Esterification of 24S-OHC induces formation of atypical lipid droplet-like structures, leading to neuronal cell death

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Abstract The 24(S)-hydroxycholesterol (24S-OHC), which plays an important role in maintaining brain cholesterol homeostasis, has been shown to possess neurotoxicity. We have previously reported that 24S-OHC esterification by ACAT1 and the resulting lipid droplet (LD) formation are responsible for 24S-OHC-induced cell death. In the present study, we investigate the functional roles of 24S-OHC esters and LD formation in 24S-OHC-induced cell death, and we identify four long-chain unsaturated fatty acids (oleic acid, linoleic acid, arachidonic acid, and DHA) with which 24S-OHC is esterified in human neuroblastoma SH-SY5Y cells treated with 24S-OHC. Here, we find that cotreatment of cells with 24S-OHC and each of these four unsaturated fatty acids increases prevalence of the corresponding 24S-OHC ester and exacerbates induction of cell death as compared with cell death induced by treatment with 24S-OHC alone. Using electron microscopy, we find in the present study that 24S-OHC induces formation of LD-like structures coupled with enlarged endoplasmic reticulum (ER) lumina, and that these effects are suppressed by treatment with ACAT inhibitor. Collectively, these results illustrate that ACAT1-catalyzed esterification of 24S-OHC with long-chain unsaturated fatty acid followed by formation of atypical LD-like structures at the ER membrane is a critical requirement for 24S-OHC-induced cell death.—Takabe, W., Y. Urano, D-K. H. Vo, K. Shibuya, M. Tanno, H. Kitagishi, T. Fujimoto, and N. Noguchi. Esterification of 24S-OHC induces formation of atypical lipid droplet-like structures, leading to neuronal cell death. J. Lipid Res. 2016. 57: 2005–2014.

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The brain contains about 25% of the total amount of cholesterol in the human body (1). As the blood-brain barrier prevents cholesterol translocation between the brain and the circulation, brain cholesterol is locally produced. To maintain cholesterol homeostasis in the brain, cholesterol 24-hydroxylase (CYP46A1) converts excess amounts of cholesterol into 24(S)-hydroxycholesterol (24S-OHC), which readily crosses the blood-brain barrier (2). CYP46A1 mRNA and protein are present in neurons, but not in support cells (3). It has been reported that free 24S-OHC is present at concentrations of about 4–15 ng/mg wet weight in the human brain (4). Another report showed that 24S-OHC in four different brain areas of healthy controls was about 20 ng/mg tissue (5); assuming a volume of 1 µl per 1 mg tissue, this would imply a concentration of 24S-OHC in brain tissue that is approximately 50 µM. Several lines of evidence suggest that dysregulation of cholesterol and 24S-OHC metabolism in the brain has been linked to the development of neurodegenerative diseases such as Alzheimer’s disease (AD) and Parkinson’s disease (6–11). Several studies reported that 24S-OHC levels increase or decrease in plasma and cerebrospinal fluid of patients with AD or mild cognitive impairment (11–13); although inconclusive, the differing degrees of progression of disease may account for the different results observed. Selective expression of CYP46A1 around neuritic plaques has also been reported.

Abbreviations: AA, arachidonic acid; AD, Alzheimer’s disease; ADRP, adipose differentiation-related protein; CYP46A1, cholesterol 24-hydroxylase; DART, direct analysis in real time; ER, endoplasmic reticulum; EtOH, ethanol; LA, linoleic acid; LD, lipid droplet; MLKL, mixed lineage kinase domain-like; OA, oleic acid; PA, palmitic acid; RIPK, receptor-interacting protein kinase; SA, stearic acid; SCD1, stearoyl-CoA desaturase 1; 24S-OHC, 24(S)-hydroxycholesterol.

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(14). Moreover, in the brains of patients with AD, increased CYP46A1 expression in astrocytes has been observed (15, 16). Furthermore, we and other groups have shown that 24S-OHC possesses a potent neurotoxicity that may be involved in the etiology of neurodegenerative disease (17, 18). We have also shown that high concentrations of 24S-OHC can induce capsaicin-independent cell death in human neuroblastoma SH-SY5Y cells and rat primary cortical neuronal cells (18).

