Abstract Withdrawing growth factors or serum from endothelial cells leads to the activation of effector caspases 3 and 7, resulting in apoptotic cell death. HDL protects against caspase induction through sphingosine-1-phosphate (SIP) receptors. This anti-caspase activity of HDL is antagonized by VLDL from apolipoprotein E4 (apoE4) (genotype, APOE4/4; apolipoprotein, apoE) targeted replacement (TR) mice, but not by VLDL from TR APOE3/3 mice, and requires the binding of apoE4-VLDL to an LDL receptor family member. In the absence of HDL, apoE4-VLDL and apoE3-VLDL from TR mice have limited antiapoptotic activity. In contrast, we show here that a high-fat/high-cholesterol/cholate diet (HFD) radically alters this biological activity of VLDL. On HFD, both apoE3-VLDL and apoE4-VLDL (HFD VLDL) inhibit caspase 3/7 activation initiated by serum withdrawal. This activity of HFD VLDL is independent of an LDL receptor family member but requires the activation of SIP3 receptors, as shown by the ability of pharmacological block of SIP receptors by VPC 23019 and by small interfering RNA-mediated downregulation of SIP3 receptors to inhibit HFD VLDL anticaspase activity.—Mihovilovic, M., J. B. Robinette, R. M. DeKroon, P. M. Sullivan, and W. J. Strittmatter. High-fat/high-cholesterol diet promotes a SIP receptor-mediated antiapoptotic activity for VLDL. J. Lipid Res. 2007. 48: 806–815.

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Plasma lipoproteins influence the clinical expression of atherosclerosis. HDLs inhibit, whereas VLDLs and LDLs promote, atherosclerotic plaque formation (1, 2). HDL inhibits apoptosis of human umbilical vein endothelial cells (HUVECs) initiated by growth factor and serum withdrawal. In vivo, HDL may inhibit dysfunction of the vascular endothelium, decreasing the subsequent infiltration of monocytes into vessel walls and retarding atherosclerosis (3). In contrast, in vivo, the sustained presence of VLDLs, chylomicron remnants, and low density lipoproteins accelerates atherosclerotic plaque formation (4, 5).

The mechanism by which HDL inhibits endothelial cell death has been investigated extensively by Nofer et al. (6, 7) and Kimura et al. (8). Nofer et al. (6, 7) have shown that HDL particles dock with the scavenger receptor class B type I (SR-BI) and activate sphingosine-1-phosphate (SIP) receptor 3 (SIP3)/endothelial differentiation gene (EDG3) through lysosphingolipids in the HDL particle. The subsequent phosphorylation of Akt inhibits caspase 3/7 activation and thereby inhibits apoptosis. Kimura et al. (8) subsequently reported the participation of SIP1 in HUVEC cell survival. Previous work by us demonstrated that apolipoprotein E4 (apoE4)-VLDL, from APOE4/4 mice on a regular chow diet, antagonizes this ability of HDL to inhibit caspase 3/7 (9). ApoE4-VLDL inhibits HDL anticaspase activity through an LDL receptor family member, because this activity of apoE4-VLDL is prevented by the receptor-associated protein (RAP), which inhibits apoE binding to these receptors (10, 11). In contrast to apoE4-VLDL, apoE3-VLDL does not inhibit HDL activity. The mechanism conferring this apoE isoform specificity is not known.

High-fat/high-cholesterol/cholate diets (HFDs) are proatherogenic and markedly alter plasma lipoprotein particle number, distribution, and composition (12, 13). We used a cholate-containing HFD previously shown to markedly increase plasma cholesterol and to accelerate atherosclerosis (14). Here, we report that VLDL from targeted replacement (TR) APOE3/3 and APOE4/4 mice on this high-fat/high-cholesterol diet (HFD VLDL), unlike VLDL from mice on a regular chow diet, drastically

Abbreviations: apoE, apolipoprotein E; EDG, endothelial differentiation gene; FCS, fetal calf serum; HFD, high-fat/high-cholesterol/cholate diet; HUVEC, human umbilical vein endothelial cell; RAP, receptor-associated protein; SFM, serum-free medium; siRNA, small interfering RNA; SIP, sphingosine-1-phosphate; SR-BI, scavenger receptor class B type I; TR, targeted replacement.

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inhibited the activation of caspase 3/7 in HUVECs initiated by acute serum withdrawal. In the HFD VLDL fraction, both apoA-I-containing chylomicron remnants and apoA-I-free particles possess anticaspase activity. Incubation of HUVECs, whose survival depends on S1P receptors, with sphingosine-1-phosphate (SIP3) antagonist VCP 23019 (17) decreased the anticaspase activity of both HDL and HFD VLDL, as it does small interfering RNA (siRNA)-mediated downregulation of SIP3 receptors. In contrast to apoE4-VLDL from APOE4/4 mice on a regular chow diet, HFD VLDL does not antagonize the antiapoptotic activity of HDL. Thus, we report that this cholate-containing HFD promotes an antiapoptotic activity of VLDL, independent of APOE genotype, through the activation of SIP3 receptors.

