Ceramide is a bioactive molecule involved in cellular responses to stress and inflammation. The major pathway for ceramide accumulation is via agonist-induced activation of cellular sphingomyelinases. It has also been shown that the ceramide level in circulating low density lipoprotein (LDL) increases during systemic inflammation, hence it is of importance to understand whether LDL-derived ceramide also contributes to the cellular ceramide homeostasis and affects cell functions. This article provides evidence of uptake of ceramide-enriched LDL by human microvascular endothelial cells in a receptor-mediated fashion. This uptake can be competed by native LDL, indicating that the LDL-binding receptor may be involved. Following uptake, part of the LDL-derived ceramide is converted to sphingosine, but more importantly, part of it accumulates inside the cells (approximately 1.44 nmol/mg of cell protein). This accumulation of ceramide correlates with an increased incidence of apoptosis. The addition of tumor necrosis factor-α further enhances the accumulation of LDL-derived ceramide and the rate of apoptosis. In contrast, inhibitors of receptor-mediated endocytosis block both, the accumulation of LDL-derived ceramide and the concurrent increase in apoptosis. We also show that LDL-delivered ceramide is a more efficient inducer of apoptosis as compared with ethanol-delivered ceramide, the same apoptotic effect being achieved by substantially smaller increases in intracellular ceramide. Taken together, the presented data indicate that increases in lipoprotein ceramide concentration may result in changes in the bioactive properties of circulating lipoproteins such as the enhanced ability to induce apoptosis in the microvascular endothelium.

The uptake and metabolism of modified low density lipoprotein (LDL) are a prerequisite for the formation of atherosclerotic plaques (1–7). Recent studies suggest that changes in the sphingolipid content of LDL also affect the development of atherosclerosis by promoting aggregation (8–10) and oxidation (11) of LDL. These studies have focused on the role of secreted, zinc-dependent, sphingomyelinase (S-SMase), which hydrolyzes LDL sphingomyelin (SM) to ceramide. It is secreted mainly by endothelial cells in vitro in response to either TNF-α or interleukin-1β (12), as well as in response to lipopolysaccharide in vivo (13). Studies using bacterial SMase or recombinant S-SMase have shown that conversion of LDL-SM to ceramide leads to aggregation (8) and fusion (14) of the LDL particles, increases their retention in subintimal space (19), and can facilitate LDL oxidation (11). The possible role of SMase has been further emphasized by data showing that LDL extracted from advanced human aortic plaques has higher ceramide content than LDL extracted from circulation or from normal aorta (19). These findings indicate that changes in the SM/ceramide ratio of LDL affect their atherogenic properties, however, they do not distinguish between the effect of ceramide increase and that of SM depletion.

Studies in animal models as well as in isolated rat hepatocytes have shown that activation of serine palmitoyltransferase, the rate-limiting step in de novo ceramide synthesis in the liver increases up to 8 times the ceramide content of very low density lipoprotein and LDL that account for 80% of the total serum ceramide (15, 16). This is attributed to specific increases in the secretion of ceramide by the liver, which is not, however, paralleled by a decrease in SM mass. Hence, these studies seem to indicate that the ceramide level is not regulated solely by activation of S-SMase, and therefore, ceramide effects on LDL properties have to be assessed independently of those of S-SMase.

Ceramide is a bioactive molecule that participates in signal transduction cascades initiated by cytokines, oxidized LDL, and chemotherapeutic drugs. In endothelial cells, regulation of the ceramide level is critical for cell functions and survival (17–19). For example, in whole animal models (19), the activation of acidic sphingomyelinase by lipopolysaccharide has been shown to be a requirement for the onset of endothelial apoptosis and endotoxic shock-mediated death. Also, removal of excess ceramide in human umbilical vein endothelial cells by activating ceramidase prevents TNF-α-induced cell death and the resulting accumulation of sphingosine and sphingosine phosphate induces the expression of the adhesion molecules

---

* This work was supported in part by American Heart Association (Ohio Valley Affiliate) Grant-in-aid 0060312B, National Institute on Drug Abuse Grant K12 DA14040-03 (Building Interdisciplinary Research Centers in Women’s Health), and National Center for Research Resources, National Institutes of Health Grant P20 RR 15592 (Center of Biomedical Research Excellence in Women’s Health). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 859-323-8210; Fax: 859-323-1070; E-mail: mnikolo@uky.edu.

† The abbreviations used are: LDL, low density lipoproteins; BSA, bovine serum albumin; Cer, ceramide; C2-Cer, C2-ceramide (N-acetylsphingosine); Cer-LDL, ceramide-enriched LDL; C2-NBD-Cer, N-hexanoyl-1-O(7-nitrobenz-2-oxa-1,3-diazol-4-ylamino)-sphingosine; HME-1, HME-1, human microvascular endothelial-1; SM, sphingomyelin; S-SMase, sphingomyelinase; S-SMase, secreted sphingomyelinase; TNF-α, tumor necrosis factor α; HPLC, high performance liquid chromatography; TUNEL, terminal deoxynucleotidyltransferase-mediated nick-end labeling.
E-selectin and vascular cell adhesion molecule-1 (17). The pathways for intracellular generation and turnover of ceramide have been the subject of extensive studies. However, the possibility for lipoprotein-derived ceramide being a significant source of ceramide has not been explored. Such a pathway could be important for vascular cells involved in active lipoprotein uptake and turnover, especially during inflammation (20) or aging, when the lipoprotein levels of ceramide are increased.