Necroptosis is a form of caspase-independent programmed necrosis (19-21). Necroptosis can be caused by death receptor ligands or stimuli that induce expression of death receptor ligands under apoptotic-deficient conditions such as absence of functional caspase-8. This regulated cell death requires three proteins, these being: receptor-interacting protein kinase (RIPK)1, RIPK3, and mixed lineage kinase domain-like (MLKL). We have shown that necrostatin-1 (a specific inhibitor of RIPK1 kinase activity) or knockdown of RIPK1 significantly suppresses 24S-OHC-induced cell death (18). We further showed that RIPK1, but neither RIPK3 nor MLKL, was expressed in SH-SY5Y cells, suggesting that 24S-OHC induces RIPK1-dependent necroptosis-like cell death (22). Interestingly, we found that the neuronal cells used did not express caspase-8, which we understood to be the reason why 24S-OHC-induced necroptosis-like cell death and not apoptosis. When we induced the expression of caspase-8 in SH-SY5Y cells by treating the cells with all-trans-retinoic acid, we found that apoptosis could be induced by 24S-OHC (23). We have also shown that 24S-OHC induces apoptosis in human T lymphoma Jurkat cells that endogenously express caspase-8, and that 24S-OHC induces RIPK1-dependent, but MLKL-independent, necroptosis-like cell death in caspase-8-deficient Jurkat cells (22). Despite clues drawn from the foregoing research, however, the specific molecular mechanisms responsible for induction of cell death have remained unclear.

ACAT1, an enzyme located in the endoplasmic reticulum (ER), forms cholesteryl esters to store or prevent cytotoxic build-up of cholesterol (24). We had previously found that ACAT1 catalyzes 24S-OHC esterification to produce at least four varieties of 24S-OHC esters, leading to formation of lipid droplets (LDs) that stained positive with the fluorescent probe, Nile red, in SH-SY5Y cells (25). Because selective ACAT1 inhibitor or ACAT1 siRNA suppresses both 24S-OHC-induced LD formation and cell death, we postulated that ACAT1-catalyzed esterification of 24S-OHC might be responsible for initiation of cell death signaling. However, even if our postulated explanation was correct, it was still unclear to us which varieties of 24S-OHC esters might have formed in the cells treated with 24S-OHC because, at the time of our previous study, no useful method had as yet been established for identifying oxysterol esters and no compounds suitable for use as 24S-OHC ester reference standards were as yet available. Moreover, it was still unclear to us at that time whether the cell death that we observed might be due to the specific cytotoxic effect of some particular 24S-OHC ester or whether it might be the accumulation of total 24S-OHC ester regardless of the variety that was responsible. It was also unknown to us at that time whether it was accumulation of 24S-OHC ester or formation of LDs (or some other unknown phenomenon) that was responsible for cell death signaling.

In the present study, we investigate the precise association between 24S-OHC-induced cell death and the ACAT1-catalyzed 24S-OHC esterification that is responsible for LD formation. We find that there are increased amounts of esters produced by esterification of 24S-OHC with four varieties of long-chain unsaturated fatty acid in 24S-OHC-treated SH-SY5Y cells. We further show that 24S-OHC induces formation of atypical LD-like structures coupled with enlarged ER lumina in SH-SY5Y cells. We conclude that formation of atypical LD-like structures and the accompanying abnormal ER morphology, both of which are caused by accumulation of 24S-OHC esters, play an important role in 24S-OHC-induced cell death.

MATERIAL AND METHODS

Materials

ACAT inhibitor F12511 was the generous gift of Dr. Ta-Yuan Chang (Dartmouth Medical School, Hanover, NH). The 24S-OHC and various forms of esterified 24S-OHC were synthesized as we previously reported (26). All fatty acids and Nile red were obtained from Sigma-Aldrich (St. Louis, MO). BODIPY 493/503 and DAPI were from Thermo Fisher Scientific (Waltham, MA). Stearoyl-CoA desaturase 1 (SCD1) inhibitor was from BioVision (Milpitas, CA). All other chemicals, of analytical grade, were obtained from Sigma-Aldrich or Wako (Osaka, Japan).

Cell culture

Human neuroblastoma cells from the SH-SY5Y cell line were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 (Thermo Fisher Scientific), which contained 10% fetal bovine serum (HyClone, Logan, UT) and antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin; Thermo Fisher Scientific). Cells were grown at 37°C in an atmosphere of 5% CO2.