MATERIALS AND METHODS

Materials

HUVECs were obtained from the American Type Culture Collection (Manassas, VA) through the Cell Culture Duke Cancer Facility; SIP3 antagonist VCP 23019 and SIP were from Avanti (Alabaster, AL). If not indicated in the text, reagents were obtained from Sigma (St. Louis, MO).

HFD

Groups of 14 to 20 TR male mice homozygous for human apolipoprotein alleles E3 or E4 (APOE3/3 or APOE4/4) (12, 13) were kept on either a regular chow diet (Prolab Isopro Agway, Inc., Dewitt, NY) or an atherogenic high-fat/cholate diet (14) (TD 88051; Harlan-Teklad, Madison, WI) for 8 weeks. At the start of the diet, mice were at least 15 weeks old, and by the end of the diet they did not exceed 37 weeks of age. All animal handling and procedures followed the directives of the institutional animal care and use committee.

Cell culture and induction of apoptosis

HUVECs were grown in EBM-2 Clonetics medium (BioWhittaker, Inc., Wakersville, MD) supplemented with 2% fetal bovine serum. Cells were grown to near confluence on 96-well plates (Phoenix Research Products, Hayward, CA). To induce apoptosis, fetal calf serum (FCS)-containing medium was removed and cells were washed with RPMI, as reported previously (9). Each well received 70 μl of a) serum-free medium (SFM), consisting of RPMI 1640 supplemented with 0.2 μg/ml hydrocortisone and 1.0 μg/ml ascorbic acid (Sigma); b) SFM supplemented with 20% characterized FCS (HyClone, Logan, UT); or c) SFM supplemented with the indicated concentrations of lipoproteins. Caspase 3/7 activity was measured after 9 h of serum withdrawal within the linear response curve for caspase induction (4–12 h).

Caspase 3/7 activity assay

Caspase 3/7 activity was measured using the Apo-ONE assay developed by Promega (Madison, WI) using a 1:200 dilution of substrate as reported previously (9). Caspase activity (%) was determined by subtracting the relative fluorescence units obtained in the presence of 20% FCS from that obtained in SFM and assigning 100% caspase activation to this difference. Caspase activity (%) of the experimental conditions was expressed relative to this difference. Cells in SFM produce photofluorescence readings of at least 750 relative fluorescence units (caspase 3/7 activity at room temperature, 12.5 pmol/ml rhodamine 111 released/h/well). Cells in 20% serum typically yield values between 5% and 10% of those obtained with cells in SFM.

Preparation of lipoprotein fractions

Lipoproteins were purified from pooled plasma of fasted adult mice through isopycnic flotation as described previously (9, 18, 19). Plasma was adjusted to 5 mM EDTA by the addition of 1.0 M EDTA, pH 7.5. VLDL fractions were floated at a density of 1.006 g/ml; intermediate density lipoprotein (corresponding to human intermediate density lipoprotein/LDL) fractions were floated at a density >1.006 g/ml and <1.063 g/ml; HDL fractions were floated at a density >1.136 g/ml and <1.25 g/ml; and total lipoproteins were floated at a density of 1.25 g/ml. Both HDL and total lipoproteins were refloated at a density of 1.25 g/ml to obtain the purified lipoproteins. VLDL was not refloated. Lipoproteins were dialyzed against 0.9% NaCl, 5 mM EDTA, pH 7.5 (for storage at 4°C) or 0.9% NaCl, pH 7.5 (for immediate use). After dialysis, lipoproteins were filtered through Millex-GV 0.22 μm filters (Millipore, Bedford, MA), kept at 4°C, and used within 8 weeks of preparation; EDTA-stored preparations were used up to 16 weeks after preparation. Lipoprotein concentrations are expressed as cholesterol concentration.

Depletion of apoA-I-rich lipoprotein particles from HFD VLDL fractions

HFD VLDL fractions were depleted of apoA-I-containing lipoproteins by incubation with anti-apoA-I magnetic beads (see below). Incubation was for 2.5 h at room temperature with gentle stirring. Nonbound lipoproteins were then recovered and characterized by SDS-PAGE and agarose electrophoresis; their concentration was assessed through cholesterol determination. Nonretained HFD apoE4-VLDL was 35.08 ± 0.66% (n = 3) of total VLDL; nonretained HFD apoE3-VLDL was 45.70 ± 5.91% (n = 2) of total VLDL. Mock apoA-I VLDL depletion was performed with anti-goat IgG magnetic beads in lieu of anti-apoA-I magnetic beads. SDS-PAGE analysis revealed complete depletion of apoA-I-containing particles after incubation with anti-apoA-I immunosorobent.

