Apolipoprotein C-I Induces Apoptosis in Human Aortic Smooth Muscle Cells via Recruiting Neutral Sphingomyelinase

Antonina Kolmakova, Peter Kwiterovich, Donna Virgil, Petar Alaupovic, Carolyn Knight-Gibson, Sergio F. Martin, Subroto Chatterjee

Objectives—Apolipoprotein C-I (apoC-I) influences lipoprotein metabolism, but little is known about its cellular effects in aortic smooth muscle cells (ASMC).

Methods and Results—In cultured human ASMC, apoC-I and immunoaffinity purified apoC-I–enriched high-density lipoproteins (HDL) markedly induced apoptosis (5- to 25-fold), compared with control cells, apoC-I–poor HDL, and apolipoprotein C-III (apoC-III) as determined by 4′, 6-diamidino-2-phenylindole dihydrochloride staining and DNA ladder assay. Preincubation of cells with GW4869, an inhibitor of neutral sphingomyelinase (N-SMase), blocked apoC-I–induced apoptosis, an effect that was bypassed by C-2 ceramide. The activity of N-SMase was increased 2- to 3-fold in ASMC by apoC-I, apoC-I–enriched HDL, and tumor necrosis factor α (TNF-α) (positive control) after 10 minutes and then decreased over 60 minutes, which is a kinetic pattern not seen with controls, apoC-III, and apoC-I–poor HDL. ApoC-I and apoC-I–enriched HDL stimulated the generation of ceramide, the release of cytochrome c from mitochondria, and activation of caspase-3 greater than that found in controls, apoC-III, and apoC-I–poor HDL. GW4869 inhibited apoC-I–induced production of ceramide and cytochrome c release.

Conclusions—ApoC-I and apoC-I–enriched HDL activate the N-SMase-ceramide signaling pathway, leading to apoptosis in human ASMC, which is an effect that may promote plaque rupture in vivo. (Arterioscler Thromb Vasc Biol. 2004; 24:264-269.)

Key Words: apolipoprotein C-I ■ apoptosis ■ sphingomyelinase ■ high-density lipoproteins ■ tumor necrosis factor-α

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Apolipoprotein C-I and ApoC-I–Enriched HDL Particles Stimulate Apoptosis in ASMC

Cells incubated with apoC-I contained many apoptotic cells, as judged by white staining of the nuclei after treatment with 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI) reagent (Figure 1A). In contrast, cells incubated with apoC-III for 24 hours had only a few apoptotic cells (Figure 1A). After fluorescence microscopic quantitative analysis of DAPI staining of the nucleus in attached cells after treatment (Figure 1B), 2.24% of normal (control) cells, 4.78% of apoC-III–treated cells, and 26.19% of apoC-I–treated cells were apoptotic. In addition, ≈50% of ASMC incubated with apoC-I–enriched HDL particles were apoptotic; whereas only ≈2% of the cells incubated with apoC-I–poor HDL were apoptotic (Figure 1B; Table 1).

Apoptotic cells undergo endonucleosomal cleavage, resulting in the fragmentation of DNA into 180 to 200 base-pair fragments that resolve as a ladder on agarose gel electrophoresis. Cells incubated with tumor necrosis factor α (TNF-α) (positive control), or with apoC-I, exhibited DNA laddering. In contrast, control cells, and those incubated with apoC-III, did not exhibit significant DNA laddering (Figure 1C).

Neutral Sphingomyelinase Inhibitor, GW4869, Abrogates ApoC-I–Induced Apoptosis

As shown in Figure 2A and the corresponding densitometric scan (Figure 2B), N-SMase inhibitor, GW4869 (20 μmol/L), completely abrogated the apoC-I–induced apoptosis (≈12% compared with control ≈2%), an effect that was also observed using a lower dose (10 μmol/L) of GW4869. However, when C2-ceramide was added to the ASMC with apoC-I and GW4869, the inhibitory effect of GW4869 on N-SMase was bypassed and apoptosis was restored (Figure 2A and B).

Methods

Please see online Methods, available at http://atvb.ahajournals.org.