Cell treatment

The 24S-OHC and oleic acids (OAs) were dissolved in ethanol (EtOH) and stored at −20°C. To examine toxicity or esterification of 24S-OHC in SH-SY5Y cells, cells were treated with 50 μM 24S-OHC (final concentration of EtOH in the medium was 0.5%) in the presence or absence of 50 μM of each fatty acid under study for the indicated period. To evaluate LD formation, cells were treated with 50 μM 24S-OHC or 200 μM OA for the indicated period. Control cells were treated with EtOH.

Knockdown of ACAT1 and RIPK1 by siRNA

Stealth siRNA targeting human RIPK1 (18), ACAT1 (25), and Stealth RNAi negative control were obtained from Thermo Fisher Scientific. Cells were transfected with siRNAs using Lipofectamine RNAiMax (Thermo Fisher Scientific) as previously described (18, 25). Forty-eight hours after transfection, cells were used for further analyses.

Neutral lipid staining

Neutral lipids were stained with Nile red as previously described (25) and fluorescence images were obtained using an inverted microscope.
fluorescence microscope (OLYMPUS IX71; Tokyo, Japan). To clarify the localization of neutral lipid-rich structures, cells were stained with BODIPY 493/503 (Thermo Fisher Scientific). Briefly, cells were fixed in a mixture of 3% formaldehyde and 0.025% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min. Cells were then stained with 0.5 μg/ml BODIPY 493/503 for 15 min at room temperature. Nuclei were counterstained by DAPI and images were obtained using confocal microscopy (Zeiss LSM710; Oberkochen, Germany).

**Lipid extraction and HPLC analysis**

Cellular lipids were extracted and HPLC analysis was performed as described previously (25). Briefly, after the treatment, the medium was removed and the cells were washed with PBS three times, lysed by 0.2 M NaOH, and neutralized immediately with 3 M HCl and 1 M KH₂PO₄. The Folch method (2:1, v/v chloroform/methanol) was thereafter used to extract lipids, the chloroform phase being evaporated in a nitrogen gas environment. The dry residue was then dissolved in eluent without water, and this was subjected to HPLC analysis. HPLC was carried out using an Inertsil ODS-3 column (5 μm, 4.6 × 250 mm; GL Science, Tokyo, Japan) in combination with a pump (PU-980 Intelligent HPLC pump; Nihon Bunko, Tokyo, Japan), an UV detector outputting a signal in units of millivolts (UV-970 Intelligent UV/VIS detector; Nihon Bunko), and a data processor (EPC-500; Eicom, Kyoto, Japan) under conditions such that flow rate of eluent (acetonitrile/2-propanol/water, 43/53/4) was 1.0 ml/min.

**Direct analysis in real time-MS analysis**

Synthesized 24S-OHC esters were dissolved in CHCl₃ at concentrations of approximately 10⁻⁴ M and the resulting solutions were subjected to measurement of mass spectra. For cellular extract, lipid extracts were fractionated by HPLC, the eluent was removed using nitrogen gas, the dry residue was dissolved in CHCl₃ (approximately 100 μl), and the resulting CHCl₃ solutions were subjected to MS analysis. Measurement of mass spectra was carried out using an LCMS 2020 instrument (Shimadzu, Kyoto, Japan) equipped with a direct analysis in real time (DART)-SVP ion source (IonSense, Inc., Saugus, MA). Helium was used as ionization gas at a temperature of 500°C. Mass spectra were obtained with the instrument set to positive ion mode.

**Determination of cell viability**

To determine cell viability, WST-8 assay was performed using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer’s protocol. MITT assay was performed as described previously (18). Data were expressed as percentages relative to cells treated with vehicle serving as control.

**Isolation of LD-enriched fraction**

Cells were resuspended in buffer A [25 mM Tris-HCl (pH 7.4), 100 mM KCl, 1 mM EDTA, 1 mM EGTA] with protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). Cells were disrupted under high pressure by nitrogen bomb at 800 psi for 15 min, and the lysate was centrifuged for 10 min at 1,000 g at 4°C. The postnuclear supernatant was mixed with an equal volume of 1.08 M sucrose in buffer A (final sucrose concentration was 0.54 M). This postnuclear supernatant (1.25 ml) in 0.54 M sucrose in buffer A, 1.25 ml of 0.27 M sucrose in buffer A, 1.25 ml of 0.135 M sucrose in buffer A, and 1.25 ml of buffer A without KCl were layered sequentially in 5 ml tubes for ultracentrifugation (Beckman Coulter, Brea, CA). Tubes were centrifuged at 34,000 rpm at 4°C for 1 h using an Optima L-90K Ultracentrifuge (Beckman Coulter) with a swing rotor (SW 55 Ti). Following centrifugation, fractionated samples were collected at 0.5 ml intervals from the top of each tube.

