Apolipoprotein CIII Induces Expression of Vascular Cell Adhesion Molecule-1 in Vascular Endothelial Cells and Increases Adhesion of Monocytic Cells

Akio Kawakami, MD; Masanori Aikawa, MD, PhD; Pilar Alcaide, PhD; Francis W. Luscinskas, PhD; Peter Libby, MD; Frank M. Sacks, MD

Methods and Results—Treatment of ECs with apoCIII or apoCIII-rich VLDL caused human mononuclear THP-1 cells to adhere to them under static condition or under laminar shear stress (1.0 dyne/cm²). ApoCIII increased EC expression of vascular cell adhesion molecule-1 (VCAM-1) protein and intercellular cell adhesion molecule-1 (ICAM-1) protein (4.9±1.5-fold and 1.4±0.5-fold versus control, respectively). Furthermore, apoCIII remarkably increased membrane-bound protein kinase C (PKC) β in ECs, indicating activation. A selective inhibitor of PKCβ prevented the rise in VCAM-1 and THP-1 cell adhesion to ECs. Moreover, exposure of ECs to apoCIII induced nuclear factor-κB (NF-κB) activation. PKCβ inhibition abolished apoCIII-induced NF-κB activation, and NF-κB inhibition reduced expression of VCAM-1, each resulting in reduced THP-1 cell adhesion. ApoCIII-rich VLDL also activated PKCβ and NF-κB in ECs and increased expression of VCAM-1. Pretreatment of ApoCIII-rich VLDL with anti-apoCIII neutralizing antibody abolished its effect on PKCβ activation.

Conclusions—Our findings provide the first evidence that apoCIII increases VCAM-1 and ICAM-1 expression in ECs by activating PKC and NF-κB, suggesting a novel mechanism for EC activation induced by dyslipidemia. Therefore, apoCIII-rich VLDL may contribute directly to atherogenesis by activating ECs and recruiting monocytes to them. (Circulation. 2006;114:681-687.)

Key Words: apolipoproteins ■ atherosclerosis ■ cell adhesion molecules ■ endothelial cells

The induction of adhesion molecules in vascular endothelial cells (ECs) and the subsequent recruitment of circulating monocytes are proinflammatory events that promote atherogenesis and plaque instability.1–2 We and others have reported previously that dyslipidemia induced by an atherogenic diet increased vascular cell adhesion molecule-1 (VCAM-1) in ECs in vivo.3–7 Patients with type 2 diabetes and hypertriglyceridemia have high plasma adhesion molecules.8 Indeed, acute hypertriglyceridemia induced by an oral fat loading increases circulating cellular adhesion molecules in healthy subjects9 and patients with type 2 diabetes.8

Apolipoprotein CIII (apoCIII) resides on the surface of a portion of VLDL and LDL and inhibits the activity of lipoprotein lipase, which metabolizes triglyceride in these lipoproteins.10 ApoCIII also impairs the clearance of apoB lipoproteins from the circulation by interfering with their binding to hepatic lipoprotein receptors.11 Thus, apoCIII causes dyslipidemia characterized by increased apoCIII-containing apoB lipoproteins. ApoCIII in apoB lipoproteins is an independent risk factor for coronary heart disease (CHD).12–14 However, the direct effects of these lipoprotein fractions on vascular cells have been unexplained. We recently showed that apoCIII alone or as a component of VLDL or LDL induces activation of protein kinase C (PKC) α as well as PKCβ and β-integrin in the human mononuclear THP-1 cells and increases their adhesion to vascular ECs under static conditions.15,16
or flow conditions. These findings suggest that apoCIII not only affects apoB lipoprotein metabolism but can also participate directly in atherogenesis by enhancing the atherogenicity of apoB lipoproteins.

The present study tested the hypothesis that apoCIII alone or apoCIII-rich VLDL (VLDL CIII) can regulate EC expression of intercellular cell adhesion molecule-1 (ICAM-1) and VCAM-1 and adhesion of human mononuclear THP-1 cells to ECs under static and flow conditions.

