not differ between the control cultures and the cultures with 0.5 and 1 µg/ml chylomicron remnants for 18 h (data not shown).

**Chylomicron Remnants Stimulate PAI-1 Secretion**

HUVEC produced measurable concentrations of PAI-1 protein in the control medium without chylomicron remnants.
Addition of chylomicron remnants significantly increased PAI-1 secretion in HUVECs (Fig. 1). Secreted protein concentration of PAI-1 by 0.5 µg/ml chylomicron remnants was similar to that by 10 nmol/l angiotensin II. Figure 2A shows the time course of the effects of chylomicron remnants on PAI-1 mRNA expression, which was stimulated by chylomicron remnants to a maximum amount at 4 h in the case of both bands (2.2 kb, 3.2 kb). Figure 2B demonstrates the effect of chylomicron remnants upon mRNA expression for PAI-1; induced PAI-1 mRNA expression by 0.5 µg/ml chylomicron remnants was similar to that by 10 nmol/l angiotensin II. These results indicated that chylomicron remnants were able to stimulate protein secretion and mRNA expression of PAI-1.

Effects of Renin-Angiotensin System Antagonists on Chylomicron Remnant-Stimulated PAI-1 Secretion

Next, we examined the effects of renin-angiotensin system antagonists on chylomicron remnant-stimulated PAI-1 secretion. Pretreatment of HUVECs with an angiotensin converting enzyme inhibitor (temocaprilat, 100 nmol/l) and an AT1 antagonist (RNH-6270, 100 nmol/l) inhibited PAI-1 secretion stimulated by 0.5 µg/ml chylomicron remnants (Fig. 3). The AT2 antagonist (PD123319) had no significant effect on secretion. Enhancement of mRNA expression by chylomicron remnants was also reduced by temocaprilat or RNH-6270 (Fig. 4). Without chylomicron remnants, neither temocaprilat nor RNH-6270 had any effect on PAI-1 protein production or mRNA expression (data not shown). These results indicated that chylomicron remnants were able to stimulate protein secretion and mRNA expression of PAI-1.
suggest that the inhibition of an AT1-mediated mechanism, as well as ACE inhibition, effectively reduce chylomicron remnant-stimulated protein secretion and mRNA expression of PAI-1.

EMSA

To investigate the possibility of chylomicron remnant-induced transcription, and more specifically to determine whether or not chylomicron remnants can induce transcription involving the VLDL response element as well as VLDL, EMSA was performed using oligonucleotides corresponding to the PAI-1 VLDL response element site. It was found that the oligonucleotides bound to a chylomicron remnant-inducible nuclear protein complex (Fig. 5). An addition of a 100-fold excess of cold probe apparently inhibited DNA-protein complex formation. An addition of temocaprilat or RNH-6270 also inhibited DNA-protein complex formation. Moreover, chylomicron remnants activated NF-κB in EMSA (Fig. 6). An addition of temocaprilat or RNH-6270 also inhibited NF-κB activation. These results suggest that nuclear extracts binding to the VLDL-response element in the PAI-1 promoter and NF-κB activation induced by chylomicron remnants were both regulated by a mechanism involving the renin-angiotensin system.

