Direct observation of lipoprotein cholesterol ester degradation in lysosomes

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We have investigated whether pyrene-labelled cholesterol esters (Pyr_nCEs) (*n* indicates the number of aliphatic carbons in the pyrene-chain) can be used to observe the degradation of low-density lipoprotein (LDL)-derived cholesterol esters (CEs) in the lysosomes of living cells. To select the optimal substrates, hydrolysis of the Pyr_nCE species by lysosomal acid lipase (LAL) in detergent/phospholipid micelles was compared. The rate of hydrolysis varied markedly depending on the length of the pyrenyl chain. Pyr₁₀CE was clearly the best substrate, while Pyr₄CE was practically unhydrolysed. Pyr₁₀CE and [³H]cholesteryl linoleate, the major CE species in LDL, were hydrolysed equally by LAL when incorporated together into reconstituted LDL (rLDL) particles, thus indicating that Pyr₁₀CE is a reliable

reporter of the lysosomal degradation of native CEs. When rLDL particles containing Pyr₇CE or Pyr₁₀CE were incubated with fibroblasts, the accumulation of bright intracellular vesicular fluorescence was observed with the former fluorescent derivative, but not with the latter. However, when the cells were treated with chloroquine, an inhibitor of lysosomal hydrolysis, or when cells with defective LAL were employed, Pyr₁₀CE also accumulated in vesicular structures. HPLC analysis of cellular lipid extracts fully supported these imaging results. It is concluded that Pyr_nCEs can be used to observe degradation of CEs directly in living cells. This should be particularly useful when exploring the mechanisms responsible for the accumulation of lipoprotein-derived CEs in complex systems such as the arterial intima.

INTRODUCTION

Elevated low-density lipoprotein (LDL) concentration in the serum is a well-established risk factor in the development of atherosclerosis in humans [1], and excessive consumption of saturated fat has been established as one of the factors responsible for increased LDL levels in serum [2]. The reasons for this diet-dependent accumulation of lipids are not well understood, but one possibility is that the saturated dietary fat alters the lipid composition of LDL particles [3,4] and thus their physical state in a manner that impairs the metabolism of the particles [5–7]. LDL is catabolized in lysosomes [8], so it is feasible that the altered physical state of the LDL particle could adversely affect the degradation of its constituent lipids by lysosomal lipases [7,9]. This possibility is supported by the following observations: (1) the physical state of LDL lipids significantly influences the hydrolysis of LDL cholesterol esters (CEs) by lysosomal acid lipase (LAL) *in vitro* [9]; (2) premature atherosclerosis is common in individuals affected by the CE storage disease due to a defective LAL [10]; (3) LAL activity is significantly diminished in mononuclear leucocytes from patients affected by early atherosclerosis [10]; and (4) mammalian species susceptible to atherosclerosis have a lower activity of LAL in their aortic cells than more resistant species [10].

The aortic intima is a complex system consisting of various types of cell and an extracellular matrix [11]; it is therefore extremely difficult to deduce the sequence of metabolic events leading to intimal lipid accumulation from biochemical experiments alone. Obviously it would be useful if there were a means of observing the metabolism and distribution of intimal lipids directly under a microscope. Fluorescence microscopy has been employed to study the accumulation of LDL in tissues or cells [12–15]. However, in none of these studies was direct information on degradation of a lipid constituent of the LDL particle obtained. Therefore we investigated whether cholesterol esters

labelled with pyrene at the acyl chain (Pyr_nCEs) could be used to achieve this. These derivatives were chosen because pyrene is hydrophobic and does not change the conformation of the parent lipid [16], as more polar fluorophores tend to do [17–19]. Another important advantage is that the pyrene derivatives display concentration-dependent excimer formation, which makes it possible to determine their local concentrations quantitatively in living cells by fluorescence imaging [20].

To choose the optimal substrates, the hydrolysis of different Pyr_nCE species by LAL was studied first in detergent/phospholipid micelles. Then selected Pyr_nCEs were incorporated into reconstituted LDL (rLDL) particles, which were incubated with fibroblasts under various conditions. The uptake and metabolism of the Pyr_nCEs were followed by fluorescent imaging and HPLC analysis of cellular lipid extracts.