In this article we describe a method for selective enrichment of native LDL with short chain ceramide. Analyses of the biophysical and biochemical properties of these ceramide-enriched particles show that ceramide alone is not sufficient to induce LDL aggregation or oxidation. Human microvascular endothelial-1 (HME-1) cells take up ceramide-enriched LDL in a receptor-mediated fashion, leading to accumulation of LDL-derived ceramide inside the cells. This induces apoptosis in HME-1 cells, which can be blocked by inhibitors of receptor-mediated endocytosis.

EXPERIMENTAL PROCEDURES

Materials—N-Hexanoyl-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-sphinganine (C6-NBD-Cer) was purchased from Matreya (State College, PA). N-Acetylsphinganine (C2-Cer) and C6-Cer were from Avanti Polar Lipids (Alabaster, AL). SMase from Staphylococcus aureus and bovine serum albumin (BSA) were from Sigma. BSA was maleylated according to the method of Butler and Hartley (21). The in situ cell death detection kit was purchased from Roche Diagnostics. Annexin V-fluorescein isothiocyanate apoptosis kit was from R&D (Minneapolis, MN). N-Acetyl-sphinganine was synthesized as described previously (22) and used as an internal standard.

Cell Cultures—HME-1 cells (Center for Disease Control and Prevention, Atlanta, GA) were used in the experiments. Cells were cultured in MCDB-131 media (Sigma) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 100 units/ml penicillin/streptomycin (Invitrogen), 50 μg/ml hydrocortisone (Sigma), and 50 μg/ml epidermal growth factor (Calbiochem). In all experiments, cells were cultured in serum-reduced medium containing 0.2% serum and 1 μg/ml epidermal growth factor for 12–16 h before treatment. The treatments also were conducted in serum-reduced medium.

Human Lipoproteins—LDL (density between 1.019 and 1.063 g/ml) was isolated from the venous blood of healthy human subjects. Plasma was obtained by low speed centrifugation. The whole blood was purchased from the Central Kentucky Blood Center (Lexington, KY). Plasma was stored at 4°C and used within 48 h for isolation of LDL. Lipoprotein fractions were isolated by sequential ultracentrifugation according to the method of Havel et al. (23). Briefly, the density of the plasma was adjusted to 1.019 g/ml with KBr solution (d = 1.34 g/ml). The plasma was centrifuged at 208,000 × g for 18 h at 10°C using Quick Seal™ ultracentrifuge tubes (Beckman-Spinco, Palo Alto, CA) and a Beckman Ti70 rotor. The top fraction was discarded and the density of the bottom fraction was adjusted to 1.063 g/ml. Ultracentrifugation was repeated and the top fraction was collected as LDL. After isolation, LDL was treated for albumin precipitation by gel filtration. LDL ran as a single protein band indicating the absence of albumin contamination. The LDL was stored under argon to prevent oxidation.

Enrichment of LDL with C6-NBD-Cer or C2-Cer—To selectively enrich LDL with ceramide without affecting the levels of SM, ceramide-containing liposomes were used as donor and native LDL was used as acceptor. In the labeling procedure, LDL was used for optimizing the enrichment procedure. Donor liposomes were prepared as follows. Total lipids were isolated from the venous blood of healthy human subjects. Plasma was isolated from the venous blood of healthy human subjects. Plasma was obtained by low speed centrifugation. The whole blood was purchased from the Central Kentucky Blood Center (Lexington, KY). Plasma was stored at 4°C and used within 48 h for isolation of LDL. Lipoprotein fractions were isolated by sequential ultracentrifugation according to the method of Havel et al. (23). Briefly, the density of the plasma was adjusted to 1.019 g/ml with KBr solution (d = 1.34 g/ml). The plasma was centrifuged at 208,000 × g for 18 h at 10°C using Quick Seal™ ultracentrifuge tubes (Beckman-Spinco, Palo Alto, CA) and a Beckman Ti70 rotor. The top fraction was discarded and the density of the bottom fraction was adjusted to 1.063 g/ml. Ultracentrifugation was repeated and the top fraction was collected as LDL. After isolation, LDL was treated for albumin precipitation by gel filtration. LDL ran as a single protein band indicating the absence of albumin contamination. The LDL was stored under argon to prevent oxidation.

Enrichment of LDL with C6-NBD-Cer or C2-Cer—To selectively enrich LDL with ceramide without affecting the levels of SM, ceramide-containing liposomes were used as donor and native LDL was used as acceptor. In the labeling procedure, LDL was used for optimizing the enrichment procedure. Donor liposomes were prepared as follows. Total lipids were extracted from native LDL (0.5 mg of protein) according to the two-phase extraction procedure of Bligh and Dyer (24), modified as described previously (25). After extraction, the lipids from the lower chloroform phase were passed through a sodium sulfate column, and mixed with 2, 5, or 10 nmol of C6-NBD-Cer for a final concentration of ceramide during the transfer of 10, 25, or 50 μm. The solvent was evaporated under a stream of argon, and the lipids were resuspended in 1 ml of KBr (d = 1.019), vortexed, and sonicated in a 0.5 mm thoron sonicator until translucent. Then 0.5 mg of native LDL (acceptor) was added and incubated with the donor vesicles for 30 min at 37°C under argon in a final volume of 0.2 ml of KBr (d = 1.019). For cell culture experiments, C2-Cer and C6-Cer-ceramide were used instead of C6-NBD-ceramide at a concentration of 50 μM. In each individual preparation, the amount of all reagents was changed accordingly to obtain the desired amount of Cer-enriched LDL. In all cases, the final volume was less than 1 ml.

