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ORIGINAL ARTICLE

The enzyme lecithin–cholesterol acyltransferase esterifies cerebrosterol and limits the toxic effect of this oxysterol on SH-SY5Y cells

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Abstract

Cholesterol is mostly removed from the CNS by its conversion to cerebrosterol (24(S)-hydroxycholesterol, 24(S)OH-C), which is transported to the circulation for bile formation in liver. A neurotoxic role of this oxysterol was previously demonstrated in cell culture. Here, we provide evidence that the enzyme lecithin-cholesterol acyltransferase, long known to esterify cholesterol, also produces monoesters of 24(S)OH-C. Proteoliposomes containing apolipoprotein A-I or apolipoprotein E were used to stimulate the enzyme activity and entrap the formed esters. Proteoliposomes with apolipoprotein A-I were found to be more active than those with apolipoprotein E in stimulating the production of oxysteryl esters. Cholesterol and 24(S)OH-C were found to compete for enzyme activity. High levels of haptoglobin, as those circulating during the acute inflammatory phase, inhibited 24(S)OH-C esterification. When highly neurotoxic 24(S)OH-C was treated with enzyme and proteoliposomes before incubation with differentiated SH-SY5Y cells, the neuron survival improved. The esters of 24(S)OH-C, embedded into proteoliposomes by the enzyme and isolated from unesterified 24(S)OH-C by gel filtration chromatography, did not enter the neurons in culture. These results suggest that the enzyme, in the presence of the apolipoproteins, converts 24(S)OH-C into esters restricted to the extracellular environment, thus preventing or limiting oxysterol-induced neurotoxic injuries to neurons in culture.

Keywords: ApoA-I, ApoE, cerebrosterol, haptoglobin, LCAT, neuron culture.

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Cell cholesterol (C) homeostasis in CNS depends on mechanisms that are regulated by the level of C and by oxygenated derivatives of C, termed oxysterols, contributing to lowering intracellular free C levels (Björkhem *et al.* 2009; Bielska *et al.* 2012; Olsen *et al.* 2012). In particular, C is partly converted to the more polar compound 24(S)-hydroxycholesterol (24(S)OH-C), also termed cerebrosterol, that can freely diffuse across the cell membranes and the blood–brain barrier (BBB) (Björkhem and Meaney 2004; Leoni and Caccia 2011) thus gaining access to the circulation, by which it is transported to the liver for catabolism and elimination from the body (Björkhem *et al.* 2001, 2009). 24(S)OH-C, together with other oxysterols, promotes C efflux and limits lipoprotein C uptake, and stimulates

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Abbreviations used: BBB, blood–brain barrier; LCAT, lecithin-cholesterol acyltransferase; ApoA-I, apolipoprotein A-I; ApoE, apolipoprotein E; Hpt, human haptoglobin; C, cholesterol; 24(S)OH-C, 24(S)-hydroxycholesterol; CYP46, cholesterol 24-hydroxylase; Aβ, amyloid-β peptide; PC, phosphatidylcholine; NBD-PC, 1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-sn-glycero-3-phosphocholine; LPDS, lipoprotein-deficient serum; HSA, human serum albumin; FBS, fetal bovine serum; RA, retinoic acid; BHT, butylated hydroxytoluene; MRFA, Met-Arg-Phe-Ala peptide; DS, dextran sulfate; $[M+H-H_2O]^+$, protonated molecular mass without a molecule of water; C-n, carbon atom at position n in the 24(S)OH-C structure; S, saponified sample; NS, non-saponified sample.

intracellular C esterification (Wang *et al.* 2008; Bielska *et al.* 2012; Olsen *et al.* 2012). This oxysterol, although playing protective roles such as inhibiting the synthesis of amyloid-β peptides (Aβ) (Björkhem *et al.* 2009; Prasanthi *et al.* 2009), was also suggested to be implicated in inflammation (Vejux and Lizard 2009) and pathogenesis of neurodegenerative processes (Poli *et al.* 2013). Neurotoxic properties of 24(S)OH-C, mediated by increased generation of free radicals, reduced mitochondrial membrane potential, and induction of necroptosis, were demonstrated on both undifferentiated and differentiated human neuronal cells in culture (Kölsch *et al.* 1999, 2001; Gamba *et al.* 2011; Yamanaka *et al.* 2011; Testa *et al.* 2012). The removal of C excess from peripheral cells, and its delivery to liver by lipoproteins, a key process preventing C accumulation and toxicity, includes a step of C esterification resulting from the activity of the enzyme lecithin-cholesterol acyltransferase (LCAT, EC 2.3.1.43) (Glomset 1968; Jonas 2000). This enzyme is present on either side of the BBB, as detected in both plasma (Jonas 2000) and CSF (Hirsch-Reinshagen *et al.* 2009), and was demonstrated to esterify in plasma both 25-hydroxycholesterol and 27-hydroxycholesterol producing monoesters from the former (Lin and Morel 1996), and both monoesters and diesters from the latter oxysterol (Szedlacsek *et al.* 1995). Because of the structural similarity between 24(S)OH-C and C, it was hypothesized that LCAT might also produce esters of this oxysterol in CSF, and that the 24(S)OH-C esterification might be functional to the removal of this oxysterol from the CNS (Papassotiropoulos *et al.* 2002; Björkhem 2006), thus preventing its neurotoxic accumulation in the brain. Most of 24(S)OH-C was actually found as fatty acid esters (24(S)OH-CE) in CSF and plasma lipoproteins (Dzeletovic *et al.* 1995; Papassotiropoulos *et al.* 2002; Burkard *et al.* 2007), but evidence that 24(S)OH-CE can be produced by LCAT is lacking to date. Therefore, an aim of our study was to demonstrate that LCAT esterifies 24(S)OH-C, and provide information on whether the produced 24(S)OH-CE are mono- or diesters or both. We also compared the abilities of apolipoprotein A-I (ApoA-I) and apolipoprotein E (ApoE), both necessarily required for LCAT activation (Zorich *et al.* 1985; Jonas 2000) in stimulating 24(S)OH-C esterification. ApoE and ApoA-I play a key role in modulating lipid and lipoprotein metabolism in the CNS, and are associated with high density lipoprotein-like lipoproteins in CSF (Pitas *et al.* 1987; Demeester *et al.* 2000; LaDu *et al.* 2000; Koch *et al.* 2001). Finally, the major aim of our study was to investigate whether 24(S)OH-C esterification affects the internalization of this oxysterol into differentiated neuroblastoma cells in culture, and limits or prevents the neurotoxic effect of this oxysterol. Our results provide evidence that LCAT produces 24(S)OH-C monoesters, ApoA-I stimulates the 24(S)OH-C esterification better than ApoE, and haptoglobin (Hpt) inhibits such an esterification activity. Furthermore, we demonstrate that the 24(S)OH-C neurotoxicity is reduced after this oxysterol incubation with LCAT, and proteoliposomes containing 24(S)OH-CE do not enter the cells.