EXPERIMENTAL

Lipids and other materials

1-Palmitoyl-2-oleoylphosphatidylcholine and 1-palmitoyl-2-oleoylphosphatidic acid were obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.) and other unlabelled lipids from Sigma (St. Louis, MO, U.S.A.). Pyr_nCEs were synthesized by established methods [21] and were purified by reverse-phase HPLC on an Ultrasphere octadecylsilica 4.6 mm × 25 cm column (Beckman Instruments, San Ramon, CA, U.S.A.), with acetonitrile/chloroform/acetic acid (600:400:25, by vol.), as eluent. The purity and identity of the synthesized Pyr_nCE species were based on the following criteria: (1) each Pyr_nCE gave a single symmetrical peak coinciding with that obtained for the respective Pyr_nCE standard (Pyr₄CE and Pyr₆CE available) obtained from Molecular Probes (Eugene, OR, U.S.A.); (2) the retention time of Pyr_nCEs increased logarithmically with the nominal acyl chain

Abbreviations used: 18:2-CE, cholesteryl linoleate; CE, cholesterol ester; LAL, lysosomal acid lipase; LDL, low-density lipoprotein; Pyr_nFA, pyrenyl fatty acid (*n* indicates the number of aliphatic carbons); Pyr_nCE, pyrenyl acyl cholesterol ester (*n* indicates the number of aliphatic carbons in the pyrene-labelled acyl chain); rLDL, reconstituted low-density lipoprotein.

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length, as expected; and (3) each Pyr_nCE gave a single band after TLC on silica gel with hexane/diethyl ether/acetic acid (80:20:1, by vol.) as the solvent. [$1\alpha,2\alpha(n)$ - ^3H]Cholesterol (specific radioactivity 46 Ci/mmol) was from Amersham (Little Chalfont, Bucks., U.K.). [^3H]Cholesteryl linoleate ([^3H]18:2-CE) was synthesized as described previously [9]. All solvents used were of HPLC grade and were obtained from Merck (Espoo, Finland); other chemicals were from Sigma.

Assay of Pyr_nCE hydrolysis

To study the hydrolysis of Pyr_nCEs in detergent/phospholipid micelles, 15 pmol of each was mixed either together or separately with 1-palmitoyl-2-oleoylphosphatidylcholine and 1-palmitoyl-2-oleoylphosphatidic acid (9 and 1 nmol respectively) in chloroform, taken to dryness and dispersed in 50 mM sodium acetate/4 mM taurocholate/1 mM EDTA buffer (pH 3.9). A sample (2 μg of protein) of a post-nuclear fibroblast homogenate [22] was then added and the mixture was incubated at 37 °C for 30 min under argon in the dark. The reaction was stopped by the addition of chloroform/methanol (1:2, v/v), after which the lipids were extracted [23] in the presence of 0.5 M acetic acid to ensure the quantitative partitioning of the released fatty acids to the chloroform phase. The lipids were then separated on an Ultrasphere 4.6 cm $\times$ 25 cm column eluted with acetonitrile/chloroform/acetic acid (600:400:25, by vol.) at a flow rate of 0.5 ml/min. For quantification, the eluent was passed through a Merck-Hitachi F-1050 fluorescence detector with the excitation and emission monochromators set to 345 and 395 nm respectively, coupled to a Merck-Hitachi D-2000 integrator. Because the cell extracts contained one or more endogenous fluorescent compounds that co-eluted with pyrenyl fatty acid (Pyr_nFA), the rate of hydrolysis was measured either (1) by comparing the peak areas of the individual Pyr_nCE species (when present simultaneously) or (2) by comparing the peak areas of the Pyr_nCE studied with that of an internal standard (Pyr_{10}CE or Pyr_{16}CE) added after the reaction. The results were corrected for minor spontaneous hydrolysis.