After the incubation, the suspension was overlayed with KBr (d = 1.019) to a final volume of 1 ml and centrifuged for 2.5 h at 265,000 × g at 10°C using a Beckman-Spinco table centrifuge. The donor liposomes were recovered floating at the surface and reentrance. The volume was adjusted again to 1 ml with KBr (d = 1.019) and the samples were spun again. The ceramide-enriched LDL (Cer-LDL) was recovered in the lower 0.5 ml of the suspension. The amount of incorporated C6-NBD-Cer was analyzed on HPLC equipped with fluorocence detector (Shimadzu, Kyoto, Japan) and calculated based on the quantum yield of the external standard with a known concentration (26). The mass of incorporated C2-Cer or C6-Cer was analyzed by TLC/HPLC as described below. The degree of enrichment was represented as nanomole of Cer per mg of LDL protein. Throughout the experiments, LDL which underwent the same procedure, but donor particles did not contain ceramide, was used as controls (control LDL). Cer-LDL was prepared fresh for each experiment.

Quality Control of Cer-LDL Particles—The oxidation of LDL was monitored by malonaldehyde bis-demethylalactol colorimetric assay and relative electrophoretic mobility in a 1.8% agarose gel. The aggregation was monitored by gel electrophoresis. As a positive control for aggregation, LDL (50 μg) was treated with bacterial SMase at various concentrations for 24 h in 50 μl of 0.1x Tris, pH 7.4. As a positive control for oxidation, LDL was treated with 50 μM CuSO4 for 24 h. Typically, 10 μg of LDL were loaded per lane. The C6-NBD-Cer in LDL was visualized by UV light. The protein was visualized using Coomassie Brilliant Blue staining.

Binding and Degradation Assay—For these experiments, 125I-labeled LDL was prepared according to Bilheimer’s modification (27) of the iodine monochloride method described by Goldstein et al. (28). Enrichment with C6-Cer was performed after the labeling procedure. HME-1 cells were cultured in 12-well plates until confluence and changed to serum-reduced medium for 12 h.

For binding studies, cells were cooled down to 4°C, and after washing with ice-cold phosphate-buffered saline, adherent cells were treated with medium containing the indicated concentrations of ligand. After incubation at 4°C for 2 h, the conditioned media was removed, and the cells were washed rapidly 3 times with washing buffer (50 mM Tris, 150 mM NaCl, and 2 mg/ml fatty acid free BSA) followed by 2 washes with washing buffer without BSA. The cells were dissolved in 1 ml of 1 N NaOH for 2 h at room temperature. Radioactivity was measured on a γ-counter (Cobra II, Packard Instrument Co.).

The uptake was assessed by monitoring the proteolytic degradation of 125I-labeled LDL or Cer-LDL (29). After serum starvation, cells were treated with the indicated ligands for 6 h at 37°C; the medium was collected and cleared from possible detached cells by centrifugation. The radioactivity was measured in a trichloroacetic acid-soluble, chloroform-unextractable fraction of the media and used as a measure for lipoprotein uptake after subtraction of the background. The background was quantified using control incubations with empty wells.

Lipid Assays—HME-1 cells were treated with C6-Cer-LDL as indicated. Then cells were harvested in 0.5 ml of phosphate-buffered saline and lysed by sonication through two passes with a 0.5 mm Glass probe. Aliquots were taken for protein determination, and the lipids were extracted from the remaining homogenate as described (24, 25). Lipids from each dish were analyzed by thin layer chromatography (TLC) on silica gel plates (Whatman, Clifton, NJ) with ether: methanol (99:1, by volume) as a developing solvent. In this mobile phase, the relative electrophoretic mobility of C2-Cer was 0.14, and for long chain ceramide 0.82. The lipids were visualized with I2. The spots migrating with standard C2-Cer or long chain ceramide were scraped and the lipids were eluted from the silica using 2 ml of chloroform:methanol (1:1 by volume) by vigorous vortexing for 1 min, and centrifugation for 10 min in a tabletop centrifuge. This procedure was repeated 3 times, and pooled supernatants were evaporated under reduced pressure. The amount of ceramide, 0.5 nmol of N-acetyl-C2-Cer-sphinganine was added to each sample as an internal standard. Samples were analyzed on HPLC after acid methanolysis and values were corrected for recovery of the internal standard (30). Free sphingolipid bases were quantified in separate
Experiments as described (31). For quantification of ceramide in LDL, lipids were extracted from 20 μg of LDL following the same procedure.

Assay of Apoptosis by Terminal Deoxynucleotidyltransferase-mediated Nick-end Labeling (TUNEL) Assay—Apoptosis in HME-1 cells was detected using the In Situ Cell Death Detection Kit (Roche Diagnostics) (32). Briefly, cells were cultured in 6-well dishes on glass coverslips in the medium described above. Before experiments, cells were serum-deprived and then treated with appropriate reagents and controls for 16 h. Labeling of 3'-free hydroxyl ends of the fragmented DNA with fluorescein-conjugated dUTP was catalyzed by terminal deoxynucleotidyltransferase, using a commercially available kit, following the manufacturer's directions. The apoptotic cells were then detected by fluorescence microscopy (Nikon Diaphot 300, Kyoto, Japan). Averages of 600 cells from random fields on each slide were analyzed. Sample indicators were concealed during scoring, and samples from three independent experiments were scored per group.