**Immunoblotting and MS analysis**

Equal aliquots from each fraction were subjected to SDS-PAGE and immunoblotting by using primary antibodies specific for adipose differentiation-related protein (ADRP; Fitzgerald Industries International, Acton, MA), ribophorin-1 (Santa Cruz Biotechnologies, Dallas, TX), and RIPK1 (BD Biosciences, San Jose, CA) with appropriate secondary antibodies. Immunoblotting was visualized with enhanced chemiluminescence (Millipore, Billerica, MA). For each set of conditions, i.e., EtOH, 24S-OHC, and OA as indicated in Fig. 6B, the top three fractions were subjected to SDS-PAGE and were detected by silver staining using a Dodeca silver stain kit (Bio-Rad, Berkeley, CA). Visible bands obtained by silver staining were manually cut and subjected to LC-MS as described previously (6).

**Electron microscopy analysis**

For electron microscopy, cells cultured on glass coverslips were fixed for more than 2 h in a mixture of 2% formaldehyde and 2.5% glutaraldehyde in 0.1 M HEPES-NaOH (pH 7.4), to which 1 mM CaCl₂ had been added, and were thereafter postfixed for 1 h in a mixture of 1% osmium tetroxide and 0.1% potassium ferrocyanide in 0.1 M sodium cacodylate buffer (27), following which they were dehydrated and embedded in epoxy resin. Ultrathin sections were observed using a JEM1011 electron microscope (JEOL, Tokyo, Japan) operated at 100 kV.

**Statistical analysis**

Data are reported as mean ± SD of at least three independent experiments. The statistical significance of the difference between the determinations was calculated by an ANOVA using Tukey’s test for multiple comparisons. The difference was considered significant at P < 0.05.

**RESULTS**

The 24S-OHC-induced neutral lipid-rich structure formation occurred upstream of RIPK1 signaling in SH-SY5Y cells

We have previously shown that siRNA knockdown of either ACAT1 or RIPK1 significantly suppresses 24S-OHC-induced cell death (18, 25). In the present study, to examine the relationship between 24S-OHC-induced LD formation and RIPK1 activation, cells were transfected with ACAT1 (siACAT1), RIPK1 (siRIPK1), or negative control siRNA oligos for 48 h, and were then treated with 50 μM 24S-OHC or vehicle (0.5% EtOH) for a further 6 h. Consistent with previous findings, knockdown of ACAT1 was found to suppress 24S-OHC-induced Nile red-positive LD formation (Fig. 1). In contrast, knockdown of RIPK1 did not affect 24S-OHC-induced LD formation. These data suggested that 24S-OHC-induced LD formation was occurring upstream of RIPK1 signaling.

**Esterified forms of 24S-OHC in which 24S-OHC was esterified with four varieties of unsaturated fatty acid were identified in SH-SY5Y cells treated with 24S-OHC**

Because we had previously observed that LD formation due to accumulation of 24S-OHC esters occurs in the early stage of 24S-OHC-induced cell death (25), we next focused on identifying which fatty acids it was that were esterifying with 24S-OHC. We previously reported being able to detect
samples that contained 24S-OHC-AA, 24S-OHC-LA, or 24S-OHC-OA showed a marked increase in the area under the respective peak 2, 3, or 4, as compared with lipid extract alone (Fig. 3, peaks 2"–4"). Note that we also measured the retention times of the peaks observed with synthesized 24S-OHC-SA and 24S-OHC-PA, and found that neither of these corresponded to any of the retention times measured for peaks 1–4 (data not shown).