To compare the hydrolysis of [^3H]18:2-CE and Pyr_{10}CE , these lipids were incorporated together in either rLDL or reconstituted high-density lipoprotein particles (0.6 μg of protein) and then incubated with post-nuclear fibroblast homogenate (0–20 μg of protein) in a buffer containing 0.1 M sodium acetate, 1 mM EDTA, 5 mM 2-mercaptoethanol and 0.5 mg/ml BSA, pH 4.3 [24], for 2 h at 37 °C. The lipids were then extracted and the reaction products were separated by reverse-phase HPLC (see above), then quantified by on-line fluorescence detection or by determining the radioactivity of collected fractions by liquid-scintillation counting.

Reconstitution of HDL and LDL particles containing pyrenyl cholesterol esters

Reconstituted HDL particles containing [^3H]18:2-CE and Pyr_{10}CE were prepared as described previously [25]. Reconstituted LDL particles containing pyrenyl-labelled cholesterol esters ($\text{Pyr}_n\text{CE-rLDL}$) were prepared from native LDL, isolated from fresh human plasma [26], by the method of Krieger et al. [27]. In brief, a heptane-extracted LDL residue (2 mg) was incubated with (1) cholesteryl linoleate (18:2-CE; 4.8 mg) and Pyr_nCE (1.2 mg), (2) 18:2-CE (4.8 mg) and Pyr_{10}CE (1.2 mg) or (3) 18:2-CE (5.97 mg), Pyr_{10}CE (0.03 mg) and [^3H]18:2-CE (0.8 nmol, 2×10^7 c.p.m.). The crude, heterogeneous rLDL preparation was then fractionated on a Superose 6HR gel-filtration column (Pharmacia, Uppsala, Sweden) with 10 mM Tris/1 mM EDTA/150 mM NaCl (pH 7.4) as the eluent. Rechromatography of the

isolated rLDL fraction gave a symmetrical peak coinciding with that of native LDL. In addition, the protein and Pyr_nCE elution profiles coincided precisely, indicating that a homogeneous population of rLDL particles had been obtained. The homogeneity was further supported by the observation that the pyrene excimer-to-monomer ratio was essentially constant over the rLDL peak. The recoveries of input LDL protein and Pyr_nCE in the final rLDL preparation were 8–10% and 3–5% respectively. The $\text{Pyr}_n\text{CE-rLDL}$ was stored at 4 °C for less than 4 weeks before use; there were no noticeable changes in properties.

Uptake and hydrolysis of fluorescently labelled rLDL particles by intact fibroblast monolayers

Normal human skin fibroblasts (GM 08333A) and Wolman fibroblasts (GM 01606A), obtained from Coriell Cell Repositories (Camden, NJ, U.S.A.), were grown in tissue culture flasks (Nunc, Roskilde, Denmark), as described previously [22]. For microscopy, the cells were grown on 32-mm circular coverslips placed in culture dishes. Before the experiments the cells were washed and maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Life Technologies Ltd, Paisley, Scotland, U.K.) containing human lipoprotein-deficient serum for 48 h at 37 °C [28]. The cells were then incubated with either $\text{Pyr}_n\text{CE-rLDL}$ or $\text{Pyr}_{10}\text{CE-rLDL}$ (10–20 μg /ml protein) in serum-free medium for 3 h at 37 °C. Where indicated, chloroquine (50 μM) and Sandoz compound 58-035 (5 μg /ml), an inhibitor of acyl-CoA:cholesterol-acyltransferase [29], were present in the medium. Thereafter the cells were washed twice with PBS and the incubation was continued in the absence of chloroquine and serum for up to 68 h. The incubation medium was then removed, the cells were washed twice with PBS, scraped into PBS, suspended in 2% (w/v) NaCl/0.5 M acetic acid and extracted as above. The fluorescent lipids were analysed by HPLC on a silica gel column by gradient elution, with on-line fluorescence detection [30].

Fluorescence microscopy

Fibroblasts on coverslips, labelled as described above, were placed in home-built aluminium/Teflon chambers and covered with PBS. The medium was then depleted of oxygen by adding glucose (0.6%), glucose oxidase (10 units) and catalase (0.06 unit); after 1 min the cells were imaged with a system consisting of a Zeiss Axiovert 10 microscope, a Plan-Neofluor $\times 100$ objective lens and a Photometrics 250 charge-coupled device camera (Photometrics, Tucson, AZ, U.S.A.) controlled by a computer. All images were obtained with a 340 nm (11 nm bandpass) excitation filter, a 480 nm (80 nm bandpass) emission filter and a 375 nm dichroic mirror. For further details see [31].