Anti-apoA-I magnetic bead preparation

Anti-apoA-I magnetic beads were prepared by incubating anti-goat magnetic beads (catalog number 605-718-002; Rockland Immunochemicals, Inc., Gilbertsville, PA) with goat anti-mouse apoA-I polyclonal antibody (catalog number 600-101-196; Rockland Immunochemicals, Inc.). Anti-goat magnetic beads were washed four times with 0.9% NaCl, pH 7.5, resuspended to 1/20th of their original volume, and incubated for 1 h at room temperature with anti-apoA-I antibody dialyzed against 0.9% NaCl, pH 7.5. Optimal ratios of bead to antibody were determined by measuring the antibody binding capacity of the beads by titration of a fixed amount of beads with increasing concentrations of antibody. Beads were added in slight excess over antibody to ensure that unbound apoA-I antibody was absent from the final eluted lipoprotein fraction. Anti-apoA-I-loaded beads were washed once with 0.9% NaCl, pH 7.5, before use. Thirty milliliters of magnetic beads bound 0.6 mg of the antibody and fully retained the apoA-I-containing particles in ~2 mg of HFD VLDL (VLDL amount expressed in cholesterol).

siRNA-mediated downregulation of SIP3 receptor

SIPA3 sense and antisense oligoribonucleotides corresponding to the open reading frame sequence 799–818 of gene
EDG3 (accession number NM_005226) and control nontargeted, nonfunctional, double-stranded oligoribonucleotide (product D-001220-01-05) were obtained from Dharmacon RNA Technologies (Chicago, IL). S1P3 oligonucleotides were annealed as recommended by the manufacturer and their concentrations determined measuring optical density at 260 nm.

HUVECs grown on six-well plates at ~80% confluence were transfected with the annealed oligoribonucleotides complexed with Oligofectamine (Invitrogen, Carlsbad, CA) to give a final concentration of oligoribonucleotide of 429 nM and using 8 μL of Oligofectamine per milliliter of transfection medium (Clonetics EBM-2, 10 mM HEPES, pH 7.5). After 3 h of incubation, transfection medium was supplemented with 0.3 volume of Clonetics EBM-2 made 2% in FCS, and cells were grown for an additional 48 h. Cells were harvested after trypsinization and plated on 96-well plates at a density of 10,000 cells per well. Replated cells were grown for 24 h before serum withdrawal. In control experiments, a mock control was also included in which neither Oligofectamine nor siRNAs were present in the transfection mixtures.

RNA purification

Total RNA was purified as described previously using STAT-60 (Tel-Test B, Friendswood, TX) (20); RNA was extracted with phenol-chloroform by adding STAT-60 directly to adherent HUVECs. RNA was precipitated by addition of isopropanol, then pellets were washed with 75% ethanol, dried, and resuspended in 1 mM Tris-Cl, pH 7.5, 0.2 mM EDTA. RNA concentration was determined through absorbance at 260 nm, based on 40 μg/ml RNA having an absorbance of 1.00 optical density unit.

S1P3 receptor and β-actin amplification of reverse-transcribed RNA (RT-PCR)

Downregulation of S1P3 receptor expression was assessed through RT-PCR. Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). S1P3 receptor primers were as follows: forward primer 57F (agggactagggctgag in exon 1) spanning nucleotides 57 through 75 of the spliced transcript of gene EDG3 (accession number NM_005226) and reverse primer 474R (ccccagcttactggtaatgct in exon 2) spanning nucleotides 455 through 474. β-Actin primers were as follows: forward primer 467F (agttgaccttagcttcagc) in exon 3) spanning nucleotides 467 through 486 of the spliced transcript of gene ACTB (accession number NM_001101) and reverse primer 655R (tggtgaggttatcaggt in exon 3) spanning nucleotides 636 through 655.

cDNA was synthesized using Multiscribe reverse transcriptase and was amplified using the GeneAmp Gold RNA PCR reagent kit (Applied Biosystems, Inc., Foster City, CA) according to the guidelines provided by the manufacturer but included a 10 min 65°C primer annealing step before cDNA synthesis. After hot start activation of the polymerase, cycling for S1P3 receptor cDNA was as follows: 5 cycles of 2 min of denaturation at 94°C, followed by 1 min of annealing at 62°C and a 1 min extension at 72°C, and 40 cycles of 1 min of denaturation at 94°C, followed by 1 min of annealing at 62°C and a 1 min extension at 72°C; cycling for β-actin cDNA consisted of 5 cycles of 2 min of denaturation at 94°C, followed by 1 min of annealing at 60°C and a 1 min extension at 72°C, followed by 20 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 60°C, and a 1 min extension at 72°C. S1P3 receptor RT-PCR was performed using 155 to 800 ng of total RNA; β-actin RT-PCR was performed using 80 to 200 ng of total RNA. Omission of reverse transcriptase did not produce amplification products. Amplicons were resolved through 1.5% agarose-ethidium bromide electrophoresis as described previously (20); gels were scanned using a Kodak Image Station 440CF (New Haven, CT), and signal intensity was quantified using ImageQuant software (GE Health Care Life Sciences, Piscataway, NJ). Experimental conditions were chosen to obtain ethidium bromide-stained electrophoretically resolved RT-PCR products whose fluorescence intensities were directly proportional to their RNA concentrations.