Methods

Materials

The fluorescent lipid 1-palmitoyl-2-{6-[(7-nitro-2-1,3- benzoxadiazol-4-yl)amino]hexanoyl}-sn-glycero-3-phosphocholine (16:0-6:0 NBD-PC) and 24(S)OH-C were purchased from Avanti Polar Lipids (Alabaster, Al, USA). [22,23-^{3}H]-24(S)OH-C (50 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO, USA). [1α,2α-^{3}H]-Cholesterol (52.5 Ci/mmol) and the scintillation cocktail Ultima Gold were obtained from Perkin-Elmer (Boston, MA, USA). Chemicals of the highest purity, human serum albumin (HSA), phosphatidylcholine (PC) from egg yolk, 2-linoleoyl-1-palmitoyl-sn-glycero-3-phosphocholine, retinoic acid (RA), glutaraldheyde, Crystal Violet, cholesterol, and the Supelcosil LC-18 column (5 μm particle size, 250 × 4.6 mm) were purchased from Sigma-Aldrich (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were from BioWhittaker (Verseviers, Belgium). L-glutamine, penicillin, and streptomycin were from Gibco (Life Technologies Italy, Monza, Italy). 96-well cell culture plates and sterile pipettes of Beckton-Dickinson (Milan, Italy) were used. Human ApoA-I and Amicon centrifugal filters from Millipore (Billerica, MA, USA) were used. Recombinant ApoE3 was purchased from Peprotech (London, UK). Rabbit monoclonal IgG anti-LCAT was from Abcam (Cambridge, UK). The Nucleodur C18 ISIS column (3 μm particle size, 250 × 4.6 mm), and Sil-G plates for TLC (thickness 0.25 mm) were from Macherey-Nagel (Duren, Germany). The Sephacryl S-200 resin was from GE-Healthcare Life Sciences (Milan, Italy). Organic solvents were purchased from Romil (Cambridge, UK). The human neuroblastoma cell line SH-SY5Y was kindly provided by IGB-CNR (Naples, Italy). CSF samples were kindly provided by the Neurology Clinic of the Second University of Naples.

Esterification of 24(S)OH-C *in vitro*

Lipoprotein-deficient serum (namely LPDS), prepared as previously reported (Ohnishi *et al.* 1990) and dialyzed (cut-off = 30 kDa) against Tris-buffered saline (TBS) (140 mM NaCl, 20 mM Tris-HCl, pH 7.4) at 4°C, was used as source of LCAT. In some experiments 24(S)OH-C was esterified by LCAT using proteoliposomes, containing ApoA-I as stimulating effector, and PC with α2-linked fluorescent hexanoyl acid (NBD-PC) or natural fatty acids as acyl donor. Fluorescent proteoliposomes (ApoA-I/NBD-PC molar ratio = 1/133; 27.5 μM ApoA-I) were prepared by the cholate dialysis method (Chen and Albers 1982) omitting sterol addition. The reaction mixture (50 μL) contained 10 μL of LPDS, 30 μM 24(S)OH-C, NBD-PC with 1.8 μM ApoA-I, 0.5% HSA, and 50 μM β-mercaptoethanol in TBS. This mixture was incubated for 0.5, 1, 2, 4, 6, or 8 h at 37°C. Incubation mixtures lacking 24(S)OH-C or LPDS were used as controls. A reaction mixture, treated with 2 μL of rabbit monoclonal IgG anti-LCAT (1 h/37°C) before incubation, was used as a further control. After incubation, the reaction was stopped by addition of 150 μL of ethanol, and the lipids were extracted by hexane. Four sequential extracts (500 μL each) were pooled. After hexane evaporation, the dried lipids were

dissolved in 60 μL of acetonitrile/methanol (60/40, v/v), and 20-μL aliquots were injected onto a Supelcosil LC-18 column for HPLC. Chromatography was carried out at 1 mL/min and 25°C, using acetonitrile/methanol/trifluoroacetic acid (60/39.9/0.1, v/v/v) for elution, and fluorescence detection (λ_{EX} = 460 nm, λ_{EM} = 534 nm) for monitoring NBD-coupled material. The areas of the fluorescent peaks were measured by Class-VP software (Shimadzu, Columbia, MD, USA), and the amounts of the fluorescent material were expressed in arbitrary units. NBD-labeled 24(S)OH-C was processed for MS and MS/MS analysis.

In experiments carried out to hydrolyze NBD-labeled 24(S)OH-CE, the reaction mixture, after incubation (8 h/37°C), was supplemented with 2 mg/mL butylated hydroxytoluene and 0.3 μM EDTA, and then rapidly divided into two aliquots of 50 μL. One aliquot (namely S) was incubated (1 h/37°C) with 150 μL of 1M KOH in ethanol, whereas the other one (namely NS) was incubated (1 h/37°C) with 150 μL of ethanol. After alkaline treatment, KOH was neutralized by 10 μL of 50% phosphoric acid (v/v). Each aliquot was then supplemented with 50 μL of 2 M NaCl, and the lipids were isolated as above for HPLC. In particular, the dried lipids were dissolved in acetonitrile and then fractionated by a Nucleodur C18 ISIS column in acetonitrile at 1 mL/min and 25°C. A UV detector (λ = 205 nm) was used to monitor the 24(S)OH-C elution. The areas of the 24(S)OH-C peaks, in samples from both S and NS, representing the amounts of total and unesterified 24(S)OH-C, respectively, were measured by the Class-VP software.

In some experiments, 24(S)OH-CE was synthesized in a reaction mixture prepared as above described, but containing proteoliposomes with 2-linoleoyl-1palmitoyl-sn-glycero-3-phosphocholine. After incubation (16 h/37°C), the lipids were processed for TLC as below described. The stationary phase between Rf = 0.65 and Rf = 0.85 was recovered by a scraper, and then extracted by 10 volumes of ethanol. The extract was dried, dissolved in 30 μL of isopropanol/acetonitrile (1/2, v/v), and analyzed by MS/MS.

The esterification of tritium-labeled 24(S)OH-C (^{3}H-24(S)OH-C) was performed by using either ApoA-I or ApoE as LCAT activator. Proteoliposome suspensions containing either apolipoprotein, prepared as above described (apolipoprotein/PC molar ratio = 1/133; 20 μM apolipoprotein), and ^{3}H-24(S)OH-C were used as substrates. The reaction mixture (170 μL) contained 16 μL of LPDS, 30 μM ^{3}H-24(S)OH-C (20 μCi/μmole), 1 μM apolipoprotein on liposomes, 0.5% HSA, and 50 μM β-mercaptoethanol in TBS. The mixture was incubated at 37°C, and 32-μL aliquots were taken on at different times (0.5, 1, 2, 4, and 8 h) and added to 150 μL of ethanol. A mixture without the enzyme was used as control. The lipids, extracted in hexane and dried as above described, were dissolved in 50 μL of chloroform and spotted onto Sil G pre-coated plastic sheets. TLC, in hexane/ethyl acetate (70/30, v/v), was used to separate ^{3}H-24(S)OH-CE (Rf = 0.75) from ^{3}H-24(S)OH-C (Rf = 0.35). After TLC, each chromatography running lane was cut into 1 cm long slices, and the radioactivity of each slice was measured by scintillation. The migration of standard ^{3}H-24(S)OH-C was used to identify the slice containing ^{3}H-24(S)OH-C in each fractionated sample. The amount of ^{3}H-24(S)OH-CE synthesized was calculated as (^{3}H-24(S)OH-CE)×100/(^{3}H-24(S)OH-CE + ^{3}H-24(S)OH-C). Studies on esterification kinetics were performed as follows. 24(S)OH-C esterification was carried out in 32-μL reaction mixtures, prepared as above described, using proteoliposomes containing ApoA-I or ApoE (1-μM final concentration) and different amounts of ^{3}H-24(S)OH-C (1.5, 5, 15, 30, 60, or 100 μM; 20 μCi/μmole) as substrates, and LPDS as LCAT source. After 3 h at 37°C, the amounts of ^{3}H-24(S)OH-CE and ^{3}H-24(S)OH-C were measured as above described. The K_m and V_{max} values of LCAT, under stimulation by ApoA-I or ApoE, were calculated by the GraphPad Prism 5.01 software (GraphPad Software Inc., San Diego, CA, USA). The Lineweaver-Burk plot was displayed by the same software.