Other methods

Protein was determined with BSA as the standard by the method of Lowry et al. [32].

RESULTS

Hydrolysis of Pyr_nCE species in detergent micelles

To select the optimal pyrene-labelled cholesterol esters for microscopic studies the Pyr_nCE species ($n = 4$ –16) were incorporated together in detergent/phospholipid micelles and incubated with a post-nuclear human fibroblast homogenate in a pH 3.9 buffer at 37 °C. After extraction the Pyr_nCE species were

Figure 1 Assay of Pyr_nCE hydrolysis by reverse-phase HPLC

An equimolar mixture of seven Pyr_nCE species were incubated with a post-nuclear fibroblast homogenate ($2\ \mu\text{g}$) at 37°C in a pH 3.9 buffer for 0 min (**A**) and 30 min (**B**); the lipids were then extracted and subjected to reverse-phase HPLC with on-line fluorescence detection as detailed in the Experimental section. The numbers above the peaks indicate the number of aliphatic carbons in the acyl chain of Pyr_nCEs . Note that most of the fluorescence in the front peak in (**A**) is not due to Pyr_nFA , because a similar peak was obtained with sample containing exclusively the homogenate, i.e. lacking the Pyr_nCEs .

separated by reverse-phase chromatography and quantified by using on-line fluorescence detection (Figure 1). As shown in Figure 2, the rate of hydrolysis was markedly dependent on the length of the pyrenyl chain. Pyr_{10}CE was hydrolysed most efficiently, followed by Pyr_8CE and Pyr_{12}CE . Pyr_6CE was hydrolysed poorly and Pyr_4CE hardly at all. Identical results were obtained when Pyr_nCE species were incorporated into the micelles separately (results not shown). No detectable hydrolysis of Pyr_nCEs was observed when the homogenate of Wolman fibroblasts lacking functional LAL [33] was used (Figure 2, $\circ$), or when the incubation was performed at pH 7.4 (results not shown), thus demonstrating that the hydrolysis of Pyr_nCEs was indeed mediated by LAL.

To exclude the slow hydrolysis of certain Pyr_nCEs results from their incomplete dissolution in detergent/phospholipid micelles, the excimer-to-monomer ratio (E/M) was recorded as a function of temperature. Plots of E/M against temperature have previously been shown to be informative about the physical state of pyrene lipids [34]. With each Pyr_nCE , E/M plot increased linearly over the whole temperature range studied ($15\text{--}50^\circ\text{C}$; results not shown), thus strongly suggesting that all Pyr_nCE species are equally solubilized in detergent/phospholipid micelles. Note also that the simultaneous presence of all Pyr_nCE species excludes the possibility that any species-dependent effects on the micellar structure would bias the results.

Figure 2 Effect of acyl chain length on LAL-mediated hydrolysis of Pyr_nCE

Pyr_nCEs co-incorporated into detergent/phospholipid micelles were incubated in the presence of post-nuclear homogenate ($2\ \mu\text{g}$) of normal human fibroblasts ($\bullet$) or Wolman fibroblasts ($\circ$) for 30 min at 37°C and pH 3.9. The extent of hydrolysis was determined by reverse-phase HPLC with on-line fluorescence detection (see Figure 1). Each data point represents an average of three parallel samples and the error bars indicate the S.D. Analogous results were obtained in several other experiments performed under somewhat different conditions or when the Pyr_nCE species were incubated separately.

Figure 3 Hydrolysis of Pyr_{10}CE and $[^3\text{H}]18:2\text{-CE}$ incorporated in reconstituted LDL particles

Pyr_{10}CE and $[^3\text{H}]18:2\text{-CE}$ were incorporated together in rLDL particles, which were then incubated with post-nuclear fibroblast homogenate ($0\text{--}20\ \mu\text{g}$) for 2 h at 37°C and pH 3.9. The lipids were extracted and analysed by reverse-phase HPLC as detailed in the Experimental section. Analogous results were obtained when Pyr_{10}CE and $[^3\text{H}]18:2\text{-CE}$ were incorporated together in reconstituted high-density lipoprotein particles.