Results

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lation of N-SMase activity with apoC-I-enriched HDL was still manifested (2.2-fold) at 30 minutes and then approached baseline at 60 minutes (Figure 3B). ApoC-I–poor HDL also stimulated N-SMase activity in a similar pattern, but to a considerably lesser extent than apoC-I–enriched HDL (Figure 3B). ApoC-I exerted a concentration-dependent increase in the activity of N-SMase in ASMC (Figure 3C), with a maximum increase in N-SMase activity (1.5-fold) at a concentration of apoC-I of 2.5 μg/mL medium). In additional experiments to examine the specificity of this inhibitor, we found that GW4869 did not alter the acid sphingomyelinase activity (control: 400 nmol/mg protein; GW4869: 340 nmol/mg protein) in cultured human ASMC.

Antibody Against N-SMase Abrogates apoC-I–Enriched HDL–Induced N-SMase Activity in Cultured ASMC

Previous studies in human renal proximal tubular cells and neuronal cells have shown that N-SMase is localized on the outer leaflet of plasma membrane. Using N-SMase antibody and FITC-conjugated secondary antibody, we made similar observations in human ASMC (data not shown). We therefore preincubated ASMC with an antibody (IgG) against N-SMase (1:500 dilution) and found that anti-N-SMase, but not rabbit IgG (control), inhibited the stimulation of N-SMase activity.

Figure 2. Effect of apoC-I, the N-SMase inhibitor, GW4869, and C-2 ceramide on apoptosis in cultured human ASMC. A, ASMC (1 x 10⁶) were seeded on sterilized glass cover slips and grown in tissue culture medium with 10% fetal calf serum for 48 hours. Next, fresh serum-free medium, with and without GW4869 (20 μmol/L in DMSO), was added to some dishes. Thirty minutes later, vehicle DMSO, apoC-I (2.5 μg/mL), or C2-ceramide (10 μmol/L) were added to dishes and the incubation continued for 24 hours. The cells were then fixed with ethanol-acetic acid (3:1 v/v), stained with DAPI reagent, assessed by fluorescent microscopy, and photographed. Top to bottom: control cells; cells incubated with apoC-I ± the N-SMase inhibitor, GW4869; and cells incubated with apoC-I, GW4869, and C2-ceramide. B, The DAPI-stained ASMC from the four experimental conditions described was analyzed for quantitative apoptosis. Mean (SD) from 4 experiments. *P<0.05.

Figure 3. Effects of apoC-I, apoC-III, TNF-α, apoC-I–enriched HDL, and apoC-I–poor HDL on the activity of N-SMase in ASMC. Confluent cultures of ASMC were preincubated for 30 minutes with serum-free (control) medium, then apoC-I (2.5 μg/mL medium), apoC-III (2.5 μg/mL medium), TNF-α (20 ng/mL medium) (A, top), or apoC-I–enriched HDL (1 μg apoA-I/mL medium), or apoC-I–poor HDL (5 μg apoA-I/mL medium) (B, middle) were added individually to cells. The cells were then incubated and harvested at the indicated time points, and the activity was of N-SMase determined using 14C sphingomyelin as substrate (see Methods). The N-SMase activity in control cells was 22 nmol/h per milligram cell protein and this value was considered as 100% activity. In a separate experiment (C, bottom), cells (0.5 x 10⁶) were seeded in P60 Petri dishes and grown in DMEM supplemented with 10% fetal bovine serum for 5 days. After preincubation of cells for 30 minutes in serum-free medium, increasing amounts of apoC-I were added. After incubation for 10 minutes, cells were harvested and N-SMase activity measured as described in Methods.
by apoC-I–enriched HDL by 77%. This result suggested that apoC-I mediated the increased activity of N-SMase in human ASMC by the apoC-I–enriched HDL particle at the cell surface.