The esterification of 24(S)OH-C in CSF was performed as follows. CSF samples were collected, after informed consent, from spinal anesthesia subjects (male Caucasians, 50–60 years old, not taking drugs) with no neurological symptoms and no previous history of any neurological deficit, and were processed for routine analysis according to current guidelines (Deisenhammer *et al.* 2011). The CSF (n = 3) were pooled and used within 2 h from sampling. Aliquots of 500 μL were analyzed, in three independent experiments, for measuring the percentage of 24(S)OH-CE according to a published procedure (Lütjohann *et al.* 1996), whereas aliquots of 100 μL were supplemented with 1 μL of 2 μM ^{3}H-24(S)OH-C and incubated for different times (2–16 h) at 37°C. Some samples, incubated for 4 or 16 h, contained 3 μL of anti-LCAT IgG. After incubation, the lipids were extracted by hexane and processed for TLC and scintillation analysis.

The effect of Hpt on 24(S)OH-C esterification was assayed by incubating reaction mixtures prepared as above described, but containing lower amount of proteoliposomes (0.5 μM ApoA-I final concentration), 15 μM ^{3}H-24(S)OH-C, and different amounts of Hpt (1, 2.5, 7.5, or 10 μM; Hpt/ApoA-I molar ratio: 2, 5, 15, or 20). Hpt (mixed phenotypes) was prepared as previously reported (Cigliano *et al.* 2009). After 2.5 h at 37°C, ^{3}H-24(S)OH-CE and ^{3}H-24(S)OH-C were extracted and processed for TLC, and their amounts were measured by scintillation counting.

Mass analysis was performed essentially according to a published procedure (Honda *et al.* 2009) using a LCQ DECA XP Ion Trap spectrometer (Thermo Fisher, Waltham, MA, USA) equipped with an Opton electrospray ionization source operating at a needle voltage of 3.5 kV and at a temperature of 300°C. Mass calibration was carried out automatically by means of selected multiple charged ions, using a commercial standard mixture of caffeine, Met-Arg-Phe-Ala peptide, and Ultramark (Thermo Fisher). The general MS/MS conditions were as follows: spray voltage = 1000 V; vaporizer temperature = 350°C; sheath gas (nitrogen) pressure = 85 psi; auxiliary gas (nitrogen) flow = 60 arbitrary units; ion transfer capillary temperature = 350°C; collision gas (argon) pressure = 1.5 mTorr; positive ion polarity. Data analyses and processing were performed using Finnigan Xcalibur software v. 1.3 (Thermo Fisher). Fragmentation was induced on the selected ion with 30 eV.

Competition between C and 24(S)OH-C for LCAT activity

The esterification assay of tritium-labeled C (^{3}H-C) was carried out essentially according to published procedures (Spagnuolo *et al.* 2005). In particular, the enzyme activity was measured using proteoliposomes (ApoA-I/PC/^{3}H-C molar contributions = 1.5/200/18) prepared by the cholate dialysis method (Chen and Albers 1982) as substrate, and LPDS as LCAT source. The reaction was carried out in TBS supplemented with EDTA (TBE; 140 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4) containing 4% LPDS (v/v),

0.2 μM ^{3}H-C (365 μCi/μmole) embedded into proteoliposomes, different amounts (0–100 μM) of 24(S)OH-C, 0.5% HSA, and 50 μM β-mercaptoethanol. After incubation (2 h at 37°C), the lipids were extracted by hexane, fractionated by TLC with petroleum ether/diethyl ether/acetic acid (90/30/1, v/v/v; C Rf = 0.3, C esters Rf = 0.9), and recovered for scintillation analysis (Spagnuolo *et al.* 2005).

Cell culture

SH-SY5Y cells (8000 cells/well in 96-well plate) were differentiated to mature neuronal phenotype by incubation in low-serum medium containing RA, as previously reported (Nordin-Andersson *et al.* 2003). The toxicity of 24(S)OH-C for the differentiated cells was evaluated as follows. The RA-containing medium was replaced by DMEM containing 0.5% HSA and different amounts of 24(S)OH-C (0 to 50 μM). After 5 h at 37°C, the 24(S)OH-C-supplemented medium was removed, and the cells were further incubated (16 h/37°C) with DMEM containing 0.2% HSA and finally fixed (15 min/37°C) with 100 μL of 1% glutaraldheyde in phosphate-buffered saline (137 mM NaCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 2.7 mM KCl, pH 7.4). Then 100 μL of 1 mg/mL Crystal Violet was added to each culture well, and incubated at 25°C for 30 minutes. The solution was then removed, the well-bound stain was solubilized with 200 μL of 10% acetic acid (v/v), and the absorbance at 595 nm was measured. The data were expressed as viability percentage, assuming the absorbance value from cells cultured without 24(S)OH-C as 100%.

24(S)OH-C internalization in the differentiated cells was analyzed as follows. The cells were incubated with fresh RA-containing medium for 2 h before treatment. The medium was then replaced by DMEM containing 0.5% HSA and 20 μM ^{3}H-24(S)OH-C (20 μCi/μmole), previously treated with or without LPDS. In detail, ^{3}H-24(S)OH-C was pre-incubated (5 h/37°C) in DMEM containing 2 μM ApoA-I on proteoliposomes, 50 μM β-mercaptoethanol, 0.5% HSA, in presence or absence of 3 mg of protein/mL LPDS. Culture medium pre-incubated with ^{3}H-24(S)OH-C alone, or LPDS plus ^{3}H-24(S)OH-C was used as control. After pre-incubation, a 60-μL aliquot of medium was used to culture the cells for different times (0.5–6 h), while another aliquot (30 μL) was treated with hexane for recovering the lipids and measuring 24(S)OH-C esterification as above described. After incubation, the medium was collected, cleared of any cellular debris by centrifugation (800 g, 10 min), and finally analyzed for its radioactivity content. The cells were repeatedly washed with DMEM (until radioactivity was no more detected in the washes), and then lysed with 0.1 M NaOH for measuring the internalized radioactivity, that was calculated as percentage of total radioactivity in lysates and media.

In experiments for analyzing 24(S)OH-CE toxicity, 20 μM 24(S)OH-C was pre-incubated (5 h/37°C) with DMEM containing 3 mg of protein/mL LPDS, 2 μM ApoA-I on proteoliposomes, 0.5% HSA, and 50 μM β-mercaptoethanol. Medium containing 24(S)OH-C alone, or LPDS alone, or HSA alone was also prepared and used as control. After pre-incubation, 60-μL aliquots from pre-incubated medium were used for culturing the cells. After incubation (5 h/37°C), the medium was removed, and the cells were further incubated (16 h/37°C) with DMEM containing 0.2% HSA before assaying cell viability.

In experiments for analyzing 24(S)OH-CE internalization, 20 μM ^{3}H-24(S)OH-C (20 μCi/μmole) was pre-incubated with 3 mg protein/mL LPDS in larger volume of reaction mixture (200 μL) as above described, but in medium containing either 2 μM ApoA-I or 2 μM ApoE on proteoliposomes. After pre-incubation, the two types of proteoliposomes were isolated by chromatography with a column of Sephacryl-S200 (1.5 × 11 cm) in DMEM, at 6 mL/h flow rate. The elution of proteoliposomes was monitored by measuring radioactivity and absorbance at 280 nm of the fractions (300 μL). Control chromatography experiments were carried out for determining the elution volumes of proteoliposomes and albumin. Fractions containing the higher level of radioactivity/absorbance were pooled, and the resulting proteoliposome suspension was analyzed for its content in ^{3}H-24(S)OH-CE and ^{3}H-24(S)OH-C by TLC. ^{3}H-24(S)OH-C was not detected in the proteoliposome suspension. Aliquots (200 μL) of labeled proteoliposomes with ApoA-I (3780 dpm and 0.3 μM ApoA-I in culture medium) or ApoE (2300 dpm and 0.3 μM ApoE in culture medium) were incubated for 5 h with the cells. Control cells were incubated in culture medium without proteoliposomes. Finally, the incubated medium was recovered, and the cells were washed by fresh medium until no radioactivity was detected in the washes that were added to the incubated medium. Cell samples were assayed by Crystal Violet staining for analyzing cell viability, while other cell samples were lysed as above described. The radioactivity of both recovered media and cell lysates was measured by scintillation analysis. Labeled proteoliposomes with ApoA-I were also prepared in 2 mL of reaction mixture. After concentration by Amicon Ultra centrifugal filters (MW cut-off = 30 000 Da), the proteoliposomes were isolated by gel filtration in DMEM, as above described. Aliquots (200 μL) of proteoliposome suspension, containing 5 μM ^{3}H-24(S)OH-CE, were incubated for 24 h with the cells. Control cells were incubated with 5 μM ^{3}H-24(S)OH-C, or without either proteoliposomes or ^{3}H-24(S)OH-C. Then the radioactivity in culture media and cell lysates was measured, and cell survival was evaluated by Crystal Violet staining.