To determine the rate of LAL-mediated hydrolysis of Pyr_{10}CE relative to that of natural CEs, rLDL particles containing both fluorescent Pyr_{10}CE and radioactively labelled $[^3\text{H}]18:2\text{-CE}$ were prepared and incubated with post-nuclear homogenate at pH 3.9. As shown in Figure 3, both CEs were hydrolysed at similar rates. Analogous results were obtained when the two CEs were incorporated in reconstituted HDL particles (results not shown). Thus Pyr_{10}CE seems to be as good a substrate for the lysosomal

Figure 4 Fluorescence imaging of Pyr₁₀CE- and Pyr₄CE-rLDL uptake by fibroblasts

Normal fibroblasts growing on glass coverslips were incubated with Pyr₁₀CE-rLDL (10 µg/ml) in the presence (A) or absence (B) of chloroquine, an inhibitor of lysosomal CE hydrolysis, for 3 h at 37 °C; they were then washed twice with PBS and imaged as specified in the Experimental section. (C) LAL-deficient Wolman fibroblasts were incubated with Pyr₁₀CE-rLDL in the absence of chloroquine. (D) Normal cells were incubated with Pyr₄CE-rLDL and without chloroquine. Identical exposures and gains were used for each image, except for the insert in (B), which displays the image in the panel proper digitally enhanced to give a 15-fold greater intensity. Representative fields are shown.

lipase as 18:2-CE, which is the major CE species in human LDL [35].

Observation of Pyr_nCE degradation in fibroblasts by fluorescence imaging

To study whether Pyr_nCEs could be used to image the degradation of LDL-derived CEs in living cell cultures, rLDL

Figure 5 Stability of Pyr_nCE-rLDL-derived fluorescence in normal and mutant fibroblasts

Normal fibroblasts (A,C) or LAL-deficient Wolman fibroblasts (B) growing on glass cover slips were incubated with either Pyr₁₀CE-rLDL (A,B) or Pyr₄CE-rLDL (C) (10 µg/ml) at 37 °C for 3 h. Chloroquine (0.5 mM) was included in the medium of normal cells when incubated with Pyr₁₀CE-rLDL (A), to inhibit LAL. The cells were then washed twice with PBS and incubated in a lipoprotein-deficient medium without chloroquine for 24 h at 37 °C, washed twice with PBS and observed as detailed in the legend to Figure 4 and in the Experimental section. The insert in (A) displays the same image as the panel proper digitally enhanced to give a 15-fold greater intensity. Representative fields are shown.

particles containing either Pyr₁₀CE or Pyr₄CE were prepared and incubated with either normal or mutant (Wolman) fibroblasts having a defective LAL. When normal fibroblasts were incubated with Pyr₁₀CE-rLDL in the presence of chloroquine, an inhibitor of LAL-mediated CE hydrolysis [36], or when mutant (Wolman) fibroblasts with defective LAL were employed, a marked accumulation of pyrene fluorescence in intracellular vesicular structures was detected (Figures 4A and 4C respectively). In contrast, very little accumulation could be detected when normal fibroblasts were incubated with Pyr₁₀CE-rLDL in the absence of chloroquine (Figure 4B). When normal cells were incubated with Pyr₄CE-rLDL, brightly fluorescent intracellular vesicles also accumulated in the absence of chloroquine (Figure 4D). Collectively, these findings indicate that Pyr_nCE-rLDL particles are taken up by the cells and delivered to lysosomes, where the Pyr_nCEs are hydrolysed by LAL unless the latter is rendered inactive owing to the presence of chloroquine or a mutation, or

Figure 6 HPLC analysis of degradation of Pyr_{10}CE and Pyr_4CE in living cell cultures