**RESULTS**

Enrichment of LDL with Ceramide—Various concentrations of native LDL (acceptor) and C6-NBD-Cer-containing liposomes (donors) were incubated for different periods of time at 37 °C. Following incubation, the donor liposomes were removed by floating through a density gradient. The loss of LDL protein during the procedure was less than 10% (data not shown). The resulting ceramide-enriched LDL was analyzed by agarose gel electrophoresis and visualized with UV light or Coomassie Blue staining (Fig. 1, panel A). The result overlapped, confirming that C6-NBD-Cer had incorporated into the LDL particles. The rate of incorporation correlated linearly with the increasing C6-NBD-Cer concentration in the donor vesicles (Fig. 1, panel B). The transfer reached equilibrium in ≈ 30 min of incubation (Fig. 1, panel C). Similar results were obtained when C2-Cer was used instead of C6-NBD-Cer.

Enrichment of LDL with Ceramide Does Not Cause Extensive Aggregation and Oxidation—Treatment of LDL with bacterial SMase induces aggregation (8) and facilitates oxidation (11) of the particles. The Cer-LDL was analyzed for aggregation and oxidation to test whether increases in ceramide levels alone are sufficient to alter these properties of LDL. SMase-treated LDL was used as a positive control. As expected, a significant portion of the SMase-treated LDL aggregated as indicated by the smeared pattern seen on an agarose gel or by their inability to enter the gel all together (Fig. 2, panel B). Some SMase-treated particles also have a shift in the electrophoretic mobility. Such shifts were typical for oxidized LDL that was incubated with CuSO4 (Fig. 2, panel C). In contrast, Cer-LDL was neither aggregated nor oxidized (Fig. 2, panel A). The lack of oxidation was confirmed further by measuring the levels of thiobarbituric reactive substances in Cer-LDL. Cer-LDL and native LDL had similar levels of thiobarbituric reactive substances (<0.1 nmol/mg), which were significantly lower than that in mildly oxidized LDL (7.25 nmol/mg).

To control for the level of ceramide in both treatments, the generation of ceramide in SMase-treated LDL was measured. As shown in Fig. 2, SMase treatment resulted in the generation of 10 to 12 nmol of ceramide, which is similar to the levels of ceramide in Cer-LDL (between 12 and 18 nmol/mg). This shows that the lack of aggregation or oxidation in Cer-LDL is not because of the lower ceramide level. Taken together, these results show that enrichment with ceramide alone does not induce oxidation or aggregation of LDL.

**Binding and Uptake of Cer-LDL by HME-1 Cells Is Receptor-mediated**—The uptake of Cer-LDL was studied in human microvascular endothelial cells that express different LDL-binding receptors, including the LDL receptor, scavenger receptor BI, and CD 36 (35, 36). The kinetics for Cer-LDL and LDL uptake and binding were very similar. Both the binding and the uptake reached saturation at a ligand concentration of 75–100 μg/ml and were competed by non-labeled LDL at 50-fold excess (Fig. 3). Taken together, these data show that the uptake of Cer-LDL like that of native LDL is receptor-mediated and most likely involves the same receptor(s).

**LDL-derived C2-Cer Accumulates in HME-1 Cells**—The uptake of native LDL does not affect the levels of endogenous sphingolipids, suggesting that the ceramide present in the native LDL is hydrolyzed by the lysosomal ceramidase to sphingosine that in turn is further metabolized to sphingosine-phosphate or re-acycated back to ceramide. Indeed, when HME-1 cells were treated with native LDL, no increases were detected in the intracellular levels of ceramide and sphingosine. On the contrary, cells treated with C2-Cer-LDL began to accumulate
C2-Cer within 60 min reaching 1.44 nmol of Cer/mg of cell protein in 4 h, whereas sphingosine concentrations increased by 1.40 nmol (Table I). This implies that although LDL-derived C2-Cer is hydrolyzed to sphingosine, the rate of hydrolysis is not sufficient for complete elimination of the excess ceramide. It is unlikely that such accumulation is caused by lower affinity of ceramidase for short chain ceramide because (i) earlier studies in cell cultures have shown efficient hydrolysis of C2-Cer and C6-ceramide to sphingosine (26, 37); and (ii) in vitro assay with purified human acid ceramidase have shown that C2-, C6-, and C18-ceramide are all hydrolyzed at comparable rates: 21, 19, and 17 nmol/h/mg protein (38).

The accumulation of free sphingosine following Cer-LDL uptake may indicate that the enzymes of sphingosine turnover are also rate-limiting. Interestingly, sphingosine generated by micelle-derived C2-ceramide was efficiently re-acylated to long chain ceramide, as indicated by the increases in the mass of long chain ceramide (Table I). In contrast, no such increases were found in Cer-LDL-treated cells. This may indicate that sphingosine generated from LDL-derived ceramide is less accessible to ceramide synthases. Alternatively, it may be because of the lower levels of accumulated sphingosine (1.40 versus 2.44 nmol/mg).

In summary, these data show that the uptake of LDL with the elevated content of ceramide may lead to accumulation of ceramide and sphingosine inside the cells. More importantly, the magnitude of ceramide accumulation is comparable with that observed in response to IL-1β/H9252 or oxidized LDL (26, 39) and implies that LDL-derived ceramide may have a significant biological function.

Ceramide-enriched LDL Induces Apoptosis in Endothelial Cells

**Uptake of Cer-LDL Increases the Incidence of Apoptosis in HME-1 Cells**—Activation of acid sphingomyelinase and lysosomal accumulation of ceramide mediate the onset of endothelial cell apoptosis in response to radiation (40) and lipopolysaccharide (19). Therefore, it is likely that increases in cellular ceramide levels because of Cer-LDL uptake will also affect cell survival. Indeed, the addition of C2-Cer-LDL to HME-1 cells induced apoptosis in a time- and dose-dependent manner, as judged by TUNEL assay (Fig. 4). The incidence of apoptosis reached maximum (6–7% of all cells) at 16 h using 100 μg/ml C2-Cer-LDL. In contrast, control LDL caused less than 1% of all cells to become TUNEL-positive. The incidence of apoptosis was further confirmed by annexin V and Hoechst staining (data not shown). As a positive control, the cells were treated with staurosporine at a concentration of 2 μM, which for most cell lines is sufficient to kill 90% of the total cell population. However, in HME-1 cells, staurosporine treatment increased the incidence of apoptosis to only 18–20% (data not shown).