Lipoprotein cholesterol and S1P and dihydro-S1P determination

Cholesterol concentration was determined using a cholesterol oxidase-based assay (Thermo Electronics, Louisville, CO) as reported previously (9) using a cholesterol standard (catalog number C7500-STD) obtained from Point Scientific, Inc. (Lincoln Park, MI).

Analyses of S1P and dihydro-S1P lipoprotein concentration were performed by the Lipidomics Core at the Medical University of South Carolina (Charleston, SC) on a Thermofinnigan TSQ 7000, triple-stage quadrupole mass spectrometer operating in multiple reaction monitoring positive ionization mode (21).

Electrophoresis and Western blots of lipoprotein

Agarose electrophoresis was performed using the Titan lipid-protein gel system (Helena Laboratories, Allen Park, MI) according to the manufacturer’s instructions. Fat Red was used to visualize lipids. SDS-PAGE was performed using Bio-Rad Criterion (Hercules, CA) 4–15% gels. Western blotting was performed as described previously (9). ApoE was detected by indirect immunostaining using goat anti-human apoE (1:2,000) (Calbiochem) followed by HRP-conjugated bovine anti-goat IgG (1:2,500) (Santa Cruz Biotechnologies, Santa Cruz, CA) and the blots developed through ECL detection (Amer sham, Piscataway,NJ). 4-Hydroxy-2-nonenal Michael adducts were detected using anti-4-hydroxy-2-nonenal Michael adducts (1:100) (Calbiochem) followed by HRP-conjugated goat anti-rabbit IgG (1/3,000) (Cell Signaling Technology, Danvers, MA).

ApoE ELISA

ApoE ELISA was performed using an apoE4/Pan-apoE ELISA kit (MBL International Corp., Woburn, MA) according to the manufacturer’s instructions.

Statistical analysis

Results are presented as means ± SEM. Data presented are the result of at least two independent experiments typically comprising a total of 12 independent determinations per experimental group. Data analysis was done with the GraphPad InStat 3 software using either paired or unpaired ANOVA depending on the experimental population. For one data set (see Fig. 7A), data did not conform to a normal Gaussian distribution; in this case, the results of the Wilcoxon matched-pairs signed-rank test are reported. For statistical analysis, data presented in Table 1 were grouped by diet (HFD VLDL, n = 7; regular diet VLDL, n = 6); the regular diet data set did not conform to a Gaussian distribution and required nonparametric analysis.

RESULTS

HFD alters lipoprotein particle distribution and composition

Feeding APOE3/3 and APOE4/4 TR mice a HFD instead of a regular chow diet markedly increased cholesterol and apoE concentrations in plasma VLDL, making this lipoprotein fraction the main contributor to the total lipoprotein cholesterol and S1P and dihydro-S1P concentration.
pool of lipoproteins (Fig. 1A, upper and middle panels). On HFD, most apoE was associated with preβ-migrating particles (Fig. 1B). In APOE3/3 mice, HFD VLDL cholesterol content increased more than apoE content, suggesting that these particles are relatively apoE-poor compared with apoE3-VLDL particles from regular chow-fed mice (Fig. 1A, lower panel). In contrast, VLDL from APOE4/4 mice on the HFD showed little change in apoE content per microgram of cholesterol, compared with VLDL from regular diet APOE4/4 mice (Fig. 1A, lower panel).

The amount of apoA-I associated with VLDL was increased markedly by the HFD in both APOE3/3 and APOE4/4 mice (Fig. 1C) (12, 13). ApoA-I associates with chylomicrons (22–24). The increase in VLDL apoA-I levels in the plasma of fasted mice on the HFD results from a large increase in chylomicron remnant particles produced by this diet.

Neither HFD VLDL nor regular diet VLDL showed detectable levels of oxidative damage, as indicated by the absence of malondialdehyde or 4-hydroxy-2-nonenal-modified proteins (data not shown).