Statistical analysis

In all the experiments, except for K_m analysis, the data were expressed as means ± SD from three independent experiments. The GraphPad Software was used for non-linear regression analysis of kinetics data (from two independent experiments). Significance of statistical differences was determined by *t*-test or, where appropriate, one-way ANOVA, followed by Tukey's test for multiple comparisons.

Results

24(S)OH-C esterification with NBD-hexanoyl chain by LCAT

Evidence on the 24(S)OH-C esterification by LCAT was obtained by incubating (4 h/37°C) this oxysterol with LPDS, as enzyme source, and proteoliposomes containing ApoA-I and NBD-PC, in TBS. In a control mixture, LPDS pre-incubated with anti-LCAT IgG was used. After incubation, the lipids were isolated and then analyzed by HPLC in acetonitrile/methanol/trifluoroacetic acid with fluorescence detection. A fluorescent peak, following the elution of faster moving material, was detected at 6.6 min retention time (Fig. 1a). The area of this peak was significantly lower ($p < 0.01$) in the chromatogram from the control mixture

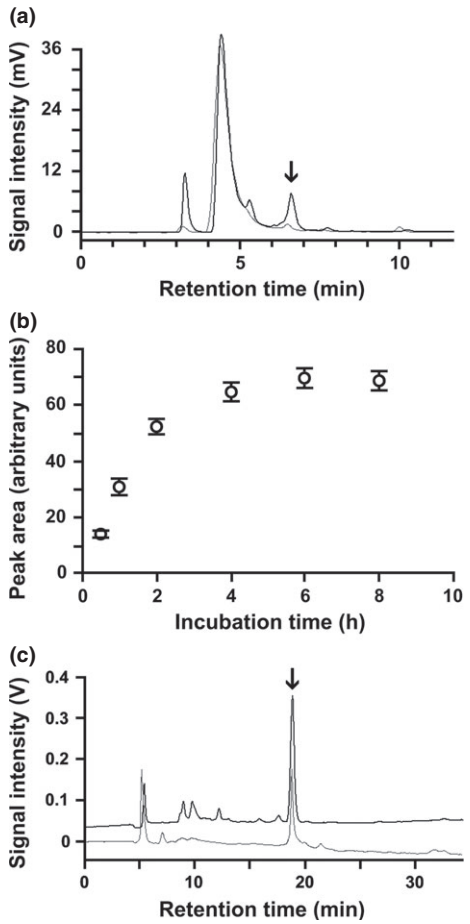

Fig. 1 HPLC analysis of products formed by lecithin-cholesterol acyltransferase (LCAT) from fluorescent phospholipids and 24(S)OH-C. 24(S)OH-C was incubated (37°C) with lipoprotein-deficient serum (LPDS) and proteoliposomes containing apolipoprotein A-I (ApoA-I) and phosphatidylcholine (as donor of NBD-labeled hexanoyl chain), and then the fluorescent lipids were processed for HPLC. (a) Elution patterns of samples incubated (4 h) with (gray line) or without (black line) anti-LCAT IgG. Fluorescence detection (λ_{EX} = 460 nm; λ_{EM} = 534 nm). The arrow indicates the peak formed by LCAT. (b) Areas of fluorescent material formed by LCAT during incubation. The data are expressed as means ± SD from three independent experiments. (c) The mixture, after incubation (8 h), was divided into two equal aliquots, one undergoing alkaline treatment, and the other one being untreated. Then, the fluorescent lipids from the two samples were separately processed for HPLC with UV detection (205 nm). The arrow indicates the 24(S)OH-C peak in the patterns from treated (black line) and untreated (gray line) samples.

containing LPDS pre-incubated with IgG (Fig. 1a). This peak was not detected if the reaction mixture lacked 24(S)OH-C or LPDS (data not shown). The rate of production of the molecules eluted at 6.6 min was studied by incubating 24(S)OH-C with LPDS and proteoliposome for different times. The amount of these products was found to linearly increase from 0 to 2 h of incubation, and did not significantly increase after 4 h (Fig. 1b). The results suggest that LCAT converts 24(S)OH-C to esters with NBD-hexanoyl chain.

It was expected that the putative 24(S)OH-C esters (24(S)OH-CE) could be hydrolyzed. The products of incubation of 24(S)OH-C with LPDS and fluorescent proteoliposomes were actually converted back to unesterified form by alkaline treatment. In particular, after incubation, each reaction mixture was divided into two aliquots, one undergoing saponification by KOH (namely S), and the other one being untreated (namely NS). The lipids were extracted from S and NS, and then processed by HPLC in acetonitrile. As shown in Fig. 1c, the amount of the 24(S)OH-C peak, eluted at about 18 min, and detected by measuring the effluent absorbance at 205 nm, was higher in the sample from S than in that from NS. The areas of the two peaks were measured, and the amount of 24(S)OH-CE in NS was calculated as difference between the areas from S and NS. Analysis of samples indicated that 54.1 ± 2.3% of 24(S)OH-C was converted into 24(S)OH-CE. This result actually shows that 24(S)OH-C, after incubation, remained partly unchanged and partly transformed in products from which 24(S)OH-C was recovered by alkaline hydrolysis, supporting the hypothesis that LPDS produced 24(S)OH-CE.

The production of NBD-labeled hexanoyl ester of 24(S)OH-C was demonstrated by mass spectrometry analysis of the peak at 6.6 min (see Fig. 1a). This analysis indicated that the mass of fluorescent compound was 663.3 u, in agreement with the protonated form of the monoester from 24(S)OH-C and NBD-hexanoic acid, without a molecule of H_2O ($[M+H-H_2O]^+$) (Figure S1a). In addition, only the 3β-hydroxyl group of 24(S)OH-C was found bound to the NBD-labeled acyl chain (Fig. 2a). The fragmentation behavior of $[M+H-H_2O]^+$ actually indicated that loss of part of the side chain (C-24 to C-27) of the oxysterol structure occurred, thus producing the fragment with 607.0 u, compatible with the formation of a carbon–carbon double bond on the remaining part of the side chain. A fragment with 551.0 u, compatible with loss of the complete oxysterol side chain and formation of a carbon-–carbon double bond was also observed. A peak at 499.9 m/z (loss of the 7-nitro-2-1,3-benzoxadiazol-4-yl group) could be easily assigned in the fragmentation pattern. Other peaks in the spectrum could not be directly assigned to ions from simple fragmentation of the monoester.

