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Analysis of cholesteryl esters and diacylglycerols using lithiated adducts and electrospray ionization-tandem mass spectrometry

John A. Bowden, Carolyn J. Albert, Omar S. Barnaby, David A. Ford *

Department of Biochemistry and Molecular Biology, School of Medicine, and Center for Cardiovascular Research, Saint Louis University, St. Louis, MO 63104, USA

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ABSTRACT

Cholesteryl ester (CE) and diacylglycerol (DAG) molecular species are important lipid storage and signaling molecules. Mass spectrometric analyses of these lipids are complicated by the presence of isobaric molecular ions shared by these lipid classes and by relatively poor electrospray ionization, which is a consequence of an inherently weak dipole moment in these lipid classes. The current study demonstrates that lithiated adducts of CE and DAG molecular ions have enhanced ionization and lipid class-specific fragmentation in tandem mass spectrometry (MS/MS) scan modes, thereby allowing the implementation of strategies capable of lipid class-specific detection. Using neutral loss (NL) mode for the loss of cholestane from cholesterol esters (NL 368.5) and specific selected reaction monitoring for DAG molecular species, the response of specific molecular species to that of internal standards was determined. CE and DAG molecular species were quantified in human coronary artery endothelial cells (HCAECs) incubated with both palmitic acid and oleic acid. Furthermore, NL 368.5 spectra revealed the oxidation of the aliphatic fatty acid residues of CE molecular species. Taken together, these studies demonstrate a new analytical approach to assessing CE and DAG molecular species that exploits the utility of lithiated adducts in conjunction with MS/MS approaches.

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Lipids are important cellular molecular constituents with critical roles in cell membrane structure and function, energy storage and production, cell signaling, and both tissue and systemic physiological functions [1]. Accordingly, the detection of specific lipid alterations using mass spectrometric methods is an important strategy to interrogate the role of lipids in cellular, tissue, and systemic physiological and pathophysiological processes [2,3]. Two lipid classes of great importance in cell signaling and lipid storage that have been implicated in human disease are cholesteryl esters (CEs)[1] and diacylglycerols (DAGs) [4,5]. However, mass spectrometry (MS) of these two lipid classes (CE and DAG) is complicated by these two classes having common isobaric molecular ions that render their identity by single stage MS incomplete.

DAGs represent an important group of intermediate molecules that are key to the biosynthesis of lipids. DAGs are principally derived from either the hydrolysis of phosphatidylinositols (PIs) or phosphatidylcholines (PCs) by specific phospholipase C (or the coupled actions of phospholipase D and phosphatidate phosphohydrolase) or by *de novo* synthesis [6,7]. The biological function of DAGs is predominantly considered to be 2-fold: as an intermediate in the formation of triacylglycerols (TAGs) and phospholipids and to regulate DAG-sensitive protein kinase C (PKC) isozymes that modulate numerous cellular processes, including cell growth and differentiation [8,9]. It should also be appreciated that DAG accumulation has been shown to be associated with diabetes, carcinoma, and coronary heart disease [10,11].

CEs represent an important group of lipids central in the regulation of cholesterol transport and storage in addition to its integral role in membrane function [4,12]. Cholesterol and CE biosynthesis is tightly regulated by intracellular cholesterol levels [13]. Intracellular CEs are synthesized by the action of acyl CoA acyl transferase (ACAT), and cholesterol is converted to CEs in high-density lipoprotein (HDL) by the action of lecithin cholesterol acyl transferase (LCAT) [14,15]. Elevated plasma CE levels are associated with atherosclerosis [16,17]. In addition, under conditions that cholesterol metabolism is altered, such as tumor promotion, elevated tissue CE levels are observed [18]. Furthermore, the appearance of CE-enriched lipid droplets in tissues is a consequence of impaired metabolism or overnutrition [19,20]. Alterations in plasma levels of specific molecular species of CE have also been speculated to have utility as predictors of diabetes mellitus, coronary heart disease, and cancer [20].

* Corresponding author.
E-mail address: fordda@slu.edu (D.A. Ford).