Normal fibroblasts (traces A and C) or LAL-deficient mutants (trace B) were first incubated with either $\text{Pyr}_{10}\text{CE-rLDL}$ (traces B and C) or $\text{Pyr}_4\text{CE-rLDL}$ (trace A) for 3 h at 37 °C, washed and then maintained in lipoprotein-free medium for 24 h. The medium contained Sandoz Compound 58-035 (5 $\mu\text{g/ml}$), an inhibitor of acyl-CoA:cholesterol acyltransferase, to prevent the possible incorporation of released Pyr_{10}FA into cytoplasmic CEs. After incubation, the cells were detached with a rubber 'policeman', washed twice with PBS and assayed for pyrene-labelled lipids as detailed in the Experimental section. Note that in trace C the appearance of a twin peak in the front is an artifact because both of the individual peaks contained only Pyr_{10}CE as revealed by re-chromatography on a reverse-phase column.

if the particular Pyr_nCE used does not serve as a substrate for LAL, as with Pyr_4CE (see Figure 2).

$\text{Pyr}_n\text{CE-rLDL}$ particles are apparently taken into the cells via receptor-mediated endocytosis because the inclusion of a 100-fold excess of unlabelled native LDL in the incubation medium completely blocked the uptake of $\text{Pyr}_4\text{CE-rLDL}$ (results not shown). It is very unlikely that transfer of Pyr_nCEs to the unlabelled LDL particles, rather than competition for the receptor by the latter, would explain the absence of intracellular fluorescence in the presence of the unlabelled LDL particles, because the translocation of Pyr_nCEs between lipoproteins is an extremely slow process ([25], S. Lusa and P. Somerharju, unpublished work).

To investigate the stability of the intracellular (lysosomal) fluorescence that accumulated in the presence of $\text{Pyr}_n\text{CE-rLDL}$, the cells were washed and then incubated in a chloroquine-free medium for up to 68 h. Normal cells that had accumulated intracellular fluorescence in the presence of $\text{Pyr}_{10}\text{CE-rLDL}$ and chloroquine lost virtually all their fluorescence within 24 h (Figure 5A). In contrast, a bright vesicular fluorescence was maintained in the mutant, LAL-deficient, cells preincubated with $\text{Pyr}_{10}\text{CE-rLDL}$ (Figure 5B), as well as in normal fibroblasts preincubated with $\text{Pyr}_4\text{CE-rLDL}$ (Figure 5C). The vesicular fluorescence was readily visible even after 68 h of incubation in both of the latter cases (results not shown).

To confirm that the observed pyrene fluorescence was indeed derived from intracellular structures, the cells were treated with trinitrophenyl lysophosphatidylethanolamine, a quencher of pyrene fluorescence that does not penetrate into cells [31] and fully quenches Pyr_nCE fluorescence in LDL (S. Lusa and P. Somerharju, unpublished work). No detectable quenching was observed, showing that rLDL particles or aggregates possibly adhering to the cells do not contribute significantly to the observed fluorescence.

Analysis of Pyr_nCE hydrolysis by HPLC

The metabolism of $\text{Pyr}_{10}\text{CE-rLDL}$ or $\text{Pyr}_4\text{CE-rLDL}$ taken up by the cells was also analysed by using an HPLC system capable of separating all major lipid species including CEs, triacylglycerols, diacylglycerols and fatty acids as well as all major phospholipid classes [37]. Figure 6 (trace A) shows the chromatogram of a lipid extract from normal fibroblast incubated for 3 h with $\text{Pyr}_4\text{CE-rLDL}$, washed and then incubated with lipoprotein-free medium for 24 h. A major peak co-eluting with Pyr_4CE marker was detectable, in accordance with the imaging results showing an accumulation of intracellular fluorescence under these conditions (Figure 5C). Similarly, a major fluorescent CE peak appeared in the chromatogram obtained for the mutant (Wolman) cells labelled with $\text{Pyr}_{10}\text{CE-rLDL}$ and then incubated for 24 h (Figure 6, trace B). In contrast, much less CE fluorescence was detected in normal cells, which had accumulated $\text{Pyr}_{10}\text{CE-rLDL}$ in the presence of chloroquine and had then been incubated for 24 h in the absence of chloroquine (Figure 6, trace C).