**HFD apoE3- and apoE4-VLDL protect endothelial cells from apoptosis**

The withdrawal of serum from HUVECs initiates the extrinsic apoptotic cascade that activates effector caspases 3 and 7 and ultimately results in apoptotic cell death (6, 25). Therefore, caspase 3/7 activity can be used as a measure of apoptosis. Withdrawal of serum from HUVECs increased caspase 3/7 activity (Fig. 2). Addition of apoE3- or apoE4-VLDL from mice on the HFD inhibited the increase in caspase 3/7 activity (Fig. 2; see supplementary Fig. 1). In contrast, VLDL from mice on a regular chow diet had little or no effect on caspase 3/7 activity.

**HFD apoE4-VLDL does not antagonize the anticaspase activity of HDL**

We reported previously (9) and also show in Fig. 3 that apoE4-VLDL (but not apoE3-VLDL) from mice on a regular chow diet antagonizes the antiapoptotic activity of HDL. We tested the activity of apoE3-VLDL and apoE4-VLDL using HDL concentrations that reduce anticaspase 3/7 activity by 30–65% (Fig. 3) (9). Regardless of the magn-

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**Fig. 1.** Effects of a high-fat/high-cholesterol/cholate diet (HFD) on the plasma lipoprotein composition of APOE3/3 and APOE4/4 targeted replacement (TR) mice. A: Cholesterol and apolipoprotein E (apoE) content of total plasma lipoproteins, VLDL, intermediate density lipoprotein (IDL), and HDL fractions from APOE3/3 and APOE4/4 mice on either a regular chow diet or the HFD. HFD increases both total cholesterol and apoE, primarily in VLDL (upper and middle panels). HFD VLDL and intermediate density lipoprotein particles from APOE3/3 mice have lower apoE content per unit of cholesterol than regular diet lipoprotein particles (lower panel). In contrast, the apoE content of APOE4/4 VLDL is not influenced by diet. B: Agarose electrophoresis and apoE Western blots of plasma from APOE3/3 and APOE4/4 mice maintained on either regular chow or the HFD (2 μl of plasma per well). Preβ-migrating particles constitute the majority of the HFD lipoproteins, and apoE is distributed almost exclusively with them. C: SDS-PAGE of regular chow and HFD VLDL (10 μg of lipoprotein cholesterol per well). HFD increases apoA-I content.
nitude of HDL anticaspase activity, VLDL derived from APOE3 mice fed a regular diet did not antagonize HDL, whereas regular diet apoE4-VLDL did (Fig. 3) (9).

In contrast, neither HFD apoE4-VLDL nor HFD apoE3-VLDL antagonized the anticaspase activity of HDL, as shown in Fig. 3 (right and left panels respectively) and supplementary Fig. II.

Both HFD apoA-I-rich remnants and HFD apoA-I-free apoE4-VLDL particles have anticaspase activity

Purified VLDL fractions from plasma of fasted mice floated at a density of 1.006 g/ml contain both apoA-I-rich chylomicron remnants and apoA-I-free VLDL. Fasted mice on a regular chow diet have small amounts of apoA-I-rich chylomicron remnants. In contrast, VLDL from mice on the HFD contains a high proportion of these apoA-I-rich remnants (on a cholesterol basis, 64.92% and 54.30% of the total HFD apoE4- and apoE3-VLDL, respectively) in addition to apoA-I-free VLDL (Figs. 1C, 4B) (13). Therefore, we investigated whether apoA-I-rich particles possessed anticaspase activity or prevented HFD apoE4-VLDL from antagonizing HDL activity. We depleted HFD VLDL fractions of the apoA-I-rich remnants using anti-apoA-I antibodies conjugated to magnetic beads. Total lipoproteins in the VLDL fraction, the apoA-I remnant-depleted VLDL fraction, and the mock-depleted VLDL fraction were analyzed by gel electrophoresis. All were preβ-migrating particles (Fig. 4A) with similar apoE content (Fig. 4B), and all showed equivalent anti-caspase 3/7 activities per unit of cholesterol (Fig. 5A). These observations indicate that both apoA-I-containing and apoA-I-free particles in the HFD VLDL fraction contribute to anticaspase activity of the HFD VLDL fraction and that HFD VLDL anticaspase activity does not require apoA-I. In addition, none of the three fractions inhibited the anticaspase activity of HDL (Fig. 5B). HFD apoE3-VLDL (data not

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**Fig. 2.** Effects of diet on VLDL anticaspase 3/7 activity. Caspase 3/7 activity was assayed after serum withdrawal and the addition of lipoprotein fractions. Compared with regular diet, HFD VLDL of either APOE3/3 or APOE4/4 genotype shows increased anticaspase activity. After the HFD, VLDL becomes the main contributor of plasma total lipoprotein anticaspase activity. VLDL from a regular chow diet contributes minimally to the anticaspase activity of the total lipoproteins. Open symbols, regular diet; closed symbols, HFD. Data shown are means ± SEM. * Comparison between caspase activities of regular chow- and HFD-derived VLDL at 200 μg cholesterol/ml gave P < 0.005 for apoE3-VLDL and P < 0.05 for apoE4-VLDL.