**ApoC-I and ApoC-I–Enriched HDL Particles Stimulate the Generation of Ceramide in Cultured Human ASMC**

ApoC-I exerted a time-dependent increase in ceramide levels, which reached a maximum effect (1.7-fold) by 5 minutes and then decreased to a value similar to that in control cells by 10 minutes (Figure 4A). Incubation of ASMC with apoC-I–enriched HDL particles with time reached a maximum level of ceramide by 30 minutes, whereas apoC-I–poor HDL did not. ApoC-I–enriched HDL particles stimulated the activity of N-SMase in human ASMC. Both apoC-I and apoC-I–enriched HDL particles stimulated the activity of N-SMase in ASMC in a time- and concentration-dependent fashion. Moreover, the kinetics of N-SMase activation by these two agonists was similar to that of TNF-α, in this study and in our previous studies. The tenet that apoC-I induced N-SMase activation and the subsequent production of ceramide were essential steps in the signaling cascade leading to apoptosis was substantiated by the following: (1) N-SMase inhibitor, GW4869, abrogated apoC-I–induced ceramide generation, cytochrome c release, and apoptosis; (2) preincubation of cells with antibody against N-SMase (but not the control rabbit IgG) prevented apoC-I–induced N-SMase activation and apoptosis; (3) incubation of cells with apoC-III, which has some structural homology with apoC-I, did not stimulate N-SMase activation and apoptosis; and (4) incubation of cells with apoC-I–poor HDL particles did not elevate N-SMase activity and apoptosis to the same extent as apoC-I–enriched HDL. These studies were performed in vitro, and future studies are needed to address the role of apoC-I in vivo.

**ApoC-I and ApoC-I–Enriched HDL Particles Stimulate the Release of Cytochrome c From Mitochondria in ASMC**

The release of cytochrome c from mitochondria into cytosol is a key pro-apoptotic event. ApoC-I stimulated the release of cytochrome c 4.1-fold. ApoC-III and apoC-I–enriched HDL particles also showed some stimulation of cytochrome c (2.3- and 1.2-fold, respectively) (Figure 5A and B). Using immunofluorescence, a marked increase in the release of cytochrome c from mitochondria into the cytoplasm with apoC-I (even greater than that with TNF-α) was noted, an effect that was inhibited by GW4869 (Figure 5C).

**ApoC-I and ApoC-I–Enriched HDL Particles Stimulate the Expression of Caspase-3 in Human ASMC**

ApoC-I stimulated the caspase-3 level 4-fold compared with control medium. ApoC-I–enriched HDL stimulated caspase-3 expression in ASMC 1.7-fold, whereas apoC-I–poor HDL did not. ApoC-III also stimulated caspase-3 expression, but to a lesser extent than apoC-I–enriched HDL (data not shown).

**Discussion**

The most striking phenotypic change in ASMC treated with apoC-I or apoC-I–enriched HDL particles was apoptosis that accompanied the activation of the sphingomyelinase signal transduction cascade. In additional studies, we observed that apoC-I induced the expression of Lyn-kinase protein (data not shown). Moreover, TNF-α–induced N-SMase activation in ASMC was inhibited by the use of tyrosine kinase inhibitors. Therefore, we postulate that by stimulating one or more membrane-bound protein kinases, apoC-I may activate N-SMase in human ASMC. Both apoC-I and apoC-I–enriched HDL particles stimulated the activity of N-SMase in ASMC in a time- and concentration-dependent fashion. Moreover, the kinetics of N-SMase activation by these two agonists was similar to that of TNF-α, in this study and in our previous studies. The tenet that apoC-I induced N-SMase activation and the subsequent production of ceramide were essential steps in the signaling cascade leading to apoptosis was substantiated by the following: (1) N-SMase inhibitor, GW4869, abrogated apoC-I–induced ceramide generation, cytochrome c release, and apoptosis; (2) preincubation of cells with antibody against N-SMase (but not the control rabbit IgG) prevented apoC-I–induced N-SMase activation and apoptosis; (3) incubation of cells with apoC-III, which has some structural homology with apoC-I, did not stimulate N-SMase activation and apoptosis; and (4) incubation of cells with apoC-I–poor HDL particles did not elevate N-SMase activity and apoptosis to the same extent as apoC-I–enriched HDL. These studies were performed in vitro, and future studies are needed to address the role of apoC-I in vivo.
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Recently, the use of GW4869, an N-SMase inhibitor, was shown in human breast cancer cells, MCF-7, to reduce agonist (TNF-α)-induced apoptosis.30 Using molecular cloning, this inhibitor was shown to be a target for N-SMase.31 Furthermore, recombinant acid sphingomyelinase activity and the activity of serine palmitoyltransferase (SPT) involved in de novo ceramide biosynthesis were shown to be unaffected in MCF-7 cells by GW4869 treatment.30 In addition, we also found that GW4869 does not alter the acid sphingomyelinase activity in cultured human ASMC. In our study, GW4869 (at 10 μmol/L and 20 μmol/L) completely inhibited apoC-I–induced apoptosis and apoC-I–induced ceramide generation (Figure 4C and D), suggesting that apoC-I may preferentially activate N-SMase to generate ceramide and induce apoptosis. Moreover, the increase in the cell level of ceramide after 24 hours of treatment with either apoC-I or GW4869 suggests that by this time, this inhibitor no longer prevented the apoC-I–induced increase in the cell ceramide level. Our findings in human ASMC are similar to those published by others using breast cancer cells.30 Clearly, additional studies are necessary to delineate further the biochemical pathways by which apoC-I can generate ceramide and induce apoptosis.