It had been previously shown that exogenous $\text{Pyr}_{10}\text{-fatty acid}$ (Pyr_{10}FA) is readily incorporated into triacylglycerols, phosphatidylcholine and phosphatidylethanolamine and some other glycerolipids of cultured cells [30,38,39]. The inability to detect significant amounts of Pyr_nFA residues in these other lipids (Figure 6, trace C) probably occurred because the amount of $\text{Pyr}_{10}\text{-FA}$ released from Pyr_{10}CE was several orders of magnitude less than that available for acylation when free exogenous Pyr_{10}FA was used as the precursor. In addition, released Pyr_{10}FA is quite rapidly oxidized to shorter homologues [30,40] that are not readily esterified to lipids but are apparently excreted to the medium [38].

In conclusion, the chromatographic data fully support the proposal that Pyr_nCEs are delivered to lysosomes where they are hydrolysed by LAL unless this enzyme is rendered inactive by a mutation or by the presence of chloroquine, or if the particular Pyr_nCE does not serve as a substrate.

DISCUSSION

Selective hydrolysis of Pyr_nCEs by LAL

The present study shows that LAL can hydrolyse pyrene-labelled CEs efficiently both in detergent/phospholipid micelles and in lysosomes of human fibroblasts. However, the length of the pyrene-labelled acyl chain had a remarkable effect on the rate of hydrolysis. Pyr_{10}CE was hydrolysed significantly faster than derivatives with a shorter or a longer acyl chain, and practically no hydrolysis of Pyr_4CE was observed (Figure 2), in accordance with a previous study in which a lymphoid cell line homogenate was used as the source of LAL [41].

Several factors could contribute to the low activity of LAL towards the short-chain Pyr_nCE species. First, it is possible that these species are not fully soluble in the detergent/phospholipid micelles, which served as the macrosubstrate when the relative rates of hydrolysis were determined. However, recordings of the pyrene excimer-to-monomer intensity ratio as a function of temperature did not support this possibility (see the Results section). In addition, the hydrolysis of Pyr_4CE incorporated in rLDL (Figures 4 and 5) and reconstituted high-density lipoprotein (results not shown) particles was also very poor compared with that of Pyr_{10}CE . Secondly, the short-chain species could be poor substrates because of their lower hydrophobicity [41]. This explanation is feasible but there is no other evidence supporting it. The third possibility, which we currently favour, is that the bulky pyrenyl group of short-chain Pyr_nCE species becomes unfavourably positioned in the (putative) acyl-binding site of

LAL. We have previously shown that (1) lysosomal phospholipases tend to discriminate against phospholipids with a short pyrenyl chain and (2) this discrimination is not due to the lower hydrophobicity of the acyl chain, but apparently results from poor accommodation of the pyrene moiety when it is closer to the carbonyl region [22]. Analogously, studies with phospholipid carrier proteins indicated that unfavourable steric interactions, rather than the lower hydrophobicity of short-chain pyrenyl acyl chain, are the main reason for their impaired interaction with the lipid-binding site of those proteins [42,43]. Accordingly we speculate that it might be a general property of the acyl-binding sites of lipases and other proteins interacting with monomeric lipids that bulky groups close to the carbonyl group are not well accommodated, indicating that the acyl-binding cavities/clefts tend to be relatively narrow in this region. This would not be unexpected, because narrow cavities/clefts should provide an optimal fit with the (non-bulky) acyl chains of natural lipids.

The observed decrease in the rate of hydrolysis when the length of the acyl chain exceeded 10 carbon atoms (Figure 2) could be due to problems of accommodating a long pyrenyl acyl chain at the putative acyl-binding site of LAL. Alternatively, this decrease could result from the fact that the hydrophobicity of the whole molecule impairs its efflux from the micelle to the active site of the enzyme, thereby slowing the hydrolysis. Although we cannot choose between these alternatives at present, the latter option is supported by the finding that the rate of LAL-mediated hydrolysis of natural CE is inversely correlated with the hydrophobicity of the CE molecule [9]. Analogously, the hydrolysis of pyrene phospholipids by lysosomal phospholipases was found to be impaired by increasing the hydrophobicity of the substrate [22].