Discussion

Triglycerides and/or fatty acids in chylomicron remnants
might induce the activation of a transcription factor for PAI-1 production. VLDL, rich in triglycerides and fatty acids, have been found to induce transcription of the human PAI-1 promoter in endothelial cells, whereas native LDL do not (14, 32). Fatty acids (33) have an effect upon the VLDL-inducible transcription factor that is similar to that of VLDL (34). The triglyceride content of lipoprotein particles influences PAI-1 expression in endothelial cells, whereas OxLDL increase PAI-1 expression by a different mechanism than that seen with triglyceride-rich lipoproteins (35). In particular, lysophosphatidylcholine, presented in OxLDL, has been
shown to stimulate PAI-1 expression (36). Gräfe et al. (15) have also reported that OxLDL enhanced PAI-1 activity and antigen secretion by human coronary artery endothelial cells. Since chylomicron remnants are triglyceride-rich and involve lysophosphatidylcholine in the process of synthesis (see Methods), both may participate in PAI-1 expression. In this study, we demonstrated that chylomicron remnants enhanced the binding of nuclear extracts to the VLDL response element in the PAI-1 promoter. On the other hand, NF-κB activation has been reported to be associated with PAI-1 production (37, 38). NF-κB is a common transcription factor involved in the regulation of many proinflammatory genes (39). Chylomicron remnants can provoke oxidative stress (40, 41), and oxidative stress can in turn activate NF-κB (42). We determined NF-κB activation induced by chylomicron remnants, and found that the NF-κB activation was consistent with the results obtained by VLDL (43). According to a recent report, remnant like lipoprotein particles from patients with type III hyperlipoproteinemia induce endothelial cell PAI-1 expression (44). Taking these findings, chylomicron remnants may induce PAI-1 production by both VLDL response element-mediated and NF-κB-mediated mechanisms.

The renin-angiotensin system plays an important role in the control of fibrinolysis (7). Angiotensin II regulates the expression of PAI-1 in several cell types (5–7). The details of receptor specificity for the regulation of PAI-1 expression remain controversial due to differences among species and among experimental conditions; however, recent experimental reports suggest that AT1 receptors regulate PAI-1 expression (9, 15). In the present study we demonstrated that an ACE inhibitor and AT1 antagonist reduced PAI-1 protein secretion and the expression of PAI-1 mRNA by the stimulation of chylomicron remnants. An AT2 antagonist did not inhibit these effects. It should be noted that the ACE inhibitor and angiotensin II antagonist both completely inhibited PAI-1 production in our study. The renin-angiotensin system is intact in cultured endothelial cells, which are capable of synthesizing and secreting angiotensins (45, 46). Napoleon et al. (47) showed that ACE inhibitors down-regulate tissue factor factor synthesis in monocytes and suggested that ACE inhibitors inhibited nuclear localization of c-Rel/p65 heterodimers, as seen in the EMSA, by reducing endogenous angiotensin II. Cook et al. (48) suggested that angiotensin II may be internalized or generated through an intracrine system and that it alters cellular properties after nuclear translocation, receptor binding, and transcriptional regulation of gene expression. Although we have no direct evidence at present, angiotensins may have been secreted into the medium by an autocrine mechanism or may have been generated through an intracrine system upon stimulation by chylomicron remnants, resulting in increased PAI-1 synthesis. Moreover, PAI-1 synthesis is regulated by several cell-specific second-messenger signaling pathways. Banfi et al. (31) have reported that VLDL-induced PAI-1 synthesis results mainly from stimulation via a signal pathway involving protein kinase C-mediated MAP kinase activation. Activation of ERK and Rho-kinase pathways plays a pivotal role in PAI-1 gene upregulation by angiotensin II (9, 10). PAI-1 mRNA stability was also increased by angiotensin II (9). We demonstrated here that chylomicron remnants stimulated the phosphorylation of ERK 1/2 in HUVECs, and that this phosphorylation was diminished by the ACE inhibitor and AT1 antagonist. The inhibition of phosphorylation observed here may also have contributed to the finding that a blockade of the renin-angiotensin system suppressed PAI-1 expression stimulated by chylomicron remnants. In addition, it is well known that ACE inhibitors exert anti-inflammatory and anti-oxidative effects (49). Taken together, the data suggest that the renin-angiotensin system is likely to be involved in the multiple processes from DNA-protein complex formation to secretion in PAI-1 production stimulated by chylomicron remnants in HUVECs. Chylomicron remnants observed in hyperlipidemia may be linked to atherosclerotic and thrombotic events by such mechanisms, underscoring the importance of chylomicron remnants and the renin-angiotensin system in designing therapeutic agents to treat or prevent vascular events in metabolic disorders. Further experiments are need-
ed to clarify the details of the regulation for PAI-1 production by chylomicron remnants.

In summary, chylomicron remnants stimulated PAI-1 production in HUVECs. Inhibition of the renin-angiotensin system reduced this stimulation.

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