Finally, we employed a novel mass spectrometric technique for yet further identification of the respective 24S-OHC esters. Prior to settling on the mass spectrometric technique that is reported below, we first tried to use gas chromatography-MS, matrix-assisted laser desorption/ionization time-of-flight-MS, and electrospray ionization-MS; despite our best attempts with each of these methods, however, we were unable to detect mass spectrographic patterns corresponding to the 24S-OHC esters due to difficulties experienced in ionizing these esters. But as we had recently established a novel technique using DART-MS that permits specific ion peaks derived from synthesized 24S-OHC esters to be detected (26), we decided to apply our DART-MS technique to identification of the 24S-OHC esters formed in SH-SY5Y cells using synthesized 24S-OHC esters as reference standards. Using a procedure similar to that described above with reference to Fig. 2, for each fatty acid under study, cells were co-treated with 50 μM fatty acid and 50 μM 24S-OHC for 6 h, and lipid extracts were thereafter subjected to HPLC analysis. For all DART-MS measurements, our instrument was operated in positive ion mode under conditions such that synthesized 24S-OHC esters were detected as [M+NH4]+ adduct ions (Fig. 4A). For example, synthesized 24S-OHC-DHA was detected by the presence of the parent ion peak at m/z 731 in the resulting spectra. The fragment ion peak at m/z 385.
was attributed to the ester linkage. Based on comparison with synthesized 24S-OHC-DHA, -AA, -LA, -OA (Fig. 4A), and 24S-OHC-PA, -SA (supplemental Fig. S2), the adduct ion of m/z 731 at peak 1’ (Fig. 4B) was found to be identical to that of synthesized 24S-OHC-DHA. Similarly, the adduct ion of m/z 707 at peak 3’, m/z 683 at peak 2’, and m/z 685 at peak 4’ were respectively found to be identical to those of synthesized 24S-OHC-AA, 24S-OHC-LA, and 24S-OHC-OA. Collectively, these data suggest that DHA, AA, LA, and OA are fatty acids that are used for esterification of 24S-OHC in 24S-OHC-treated SH-SY5Y cells.

**Effect that cotreatment with fatty acid had on 24S-OHC-induced cell death in SH-SY5Y cells**

Because it was our observation that at least four varieties of 24S-OHC ester accumulate in 24S-OHC-treated cells, we considered it important to determine whether any particular 24S-OHC ester had specific cytotoxic effect or whether it was the formation of LD that triggered cell death signaling regardless of the variety of 24S-OHC ester. Unfortunately, synthesized 24S-OHC esters could not be introduced directly into cells due to their high hydrophobicity (data not shown). To evaluate the cytotoxic properties of these four varieties of 24S-OHC ester, for each unsaturated fatty acid under study, we therefore evaluated the effect of cotreatment with 24S-OHC and 50 μM unsaturated fatty acid for 24 h (Fig. 5) in SH-SY5Y cells, because in the present study we had already confirmed that there was increase of the corresponding 24S-OHC ester under the same treatment for 6 h (Fig. 2). WST-8 assay was performed to determine the effect of cotreatment with 24S-OHC and fatty acid on cell death. As a result, we found that cotreatment with 24S-OHC and each of the fatty acids under study, except DHA, decreased cell viability as compared with treatment with 24S-OHC alone. Because we found that, at the concentration employed, none of the fatty acids induced cell death in SH-SY5Y cells, this supports our conclusion that, for all fatty acids under study for which reduction of cell viability was observed, it was cotreatment with the fatty acid and not any cytotoxicity on the part of the fatty acid itself that was responsible for the reduction of cell viability that we observed. These data suggest that it may be the accumulation of total esterified 24S-OHC and the resulting LD formation that causes cell death, independent of any specificity on the part of the ester employed for cotreatment.

**The 24S-OHC induced LD-like structures along with swollen ER in SH-SY5Y cells**

It is generally believed that LDs are formed to store excess lipids and protect cells against toxicity of lipids (28, 29). However, our present study showed that 24S-OHC-derived LD formation might actually be a cause of cell death. To compare 24S-OHC-induced LDs that we observed with fatty acid-induced TG-rich typical LDs, treatment with OA at high concentration (200 μM) was employed to induce LD formation. We first investigated the localization of LDs in SH-SY5Y cells in response to 50 μM 24S-OHC or 200 μM OA for 6 h by using Nile red (Fig. 6A) or the fluorescent probe, BODIPY 493/503, both of which stain neutral lipids (supplemental Fig. S3). We found as a result of our investigation that OA-treated cells had abundant LDs, these being dispersed throughout the cytoplasm, while 24S-OHC-treated cells had only a limited number of LDs, these being present near the nuclei. Cotreatment with 24S-OHC and 50 μM OA, which decreased cell viability as
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Time [min.]