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**Fig. 3.** HFD apoE4-VLDL is unable to antagonize HDL. HDL alone, or HDL and VLDL, was added to cells and caspase 3/7 activity was measured. Regular chow apoE4-VLDL antagonizes HDL anticaspase activity but HFD apoE4-VLDL does not (right panel), even though the two lipoproteins have equivalent apoE content per milligram of cholesterol (see Fig. 1A, lower panel). In comparison, neither HFD nor regular chow apoE3-VLDL antagonizes HDL anticaspase activity (left panel). Final concentration is 200 μg cholesterol/ml VLDL and either 17 or 13 μg cholesterol/ml apoE4- or apoE3-HDL, respectively. Data shown are means ± SEM.
shown) showed equivalent biochemical characteristics and biological activity to that of HFD apoE4-VLDL. (Figs. 4, 5).

**Anticaspase activity of HFD VLDL does not require apoE interaction with LDL receptor family members**

To determine whether HFD apoE-containing VLDL inhibits caspase 3/7 activity through interaction with a member of the LDL receptor family, we determined whether RAP inhibited this activity of HFD VLDL. As shown in Fig. 6, RAP at 10 μg/ml [a concentration well above that required to block apoE-mediated interactions with LDL receptor family members (9–11)] did not inhibit the anti-caspase activity of either apoE3- or apoE4-VLDL, suggesting that HFD VLDL does not require a member of the LDL receptor family to inhibit caspase activation. RAP biological activity has been demonstrated by its ability at 10 μg/ml to antagonize regular diet apoE4-VLDL inhibition of HDL anticaspase activity (9).

**Anticaspase activity of HFD VLDL is inhibited by the S1P receptor antagonist VPC 23019**

VPC 23019 (6, 8, 15, 17), an antagonist of prosurvival S1P1 and S1P3 receptors (6, 8, 15, 16), did not alter caspase 3/7 induction by serum withdrawal. Caspase 3/7 activity of VPC 23019-treated cells in SFM was 94.83 ± 7.12% of that in nontreated cells (100 ± 6.03%). This antagonist, however, did inhibit the ability of both HDL and HFD VLDL (Fig. 7A–C) to decrease caspase 3/7 activation. These observations support the conclusion by Nofer et al. (6, 7) that HDL inhibits caspase activation through S1P receptors and demonstrate that HFD VLDL, regardless of apoE phenotype, also inhibits caspase activation through these receptors (Fig. 7B, C).

**Anticaspase activity of HFD VLDL is reduced after siRNA-mediated downregulation of S1P3 receptor expression**

siRNA transfection of HUVECs with double-stranded oligoribonucleotide encoding the spliced transcript sequence 799–818 of gene EDG3 decreased S1P3 receptor expression by 73.49 ± 9.81% (n = 3) compared with HUVECs transfected with a nontargeted siRNA (Fig. 8B). Serum withdrawal increased caspase 3/7 induction equivalently in both control and S1P3 receptor siRNA-transfected HUVECs: S1P3 receptor siRNA-transfected cells showed 95.67 ± 6.84% caspase 3/7 induction compared with control-transfected cells. S1P3 receptor downregulation, however, drastically inhibited the ability of both HDL and HFD VLDL (Fig. 8A) to decrease caspase 3/7 activation.
SIP content of HFD VLDL can explain its anticaspase activity

SIP and dihydro-SIP are both agonists for SIP receptors on endothelial cells (15) and are the major phosphorylated sphingolipids in mammalian cells (16). As shown in Table 1, HFD apoE3- and HFD apoE4-VLDL contain 36.27 ± 9.00 and 41.08 ± 22.14 pmol SIP/mg cholesterol, respectively. Thus, HFD VLDL provides, under our experimental conditions, SIP concentrations of 4 to 12 nM, within the range of the dissociation constants for SIP1 and SIP3 receptors (~0.2–9 nM) (17, 26). HFD VLDL content of dihydro-SIP (30.80 ± 6.61 and 18.60 ± 4.36 pmol/mg cholesterol for HFD apoE3- and HFD apoE4-VLDL, respectively) is also within the concentration range to contribute to this anticaspase activity of HFD VLDL.

DISCUSSION

The high fat and cholesterol contents of the proatherogenic diet modified the distribution (Fig. 1A), composition (Fig. 1A, C), and biological activity (Figs. 2, 3) of lipoprotein particles. With this diet, VLDL becomes the major component of the plasma lipoprotein pool (Fig. 1A, upper panel). Remarkably, this HFD radically altered the activity of VLDL in its regulation of endothelial cell apoptosis (Fig. 2).