[1] *Abbreviations used:* CE, cholesteryl esters; DAG, diacylglycerol; MS, mass spectrometry; PI, phosphatidylinositol; PC, phosphatidylcholine; TAG, triacylglycerol; PKC, protein kinase C; GC, gas chromatography; ESI, electrospray ionization; MS/MS, tandem MS; CAD, collisionally activated dissociation; HCAEC, human coronary artery endothelial cell; HPLC, high-performance liquid chromatography; NL, neutral loss; SRM, selected reaction monitoring; BSA, bovine serum albumin.

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Gas chromatography (GC)/MS is frequently used for the analysis of esterified glycerolipid species [21]. However, GC/MS analysis of CEs and DAGs requires a derivatization step (e.g., silyl reagents for electron impact ionization (EI) [22–24] and pentafluorobenzoyl reagents for negative ion chemical ionization (NICI) [22,25,26]) to impart the necessary volatility and detection enhancement required for sensitive GC/MS analysis. Thus, attention has focused on the utility of softer ionization techniques for CE and DAG analyses, specifically the use of matrix-assisted laser desorption/ionization (MALDI) [27] and electrospray ionization (ESI) [28–30]. In particular, ESI is compatible with the rapid and sensitive quantification of lipid species in complex matrices without the need for derivatization. Although both ammoniated and sodiated adducts of CE and DAG have been used for detection of molecular species using ESI-MS, these adducts produce only moderate (for CEs) to inconsequential (for DAGs) levels of tandem MS (MS/MS) fragmentation [21,31]. In contrast, lithiated adducts have been described for the analysis of lipids with ESI due to the ability of these adducts to provide enhanced fragmentation in comparison with other adducts [32–34]. However, the utility of lithiated adduct formation for CEs and DAGs has not been examined and contrasted with the use of other adducts.

In this study, ESI-MS/MS strategies were developed and optimized for the species-selective analysis of multiple CE and DAG molecular species. In comparison with ammoniated adducts of CEs and DAGs, lithiated adducts exhibit enhanced ion intensity and fragmentation. Based on collisionally activated dissociation (CAD) analyses of CEs and DAGs, specific MS/MS scanning modes were developed to quantify CE and DAG molecular species. These strategies were applied to examine CE and DAG molecular species in human coronary artery endothelial cells (HCAECs) that were grown in the presence and absence of specific fatty acid supplementation.

Materials and methods

Standards, salts, and solvents

The standards consisted of eight CEs, including myristate (14:0), palmitate (16:0), palmitoleate (16:1), stearate (18:0), oleate (18:1), linoleate (18:2), arachidonate (20:4), and docosahexaenoate (22:6), and seven DAGs, including dipalmitin (16:0–16:0 or 32:0), 1-palmitoyl-2-oleoyl-*sn*-glycerol (16:0–18:1 or 34:1), distearin (18:0–18:0 or 36:0), diolein (18:1–18:1 or 36:2), ditranseicosenoin (20:1–20:1 or 40:2), 1-stearoyl-2-linolenoyl-*sn*-glycerol (18:0–18:2 or 36:2), and 1-stearoyl-2-arachidonyl-*sn*-glycerol (18:0–20:4 or 38:4). The lipid standards were purchased from Nu-Chek Prep (Elysian, MN, USA) or Avanti Polar Lipids (Alabaster, AL, USA) except for 1-palmitoyl-2-oleoyl-*sn*-glycerol, which was synthesized as described previously [35]. Individual stock solutions of all the standards were prepared (20 μM in methanol/chloroform, 4:1). The internal standards were cholesteryl heptadecanoate (17:0) and diarachidin (20:0–20:0 or 40:0) (NuChek Prep). The salts used for adduct formation and the Bligh–Dyer extraction were NaOH, LiOH, LiCl, and ammonium acetate, all purchased from either Fisher Scientific (Fair Lawn, NJ, USA) or Sigma (St. Louis, MO, USA). The methanol and chloroform solvents were of high-performance liquid chromatography (HPLC) grade and were purchased from Burdick & Jackson (Muskegon, MI, USA).