- 0 10 20 30 40

24S-OHC 6 h + 24S-OHC-OA
24S-OHC 6 h + 24S-OHC-LA
24S-OHC 6 h + 24S-OHC-AA
24S-OHC 6 h + 24S-OHC-DHA
24S-OHC 6 h

Fig. 3. Retention times of four synthesized 24S-OHC esters corresponded to those of four peaks observed in 24S-OHC-treated cells. SH-SY5Y cells were treated with 50 μM 24S-OHC for 6 h. Lipid extracts were mixed with synthesized 4 nmol 24S-OHC-DHA, -LA, -OA, or 2 nmol 24S-OHC-AA for spiking and were thereafter subjected to HPLC analysis.

compared with treatment with 24S-OHC alone (Fig. 5), showed increases in both the number and the size of Nile red-positive LDs (Fig. 6A).

To further evaluate 24S-OHC-treated cells versus OA-treated cells with respect to formation of LDs, we applied sucrose density gradient centrifugation to attempt to isolate LD-enriched fractions from 24S-OHC-treated cells and OA-treated cells. ADRP, which is an LD-associated protein (28), was used as marker for LDs. LDs were generally recovered at the top surface, while other organelles were recovered at lower fractions along the gradient. The results showed that, for OA-treated cells, ADRP was detected at the top fraction (fraction #1), whereas the ER marker protein, ribophorin-1, and the cytosolic protein, RIPK1, were enriched in the bottom fractions (fractions #9, #10) (Fig. 6B, right panel). However, no ADRP was observed in any fraction recovered from 24S-OHC-treated cells (Fig. 6B, center panel), even though Nile red-positive and BODIPY-positive structures were observed in those cells (Figs. 1, 6A). Three fractions from the top in each condition were further subjected to silver staining and MS analysis (supplemental Fig. S4). The results showed that various proteins reported to exist in LDs (29) were detected in the top fraction obtained from OA-treated cells, but were not detected in the top fraction obtained from 24S-OHC-treated cells. Cell viability assays of 24S-OHC-treated cells and OA-treated cells showed that while significant cell death occurred in cells treated with 24S-OHC for 24 h, treatment with OA for 24 h did not induce cell death (Fig. 6C), suggesting that typical LD formation itself was not a cytotoxic event in SH-SY5Y cells.

To further examine 24S-OHC-induced LDs in SH-SY5Y cells, we performed morphological analysis using electron microscopy. Cells were treated with either 50 μM 24S-OHC or 200 μM OA for 6 h, following which conventional ultrathin sections were prepared. While no LD structure was observed in EtOH-treated control cells (Fig. 7A), typical LD structures were observed in the cytoplasm of OA-treated cells (Fig. 7B). In contrast to the typical LD structures observed in OA-treated cells, in 24S-OHC-treated cells we observed atypical LD-like structures (Fig. 7C, white asterisk) that appeared to be attached to some sort of enlarged membrane structure (black star). Because our observations suggested that these enlarged membrane structures were connected to the ER membrane (Fig. 7C, arrow), we theorized that these structures might have formed as a result of enlargement of the ER lumen. Cells cotreated with 50 μM OA and 24S-OHC showed an increased amount of 24S-OHC-OA (Fig. 2A) and decreased cell viability (Fig. 5), and it was further observed that there was an increase in the size of the atypical LD-like structures (Fig. 7D, white asterisk) that were attached to the enlarged membrane structures (Fig. 7D, black star). When cells were treated with 24S-OHC in the presence of F12511, an ACAT inhibitor, the 24S-OHC-induced atypical LD-like structures disappeared, but unknown multilamellar structures were observed (Fig. 7E, arrowhead). These observations led us to conclude that 24S-OHC induces ACAT-dependent atypical LD-like structures along with enlarged ER lumina, these being features that are different from typical OA-induced LDs, in SH-SY5Y cells.