HDL particles contain several other apolipoproteins, as well as lipids, in addition to apoC-I (Table 1). We therefore cannot exclude the possibility that other apolipoprotein and lipid moieties, independently or collectively, contribute to N-SMase activation and apoptosis. In addition, the presence of oxidized cholesterol in caveolae may activate a signaling pathway leading to apoptosis.32 However, our studies included a control, namely immunoaffinity-purified apoC-I–poor HDL particles, whose lipid composition was quite similar to that of the immunoaffinity-purified apoC-I–enriched HDL (Table 1).

Although several studies point to the role of oxidized LDL and its components in inducing apoptosis in cells of the vascular wall, little is known about various apolipoproteins and their effect on apoptosis. Our previous study showed that apolipoproteins B, A-I, A-II, and E did not alter apoptosis in cultured human ASMC. Only very high doses of LDL (>200 μg/mL medium) induced cell death, an action mostly caused by a necrotic/cytotoxic effect. HDL had either no effect on cell death or rescued cells from LDL induced necrosis.33,34 In humans, apoC-I may be atherogenic by delaying the clearance of remnant lipoproteins.20,21 Based on our present report, we postulate that increased plasma levels of apoC-I, perhaps associated with HDL, might also contribute to the complications of atherosclerosis by inducing apoptosis in ASMC, a biochem-

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ApoC-I–enriched HDL and apoC-I–poor HDL were isolated from the heparin manganese supernatants of human plasma from umbilical cord blood by immunoaffinity chromatography as described in Methods. ApoC-I–enriched HDL is the apoA-II–retained, apoC-I–retained fraction of LpA-I:A-II particles. ApoC-poorest HDL is the apoA-II–retained, apoC-I–unretained fraction of LpA-I:A-II particles.
A physiological mechanism that might contribute to plaque rupture and coronary artery disease.

References


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METHODS

Materials

$^{14}$C Sphingomyelin (specific activity 50 mCi/mmol) was from American Radiolabeled
Chemicals (St.Louis, MO), $^{32}$γ ATP (specific activity 3000 Ci/mmol) was from New
England Nuclear Company (Boston, MA). Diacylglycerol kinase from Streptomyces
species was from Calbiochem (San Diego, CA). Other chemicals were from Sigma
Chemical Company (St.Louis, MO). A rabbit polyclonal antibody (IgG) against human
urinary neutral sphingomyelinase was prepared and characterized as described previously
(1). This antibody specifically immunoprecipitates N-SMase from both human kidney
and cultured ASMC in Western immunoblot assay, and inactivates ~80% of enzymatic
activity in cultured human kidney cells (2). A neutral sphingomyelinase inhibitor,
GW4869, was purchased from Calbiochem (San Diego, CA). Human apoC-I and apoC-
III were purchased from Academy Bio-Medical Company, Houston, TX and were >99%
homogenous by SDS gel-electrophoresis and were lipopolysaccharide free. Antibody
against ceramide (IgM enriched mouse anti-ceramide) was purchased from Glycobiotech
(Germany).