The rate of receptor-mediated endocytosis can be augmented by different cytokines, including TNF-α (41). Therefore, we hypothesized that TNF-α should enhance the accumulation of LDL-derived ceramide and the correlating rate of apoptosis. To test this, cells were treated with C2-Cer-LDL in the presence or...
Ceramide-enriched LDL Induces Apoptosis in Endothelial Cells

HME-1 cells were cultured in serum-reduced medium for 12 h and were either left untreated or treated with control LDL (100 μg/ml), C2-Cer-LDL, (100 μg/ml), ethanol vehicles, or C2-ceramide in ethanol (C2-Cer-micelles, 30 μl). In the last two treatments the final ethanol concentration was 0.1%. After 4 h of incubation, the cells were harvested, the lipids were extracted, and the levels of C2-ceramide, sphingosine, and long chain (endogenous) ceramide were quantified by TLC/HPLC for ceramides and HPLC for the free sphingoid bases. The data are average ± S.D., n = 3 and are representative for two independent experiments. The ceramide and sphingosine measurements were done in separate experiments.

| Cell treatment  | C2-ceramide | Sphingosine | Long chain ceramide |
|-----------------|-------------|-------------|---------------------|
| No treatment    | <0.03 ± 0.03| 2.13 ± 0.49 | 2.19 ± 0.39         |
| Control LDL     | <0.04 ± 0.07| 1.91 ± 0.65 | 2.69 ± 0.30         |
| C2-Cer-LDL      | 1.44 ± 0.77  | 3.45 ± 0.16<sup>a</sup> | 2.99 ± 0.41         |
| Ethanol vehicles| <0.01 ± 0.02 | 2.53 ± 0.27 | 2.31 ± 0.42         |
| C2-Cer-micelles | 15.87 ± 1.57<sup>b</sup> | 4.94 ± 0.78<sup>b</sup> | 5.52 ± 1.07<sup>b</sup> |

<sup>a</sup> p < 0.01, as compared with the respective control treatments.
<sup>b</sup> p < 0.05 as compared with respective control treatments.

FIG. 4. Cer-LDL increases the incidence of apoptosis in HME-1 cells in a time- and dose-dependent manner. HME-1 cells were incubated in serum-reduced medium for 12 h before the experiments. C2-Cer-LDL (15 nmol of C2-Cer/mg of protein) or control LDL were added at a concentration of 100 μg/ml for the indicated times (panel A) or at the indicated concentrations for 16 h (panel B). The incidence of apoptosis was assayed by TUNEL and examined by fluorescence microscopy. The percent apoptosis was quantified in a double-blinded manner. An average of 600 nuclei from four to six random fields from each sample were counted. Results are presented as a percent of TUNEL-positive cells per total cell number in the field and are average ± S.D., n = 3 tissue culture dishes. Data are representative for three independent experiments. * p < 0.05; **, p < 0.01.

Because LDL-derived ceramide is metabolized to sphingosine, the increased incidence of apoptosis could be because of sphingosine generation. Exogenously added sphingosine, however, did not replicate the C2-Cer-LDL effects (data not shown), suggesting that this is not the case.

Cer-LDL-induced Apoptosis Is Not Because of Spontaneous Transfer of Ceramide from Cer-LDL to HME-1 Cell Membrane—The rate of C2-Cer spontaneous diffusion is significant (42, 43) and could, at least in part, be responsible for the observed cellular responses to Cer-LDL. One way to test whether this is the case is to compare the rate of uptake for the

Table I

| Cell treatment          | C2-ceramide (nmol/mg cell protein) | Sphingosine (nmol/mg cell protein) | Long chain ceramide (nmol/mg cell protein) |
|-------------------------|-----------------------------------|------------------------------------|------------------------------------------|
| No treatment            | 0.03 ± 0.03                       | 2.13 ± 0.49                        | 2.19 ± 0.39                              |
| Control LDL             | 0.04 ± 0.07                       | 1.91 ± 0.65                        | 2.69 ± 0.30                              |
| C2-Cer-LDL              | 1.44 ± 0.77                       | 3.45 ± 0.16<sup>a</sup>            | 2.99 ± 0.41                              |
| Ethanol vehicles        | 0.01 ± 0.02                       | 2.53 ± 0.27                        | 2.31 ± 0.42                              |
| C2-Cer-micelles         | 15.87 ± 1.57<sup>b</sup>          | 4.94 ± 0.78<sup>b</sup>            | 5.52 ± 1.07<sup>b</sup>                  |

<sup>a</sup> p < 0.01, as compared with the respective control treatments.
<sup>b</sup> p < 0.05 as compared with respective control treatments.