Work by Nofer et al. (6, 7) and Kimura et al. (8) studying lipoproteins in humans, and our own previous observations (9) studying lipoproteins from APOE transgenic and TR mice, demonstrated prosurvival activity of HDL. We previously reported that VLDL from mice on a regular chow diet provides little protection from apoptosis and that apoE4-VLDL actually promotes apoptosis by inhibiting the antiapoptotic activity of HDL. This observation can be explained by the ability of apoE4-VLDL to inhibit HDL-mediated Akt activation (27), thus reducing the inherent antiapoptotic effect of HDL. Testing the effect of a HFD on the VLDL modulation of caspase activity, we observed that a HFD does not alter the anticaspase activity of HDL but does enhance the anticaspase activity of VLDL (Figs. 2, 3).

Our observation that a HFD promotes an antiapoptotic activity for VLDL, similar to the antiapoptotic activity of HDL, may seem paradoxical because HFD VLDL is considered proatherogenic and HDL is considered antiatherogenic. The atherogenic process, however, requires the participation of multiple cell types (endothelial, macrophages, smooth muscle), oxidative damage (of lipoproteins), and involves prolonged evolution over months to years. Our observations do not contradict the well-established notion that chronic exposure of HFD lipoproteins leads to vascular injury but rather describes an acute response (hours) of endothelial cells to nonoxidized lipoproteins. Therefore, our observations support the idea that compensatory molecular and cellular responses to changes in lipoprotein distribution and composition may be critical in atherosclerosis.

The anticaspase activity of HFD VLDL is of potential physiological significance in that it saturates, in vitro, at concentrations below or within the range of plasma VLDL concentrations of mice fed the HFD or the regular diet, respectively. A concentration of 300 µg/ml VLDL, the highest concentration shown in Fig. 2, corresponds to ~20% of the HFD VLDL concentration in blood of APO3/3 mice and to 12% of that of APO4/4 mice fed HFD. In addition, no change in the HFD VLDL anticaspase activity is observed from 300 µg/ml to the highest tested concentration of 750 µg/ml (see supplementary Fig. I). In mice fed regular chow, 300 µg/ml HFD VLDL is at least 120% and 45% of the VLDL plasma concentration of TR3/3 and TR4/4 mice, respectively. Thus, the gradual increase in plasma VLDL after a change from regular chow to a HFD produces HFD VLDL particles that could exert in vivo the anti-apoptotic activity demonstrated here in vitro. In contrast, VLDL derived from normal chow-fed mice pos-

Fig. 6. HFD VLDL anticaspase activity is not mediated by an LDL receptor family member. Caspase activity was assayed after 30 min of preincubation of cells with receptor-associated protein (RAP) (rat recombinant; Research Diagnostics, Inc., Flanders, NJ) before the addition of HFD VLDL. Final VLDL cholesterol concentration was 300 µg/ml, and final RAP concentration was 10 µg/ml. RAP did not inhibit HFD VLDL activity, indicating that this activity of VLDL is independent of apoE interaction with a member of the LDL receptor family. Data shown are means ± SEM.
sesses little antiapoptotic activity in vitro, is found at low concentrations in plasma (Fig. 1A, upper panel), and would have an even smaller antiapoptotic activity in the vascular endothelium in situ.

The S1P antagonist VPC 23019 partially inhibits HFD VLDL and HDL anticaspase activity. This antagonist has dissociation constants of \(1.38 \times 10^{-8}\) M and \(1.17 \times 10^{-6}\) M for S1P1 and S1P3 receptors, respectively; thus, at \(1.00 \times 10^{-6}\) M, it is expected to block >98% of S1P1 receptors and approximately half of S1P3 receptors (17) (Fig. 7; see supplementary Fig. III). Therefore, partial inhibition at \(1.00 \times 10^{-6}\) M VPC 23019 that is further increased at \(10.00 \times 10^{-6}\) M (see supplementary Fig. III) suggests that HFD VLDL and HDL activate S1P3 receptors. S1P3 receptor downregulation by siRNA inhibits the anticaspase activity of both HFD VLDL and HDL (Fig. 8), strengthening the conclusion that S1P3 receptors have a primary role in mediating both HFD VLDL and HDL anticaspase activity. RT-PCR analyses of S1P3 receptor gene expression in three independent experiments show a large (73.49 ± 9.81%), but not total, reduction of S1P3 receptor transcription in siRNA-treated cells, suggesting that low levels of receptor expression account for residual lipoprotein-associated anticaspase activity (S1P3 receptor siRNA-treated HUVECs show 98.47 ± 5.06% and 91.08 ± 4.24% caspase 3/7 activity for HFD VLDL and HDL, respectively; control siRNA-treated cells show 63.04 ± 6.07% and 68.84 ± 6.07% activity). At present, however, we cannot rule out the possibility that a S1P1 receptor could also contribute to lipoprotein-mediated anti-caspase activity. Additional studies are needed to assess S1P1 receptor participation in this process.