Observation of CE degradation in cells and tissues

Both the imaging and biochemical data provide clear evidence that when rLDL particles containing Pyr_nCEs are incubated with fibroblasts, Pyr_nCEs are delivered into lysosomes, where they are subject to LAL-mediated hydrolysis. Those species, such as Pyr₁₀CE, that are good substrates are rapidly degraded and the products are cleared from the lysosome, whereas the poorly degradable species (such as Pyr₄CE) accumulate in lysosomes and are cleared only very slowly. There is also evidence that natural CE species are not equally degraded by LAL [9,44]. For instance, 18:2-CE, the major CE species of human LDL, is degraded about twice as rapidly as cholesteryl oleate [9,44]. Because Pyr₁₀CE is degraded similarly to 18:2-CE (Figure 3), it could be used as a marker for 18:2-CE, whereas Pyr₈CE or Pyr₁₂CE could serve as marker for the more slowly hydrolysed cholesteryl oleate.

CEs are known to accumulate in the intimal matrix during the progression of atherosclerosis [45]. One possibility is that these intimal CE deposits derive from lysed foam cells, which are typical of atherosclerotic lesions. In these cells CE are stored in cytoplasmic lipid droplets and, to varying degrees, in lysosomes [46,47]. CE in cytoplasmic lipid droplets are derived from LDL CE via lysosomal hydrolysis and subsequent reacylation of the released cholesterol, whereas the CE accumulating in lysosomes probably represent undegraded LDL CE. When the foam cell dies, both the cytoplasmic and lysosomal CE would be released into the intimal matrix. An alternative model suggests that CE are deposited directly, i.e. without the involvement of cellular uptake and processing, into the intimal matrix as a result of aggregation/fusion of proteolytically and/or lipolytically modified LDL particles [48,49]. It is still unclear which of these

alternative mechanisms is the most relevant with respect to the accumulation of CE in the intimal matrix and therefore for the progression of atherosclerosis. A major reason for this lack of information is that the current biochemical approaches are not well suited to the study of metabolic events in such a complex system as the arterial intima. In this respect, Pyr_nCEs could be very useful in allowing the direct observation of the metabolic pathways involved.

For such studies, LDL labelled with a Pyr_nCE could be injected into the circulation of an experimental animal and the distribution and metabolism of the fluorescent LDL particles in intimal sections could then be followed by using a confocal microscope [50]. By employing Pyr₄CE-LDL and Pyr₁₀CE-LDL in parallel, one might be able to assess to what extent the mechanisms discussed above contribute to the accumulation of CE in the intima. Thus the accumulation of extracellular fluorescent particles with both Pyr₄CE- and Pyr₁₀CE-LDL would indicate that a direct deposition of LDL particles to the intima takes place. In contrast, if fluorescent particles were to accumulate in the intimal matrix when Pyr₄CE-LDL, but not Pyr₁₀CE-LDL, was employed, the route involving (1) lysosomal processing of LDL and (2) subsequent release of undegraded CE to the extracellular space would be indicated (see above). In principle, some of the Pyr₁₀FA released from the ester in the lysosomes could be re-esterified to Pyr₁₀CE [30] in the cytoplasmic CE droplets, which, after their release from lysed cells, could be confused with lipoprotein particles directly deposited to the intima (see above). Note, however, that the reformed Pyr₁₀CE molecules in those CE droplets would probably be invisible simply because the excess of unlabelled CE present would dilute the pyrenyl derivatives to the extent that hardly any excimer formation (which is proportional to the local probe concentration) could take place and thus very little excimer fluorescence would be emitted.

The apolipoprotein E-deficient mouse, which rapidly develops advanced atherosclerosis lesions very similar to those in the human disease [51,52], could provide an ideal animal model for this type of study.

In conclusion, the present results show that pyrene-labelled CE can be used to for the direct observation of the degradation of LDL cholesterol esters in living cells. This could be particularly useful when studying the fate of LDL cholesterol esters in complex systems such as the arterial intima, where biochemical methods are not readily applicable.

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