FIG. 5. TNF-α augments Cer-LDL-mediated apoptosis in HME-1 cells. HME-1 cells were incubated in serum-reduced medium for 12 h before the experiments. Then they were treated for 16 h with: panel A: C2-Cer-LDL (100 μg/ml); panel B, control LDL (100 μg/ml); panel C, C2-Cer-LDL (100 μg/ml) and TNF-α (200 units/ml); panel D, control LDL (100 μg/ml) and TNF-α (200 units/ml); panel E, C2-Cer-LDL (100 μg/ml) or control LDL (100 μg/ml) and the indicated concentrations of TNF-α. The incidence of apoptosis was quantified as described in the legend to Fig. 4. Results are mean ± S.D., n = 3. *, p < 0.05; **, p < 0.01. Statistical significance is given for the effects of C2-Cer-LDL over the respective control LDL treatments, unless indicated differently. Data are representative for three independent experiments.
Ceramide-enriched LDL Induces Apoptosis in Endothelial Cells

**Fig. 6.** Cer-LDL complexes are stable in the presence of cells. C_{6}-NBD-LDL (6.5 nmol/mg of protein) was incubated with HME-1 cells or in wells containing medium only for 4 h. Conditioned medium was collected and the LDL was re-isolated by density gradient as described under “Experimental Procedures.” Panel A, C_{6}-NBD-Cer mass was measured by HPLC and the protein level was determined by the Lowry method. Panel B, the re-isolated particles were run on 1.8% agarose gel and visualized with UV light for C_{6}-NBD-Cer or Coo massie Blue for LDL protein.

Lipid and protein components of Cer-LDL. This, however, may prove futile because LDL lipids and proteins are processed in the cells by separate mechanisms and following turnover to smaller metabolites, a significant portion of the uptaken LDL protein and C_{6}-NBD-ceramide is secreted. As an alternative approach, we analyzed the composition of C_{6}-NBD-Cer-LDL remaining in the medium after 4 h of incubation with HME-1 cells and compared it with that in control incubations of C_{6}-NBD-Cer-LDL with medium only. In these analyses, C_{6}-NBD-Cer-LDL was re-isolated from the conditioned medium, run on a gel, and the amounts of C_{6}-NBD-Cer and protein were measured. Aliquots of the starting C_{6}-NBD-Cer-LDL were used for comparison. These experiments show that the concentration of C_{6}-NBD-Cer in LDL incubated with or without HME-1 cells was similar (Fig. 6), confirming the lack of spontaneous transfer of C_{6}-NBD-Cer to the cells. Furthermore, as expected, the recovery of LDL particles was lower from the cell-containing samples because of their uptake. A small decrease in the C_{6}-NBD-Cer fluorescence was seen in all samples, which was probably because of instability or decay of the NBD group over time.

**Inhibition of Receptor-mediated Endocytosis Prevents C_{2}-Cer-LDL-induced Apoptosis**—If Cer-LDL uptake is receptor-mediated, then inhibition of receptor-mediated endocytosis should prevent the accumulation of ceramide and the increases in apoptosis resulting from Cer-LDL treatment. Maleylated BSA is known to bind polyanionic receptors from the scavenger receptor family. PRO61049 is an antibody directed against extracellular domains I, II, and III within the ligand binding region of human LDL receptor. We used maleylated BSA and PRO61049 to prevent LDL and Cer-LDL uptake. Both treatments reduced the accumulation of C_{2}-Cer in cells by more than 70% (Fig. 7, panel A). This was paralleled by a corresponding reduction in the rate of apoptosis. In contrast, nonspecific IgG that was used as a negative control had no effect (Fig. 7, panel B). These data provided strong evidence that the induction of apoptosis by Cer-LDL is receptor mediated. At the same time, however, it is still not possible to identify the exact receptor(s) involved because further characterization of the specificity of the antibody for the LDL receptor and other LDL-binding receptors has to be done.

**LDL-delivered Ceramide Is More Efficient in Inducing Apoptosis Than Micelle-derived Ceramide—C_{2}-Cer-LDL (100 μg/ml) and C_{2}-Cer delivered as micelle (30 μM) cause similar increases in the rate of apoptosis, despite that the amount of C_{2}-Cer added exogenously is quite different. To decipher the reasons for this disparity, the rate of apoptosis was compared with the increases in intracellular ceramide. When C_{2}-Cer was delivered as micelle, increases in the number of apoptotic cells were detected at exogenous ceramide concentrations of 10 and 30 μM. These increases were paralleled by accumulation of 3.9 and 15.8 nmol of ceramide per milligram of cell protein (Fig. 8, right-hand panels). In sharp contrast, treatment with 50 and 100 μg/ml C_{2}-Cer-LDL resulted in similar increases in the rate of apoptosis but a lower accumulation of ceramide, 1.0 and 1.4 nmol/mg cell protein, respectively (Fig. 8, left-hand panels).

Taken together, these results suggest that LDL-derived ceramide is a more efficient effector of apoptosis than C_{2}-Cer delivered via micelles. It is possible that this higher efficiency reflects the more appropriate subcellular localization of LDL-derived ceramide.

**DISCUSSION**

This article provides evidence that LDL-derived ceramide contributes to the cellular ceramide homeostasis and affects the viability of HME-1 cells. The cellular uptake of LDL having...
Ceramide-enriched LDL Induces Apoptosis in Endothelial Cells

Fig. 8. LDL-derived ceramide is a more efficient inducer of apoptosis than micelle-derived ceramide. Cells were treated with C2-Cer-LDL (100 µg/ml) or with the indicated concentrations of micellar C2-Cer delivered in ethanol. The final ethanol concentration was 0.05% and was used for vehicle controls. After 4 h, a set of cells was harvested and the levels of C2-Cer were quantified by the TLC/HPLC method (upper panels). The rest of the cells were incubated for 16 h and stained for TUNEL (lower panels). The data are average ± S.D. from triplicates and the experiment was repeated twice with identical results.

elevated ceramide content leads both to accumulation of ceramide in the cells and the concurrent increase in the incidence of apoptosis, processes that are further enhanced by the presence of TNF-α. Our observations may provide new insight into the mechanisms leading to endothelial cell death during the host response to infections because increases in LDL ceramide content and plasma levels of TNF-α have been reported in experimental conditions of inflammation.