24(S)OH-C was also esterified by linoleic acid in a reaction mixture containing LPDS and proteoliposomes with ApoA-I and 2-linoleoyl-1-palmitoyl-sn-glycero-3-phosphocholine. After incubation (16 h at 37°C), the lipids were processed by TLC and the fraction containing 24(S)OH-CE was isolated and analyzed by mass spectrometry. The protonated form of the monoester of 24(S)OH-C, namely, $[M+H]^+$, was detected as peak at 666 m/z, whereas the diester of 24(S)OH-C (927 u, if present) was not found (Figure S1b). The MS/MS spectrum of $[M+H]^+$, obtained with sensitivity lower than that of the MS/MS spectrum of

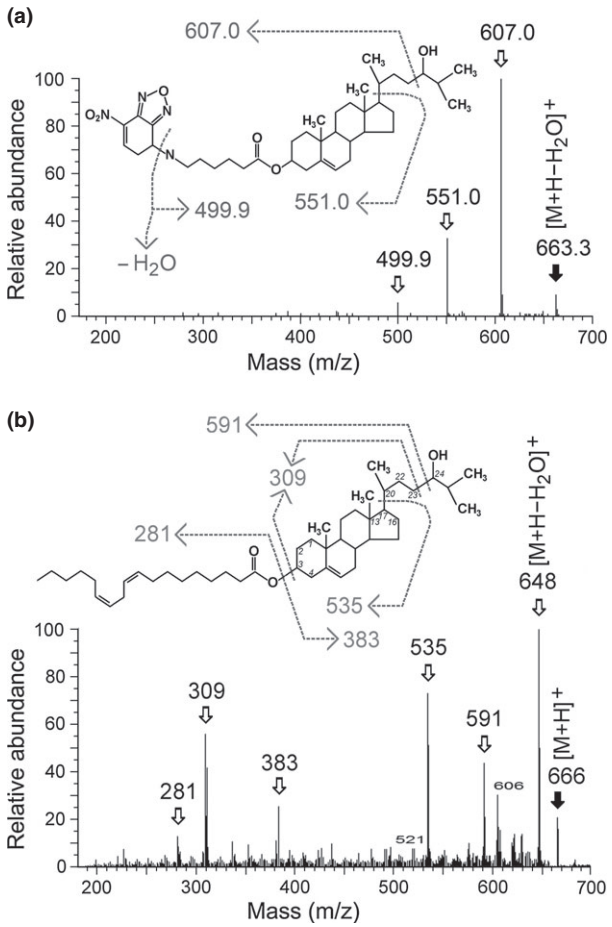

Fig. 2 LC-MS analysis of lecithin-cholesterol acyltransferase (LCAT) products. 24(S)OH-C was incubated with lipoprotein-deficient serum (LPDS) and proteoliposomes containing apolipoprotein A-I (ApoA-I) and phosphatidylcholine. (a) phosphatidylcholine with NBD-labeled hexanoyl chain was used in the reaction mixture. After 8 h at 37°C, the lipids were isolated by HPLC and analyzed by MS-MS. The mass of protonated ester without one molecule of water (full arrow) is indicated. (b) phosphatidylcholine with linoleyl chain was used in the reaction mixture. After 16 h at 37°C, the lipids were isolated by TLC and analyzed by MS/MS. The mass of protonated ester (full arrow) is indicated. In both Panels, fragments originated by cleavages of bonds in the ester structure are indicated (open arrows). The dotted lines represent such cleavages and refer to their masses in the spectrum.

the NBD-labeled monoester owing to higher dilution of the sample, indicated primarily the loss of H_2O as fragment $[M\text{-}H_2O+H]^+$ with 648 u (Fig. 2b). Cleavage between C-23 and C-24 produced the 591 u fragment, compatible with formation of carbon–carbon double bond between C-20 and C-22. The 535 u fragment is compatible with the cleavage between C-17 and C-20 bond (Szedlacsek *et al.* 1995), causing loss of the complete side chain from 24(S)OH-CE, and formation of a double bond between C-16 and C-17. Loss of the side chain could be associated with loss of the C-21 methyl group and formation of a double bond between C-13 and C-17, thus producing the peak at 521 m/z. A peak at 383 m/z is compatible with loss of linoleic acid and formation of alternate carbon–carbon double bonds (between C-1 and C-2, and C-3 and C-4) as already described in fragmentation analysis of cholesterol acetate (Partridge and Djerassi 1977). Further cleavage of this fragment (between C-23 and C-24) and formation of terminal carbon–carbon double bond on the resulting shortened side chain might explain the peak at 309 m/z. Other peaks in the spectrum could not be directly assigned to ions from simple fragmentation of the monoester.

Stimulation of 24(S)OH-C esterification by ApoA-I or ApoE

The ApoA-I or ApoE stimulation on LCAT for the 24(S)OH-C esterification was studied by following the conversion of ^{3}H-24(S)OH-C to ^{3}H-24(S)OH-CE. The labeled oxysterol was incubated with LPDS (as LCAT source) at 37°C for different time lengths, in presence of proteoliposomes containing ApoA-I or ApoE. After lipid extraction, ^{3}H-24(S)OH-CE were separated from ^{3}H-24(S)OH-C by TLC for measuring the amount of esters produced. The ^{3}H-24(S)OH-CE percentage was found dependent on incubation time in presence of both ApoA-I and ApoE (Fig. 3a). In particular, the ^{3}H-24(S)OH-C esterification was found stimulated more by ApoA-I than by ApoE.

To measure the kinetic parameters K_m and V_{max} for the reaction of LCAT with ^{3}H-24(S)OH-C, different amounts of this oxysterol were incubated with LPDS and proteoliposomes containing ApoA-I or ApoE. After lipid extraction and TLC, the relationship between substrate concentration and ApoA-I or ApoE-dependent esterification was analyzed, and the data were displayed by the Lineweaver-Burk plot (Fig. 3b). Both the K_m and the V_{max} values obtained under ApoA-I stimulation were found different from those under ApoE stimulation. In particular the data, processed for non-linear regression analysis, indicated that the K_m values were 125.5 μM ($n = 5$; $r^2 = 0.9867$) and 417.3 μM ($n = 5$; $r^2 = 0.9694$), respectively.

Esterification of 24(S)OH-C in CSF

Freshly collected CSF were pooled and analyzed for the level of 24(S)OH-C esterification by measuring the amounts of 24(S)OH-C before and after alkaline hydrolysis. The level of 24(S)OH-CE was calculated as difference between these two amounts, and expressed as percent of total 24(S)OH-C (i.e., the amount of 24(S)OH-C in saponified samples). The data indicated that 68.0 ± 3.2% of 24(S)OH-C was present as ester form in the CSF pool. Aliquots of this pool were supplemented with ^{3}H-24(S)OH-C in presence or absence of anti-LCAT antibodies, incubated at 37°C, and then processed by TLC and scintillation analysis for measuring the level of *in vitro* synthesized esters (Fig. 4). 10.47 ± 2.68% of ^{3}H-24(S)OH-C was found esterified after 16 h of incubation, and the antibodies inhibited the esterification. These results, taken together, indicate that most of 24(S)OH-C was already

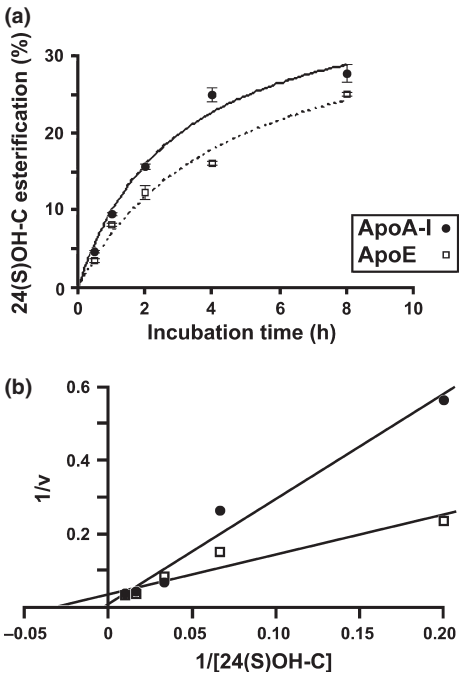

Fig. 3 Esterification of ^{3}H-24(S)OH-C by lecithin-cholesterol acyltransferase (LCAT) with apolipoprotein A-I (ApoA-I) or apolipoprotein E (ApoE) stimulation. ^{3}H-24(S)OH-C was incubated with lipoprotein-deficient serum (LPDS) and proteoliposomes containing either ApoA-I or ApoE. After incubation, ^{3}H-24(S)OH-C and ^{3}H-24(S)OH-CE were separated by TLC and their radioactivity was measured. The data are expressed as ^{3}H-24(S)OH-CE percentages (means ± SD) from three independent experiments. (a) ^{3}H-24(S)OH-CE produced under stimulation of ApoA-I (full circles) o ApoE (open squares). (b) Lineweaver-Burk plot of values obtained by incubating (3 h) different concentrations of ^{3}H-24(S)OH-C with LPDS and ApoA-I (full circles) or ApoE (open squares). [24(S)OH-C] indicates the oxysterol μmolar concentration. The data are expressed as means from two independent experiments.

