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Radiolabeled cholesteryl ethers: A need to analyze for biological stability before use

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ABSTRACT

Radiolabeled cholesteryl ethers are widely used as non-metabolizable tracers for lipoproteins and lipid emulsions in a variety of *in vitro* and *in vivo* experiments. Since cholesteryl ethers do not leave cells after uptake and are not hydrolyzed by mammalian cellular enzymes, these compounds can act as markers for cumulative cell uptakes of labeled particles. We have employed [^{3}H]cholesteryl oleoyl ether to study the uptake and distribution of triglyceride-rich emulsion particles on animal models. However, questionable unexpected results compelled us to analyze the stability of these ethers. We tested the stability of two commercially available radiolabeled cholesteryl ethers - [^{3}H]cholesteryl oleoyl ether and [^{3}H]cholesteryl hexadecyl ether from different suppliers, employing *in vitro*, *in vivo* and chemical model systems. Our results show that, among the two cholesteryl ethers tested, one ether was hydrolyzed to free cholesterol *in vitro*, *in vivo* and chemically under alkaline hydrolyzing agent. Free cholesterol, unlike cholesteryl ether, can then re-enter the circulation leading to confounding results. The other ether was not hydrolyzed to free cholesterol and remained as a stable ether. Hence, radiolabeled cholesteryl ethers should be analyzed for biological stability before utilizing them for *in vitro* or *in vivo* experiments.

1. Introduction

Radiolabeled cholesteryl esters (CE) have been used as markers for lipid and lipoprotein metabolic experiments, but CE are degradable following uptake by cells and the radiolabel can be released as free cholesterol (FC) into the cell itself or into blood. FC may readily exchange between lipoproteins and membrane surfaces and re-enter cells leading to confounding results [1,2].

Availability of CE analogs that are both radiolabeled and not metabolized in mammalian cells have contributed to understanding the metabolism of plasma lipoproteins and lipid emulsions. The structural and thermodynamic similarities of the cholesteryl ethers to the esters allow the use of ethers as non-metabolizable analogs for CE in biological systems [3–7]. The stable ether bond is not hydrolyzed by mammalian cellular enzymes and ethers do not readily escape from cells. This results in accumulation of cholesteryl ethers in cells and provides a cumulative marker of the labeled lipid particle uptake [8]. Radiolabeled cholesteryl ethers have been proven as useful tracers on a variety of *in vitro* and *in vivo* investigations employing lipoproteins [9–11], lipid emulsions [12], and liposomes [13,14].

Our research group is involved in experimental projects employing laboratory made triglyceride (TG) emulsions of docosahexaenoic acid (triDHA) [15] and commercially available fish oil and soy oil emulsions in studies in neonatal/ adult mice and *in vitro* models [16–18]. A major area of interest within our group is studies on transport and delivery of TG-rich emulsion particles in animal models. We have employed radiolabeled cholesteryl ethers as tracers for these studies [19,20]. Recently, after obtaining a new supplier for the radiolabeled cholesteryl ether, unexpected results were observed in our well established model systems. These prompted us to investigate the biological stability of commercially purchased radiolabeled cholesteryl ethers. In the present investigation, we have compared the biological stability of two commercial radiolabeled cholesteryl ethers widely used in studies on lipid metabolism, employing previously established *in vitro* [18,21] and *in vivo* [12,22] model systems from our laboratory. Further, we also tested the chemical stability of radiolabeled cholesteryl ethers under

Abbreviations: CE, cholesteryl ester; [^{3}H]CEt-ARC, [^{3}H] cholesteryl oleoyl ether-American Radiolabeled Chemicals; [^{3}H]CEt-PE, [^{3}H] cholesteryl hexadecyl ether-PerkinElmer; FC, free cholesterol; hrs, hours; TLC, thin layer chromatography; TG, triglycerides

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conditions of alkaline hydrolysis.

2. Materials and methods

2.1. Ethics statement

All research studies were carried out according to protocols approved by the Columbia University Institutional Animal Care and Use Committee and in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care guidelines.

2.2. Materials

TriDHA was purchased from Nu-Chek Prep, Inc. (Elysian, MN). Egg yolk phosphatidylcholine was obtained from Avanti Polar-Lipids, Inc. (Alabaster, AL). The soy oil emulsion (Intralipid^{Rx}) was manufactured by Fresenius Kabi (Sweden) and was purchased from Baxter Healthcare Corporation (Deerfield, IL). The fish oil emulsion (Omegaven^{Rx}) was obtained from Fresenius Kabi (Austria). We used radiolabeled cholesteryl ethers from two companies; cholesteryl oleoyl ether (ART 1319) from American Radiolabeled Chemicals (St. Louis, MO) herein named as ([^{3}H]CEt-ARC) and cholesteryl hexadecyl ether (NET 859001MC) from PerkinElmer (Boston, MA) herein named as ([^{3}H]CEt-PE).

2.3. Lipid emulsions

TriDHA emulsions (10% by TG weight/100 mL emulsion) were laboratory made using sonication and centrifugation with TG oil and egg yolk phospholipid as previously detailed [15]. The emulsions were analyzed for the amount of TG and phospholipids (PL) by enzymatic procedure using a commercial kit according to the accompanying instructions (Wako Chemicals USA, Inc., Richmond, VA). The TG: phospholipid mass ratio was 5.0 ± 1.0, similar to very low density lipoprotein (VLDL)-sized particles.