Nofer et al. (7) demonstrated that HDL anchors to the SR-BI, permitting the presentation of HDL lysosphingolipids to S1P3 receptors. VLDL interacts with SR-BI (28), CD36 (29, 30), and proteoglycans (31–33). Any of these cell surface molecules could anchor VLDL particles for lipid presentation to S1P3 receptors. We also cannot rule out the possibility that VLDL particles may directly deliver their lysosphingolipids to these receptors. Our results demonstrate that a cholate-containing HFD changes both the apolipoprotein and lipid compositions of VLDL particles (Fig. 1A, C) and profoundly influences the interactions between VLDL particles and cell surface receptors (Figs. 6–8).

ApoA-I, apoA-IV, apoB, and apoE associate with the HFD VLDL fraction (Fig. 1C) (12, 13); among these, apoA-I is most markedly affected by diet. HFD increased apoA-I content in the VLDL fraction of both APOE3 and APOE4 mice (Fig. 1C). Indeed, the VLDL fraction of fasted mice consists of two distinct lipoprotein populations: apoA-I-free VLDL and apoA-I-containing chylomicron remnants. Eliminating apoA-I-containing remnants from the HFD VLDL fraction (representing ~60% of the total VLDL cholesterol content) did not alter the anticaspase activity of the remaining HFD VLDL particles (normalized per unit of cholesterol) (Fig. 5A, B), indicating that both apoA-I-free VLDL and apoA-I-containing particles in the HFD VLDL fraction possess anticaspase activity and suggesting that the antiapoptotic activity of HFD VLDL is independent of apoA-I. In addition, the anticaspase activity of HFD VLDL is independent of apoA-IV, because HFD apoE3-VLDL contains nondetectable levels of apoA-IV (Fig. 1C) but does show anticaspase activity (Fig. 2). Finally, the inability of RAP to alter the
anticaspase activity of HFD VLDL (Fig. 6A, B) rules out a role for an apoE-mediated interaction with an LDL receptor family member. ApoE-containing lipoproteins, however, bind other cell surface receptors, including members of the scavenger receptor family (28–30), which are not inhibited by RAP. Thus, the anticaspase activity of HFD VLDL does not depend on the apolipoprotein content or composition of the particle but rather on its lysosphingolipid content (Figs. 7, 8). HFD apoE3- and HFD apoE4-VLDL contain S1P and dihydro-S1P (Table 1), providing, under our experimental conditions, concentrations of these sphingolipids within the range of their dissociation constants for S1P1 and S1P3 receptors (17, 26, 34). The regular diet VLDL and HFD VLDL content of S1P per unit of cholesterol are similar; however, the concentration of dihydro-S1P is increased significantly in VLDL from mice on the HFD. In addition, the presentation of VLDL-associated sphingolipids at the cell surface varies between regular diet VLDL and HFD VLDL, because the regulation of caspase 3/7 activity by regular diet VLDL and HFD VLDL is mediated through different receptors. In blood vessels, depending on cell type, receptor type, and dosage, S1P and related sphingolipids participate in both vessel wall repair and atherogenesis (35, 36). Because lipoproteins are effective carriers of these compounds, the development of S1P analogs for clinical intervention should not only seek S1P receptor and cellular specificity (37) but also take into account their interactions with lipoproteins, which bind to different receptors and whose plasma composition and content are highly dependent on diet.

In conclusion, the HFD a) alters the concentration, distribution, and apolipoprotein composition of plasma lipoproteins; b) strongly enhances the ability of apoE3- and apoE4-VLDL to protect HUVECs from apoptosis initiated by short-term serum withdrawal; and c) eliminates the apoE4 isoform-specific ability of VLDL to antagonize the anticaspase activity of HDL. We also show that the antiapoptotic activity of HFD VLDL involves the participation of S1P3 receptors.

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TABLE 1. S1P and dihydro-S1P contents of apoE3- and apoE4-VLDL

|                  | apoE3-VLDL | apoE4-VLDL | Regular diet apoE3-VLDL | Regular diet apoE4-VLDL |
|------------------|------------|------------|-------------------------|-------------------------|
| S1P pmol/mg cholesterol | 36.27 ± 9.00 | 41.08 ± 22.14 | 56.56 ± 15.61 | 24.47 ± 3.49 |
| Dihydro-S1P pmol/mg cholesterol | 30.80 ± 6.61 | 18.60 ± 4.36 | 6.79 ± 13.57 | 5.20 ± 7.35 |

*aSignificant difference between HFD and regular diet VLDL (P < 0.05). Comparison was done grouping regular diet and HFD VLDLs regardless of genotype.

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