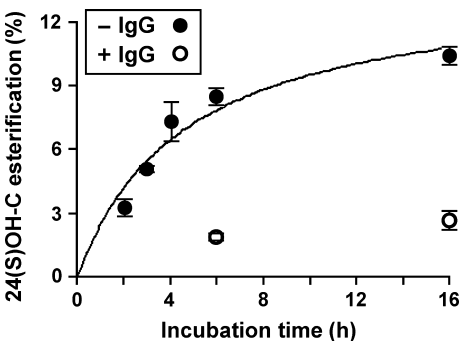

Fig. 4 Esterification of 24(S)OH-C in CSF. CSF was supplemented with 1/100 vol of 2 μM ^{3}H-24(S)OH-C, and incubated at 37°C with (full circles) or without (open circles) anti-lecithin-cholesterol acyltransferase (LCAT) IgG. Then, ^{3}H-24(S)OH-CE and ^{3}H-24(S)OH-C were separated by TLC and their radioactivity was measured. The data are expressed as ^{3}H-24(S)OH-CE percentages (means ± SD) from three independent experiments.

present as ester form in freshly collected CSF, part of unesterified 24(S)OH-C was converted into ester form during CSF incubation, and such an *in vitro* esterification depended on CSF LCAT.

Competition of 24(S)OH-C with C for LCAT activity

The ability of 24(S)OH-C to compete with ^{3}H-C for LCAT activity was investigated by incubating 0.2 μM ^{3}H-C, embedded into proteoliposomes containing ApoA-I, with LPDS and different amounts of 24(S)OH-C. After lipid extraction and TLC, the amount of labeled cholesteryl esters was measured. As shown in Fig. 5, ^{3}H-C esterification significantly dropped down ($p < 0.01$) to 76.3, 75.0, 64.6, 60.2, or 44.2% in presence of 5, 10, 25, 50, or 100 μM 24(S)OH-C, respectively. The enzyme activity for ^{3}H-C esterification was halved by 75 μM 24(S)OH-C ($p = 0.003$), a 375-fold molar excess over ^{3}H-C. The ^{3}H-C esterification in presence of 0.5 μM 24(S)OH-C was higher than with greater concentrations of 24(S)OH-C ($p < 0.01$). Similarly, ^{3}H-C esterification in presence of 5 or 10 μM 24(S)OH-C was higher than with 25, 50, 75, or 100 μM 24(S)OH-C ($p < 0.01$). This experiment indicates that 24(S)OH-C and C compete for the LCAT activity, and suggests that LCAT exhibits, in our *in vitro* conditions, higher affinity for C than for 24(S)OH-C, yet no conclusion on difference in the enzyme activity with the two substrates *in vivo* can be drawn.

Hpt influence on 24(S)OH-C esterification by LCAT

High levels of Hpt were previously reported to limit the availability of ApoA-I and ApoE for their stimulation on the LCAT activity for C esterification (Spagnuolo *et al.* 2005; Cigliano *et al.* 2009). It was therefore conceivable that also the 24(S)OH-C esterification might be inhibited by high levels of Hpt. To verify this hypothesis, the enzyme activity

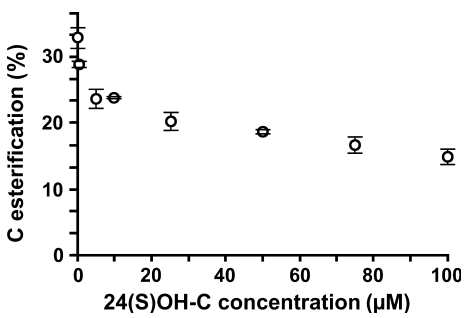

Fig. 5 Competition of 24(S)OH-C with C for the lecithin-cholesterol acyltransferase (LCAT) activity. Proteoliposomes containing ^{3}H-C, phosphatidylcholine, and apolipoprotein A-I (ApoA-I) were incubated with lipoprotein-deficient serum (LPDS) and different concentrations of 24(S)OH-C. Esterified and unesterified ^{3}H-C were separated by TLC and their radioactivity was measured. The data are expressed as esterification percentages (means ± SD) from three independent experiments.

was assayed in mixtures containing 15 μM ^{3}H-24(S)OH-C and proteoliposomes with 0.5 μM ApoA-I, in presence of different amounts of Hpt (1 to 10 μM; Hpt/ApoA-I molar ratio: 2, 5, 15, or 20). The 24(S)OH-C esterification was found significantly inhibited as Hpt/ApoA-I ratio increased (Figure S2). In particular, 2.5, 7.5, or 10 μM Hpt reduced the percentage of esterification of about 26% ($p < 0.05$), 39% ($p < 0.005$), or 58% ($p < 0.005$), respectively. Likewise, these Hpt concentrations reduced the LCAT stimulation by proteoliposomes containing ApoE (data not shown). These results indicate that Hpt, at concentrations similar to those occurring during the acute phase of inflammation, impairs the apolipoprotein stimulation on 24(S)OH-C esterification *in vitro*. Moreover, the results suggest that Hpt, if present at enhanced level in the brain, might contribute to accumulation of unesterified 24(S)OH-C in this body compartment.

Effect of 24(S)OH-C esterification on SH-SY5Y cells

The neurotoxic effect of 24(S)OH-C on SH-SY5Y cells was previously reported (Kölsch *et al.* 1999, 2001; Yamanaka *et al.* 2011). The inverse relationship between 24(S)OH-C concentration and survival of differentiated SH-SY5Y was confirmed by incubating the cells with different amounts of 24(S)OH-C for 5 h. After medium removal, the cells were further incubated (16 h) with oxysterol-free medium, and their viability was assayed by Crystal Violet staining. The treatment with 10 or 20 μM 24(S)OH-C reduced cell viability to 58% ($p < 0.05$) or 43% ($p < 0.01$), respectively (Fig. 6a). The 20-μM 24(S)OH-C concentration was therefore chosen for further experiments with LPDS (as LCAT source) in culture medium. In particular, the incubation time for maximal esterification of 24(S)OH-C in culture medium was determined by treating 20 μM ^{3}H-24(S)OH-C with LCAT and proteoliposomes containing ApoA-I (2 μM final concentration), as above described. After incubation (4 h), the level of ^{3}H-24(S)OH-CE produced (49.3 ± 2.9%) did not further increase (Fig. 6b). Therefore, 5 h was assumed to be enough incubation time for the highest conversion of oxysterol into ester form. Then, we analyzed how much oxysterol can be internalized by the cells. In particular, after pre-incubation of 20 μM ^{3}H-24(S)OH-C in culture medium with/without LPDS and proteoliposomes, the mixtures were incubated with the cells for different time lengths. The medium was removed and analyzed for its radioactivity content. The treated cells were washed, and the amount of internalized oxysterol was measured as radioactivity level in the cell lysates. The percentage of ^{3}H-24(S)OH-C incorporated by the cells increased until 2 h of incubation, then reaching a plateau. Also, the internalization of ^{3}H-24(S)OH-C by the cells was significantly reduced when the oxysterol was pre-incubated with LCAT. In particular, 13.5 ± 0.4% or 5.3 ± 0.4% ($p = 0.0007$) of total 24(S)OH-C, from mixtures pre-incubated without or with LPDS and proteoliposomes, respectively, was found into the cells after 4 h of incubation