We labeled commercially available fish oil/ soy oil emulsions and laboratory-made triDHA emulsions with radiolabeled cholesteryl ethers as we previously described [12]. Briefly, in a small glass vial, desired amounts of [^{3}H]CEt-ARC or [^{3}H]CEt-PE were mixed with 20–50 μL of 100% of ethanol. The vial was rotated to coat the vial wall evenly under N_2 gas until the solvents evaporated completely. Immediately upon the vial becoming dry, 150 μL of the emulsion was added to the vial. The vial was mixed with gentle non-vortex shaking and allowed to sit for 30 min. After the same procedure, another two portions of emulsion were added to a total volume of 500 μL. The emulsion was sonicated three times on ice for 20 s, each at a power setting of 40 W using a Branson Sonifier Cell Disruptor (model W185) (Branson Scientific, Inc., Plainview, NY) to incorporate the [^{3}H]cholesteryl ethers into the core of the emulsion particles.

2.4. Cells

Monolayer cultures of J774 A2 macrophage were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum, streptomycin (100 μg/mL), penicillin (100 U/mL), and glutamine as described previously (21). For each experiment, the cells were plated onto 6 well plates at 37 °C in an atmosphere containing 5% CO_2, 95% air. Experiments were performed at 24 h after plating and 80% confluent cells were used for these studies.

2.5. Incubations

Prior to incubation, cells were washed with PBS at 37 °C. Then PBS was exchanged with experimental media. Cells were incubated for 4 & 24 h with commercially available soy oil emulsions labeled with [^{3}H]CEt-ARC or [^{3}H]CEt-PE. The incubation was performed at 37 °C on a rocker. At the end of incubation, medium was removed. The cells were chilled on ice and washed twice with ice cold PBS containing 0.2% BSA (1 and 5 min washes) and twice with ice cold PBS alone. Cell lipids were extracted by hexane/isopropanol (3:2) as described previously [23].

2.6. Animals

Wild type C57BL/6 J adult and neonatal mice were purchased from Jackson Laboratories (Bar Harbor). Adult mice were anesthetized by intraperitoneal injection of ketamine/xylazine. The mice were intraperitoneally administered fish oil emulsion labeled with [^{3}H]CEt-ARC. After emulsion injection, retro-orbital blood was drawn at fixed time intervals (0, 1.5, 6, 8 and 24 h) by heparinized capillary tubes. Mice were sacrificed immediately after collection of the final blood sample at 24 h. Liver was dissected out after perfusion with 0.9% NaCl-containing heparin (2 units/mL). Total plasma TG was enzymatically measured by Wako TG determination kit. The amount of radioactivity in blood was measured by liquid scintillation spectrometry and was expressed as the percent (%) of the injected dose remaining in the whole blood [12].

Neonatal mice (p10) were intraperitoneally injected with laboratory-made triDHA emulsions labeled with [^{3}H]CEt-ARC or [^{3}H]CEt-PE. The animals were sacrificed at 1, 2, 4 and 24 h after the injection of radiolabeled triDHA emulsion. Blood was collected by intracardiac puncture. Liver was dissected out following perfusion with 0.9% NaCl-containing heparin (2 units/mL).

Liver samples were homogenized using a Polytron Tissue Disruptor (Omni TH, Kenneswa, GA). Chloroform/methanol (2:1) was used to extract lipids from liver homogenates, plasma and radiolabeled emulsions as described previously [16].

2.7. Chemical hydrolysis of radiolabeled cholesteryl ethers

To check the stability of radiolabeled cholesteryl ethers under conditions of alkaline hydrolysis, [^{3}H]CEt-ARC or [^{3}H]CEt-PE were mixed with 40 μg cholesteryl oleate and incubated with 0.1 mL of 1 M ethanolic KOH at 80 °C for 1 h [24]. Lipids were extracted using chloroform/methanol (2:1) as described previously [16].

2.8. Analyses

2.8.1. Thin layer chromatography

Cells/ liver extracts, radiolabeled emulsions and chemically hydrolyzed radiolabels were assayed for hydrolysis of cholesteryl ethers by determining radioactivity in the FC and CE bands on thin layer chromatography (TLC) with the solvent system hexane: diethyl ether: acetic acid (70:30:1) [23]. Samples were run in parallel lanes with standards and individual spots identified by iodine vapour, which were then scraped to determine the radioactivity in each band region.

2.8.2. Liquid scintillation counting

Tritium in blood and TLC samples were determined with liquid scintillation counting procedure. In brief, the samples were suspended in the scintillation fluid (Ultima Gold scintillation fluid, PerkinElmer, Boston, MA), mixed well and ^{3}H dpm were assayed in a Perkin Elmer Tri-Carb liquid scintillation spectrometer 5110 TR.

2.9. Statistical analysis

Data are presented as mean ± SE. We compared plasma TG and radioactivity levels between different time points after intraperitoneal (i.p.) injection of radiolabeled fish oil emulsion. Paired *t*-tests were used to determine significant differences between time points. To compare the differences between radiolabel recovered in different TLC band regions from emulsions and cells, one-way ANOVA followed by post hoc Tukey test was used. Unpaired *t*-tests were used to compare the