**Methods**

**Cell Culture and Reagents**

Human saphenous vein endothelial cells (HSVECs) and human peripheral monocytes were collected and cultured under a protocol approved by the Human Research Committee of the Brigham and Women’s Hospital, THP-1 cells, a human monocytic cell line (ATCC, Manassas, Va), HSVECs, and human peripheral monocytes were cultured as described previously. HSVECs at passage 3 were used for the assays. Human apoCIII was purchased from Academia Biomedical (Houston, Tex). Antibodies used in the present study include the following: mouse anti-PKCα antibody, mouse anti-PKCβ antibody (BD Biosciences, San Jose, Calif), mouse anti–VCAM-1 (C3P4), mouse anti–ICAM-1 (P2A4) (Chemicon International, Temecula, Calif), rabbit anti–NF-κB p65 antibody, rabbit anti–Ikβα antibody, rabbit anti–β-actin antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), and goat anti-apoCIII antibody (Academy Biomedical). Selective PKCβ inhibitor [3-[(1-(3-imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrrole-2,5-dione] as well as NF-κB inhibitor cell permeable peptide SN50 and its control scrambled peptide were purchased from Calbiochem (San Diego, Calif).

**Lipoprotein and Lipid Preparation**

This study was approved by the institutional review board of Harvard School of Public Health. Blood was drawn in tubes containing EDTA from 18 healthy volunteers after 12 hours of fasting. The subjects were not taking cardiovascular medications, antioxidants, or estrogen. VLDL (d<1.006) with apoCIII (VLDL CIII) or without (VLDL CII) was isolated from plasma as described previously. The molecular ratios of apoC, apoCII, apoCIII, and apoE to apoB (apoC/B mol, apoCII/B mol, apoCIII/B mol, apoE/B mol, respectively) of lipoprotein preparations that reflect their enrichment on the particles were calculated by using their respective molecular mass (apoB, 550 kDa; apoC, 6.6 kDa; apoCII, 8.8 kDa; apoCIII, 8.8 kDa; apoE, 13.4 kDa) with the use of ELISA as described previously. Some experiments pretreated VLDL CIII with anti-apoCIII antibody (50 μg/mL) for 30 minutes before addition to ECs. Endotoxin levels in the apoCIII and lipoprotein fractions measured with a Limulus amoebocyte lysate chromogenic test (Associates of Cape Cod, East Falmouth, Mass) were <0.03 EU/mL.

**Adhesion Assay**

**Static Conditions**

THP-1 cells were labeled with BCECF-AM (Calbiochem), placed on a confluent HSVEC monolayer (1×10⁴ per well) in a 96-well plate (1×10⁴ THP-1 cells per well), and allowed to adhere for 10 minutes. After nonadherent cells were removed, the fluorescent intensity of adhered and total cells applied to the well was measured by fluorescence plate reader (CytoFleur II, Perceptive Biosystems, Cambridge, Mass). The ratio of adherent to total cells was expressed as adhesion (%). Some experiments used freshly isolated human peripheral monocytes with apoCIII led to β₁-integrin activation and increased adhesion to vascular ECs under flow conditions. Here we examined whether pretreatment of ECs with apoCIII activates ECs to become proadhesive for leukocytes. Treating nonactivated ECs with apoCIII for 16 hours significantly increased THP-1 cell adhesion in a dose-dependent manner (Figure 1A). ApoCIII-induced THP-1 cell adhesion plateaued after 16 hours of incubation (Figure 1B). ApoCIII also induced the adhesion of human peripheral monocytes to ECs (Figure 1C). On the basis of these findings, all subsequent experiments were performed with the use of apoCIII at 100 μg/mL for 16 hours, unless indicated otherwise.

**Flow Conditions**

Adhesion experiments used a parallel plate flow chamber as previously described. Briefly, confluent HSVEC monolayers, grown on 25-mm glass coverslips (Carolina Biological Supply, Burlington, NC), were inserted into the flow chamber. THP-1 cells (0.5×10⁵/mL) suspended in flow buffer (PBS containing 0.1% human serum albumin) were drawn through the chamber at flow rates corresponding to an estimated shear stress of 1.0 dyne/cm². A video microscope determined THP-1 cell accumulation (rolling and firm adhesion) on ECs after 2 minutes of cell perfusion by counting the number of cells in 4 different fields.

