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21-Methylpyrenyl-cholesterol stably and specifically associates with lipoprotein peripheral hemi-membrane: A new labelling tool

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ABSTRACT

Lipoproteins are important biological components. However, they have few convenient fluorescent labelling probes currently reported, and their physiological reliability can be questioned. We compared the association of two fluorescent cholesterol derivatives, 22-nitrobenzoxadiazole-cholesterol (NBD-Chol) and 21-methylpyrenyl-cholesterol (Pyr-met-Chol), to serum lipoproteins and to purified HDL and LDL. Both lipoproteins could be stably labelled by Pyr-met-Chol, but virtually not by NBD-Chol. At variance with NBD-Chol, LCAT did not esterify Pyr-met-Chol. The labelling characteristics of lipoproteins by Pyr-met-Chol were well distinguishable between HDL and LDL, regarding dializability, associated probe amount and labelling kinetics. We took benefit of the pyrene labelling to approach the structural organization of LDL peripheral hemi-membrane, since Pyr-met-Chol-labelled LDL, but not HDL, presented a fluorescence emission of pyrene excimers, indicating that the probe was present in an ordered lipid micro-environment. Since the peripheral membrane of LDL contains more sphingomyelin (SM) than HDL, this excimer formation was consistent with the existence of cholesterol- and SM-enriched lipid microdomains in LDL, as already suggested in model membranes of similar composition and reminiscent to the well-described "lipid rafts" in bilayer membranes. Finally, we showed that Pyr-met-Chol could stain cultured PC-3 cells via lipoprotein-mediated delivery, with a staining pattern well different to that observed with NBD-Chol non-specifically delivered to the cells.

1. Introduction

Physiologically, lipoproteins fulfill the role of systemic shuttles in biological fluids for lipids delivery to target cells via specific receptors. Lipoproteins are multi-molecular assemblies that associate various lipids with apolipoproteins maintaining their structural stability, and they are usually classified according to their density, corresponding to various lipid/protein ratios [1]: the main ones are LDL (low-density lipoproteins, size 18–25 nm) and HDL (high-density lipoproteins, 5–12 nm). It is widely admitted that the lipoprotein core is made of the most hydrophobic lipids, essentially triglycerides and esterified cholesterol, while the particle periphery is a monolayer of less hydrophobic lipids, mainly phospholipids and unesterified cholesterol [2]. However, the structural features of lipoproteins periphery are rather poorly described, in particular regarding the lipid molecules arrangement. Among the phospholipids, sphingomyelin (SM) content is higher in LDL than in HDL (24% and 10% mol/mol, respectively [3]), which can have significant consequences on the structural organization of their peripheral hemi-membrane, in particular in relation with the presence of cholesterol. Indeed, in bilayer biological membranes, cholesterol and SM are known to be the two main components of the membrane microdomains, so-called "lipid rafts" [4].

Abbreviations: HDL, high-density lipoproteins; (V)LDL, (very) low-density lipoproteins; apo, apolipoprotein; NBD, nitrobenzoxadiazole; Pyr-met, methylpyrenyl; Chol, cholesterol; SM, sphingomyelin; LCAT, lecithin:cholesterol acyltransferase.

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It is thus essential to be able to quantify and qualitatively characterize lipoproteins, *in vitro* and *in vivo*. However, few biochemical tools are available, including apolipoprotein radiolabelling, fluorescent lipophilic dyes [5,6] and lipid derivatives [7,8], and immunoassays [9]. Fluorescent markers present many technical and practical advantages over radiolabelled compounds [10], and they also allow imaging approaches [6]. This is illustrated by the wide use of DiI for *in vitro* experiments [10,11], but since this lipophilic dye from carbocyanine family has only a very partial analogy with a physiologic lipid, its use as a lipoprotein marker can be questioned. Indeed, beyond lipoprotein fluorescent labelling, the question of the relevancy of the cell staining obtained is pivotal.

Fluorescent derivatives of cholesterol are useful molecular tools for investigating the structure of biological lipid membranes. Among them, 21-methylpyrenyl-cholesterol (Pyr-met-Chol) and 22-nitrobenzoxadiazole-cholesterol (NBD-Chol) have been previously characterized on model bilayer membranes, and they were shown to present contrasting behaviors. Indeed, Pyr-met-Chol can insert into model bilayers just like cholesterol does with respect to its intramembrane distribution [12], whereas NBD-Chol inserts into them with a behavior largely differing from that of cholesterol [13,14], in particular not preserving the raft-type membrane microdomains. We thus investigated the respective interactions of these two molecular probes with lipoproteins, particularly HDL and LDL.

Here we report that (i) Pyr-met-Chol, at variance with NBD-Chol, can stably label serum lipoproteins without being esterified by LCAT, (ii) Pyr-met-Chol labelling is quantitatively and qualitatively distinguishable between HDL and LDL, (iii) the pyrene group senses an ordered membrane environment in Pyr-met-Chol-labelled LDL but not HDL, and (iv) Pyr-met-Chol-labelled lipoproteins can be used for staining cultured cells.