ESI-MS analysis

Direct infusion–ESI-MS analysis of standards and biological samples was performed in positive ion mode using a Thermo Fisher TSQ Quantum Ultra with Xcalibur data acquisition software. Samples were analyzed at a flow rate of 3 μl/min. Tune parameters were optimized using standards and were set at spray voltage = 3800 V, sheath gas = 8 (arbitrary units), ion sweep gas pressure = 0.5 (arbitrary units), auxiliary gas pressure = 5 (arbitrary units), and capillary temperature = 270 °C. Spectra for survey scans were acquired for 5 min with a scan rate of 0.5 scan/s. For MS/MS analyses, several scan modes were investigated, including product ion (i.e., CAD), parent ion, neutral loss (NL), and selected reaction monitoring (SRM). In each MS/MS mode, the collisional energy for the CE and DAG molecular species were optimized and set to 25 and 35 eV, respectively. Initially, the fragmentation of parent ions into its fragments was investigated to determine the most abundant fragments associated with each CE and DAG molecular species. Spectra for each of these MS/MS scan modes were acquired over 3 min with a scan rate of 0.5 scan/s. For the SRM scan mode, all of the CEs and DAGs were examined with an isolation width of 2 amu. The NL and SRM scans for the different CE and DAG molecular species as lithiated adducts, respectively, are shown in Table 1.

Fatty acid supplementation of HCAECs

HCAECs (Cell Applications, San Diego, CA, USA) were grown to more than 90% confluency (passages 4–9) in 60-mm tissue culture dishes. The HCAECs were grown in EGM-2 MV BulletKit medium containing 5% serum (Lonza, Walkersville, MD, USA). Prior to fatty acid supplementation, the normal growth medium was removed from the dishes and replaced with growth medium containing only 2% fetal bovine serum. For the fatty acid supplementation, bovine serum albumin (BSA)-bound palmitic acid (16:0) and BSA-bound oleic acid (18:1) [36] were added to the medium to final concentrations of 200 and 100 μM palmitic acid and oleic acid, respectively. Negative control conditions for the fatty acid supplementation condition included incubating HCAECs with 2% growth medium containing BSA with no added fatty acid. The cells were incubated with and without the fatty acid supplementation for 24 h. To enhance lithium adducts of CE and DAG molecular species extracted from the HCAECs treated with or without fatty acid, the medium

Table 1
Calibration line parameters for lithiated adducts of CE and DAG molecular species.

CE	NL 368.5 $[M+Li]^+$	Line parameters
14:0	603.80	$y = 0.7189x + 0.0362$
16:0	631.84	$y = 1.2001x + 0.0127$
16:1	629.84	$y = 1.8776x + 0.0417$
18:0	659.88	$y = 1.1370x + 0.0061$
18:1	657.88	$y = 1.9831x + 0.0031$
18:2	655.84	$y = 2.6063x + 0.0192$
20:4	679.96	$y = 3.6454x + 0.4447$
22:6	703.88	$y = 4.0834x + 0.1929$
DAG	**SRM**	**Line parameters**
16:0–16:0	575.47 → 313.28	$y = 0.7757x + 0.094$
16:0–18:1	601.50 → 313.28[a]	$y = 0.9112x + 0.0130$
16:0–18:1	601.50 → 339.31	$y = 0.8205x + 0.0556$
18:0–18:0	631.53 → 341.27	$y = 1.0034x - 0.0641$
18:1–18:1	627.53 → 339.27	$y = 1.5439x - 0.040$
18:0–18:2	627.53 → 341.32[a]	$y = 0.9139x + 0.0432$
18:0–18:2	627.53 → 337.34	$y = 0.519x + 0.012$
18:0–20:4	651.53 → 341.10[a]	$y = 1.7066x + 0.1907$
18:0–20:4	651.53 → 361.23	$y = 0.0651x + 0.0061$
20:1–20:1	683.58 → 367.27	$y = 1.973x - 0.0431$

Note. Linear regression of ion intensity responses for each CE and DAG molecular species over the concentration range of 0.1, 0.5, 2, 5, and 10 μM was determined. In all cases, the coefficient of determination (R^2) was greater than 0.99. The internal standards, 17:0 CE and 20:0–20:0 DAG (SRM 687.58 → 369.31), were constant at 2 μM.

[a] Preferred SRM to detect DAG molecular species, which were used in HCAEC studies.

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