In healthy subjects ceramide is present in the circulating LDL at levels between 3 and 5 nmol/mg of protein. The ceramide content of LDL can be regulated through the action of S-SMase (13) and by de novo ceramide synthesis in liver (15, 16). Our results suggest that although both mechanisms lead to increases in LDL ceramide, their impact on LDL properties may be different. Ceramide increases because of S-SMase are paralleled by depletion of LDL SM, causing aggregation and increased oxidation of the LDL particles. In contrast, elevation of ceramide resulting from activation of de novo synthesis is not accompanied by decreases in SM, and does not affect LDL aggregation and oxidation. Having in mind that aggregated LDL particles are found mainly trapped in the subendothelium, these observations correspond well to earlier findings that S-SMase acts only on LDL immobilized in the subendothelial space (9).

The data in this article suggests that LDL with elevated ceramide content may affect the survival of vascular endothelial cells by providing them with an additional source of ceramide. In our studies apoptosis ensued following a 4–5-fold increase in LDL ceramide content. However, smaller increases may be sufficient in vivo, because the natural long chain ceramide is a more potent effector of cell functions than the short chain ceramide used in our studies (37). It should be noted that elevation in serum ceramide content as high as 8-fold have been described in humans (20) and hamsters (15) under experimental inflammatory conditions.

The ability of Cer-LDL to affect endothelial functions is dependent on the presence of the appropriate receptor, which most likely is the LDL receptor. Having in mind that LDL receptor expression is affected by different pathophysiological conditions, such as cancer, it is tempting to speculate that LDL-derived ceramide may play a role in the progression and outcome of such disorders. In addition, the presence of TNF-α potentiates the uptake of Cer-LDL, accumulation of ceramide, and rate of apoptosis suggesting a major role for LDL-derived ceramide in endothelial function regulation during conditions associated with elevated levels of TNF-α.

Both, in vitro and in vivo studies have shown that the endogenous levels of ceramide in the endothelial cells are a critical factor determining cell survival or death. Increases in cellular ceramide levels induced by γ-irradiation (44) or lipopolysaccharide-induced septic shock (19) have detrimental effects. In contrast, the removal of excess ceramide through the activation of ceramidase and sphingosine kinase prevents endothelial cell death in vitro (18). Our results suggest that extracellularly derived ceramide and not only intracellularly generated ceramide may cause elevation of cellular ceramide levels and may in some inflammatory conditions be involved in the regulation of endothelial survival in vivo. The mechanisms by which LDL-derived ceramide induces endothelial apoptosis is unclear and is a subject to ongoing investigations. One possibility is that by providing ceramide in a specific subcellular localization, probably the endosomal/lysosomal compartment, Cer-LDL triggers a response similar to that seen when acid sphingomyelinase is activated.

In summary, this study provides new insight into the mechanisms controlling the homeostasis of cellular ceramide. Clearly in this process the enzymes determining the intracellular generation of ceramide are critical, but for some cell types like those of the vasculature, ceramide supplied in the form of lipoprotein particles also has an important role.

Acknowledgment—We thank the Cardiovascular Research Group for invaluable help and advice.

REFERENCES

1. Steinberg, D., Parthasarathy, S., Carew, T., Khoo, J. C., and Witztum, J. L. (1989) N. Engl. J. Med. 320, 915–924
2. de Boer, O. J., Van der Wal, A. C., and Becker, A. E. (2000) J. Pathol. 190, 237–243
3. Xu, X. X., and Tabas, I. (1991) J. Biol. Chem. 266, 24849–24858
4. Steenberg, B. (1987) Circulation 76, 508–514
5. Libby, P., Miao, P., Ordovas, J. M., and Schaeffer, E. J. (1985) J. Cell. Physiol. 124, 1–8
6. Scott-Burden, T., Resink, T. J., Hahn, A. W., Baur, U., Box, R. J., and Buhrer, F. R. (1989) J. Biol. Chem. 264, 12582–12589
7. Harada-Shiba, M., Kinosita, K., Kimura, H., and Shimokado, K. (1998) J. Biol. Chem. 273, 9681–9685
8. Tabas, I., Li, Y., Brocio, R. W., Xu, S. W., Swenson, T. L., and Williams, K. J. (1989) J. Biol. Chem. 264, 20419–20432
9. Schissel, S. L., Tweddle-Hardman, J., Rapp, J. H., Graham, G., Williams, K. J., and Tabas, I. (1996) J. Clin. Invest. 98, 1455–1464
10. Schissel, S. L., Jiang, X., Tweddle-Hardman, J., Jeong, T., Camejo, E. H., Najib, J., Rapp, J. H., Williams, K. J., and Tabas, I. (1998) J. Biol. Chem. 273, 2738–2746
11. Subbaiah, P. V., Subramanian, V. S., and Wang, K. (1999) J. Biol. Chem. 274, 36409–36414
12. Maratha, S., Schissel, S., Yellin, M. J., Beutini, N., Mintzer, R., Williams, K. J., and Tabas, I. (1997) J. Biol. Chem. 272, 4981–4988
13. Wang, M. L., Xue, B., Beutini, N., Phu, P., Maratha, S., Johns, A., Gold, P. W., Hirsch, K., Williams, K. J., Licinio, J., and Tabas, I. (2001) Proc. Natl. Acad. Sci. U. S. A. 97, 8861–8866
14. Ozen, K., Hakala, J. K., Annila, A., Ala-Korpela, M., and Kovanen, P. T. (1998) J. Biol. Chem. 273, 29137–29144
15. Memon, R. A., Holleran, W. W., Moser, A. H., Uchida, Y., Fuller, J. Shigenaga, J. K., Grunfeld, C., and Feingold, K. R. (1998) Arterioscler. Thromb. Vasc. Biol. 18, 1257–1265
16. Merrill, A. H., Jr., Lingrell, S., Wang, E., Nikolova-Karakashian, M., Vales, N., and Tabas, I. (1997) J. Biol. Chem. 272, 1257–1265
17. Xia, P., Gamble, J. R., Rye, K. A., Wang, L., Hui, C. S., Cockerill, P., Khew-
Ceramide-enriched LDL Induces Apoptosis in Endothelial Cells