2. Materials and methods

2.1. Chemicals

Pyr-met-Chol was a kind gift from Dr Lopez (CNRS, France; patent WO/2006/100388), NBD-Chol (22-nitrobenzoxadiazole-cholesterol) was from Sigma-Aldrich, and stock solution were solubilized in chloroform/methanol (9:1 and 2:1, respectively).

2.2. Fluorescent cholesterol association to lipoproteins

F12 or DMEM cell culture medium containing 10-20% fetal calf serum was incubated with Pyr-met-Chol or NBD-Chol, each at 5 μM, for 48 hours at room temperature under gentle stirring and protection from light. Separation of the serum components was performed on a sepharose molecular sieve column (SuperoseTM6 10/300GL) with simultaneous recordings of emitted fluorescence (Pyr-met-Chol: excitation at 330 nm and emission at 385–400 nm; NBD-Chol: 490 nm and 535–560 nm respectively) and protein UV absorption (at 280 nm). LCAT activity was determined on human or fetal calf serum incubated for 48–72 h with either Pyr-met-Chol or NBD-Chol (20 μM).

Purified LDL and HDL were prepared from plasma of healthy donors by ultracentrifugation (120,000*g*, 4 °C, 66 h) onto a KBr layer of density 1.06 and 1.19, respectively. For fluorescent labelling, 1-7 mg/ml of lipoprotein was incubated with Pyr-met-Chol (using ethanol as a vehicle), either at 10 μM or at 5% mol/mol of the non-esterified cholesterol present in the lipoprotein fraction, corresponding to 20–50 μM for HDL and 130–300 μM for LDL, under gentle stirring and protection from light. Labelled lipoproteins were then submitted to ultracentrifugation in KBr (120,000*g*, 4 °C, 66 h), to eliminate unbound fluorescence, and then dialyzed using a semi-permeable membrane with 10 kDa cutoff, to eliminate KBr and weakly bound fluorescence. Bound Pyr-met-Chol fluorescence was measured by a fluorometric assay, using a Varioskan Flash fluorescence plate reader (Thermo-Electron), at 330 and 400 nm for excitation and emission, respectively. Protein concentration was determined by the Bradford protein assay, and cholesterol assay was performed using the Biolabo colorimetric kit. Quantitative experiments have been performed two to four times, each point being in triplicate.

To analyze the stability of fluorescent labelling, apoB-containing lipoproteins were precipitated with the phosphotungstene-containing Randox Laboratories kit, according to the manufacturer recommendations, and the fluorescence remaining in the supernatant was measured.

2.3. Fluorescent labelling of oxidized lipoproteins

Purified LDL (2–3 mg/ml) were oxidized by incubation with 5 μM $CuSO_4$ during 4 or 8 h at room temperature, and then fluorescently labelled by incubation with 10 μM Pyr-met-Chol according to the standard procedure. It is worth noting that Pyr-met-Chol labelling of oxidized lipoproteins was performed after the oxidating incubation, in contrast to other procedures reported in the literature that monitored the oxidation process by taking benefit of the simultaneous oxidation of the fluorescent probe [15,16].

2.4. Spectroscopic analysis of fluorescently labelled lipoproteins

For the spectroscopic analysis of Pyr-met-Chol associated to lipoproteins, we used labelled purified lipoproteins diluted to 0.9 mg/ml for HDL and 0.4 mg/ml for LDL, and analyzed by recording fluorescence emission spectra (excitation at 335 nm) on an Aminco500 or a QuantaMaster-4 spectrofluorometer.

2.5. Cell culture, fluorescent staining and microscopy imaging

PC-3 cells [17], provided from ATCC, were cultured in Ham's F12 medium with 10% fetal calf serum and penicillin (100 U/ml) plus streptomycin (10 μg/ml) (Invitrogen) at 37 °C in 5% CO_2. PC-3 cells were seeded onto glass slides put in wells, at 250,000 per well in 12-well plastic plates, and cultured until confluency, washed and then incubated with 5 μM of both Pyr-met-Chol and NBD-Chol, using ethanol as a vehicle, for 48 h. Pyr-met-Chol and NBD-Chol were observed using a two-photon excitation (TPE) LSM 7MP Zeiss (Carl Zeiss, Germany) microscope equipped with a Chameleon Ultra II Ti:sappire laser (Coherent, CA, USA). Pyr-met-Chol was excited at 710 nm (TPE), and emission was recorded using a 370–485 nm band-pass filter; for NBD-Chol, excitation was at 940 nm (TPE), and emission recorded with a 500–550 nm filter. Acquisitions were performed with a 63× oil objective (1.4 NA).

3. Results

3.1. Preferential association of Pyr-met-Chol over NBD-Chol to serum lipoproteins

We first evaluated the capacity of Pyr-met-Chol to associate to serum components by following their separation on sepharose with the simultaneous detection of fluorescence emission and protein content. When incubated for 48 h in the presence of 20% fetal calf serum, Pyr-met-Chol (5 μM) was found in all three major lipoprotein fractions: VLDL, LDL and HDL (Fig. 1A). Part of the fluorescence was also found in the peak corresponding to albumin, the major protein component of serum. In contrast to Pyr-met-Chol, NBD-Chol associated only marginally with the HDL peak among