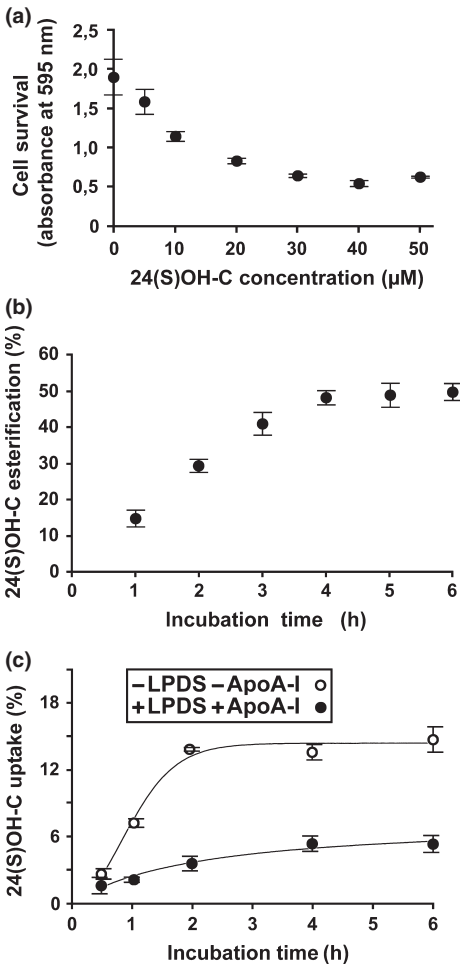

Fig. 6 24(S)OH-C cytotoxicity, esterification in culture medium, and internalization in SH-SY5Y cells. (a) The cells were challenged with different amounts of 24(S)OH-C (5 h). The cell survival was analyzed by Crystal Violet. The data are expressed as means ± SD from three independent experiments. (b) ^{3}H-24(S)OH-C was incubated for different times in culture medium with apolipoprotein A-I (ApoA-I)-containing proteoliposomes and lipoprotein-deficient serum (LPDS). After incubation, ^{3}H-24(S)OH-C and ^{3}H-24(S)OH-CE were separated by TLC, and their radioactivity was measured. The data are expressed as ^{3}H-24(S)OH-CE percentages (means ± SD) from three independent experiments. (c) ^{3}H-24(S)OH-C was pre-incubated (5 h) in culture medium with ApoA-I-containing proteoliposomes, with/without LPDS. The pre-incubation mixtures were used to culture the cells for different times. The radioactivity in both medium and cell lysate was measured. The percentage of ^{3}H-24(S)OH-C internalized by the cells, following pre-incubation with (full circles) or without (open circles) LPDS, was calculated by measuring the radioactivity in both medium and lysate. The data are expressed as means ± SD from three independent experiments.

(Fig. 6c). No significant difference between the uptake, at each incubation time, from medium containing 24(S)OH-C alone and that from medium containing both 24(S)OH-C and LPDS was observed (data not shown).This experiment

indicates that oxysterol internalization into the cells is significantly affected by the LCAT-dependent production of oxysteryl esters, and suggests that the oxysterol neurotoxic effect might be limited by conversion of unesterified oxysterol into oxysteryl esters.

To evaluate the effect of 24(S)OH-C esterification on cell survival, this oxysterol was pre-incubated in culture medium with proteoliposomes containing ApoA-I, in presence or absence of LPDS. The cells were challenged with the pre-incubated medium, and then analyzed by Crystal Violet staining. Cell viability was found significantly higher ($p < 0.0001$) when they were treated by 24(S)OH-C pre-incubated with both LCAT and proteoliposomes than by 24 (S)OH-C alone (Fig. 7). In particular, only 38% of cells survived after treatment with 24(S)OH-C alone, while 68% survived after treatment with the mixture containing 24(S) OH-C, LCAT, and proteoliposomes. This result suggests that 24(S)OH-C entered cells and caused their death, whereas 24 (S)OH-CE was not neurotoxic because did not enter. According to this hypothesis, LCAT might reduce the lethal effect of 24(S)OH-C on neurons by converting part of this oxysterol to 24(S)OH-CE. This hypothesis was proven by purifying proteoliposomes containing ^{3}H-24(S)OH-CE, and then incubating them with the cells. In detail, ^{3}H-24(S)OH-CE was prepared in the above described pre-incubation mixture, and then the labeled proteoliposomes were isolated by gel filtration chromatography. A column of Sephacryl S-200, equilibrated in culture medium, was used to separate ^{3}H-24(S)OH-CE-containing proteoliposomes from albumin-bound or free ^{3}H-24(S)OH-C (Figure S3). Aliquots from the labeled proteoliposome suspension were processed for analyzing their content in ^{3}H-24(S)OH-C and ^{3}H-24(S)OH-CE, and only ^{3}H-24(S)OH-CE were detected in this suspension. Then, the cells were incubated with the isolated proteoliposomes. Control cells were incubated in culture medium without proteoliposomes. After incubation the medium was recovered, and the cells were processed for measuring their viability and radioactivity content. No significant difference in viability was found between cells treated with proteoliposomes and control cells (96.8 ± 3.2 vs. 100 ± 3.4% survival). Radioactivity (3700 ± 177 dpm) was found exclusively in the medium from treated cells. The same result was obtained when the cells were incubated with labeled ApoE-containing proteoliposomes, prepared and used as those containing ApoA-I. Cell viability was 97.2 ± 3.7%, as compared to control cells, and radioactivity was found exclusively in the recovered medium (1900 ± 152 dpm). This experiment was also carried out with 5 μM ^{3}H-24(S)OH-CE in ApoA-I-containing proteoliposomes. These labeled proteoliposomes were incubated with the cells for 24 h, and cell cultures with 5 μM ^{3}H-24(S) OH-C alone or without proteoliposomes were used as controls. Also in this case, radioactivity was found only in medium from culture with proteoliposomes (24.730 ± 1320 dpm). Conversely radioactivity was found, as expected, in both medium (22.460 ± 512 dpm, 86.6%) and lysate (3480 ± 255 dpm, 13.4%) from culture with ^{3}H-24(S)OH-C. Cell viability was 100 ± 6.6, 106.7 ± 2.7 or 82.0 ± 7.5% after incubation with only medium, proteoliposomes, or ^{3}H-24(S)OH-C, respectively. These experiments demonstrate that 24(S)OH-CE, embedded into proteoliposomes, is restricted to extracellular environment and does not affect cell viability. Therefore, this result indicates that LCAT disarms 24(S)OH-C by esterification, and proteoliposome-entrapped 24(S)OH-CE is harmless to neurons in culture.