Isolation of human apoC-I-enriched HDL and apoC-I-poor HDL

Two plasma pools (A and B) were prepared from cord blood. Pool A and pool B
consisted of plasma from normal infants with increased or decreased amounts of large
HDL particles, respectively. The levels of apoC-I in pool A and B were 7.2 mg/dL and
3.9 mg/dL, respectively. The apoB-containing lipoproteins (VLDL, IDL, LDL, and Lp
(a) lipoprotein) were then precipitated with heparin manganese (3). No apoB was
detected in the heparin manganese supernatant. All of the apoC-I remained with HDL in
the supernatants. The supernatants were then dialyzed against the running buffer, aliquots applied to an antiapoA-II immunosorber, and the unretained plasma proteins and Lp-A-I particles separated from the retained Lp-A-I: AII particles as described (4). The retained Lp-A-I: A-II were eluted with the running buffer, the sodium thiocyanate removed, and the concentrated Lp-A-I: A-II particles applied to an anti-apoC-I-immunosorber (prepared with affinity purified apoC-I antibodies (4). The apoA-II retained-apoC-I unretained fraction was separated from the apoA-II retained, apoC-I retained fraction as described (4). The apoA-II retained, apoC-I retained particles from pool A, and the apoA-II retained, apoC-I unretained particles from pool B constituted the apoC-I-enriched HDL and the apoC-I-poor HDL, respectively. Following dialysis against the starting buffer, aliquots of both fractions were taken for determining the lipid and apolipoprotein composition (see also below). These two fractions were then stored frozen at – 80 °C until used for cell experiments. The fractions were thawed at that time, dialyzed against the culture medium (see below), and aliquots added to the cell culture dishes.

**Lipid and apolipoprotein composition of apoC-I-enriched and apoC-I-poor HDL**

Neutral lipids were determined by gas chromatography in apoC-I-enriched HDL and apoC-I-poor HDL by a modified procedure (5) of the method of Kuksis et al (6). Phospholipids were measured by the method of Gerlach and Deuticke (7). The quantitative determination of apolipoproteins was performed by electroimmunoassay for apolipoprotein A-I and apolipoprotein A-II (8), apolipoprotein B (9), apoC-I and apolipoprotein C-II (10), apoC-III (11), apolipoprotein D (12), and apolipoprotein E (13).
**Cultured Cells**

Human ASMC were purchased from Cambrex (Walkersville, MD) and cultured in Dulbecco’s minimal essential medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Hyclone, Salt Lake City, UT), 100-units/ml penicillin, and 100-µg/ml streptomycin. The cells were incubated in serum-free medium for 30 min prior to initiating the experiments. Cells were then washed and harvested in PBS, and centrifuged at 1500 rpm for 5 min.

**Assessment of apoptosis.**

*Fluorescence microscopic-quantitative assay of apoptotic death.* 1 x 10^3 cells were grown on sterilized glass cover slips in 6-well trays and treated with apoC-I (2.5 µg/ml medium), apoC-III (2.5 µg/ml medium), tumor necrosis factor TNF-α (20 ng/ml), apoC-I-enriched HDL (1 µg apoA-I/ml medium) and apoC-I-poor HDL (5 µg apoA-I/ml medium). After 24 h incubation, the medium was removed, cells fixed with ethanol: acetic acid 3:1 (v/v), stained with DAPI (4’, 6-diamidino-2-phenylindole dihydrochloride) reagent and nuclei were visualized by fluorescence microscopy (Zeiss Axiovert 25). In some experiments, fresh serum free medium, with or without the N-SMase inhibitor, GW4869 (20 µM), was added to some dishes. Thirty min later, vehicle DMSO, apoC-I (2.5 µg/ml), or C2-ceramide (10 µM), were added to dishes and the incubation continued for 24 h.