**Immunoblotting**

To detect PKC activation, cytosol and membrane fractions of THP-1 cell lysates (1×10⁷/mL) were prepared as described previously. To detect NF-κB nuclear translocation and Ikβα cytosol degradation, cytosol and nuclear fractions of THP-1 cells (1×10⁷/mL) were prepared with the use of Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, Ill). An equal amount of protein (30 μg) from each fraction was subjected to 12% SDS-PAGE. Immunoreactive protein was detected with ECL Plus (Amersham Biosciences, Piscataway, NJ). Blots were quantified by densitometry, and their intensities were shown as percentage of those of β-actin, unless indicated otherwise.

**Statistical Analysis**

Results are presented as mean±SD. Data were analyzed with unpaired t test or 1-way ANOVA, with a value of P<0.05 considered significant.

**Results**

**ApoCIII Induces the Adhesion of THP-1 Cells or Human Peripheral Monocytes to ECs**

Our previous study showed that pretreatment of human monocytes with apoCIII led to β₁-integrin activation and increased adhesion to vascular ECs under flow conditions. Here we examined whether pretreatment of ECs with apoCIII activates ECs to become proadhesive for leukocytes. Treating nonactivated ECs with apoCIII for 16 hours significantly increased THP-1 cell adhesion in a dose-dependent manner (Figure 1A). ApoCIII-induced THP-1 cell adhesion plateaued after 16 hours of incubation (Figure 1B). ApoCIII also induced the adhesion of human peripheral monocytes to ECs (Figure 1C). On the basis of these findings, all subsequent experiments were performed with the use of apoCIII at 100 μg/mL for 16 hours, unless indicated otherwise.

**ApoCIII Induces Expression of ICAM-1 and VCAM-1 in ECs**

To investigate the underlying mechanism of apoCIII-induced THP-1 cell adhesion, we tested for induction of known leukocyte adhesion molecules on HSVECs. Incubating resting HSVECs with apoCIII increased VCAM-1 protein expression (4.9±1.5-fold increase versus control). ApoCIII also induced ICAM-1 in nonactivated ECs, albeit to a lesser extent (1.4±0.5-fold increase versus control) (Figure 2A). Function-blocking anti–VCAM-1 antibody essentially abolished apoCIII-induced THP-1 cell adhesion to ECs, whereas function-blocking antibody to ICAM-1 reduced adhesion by only 30%, indicating that VCAM-1 plays a dominant role in this process (Figure 2B).
Role of PKCβ in ApoCIII-Induced VCAM-1 Expression

We recently showed that apoCIII activated β1-integrins via a PKCα- and PKCβ-dependent mechanism in THP-1 cells. We therefore studied the effect of apoCIII on PKC activation in ECs. ApoCIII increased membrane-bound PKCβ in ECs, suggesting that activation had occurred. In contrast, apoCIII minimally affected PKCα activation (Figure 3A). Treatment of ECs with a selective PKCβ inhibitor prevented increased expression of VCAM-1 by apoCIII by 82% (Figure 3B). Hence, apoCIII activates PKCβ in ECs and correlates with induction of VCAM-1 expression.

Effect of ApoCIII on NF-κB Activation and IkBα Degradation in ECs

We next examined the effect of apoCIII on NF-κB activation in ECs. Incubation with apoCIII decreased cytosol IkBα and induced NF-κB nuclear translocation, reflecting NF-κB activation (Figure 4A). To investigate whether PKCβ was upstream of NF-κB activation, we pretreated EC with a PKCβ inhibitor and monitored NF-κB distribution. Pharmacological inhibition of PKCβ substantially reduced NF-κB p65 nuclear translocation and degradation of cytosolic IkBα in response to apoCIII (Figure 4B). NF-κB inhibitor peptide SN50, but not control peptide, inhibited increased expression of VCAM-1 by apoCIII (Figure 4C). Taken together, these results suggest that in ECs apoCIII triggers PKCβ activation, which leads to activation of NF-κB and induction of VCAM-1 expression.