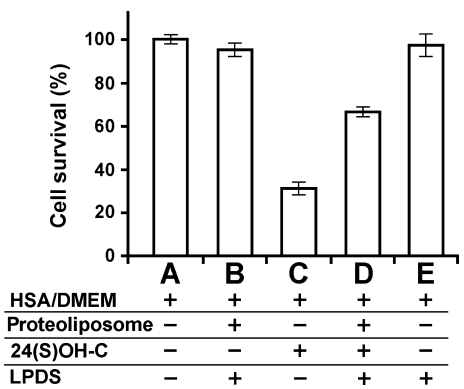

Fig. 7 Survival of SH-SY5Y cells after incubation with lecithin-cholesterol acyltransferase (LCAT)-treated 24(S)OH-C. Five mixtures were pre-incubated (5 h at 37°C): A [human serum albumin (HSA)-supplemented Dulbecco's modified Eagle's medium (DMEM)], B (lipoprotein-deficient serum (LPDS), as LCAT source, and apolipoprotein A-I (ApoA-I)-containing proteoliposomes in HSA-supplemented DMEM), C (24(S)OH-C in HSA-supplemented DMEM), D (LPDS, proteoliposomes and 24(S)OH-C in HSA-supplemented DMEM), and E (LPDS in HSA-supplemented DMEM). The mixtures were used for culturing SH-SY5Y cells (5 h). Then the mixtures were removed, the incubation was carried on for further 16 h in HSA-supplemented DMEM, and cell survival was analyzed by Crystal Violet staining. The data are expressed as means ± SD from three independent experiments.

Discussion

In this study, we demonstrate that LCAT catalyzes the 24(S) OH-C esterification by detecting a fluorescent monoester of this oxysterol that can be hydrolyzed to recover the unesterified form.

We used LPDS as LCAT source, and effective involvement of this enzyme in the 24(S)OH-C esterification was supported by different experimental approaches including failure to detect esters in reaction mixtures containing anti-LCAT antibodies, stimulation of esterification by the enzyme major effectors ApoA-I and ApoE, competition of 24(S)OH-C with C for the esterification activity, and esterification inhibition by haptoglobin. Our results actually demonstrate

that 24(S)OH-C esterification is stimulated by ApoA-I more than by ApoE, in agreement with previously published data about different stimulatory efficiency of these apolipoproteins on LCAT for C esterification (Zorich *et al.* 1985; Jonas 2000). ApoA-I, as a better LCAT stimulator, was used in experiments of competition of 24(S)OH-C with C for the enzyme activity. No conclusion can be drawn on what is the preferred substrate of LCAT, because kinetic parameters for C could not be measured. In fact, in the procedure we used, C was contained in proteoliposomes that very likely were multilamellar particles, and the amount of C available for LCAT was restricted to molecules exposed onto the proteoliposome surface. Therefore, exact concentration of this substrate for determining the enzyme K_m was unknown, and thus comparison of LCAT affinities for the two sterols could not be done. However, similar levels of C esters and 24(S)OH-CE were found in human CSF, although C concentration is far higher than that of 24(S)OH-C (Papassotiropoulos *et al.* 2002). It cannot be excluded that *in vivo* (where C effluxes from cell transporters directly to lipoproteins and LCAT, whereas the oxysterol freely diffuses from cell to interstitial fluid) the LCAT amount might be high enough for esterifying the substrates with similar efficiency.

It is well known that high density lipoprotein-like particles represent the most abundant lipoproteins in brain, and mostly contain ApoE or ApoA-I. ApoA-I, although not synthesized in brain (Beffert *et al.* 1998), yet originated from vascular endothelial cells (Möckel *et al.* 1994) or circulation (Dietschy and Turley 2001), was actually detected in human CSF (Pitas *et al.* 1987; Demeester *et al.* 2000; Koch *et al.* 2001). ApoE concentration in CSF was reported to be higher (Harr *et al.* 1996; Demeester *et al.* 2000) or comparable to that of ApoA-I (Koch *et al.* 2001), but ApoE is less effective in stimulating LCAT. Therefore, we suggest that the two apolipoproteins might similarly contribute to regulate the enzyme activity in brain. Hpt binds both apolipoproteins and protects them from oxidative damages (Salvatore *et al.* 2007, 2009) but, at high concentration, can limit their availability for LCAT stimulation, thus inhibiting the enzyme activity (Spagnuolo *et al.* 2005; Cigliano *et al.* 2009). Hpt was detected in brain, as synthesized by astrocytes (Lee *et al.* 2002; Zhao *et al.* 2009) or transported through damaged BBB (Mattila *et al.* 1994; Conti *et al.* 2004), and its level in CSF was suggested to be associated with neurodegeneration (Argüelles *et al.* 2010; Huang *et al.* 2011), but its role in CNS has not been investigated to date. We show here that high levels of Hpt, as those circulating during the acute phase of inflammation, can inhibit 24(S)OH-C esterification *in vitro*. Although no information is presently available on the Hpt amount in brain compartments locally affected by inflammation, this protein might be secreted by activated astrocytes at high levels in the interstitial fluid. Therefore, it cannot be excluded that, in inflammatory conditions, the 24(S)OH-C esterification might be impaired in specific brain compartments, and consequent local accumulation of this oxysterol in neurotoxic amounts might represent a missing link between inflammation and neurodegeneration.

As the esterification of oxysterols was suggested to influence both the delivery to the tissues and the uptake by the cells (Lin and Morel 1996), we investigated whether 24(S)OH-C internalization in differentiated SH-SY5Y cells is affected by the esterification process. Neuron viability was found higher when a toxic amount of 24(S)OH-C was pre-incubated with LCAT. This finding suggested that LCAT might counteract the 24(S)OH-C neurotoxicity effect just by converting the unesterified oxysterol into ester form, which was then embedded into the lipoproteins. The 24(S)OH-C uptake by neurons in culture was actually reduced after esterification, and 24(S)OH-CE-containing lipoproteins did not enter the cells. These results suggest that the 24(S)OH-C esterification limits the amount of freely diffusible oxysterol in the culture medium, thus improving cell survival. The LCAT activity might therefore represent a protection against 24(S)OH-C toxicity *in vivo*, as reducing the oxysterol access to cells and contributing to its massive removal, mostly as 24(S)OH-CE in lipoproteins, from CNS. A body of literature actually reports that lipoproteins can translocate from brain to circulation. In particular, CNS lipoproteins were found to bind ApoE receptors on vascular endothelial cells, and undergo transcytosis for their transport to blood in mouse (Deane *et al.* 2008). Therefore, 24(S)OH-CE are expected to be transported across the BBB. In this frame, LCAT activity and lipoprotein-mediated transport might represent an important pathway for removing 24(S)OH-C from CNS. Although some studies reported that a reduced LCAT activity was detected in patients affected by Alzheimers's disease (Demeester *et al.* 2000) or Multiple Sclerosis (Albers *et al.* 1992), unfortunately epidemiological studies on onset or progression of neurodegeneration in patients with LCAT deficiency were not done, and a neuroprotective role of LCAT was suggested by finding this enzyme over-expressed in a mouse strain resistant to neurodegeneration (Swanberg *et al.* 2006). Moreover, enhanced levels of 24(S)OH-C, a condition triggered in CNS by oxidative stress (Ohyama *et al.* 2006) occurring in LCAT-deficient mice (Ng *et al. 2002*), were found in humans with mild cognitive impairment (Papassotiropoulos *et al.* 2002). However, although the synthesis of freely diffusible 24(S)OH-C is a widely recognized mechanism for removing cholesterol excess from CNS, compensatory pathways may work to prevent cholesterol accumulation in brain, as found in LCAT-deficient mice (Hossain *et al.* 2009), and promote cholesterol transport across the BBB (Umeda *et al.* 2010).

In conclusion, our results provide evidence that LCAT limits toxic injuries of 24(S)OH-C to neurons in culture by converting this oxysterol into monoester forms that do not enter the cells. Further research is required to assess whether low levels of LCAT in CNS are associated with 24(S)OH

accumulation or toxicity, and whether high levels of Hpt inhibit the enzyme activity *in vivo*.

Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines. The authors have no conflict of interest to declare.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1. MS spectra of 24(S)OH-CE.

Figure S2. Effect of Hpt on the 24(S)OH-C esterification.

Figure S3. Isolation of proteoliposomes containing ^{3}H-24(S)OH-CE.

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