*DNA Laddering assay.* Cells were treated with either TNF-α (20 ng/ml), apoC-I (2.5 µg/ml) or apoC-III (2.5 µg/ml) for 6 h. Genomic DNA was subjected to electrophoresis, stained with ethidium bromide and the gel was photographed. The gel was calibrated using DNA fragments of known molecular weight.
**Neutral sphingomyelinase assay.** After stimulation with apoC-I, apoC-I-enriched HDL and apoC-I-poor HDL, the N-SMAse assay was performed employing $^{14}$C sphingomyelin as substrate as described (14).

**Measurement of the ceramide level.** Ceramide was measured using the sn-1, 2-diacylglycerolkinase-assay and labeling for 30 min with 1 mCi of $^{32}$γ ATP in 10 µl of 5mM ATP (15). Ceramide-1-phosphate and diacylglycerol-1-phosphate were resolved by thin-layer chromatography on Silica Gel plates (Whatman, Clinton, NJ), using a solvent composed of chloroform-acetone-methanol-acetic acid-water 10:4:3:2:1(v/v) and detected by autoradiography using an Instant Imager (Packard Canberra Company). Ceramide was expressed as pmol ceramide/nmol phosphate.

**Measurements of cytochrome c release and caspase activation**

After incubation with apoC-I (2.5 µg/ml medium), apoC-III (2.5 µg/ml medium), apoC-I-enriched HDL (1 µg apoA-I/ml medium), or apoC-I-poor HDL (5 µg apoA-I/ml medium) the cells were subjected to extraction with a buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl$_2$, 5 mM DTT, 2 mM phenyl-methylsulfonyl fluoride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 µg/ml leupeptin, 2 µg/ml aprotinin, 0.5 µg/ml benzamidine and 250 mM sucrose) on ice for 30 min. Next, the samples were centrifuged at 10,000 x g at 4°C. The supernatant was utilized as a source of cytochrome c in Western immunoblot assay. The pellets were lysed in RIPA buffer (phosphate buffer saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS and cocktail of inhibitors described above) and the incubation continued for 60 min on ice. The supernatants were used as a source of protein for Western immunoblot assays to determine caspase activation. Proteins were separated using 4-15% SDS-polyacrylamide
gel under denaturing conditions and electro-transferred onto nitrocellulose. Membranes were then incubated with primary antibody (anti cytochrome c and anti caspase-3 rabbit polyclonal antibody 1:200 (Santa Cruz Biotechnology) and horseradish labeled anti-rabbit antibody (1:2000). In some experiments, confluent cells were grown on cover-slips and pretreated with serum-free medium for 30 min. The cells were then incubated with control medium, apoC-I (2.5 µg/ml medium), TNF-α (20 ng/ml), or apoC-I (2.5 µg/ml) + GW4869 (20 µM) for 24 hr.

**Immunofluorescence of ceramide and cytochrome c in cultured human aortic smooth muscle cells.** ASMC (5x10³) were grown on glass cover-slips and then incubated with apoC-I or TNF-α for 24 h, fixed for 15 min in 4% paraformaldehyde, washed with PBS and permeabilized during a 5 min incubation in 1% Triton X-100 in PBS, washed again and treated for 1h with a IgM-enriched anti-ceramide or anti-cytochrome c 1:100. The cover slips were then washed and incubated for 1h with a fluorescein isothiocyanate-conjugated goat-anti-mouse IgG (1:100)(Sigma Chemical Company, St.Louis, MO) or fluorescein isothiocyanate-conjugated anti-rabbit IgG for 1 h. The cells were photographed on a Zeiss Axiovert 25 fluorescent microscope.

**Statistical analysis**

Results are expressed as mean ± SD. Statistical analysis was performed with Student’s t-test and values of * p ≤ 0.05 were considered significant.
References


9. Curry MD, Gustafson A, Alaupovic P, McConathy WJ. Electroimmunoassay,