VLDL CIII+ Induces Adhesion of THP-1 Cells to ECs

We next tested whether VLDL containing apoCIII (VLDL CIII+) also induces THP-1 cell adhesion. Preincubation of ECs with VLDL CIII+ increased THP-1 cell adhesion. In contrast, VLDL lacking apoCIII (VLDL CIII−) did not affect THP-1 cell adhesion (Figure 5A). The degree of THP-1 cell adhesion by VLDL CIII+ correlated with apoCIII concentration (figure in online-only Data Supplement). We then evaluated the ability of VLDL CIII+ to activate PKCβ and NF-κB in ECs and found that both molecules could be activated (Figure 5B, 5C). Preincubating VLDL
CIII\(^{-}\) with anti-apoCIII antibody inhibited VLDL CIII\(^{-}\)-induced PKC\(\beta\) activation (Figure 5B), whereas an isotype-matched IgG did not affect PKC\(\beta\) activation (data not shown). VLDL CIII\(^{-}\) activated NF-\(\kappa\)B and increased expression of VCAM-1 in ECs, which was inhibited by PKC\(\beta\) inhibitor (Figure 5C, 5D). VLDL CIII\(^{-}\) had a minimal effect on ICAM-1 expression in ECs (data not shown).

ApoCIII or VLDL CIII\(^{-}\) Induces the Adhesion of THP-1 Cells to ECs Under Flow Conditions

Finally, we examined the effects of apoCIII or VLDL CIII\(^{-}\) on THP-1 cell adhesion to ECs under flow conditions (Figure 6 and videos in the online-only Data Supplement). Few if any THP-1 cells accumulated on control (PBS)-treated ECs under laminar shear stress (1.0 dyne/cm\(^2\)). After incubation of ECs with apoCIII or VLDL CIII\(^{-}\), THP-1 cell adhesion increased significantly. Most of accumulated THP-1 cells adhered firmly rather than rolling (videos in the online-only Data Supplement). Accumulation of THP-1 cells induced by apoCIII or VLDL CIII\(^{-}\) was attenuated significantly in ECs pretreated with anti-VCAM-1 blocking antibody. In contrast, VLDL CIII did not affect THP-1 cell accumulation.

Discussion

This study tested the hypothesis that apoCIII exerts an atherogenic effect that is beyond its previously described effect on apoB lipoprotein metabolism. We demonstrated that apoCIII increases expression of adhesion molecules, especially VCAM-1, in nonactivated ECs, thus enhancing adhesion of THP-1 cells under static and laminar flow condition. Anti–VCAM-1 antibody inhibited THP-1 cell accumulation, thus validating a contribution of VCAM-1 to this process, as previously reported.\(^{3,6,22}\) Average plasma concentrations of apoCIII-containing apoB lipoproteins are 50 \(\mu g\) apoB/mL in normolipidemic persons and 100 \(\mu g\) apoB/mL in hypertriglyceridemic persons or those with CHD. ApoCIII mainly resides on VLDL fraction among apoB lipoproteins. In the present study, VLDL CIII\(^{-}\), but not VLDL CIII\(^{+}\), increased VCAM-1 expression in ECs and increased THP-1 cell adhesion at 100 \(\mu g\) apoB/mL, suggesting the clinical relevance of apoCIII effect on ECs.\(^{13}\) Although VLDL CIII\(^{-}\) was also rich in apoCI, apoCII, and apoE compared with VLDL CIII\(^{+}\) (apoCI/B mol, 26.7 versus 2.0; apoCII/B mol, 49.8 versus 4.3; and apoE/B mol, 9.2 versus 2.0 for VLDL CIII\(^{-}\) versus VLDL CIII\(^{+}\)), these apolipoproteins did not affect adhesion molecule expression activation (data not shown).

PKC participates importantly in several mechanisms that promote atherosclerosis.\(^{23}\) We recently showed that apoCIII activates PKC\(\alpha\) and PKC\(\beta\), but not PKC\(\delta\) or PKC\(\zeta\), in
THP-1 cells and induces their adhesion to ECs via activation of β integrins.15 Moreover, remnant lipoproteins rich in apoCIII activate PKC isoforms in human monocytic U937 cells16 and rat aortic smooth muscle cells.24 In the present study, VLDL CIII+ but not VLDL CIII− activated PKCβ in ECs, and anti-apoCIII antibody inhibited VLDL CIII−-induced PKCβ activation, suggesting that apoCIII in VLDL plays a pivotal role in PKCβ activation. PKCβ, which plays a role in inflammation in various types of cells, increases monocyte-endothelial interaction by mediating increase in VCAM-1 in ECs.25 We found that selective inhibition of PKCβ abolished induction of VCAM-1 by apoCIII, indicating its central role in apoCIII-induced EC activation.