DISCUSSION

ACAT generally catalyzes the esterification of free cholesterol with fatty acid to yield cholesteryl ester in the ER (19). Two ACAT isoenzymes, ACAT1 and ACAT2, have been identified. ACAT1 is the main isoenzyme in the brain (30). We have found that ACAT1 mRNA, but not ACAT2 mRNA, is expressed in SH-SY5Y cells (25). One study reported that ACAT1 showed a slight preference for oleoyl-CoA, compared with palmitoyl-, linoleoyl-, or arachidonyl-CoA, as substrate in insect cells expressing human ACAT1 (31). Another study has shown that microsomes isolated from human ACAT1-expressing CHO cells showed strong substrate preference for oleoyl-CoA, compared with linolenoyl-, arachidonoyl-, or eicosapentaenoyl-CoA (32). In our present study, we showed that ACAT1 preferentially utilizes long-chain unsaturated fatty acids (OA, LA, AA, and DHA) rather than saturated fatty acids (PA and SA) for esterification of 24S-OHC. Our previous (25) and present results suggest that 24S-OHC ester forms in SH-SY5Y cells in relative amounts such that 24S-OHC-OA > 24S-OHC-LA > 24S-OHC-AA > 24S-OHC-DHA, but there was no significant difference among these four fatty acids in terms of any observed preference as substrate for ACAT1. The present study showed that at least four varieties of 24S-OHC ester
accumulated in 24S-OHC-treated cells, though it is possible that other minor esterified forms of 24S-OHC may also have formed in the cells. We also found that cotreatment with 24S-OHC and any of the four unsaturated fatty acids tested resulted in a cell death that was enhanced as compared with that which was induced by 24S-OHC alone. Specific enhancement of 24S-OHC-induced cell death as a result of cotreatment with any particular one of the unsaturated fatty acids tested was not observed, suggesting that it was the accumulation of total 24S-OHC ester regardless of variety, and not the specific cytotoxicity of any particular 24S-OHC ester, that was responsible for the 24S-OHC-induced cell death that we observed. We also found that 24S-OHC-induced cell death was significantly reduced when cells were cotreated with an inhibitor of SCD1 (supplemental Fig. S5); because SCD1 catalyzes the synthesis of monounsaturated fatty acids from saturated fatty acids, this supported the idea that esterification of 24S-OHC with unsaturated fatty acid is responsible for 24S-OHC-induced cell death. Interestingly, extracellular esterification of 24S-OHC by lecithin:cholesterol acyltransferase reduces the cytotoxic effect of 24S-OHC by limiting uptake of 24S-OHC esters by cells (33), suggesting that ACAT1-catalyzed intracellular esterification of 24S-OHC plays an important role in 24S-OHC-induced cell death.

In our present study, biochemical and morphological analysis revealed that ACAT1-mediated 24S-OHC esterification induced formation of atypical LD-like structures coupled with what appeared to be a swollen ER structure, but that the presence of OA induced typical LD formation without affecting cell viability in SH-SY5Y cells. Many lines of evidence suggest that LDs are formed within the ER membrane (28, 29). Most conventional models have assumed that formation of LDs occurs with an initial accumulation of TG or sterol ester between the two leaflets of the ER membrane. It is believed that at some point the prenascent LD structure presumably buds off from the ER, forming a nascent LD, followed by growth of the LD. Because ACAT1 is localized in the ER membrane, the conventional thinking has been that newly produced 24S-OHC esters might accumulate between the two membrane leaflets of the ER in similar fashion as would be the case with a cholesteryl ester. However, unlike cholesteryl esters, which are entirely hydrophobic, 24S-OHC esters have a hydroxyl group at position 24 in their steroid side chain (supplemental Fig. S1). It is therefore plausible that the polar moiety at the side chain and the nonpolar moiety at the sterol ring in combination with the fatty acid portion of the 24S-OHC ester might act together to upset orientation of the 24S-OHC

Fig. 4. DART-MS spectra of synthesized 24S-OHC esters and of fractions collected by HPLC from 24S-OHC-treated cells. For each unsaturated fatty acid under study, SH-SY5Y cells were treated with 50 µM 24S-OHC and 50 µM fatty acid for 6 h as in Fig. 2. Lipid extracts were subjected to HPLC analysis. Synthesized 24S-OHC esters (A) and fractions collected by HPLC (B) were further subjected to DART-MS analysis.