Goodall, Y., Bert, A. G., Barter, P. J., and Vadas, M. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 14196–14201
18. Xia, P., Wang, L., Gamble, J. R., and Vadas, M. A. (1999) J. Biol. Chem. 274, 34499–34505
19. Haimovitz-Friedman, A., Cordon-Cardo, C., Bayoumy, S., Garzotto, M., McLaughlin, M., Gallily, R., Edwards, C. K., 3rd, Schuchman, E. H., Fuks, Z., and Kolesnick, R. N. (1997) J. Exp. Med. 186, 1831–1841
20. Lightle, S., Tosheva, R., Lee, A., Queen-Baker, J., Boyanovsky, B., Shedlofsky, S., and Nikolova-Karakashian, M. (2003) Arch. Biochem. Biophys., in press
21. Butler, P., and Hartley, B. S. (1972) Methods Enzymol. 25, 191–199
22. Nimkar, S., Menaldino, D., Merrill, A. H., and Liotta, D. C. (1988) Tetrahedron Lett. 29, 3037–3040
23. Havel, R. J., Eder, H. A., and Bragdon, J. H. (1955) J. Clin. Invest. 34, 1345
24. Bligh, E., and Dyer, W. (1959) Can. J. Biochem. 37, 911–917
25. Williams, R., Wang, E., and Merrill, A. H., Jr. (1988) Arch. Biochem. Biophys. 228, 282–291
26. Nikolova-Karakashian, M. N., Morgan, E. T., Alexander, C., Liotta, D. C., and Merrill, A. H., Jr. (1997) J. Biol. Chem. 272, 18718–18724
27. Bilheimer, D. W., Eisenberg, S., and Levy, R. I. (1972) Biochim. Biophys. Acta 260, 212–221
28. Goldstein, J. L., Ho, Y. K., Basu, S. K., and Brown, M. S. (1983) Methods Enzymol. 98, 241–260
29. Goldstein, J. L., Basu, S. K., and Brown, M. S. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 333–337
30. Chen, J., Nikolova-Karakashian, M., Merrill, A. H., Jr., and Morgan, E. T. (1995) J. Biol. Chem. 270, 25233–25238
31. Merrill, A. H., Jr., Wang, E., Mallins, R. F., Jamison, W. C., Nimkar, S., and Liotta, D. C. (1988) Anal. Biochem. 171, 373–381
32. Gavrieli, Y., Sherman, Y., and Ben-Sasson, S. A. (1993) J. Cell Biol. 119, 493–501
33. Padok, V. A., Vqelker, D. R., Campbell, P. A., Cohen, J. J., Batton, D. L., and Henson, P. M. (1992) J. Immunol. 148, 2207–2216
34. Vermes, I., Haenen, C., Steffens-Nakken, H., and Reutelingsperger, C. (1995) J. Immunol. Methods 184, 39–51
35. Blair, A., Shaul, P. W., Yuhanna, I. S., Conrad, P. A., and Smart, E. J. (1999) J. Biol. Chem. 274, 32512–32519
36. Swerlick, R. A., Lee, K. H., Wick, T. M., and Lawley, T. J. (1992) J. Immunol. 148, 78–83
37. Ogeretmen, B., Pettus, B. D., Rossi, M. J., Wood, R., Usta, J., Szulc, Z., Bielaw- ska, A., Obeid, L. M., and Hannun, Y. A. (2002) J. Biol. Chem. 277, 12960–12969
38. Bernardes, K., Hurvitz, R., Zenk, T., Desnick, R. J., Ferlinz, K., Schuchman, E. H., and Sandhoff, K. (1995) J. Biol. Chem. 270, 11089–11102
39. Auge, N., Nikolova-Karakashian, M., Carpentier, S., Parthasarathy, S., Negre-Salvayre, A., Salvayre, R., Merrill, A. H., Jr., and Levade, T. (1999) J. Biol. Chem. 274, 25533–25538
40. Paris, F., Fuks, Z., Kang, A., Capodieci, P., Juan, G., Ehleiter, D., Haimovitz-Friedman, A., Cordon-Cardo, C., and Kolesnick, R. (2001) Science 293, 293–297
41. Descamps, L., Cecchelli, R., and Torpier, G. (1997) J. Neuroimmunol. 74, 173–184
42. Simon, C. G., Jr., Holloway, P. W., and Gear, A. R. L. (1999) Biochemistry 38, 14676–14682
43. Venkataraman, K., and Futerman, A. H. (2000) Trends Cell Biol. 10, 408–412
44. Haimovitz-Friedman, A., Kan, C. C., Ehleiter, D., Persaud, R. S., McLaughlin, M., Fuks, Z., and Kolesnick, R. N. (1994) J. Exp. Med. 180, 525–535