This study identifies NF-κB as the molecular link between apoCIII-induced PKCβ activation and increased expression of VCAM-1. Distinct PKC isoforms stimulate NF-κB in different ways. Recently, Kouroedov et al26 reported that PKCβ activation by high glucose induces activation of NF-κB and increased expression of VCAM-1 in ECs. We show here that apoCIII induces 1β integrin degradation in the cytosol and translocation of NF-κB p65 to the nucleus in ECs. Dichtl et al5 showed that native VLDL from healthy subjects activated NF-κB in ECs in vitro and in vivo. Because a portion of VLDL ordinarily contains apoCIII,27 our results provide novel mechanistic insight into the activation of NF-κB by VLDL that is independent of lipid moieties and their oxidation.

The mechanism used by apoCIII to activate PKCβ in ECs remains unclear. Ca2+, phospholipids, and diacylglycerol activate conventional PKC enzymes including PKCβ23; however, we currently have little information about the direct effects of apoCIII on these molecules. The exact mechanism(s) for apoCIII-induced PKCβ activation in ECs will require further investigation. Our results (Figures 3A and 5B and figure in the online-only Data Supplement) suggest that apoCIII itself induces PKCβ activation. However, apoCIII delays hydrolysis of triglycerides and phospholipids in lipoproteins.10,28 Thus, VLDL with apoCIII may also contain distinct lipid components that activate PKCβ compared with VLDL without apoCIII.
Our results indicate that apoCIII and VLDL CIII induce VCAM-1 in ECs via a PKCβ and NF-κB activation pathway and increase THP-1 cell adhesion to ECs, suggesting a novel mechanism for EC activation by dyslipidemia. We also recently demonstrated that VLDL CIII activates β1-integrin in THP-1 cells, a ligand for VCAM-1.15 Thus, elevated levels of VLDL CIII may contribute to the firm adhesion of monocytes to vascular endothelium through an interaction between β1-integrin and VCAM-1.29,30 Our recent studies and the present studies, taken together, indicate that apoCIIIs exerts proinflammatory effects on both monocytes and ECs and suggest that lowering apoCIIIs may not only improve plasma VLDL metabolism but also may prevent the development of inflamed atherosclerotic plaques and their acute thrombotic complications.

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Disclosures

None.

References

The induction of adhesion molecules in vascular endothelial cells (ECs) and the subsequent recruitment of circulating monocytes are proinflammatory events that promote atherogenesis and plaque instability. Recent studies showed that patients with hypertriglyceridemia have high plasma levels of adhesion molecules and that dyslipidemia induced by an atherogenic diet or an oral fat loading increases endothelial and circulating cellular adhesion molecules. Apolipoprotein CIII (apoCIII) is a central component of dyslipidemia, and it circulates on apoCIII-containing apolipoprotein B lipoproteins such as VLDL and LDL. ApoCIII is an independent risk factor for coronary heart disease (CHD). We recently showed that apoCIII alone or as a component of VLDL or LDL induces the activation of protein kinase C (PKC) α and β2-integrin in human monocytes and increases their adhesion to ECs that had been exposed to interleukin-1β to induce adhesion molecules. The present study investigated whether apoCIII could itself induce adhesion molecules in ECs. The results were that apoCIII alone or apoCIII-rich VLDL increases EC expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 and recruitment of human monocytic THP-1 cells through the activation of PKCβ and nuclear factor-κB, demonstrating a novel mechanism for EC activation by VLDL that is independent of lipid moieties and their oxidation. Taken together, our recent studies indicate that apoCIII exerts proinflammatory effects on both monocytes and ECs through distinct PKC activation and suggest that lowering apoCIII may not only improve plasma VLDL metabolism but also may prevent the development of the inflamed atherosclerotic plaques and their acute thrombotic complications.
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