Fig. 5. Cotreatment with unsaturated fatty acid and 24S-OHC enhanced 24S-OHC-induced cell death. For each fatty acid under study, SH-SY5Y cells were treated with 50 µM 24S-OHC in the presence or absence of 50 µM fatty acid for 24 h. Cell viability was measured by WST-8 assay. **P < 0.01, when compared with cells with 24S-OHC and EtOH.
ester in the membrane bilayer, thereby disturbing ER membrane integrity and causing LD formation to become atypical. Because it has been reported that dysregulation of ER function, such as that which is caused by severe ER stress, can induce cell death (34–36), we postulated that disturbance of ER homeostasis by accumulation of 24S-OHC ester could trigger cell death signaling. Another possibility is that the atypical LD-like structures and/or swollen ER structures that we observed could be providing an intracellular platform to permit association of certain proteins that promote cell death signaling. Further experiments will be required to address these possibilities. It is noted that unknown multilamellar structures were observed in 24S-OHC-treated cells that had been cotreated with ACAT inhibitor. It is thought that formation of such unknown multilamellar structures might occur as a result of accumulation of free 24S-OHC. Because ACAT inhibitor suppressed 24S-OHC-induced cell death, these unknown multilamellar structures were not thought to be associated with cell death signaling. Interestingly, it has been reported that treatment with polar oxysterol (7-ketocholesterol, 7β-hydroxycholesterol, cholesterol-5β, 6β-epoxide) induced a multilamellar cytoplasmic structure that was predominantly lysosomal in origin in various cell types (37, 38). Further studies are needed to characterize the multilamellar structures that were observed in the present study.

With respect to methodology, in our present study we found that we were able to effectively ionize 24S-OHC esters using the DART method. Although mass spectrometric analysis is a powerful tool to investigate unknown biochemical compounds, inability to ionize esterified forms of sterols and sterol derivatives has forced many researchers to employ the saponification method, which unfortunately only permits evaluation of total sterol content (25, 39). In
contrast, with DART analysis, it is possible to detect the parent ion peak of molecules as they exist in their esterified form, without the need for additives, pretreatment, or other such potentially sample-altering methods of preparation. Because DART-MS analysis cannot easily distinguish between different isomers under current conditions, DART-MS analysis must be coupled with a chromatographic technique, such as HPLC, using a suitable column and the synthetic standard compounds if it to be used for identification of compounds. We propose that DART-MS analysis may serve as a useful tool for investigation not just of 24S-OHC esters, as in our present study, but of a wide variety of esters of sterols and fatty acids. For example, there has been growing interest in oxysterol research because there are a number of bioactivities of oxysterols that have received much attention (40, 41), and oxysterols also have potential for use as biomarkers of neurodegenerative diseases (10, 42, 43). Although it will be necessary to devise appropriate techniques before DART-MS can be used for precise quantitative analysis, it is thought that the DART-MS method will ultimately be valuable for evaluation not only of free oxysterol, but also of oxysterol esters in the tissue, plasma, and cerebrospinal fluid of patients suffering from neurodegenerative disease.

Because we have previously shown that ACAT1-mediated 24S-OHC esterification occurs upstream of the switching point at which the determination is made as to whether cell death will be apoptotic or necroptosis-like (22, 25), the atypical LD-like structures and swollen ER that we observed to form as a result of treatment with 24S-OHC should also play an important role in 24S-OHC-induced caspase-dependent apoptosis. Unlike the extrinsic pathway for programmed cell death in which the cell surface death receptor is stimulated by its ligand (44), the morphological abnormalities that we observed to occur as a result of treatment with 24S-OHC should also play an important role in 24S-OHC-induced cell death. In line with our observations, it has recently been reported that ER stress induces ligand-independent necroptosis in L929 mouse fibroblast cells (45). It should be noted, however, with regard to physiological relevance, that it is not clear that the changes observed in our present study would necessarily occur in accompaniment to the increase in CYP46A1 expression (14–16) or the increase in brain 24S-OHC level that has been observed in early- and middle-stage AD (11–13). Whereas LD structures have been observed in brain tissue from AD patients and in AD mouse models (46–48), there remains a need to elucidate whether an increase in 24S-OHC esters and formation of atypical LD-like structures, as reported in our present study, occur in vivo as a consequence of the normal progression of AD. In conclusion, our present study suggests that ACAT1-mediated esterification of 24S-OHC with long-chain unsaturated fatty acid and formation of atypical LD-like structures with abnormal ER morphology play important roles in 24S-OHC-induced cell death in SH-SY5Y cells.

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