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Simultaneous Quantitative Determination of Different Ceramide and Diacylglycerol Species in Cultured Cells by Using Liquid Chromatography–Electrospray Tandem Mass Spectrometry

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Abstract

A sensitive, reproducible reverse-phased high performance liquid chromatography electrospray tandem mass spectrometry (HPLC-ESI-MS/MS) method with simple sample preparation was developed for the simultaneous determination of a wide range of ceramides, diacylglycerols (DAGs) in cultured cells. Chromatographic separation of the compounds was achieved in a 14-minute run using a C8 column with a gradient elution by methanol and 10 mM ammonium acetate buffer as mobile phase at a flow rate of 0.5 ml/min. Various ceramides, DAGs were detected with a triple quadrupol system in multiple reaction monitoring mode, which is based on a soft positive electrospray ionization. The usual sample preparation process was shortened by the application of pure methanol for the extraction instead of the widely used methanol/chloroform mixture. C17:0 ceramide which does not occur in the cell samples, was used as an internal standard. The sample preparation process was optimized and the methodology was tested on a human hepatocarcinoma cell culture. Our results clearly showed accumulation of some ceramides and DAGs in the cells treated with BSA-conjugated palmitate for 8 hours. Since both ceramides and DAGs are important lipid intermediates and signal messengers, alteration in their cellular levels have major impact on cell functions, and thus our novel analytic method can be widely used in lipotoxicity research. The presented technique can be further developed to measure other intermediates of ceramide synthesis and other derivatives of DAGs as well.

Keywords

mass spectrometry, liquid chromatography, ceramides, glycerolipids, lipotoxicity

1 Introduction

Depot fat stored in the adipocytes contains fatty acids (FAs) ingested with animal fat [1] and with natural or hydrogenated plant oils [2, 3] as well as FAs synthesized endogenously in the fed state. As obesity and related diseases, such as cardiovascular diseases, insulin resistance and nonalcoholic fatty liver disease have become epidemic, there is a strong emphasis on investigating their pathology. Adipocyte hypertrophy is an indispensable component of obesity in adults, and it often leads to a local inflammation in the overgrown adipose tissue, which in turn enhances triglyceride turnover and elevates serum free fatty acid (FFA) levels [4].

Oversupply of FFAs stimulates both lipid biosynthesis and FA degradation. Deleterious effects of excessive FFAs were mainly attributed to ectopic fat deposition in various cells, such as hepatocytes [4]. However, growing evidence shows that lipotoxic oxidative and endoplasmic reticulum (ER) stress [5], inflammation and apoptosis are mostly due to fatty acyl coenzyme A (acyl-CoA) build-up and to the consequent accumulation of certain lipid intermediates in the cells. Ceramides, which are amides of a sphingosine and a FA chain, constitute the hydrophobic backbone of a wide array of sphingolipids, play an important role in signaling.

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They influence cell growth and proliferation [6] and cellular senescence [7], and cell death as they can induce apoptosis [8, 9]. Recently, many publications have also suggested the role of ceramides in the development of insulin resistance and in β-cell failure, and thus their importance in type 2 diabetes [10]. Intracellular ceramide production (Fig. 1) is potentially affected by fatty acyl-CoA supply at different acylations in the cell. *De novo* synthesis of ceramides starts with the condensation of palmitoyl-CoA and serine to 3-dehydrosphinganine, and its subsequent reduction. The product, sphinganine is *N*-acylated with another acyl-CoA by ceramide synthase. Sphingosine, an intermediate of sphingolipid degradation can be recycled through *N*-acylation by sphingosine acyltransferases which can use various fatty acyl-CoA species for substrates. Diacylglycerols (DAGs) are also important lipid species with signaling functions. These compounds have two fatty acyl groups in ester bonds with two hydroxyl groups of glycerol. As it is presented in Fig. 2, they can serve as precursors in the synthesis of triacylglycerols and complex glycerophospholipids such as phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), etc. or galactolipids such as monogalactosyldiacylglycerol (MGDG). When 1,2-DAGs are formed by the hydrolytic cleavage of plasma membrane glycerophospholipids, mostly phosphatidylinositols by phospholipase C, they serve as signal messengers through the

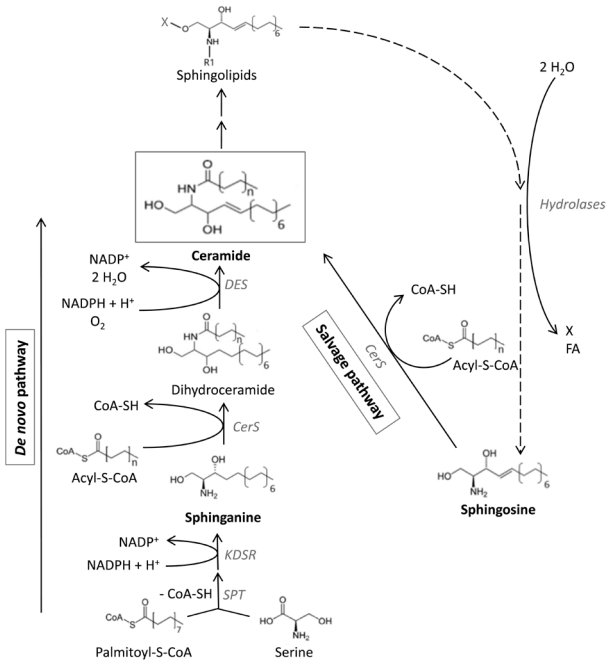

Fig. 1 *De novo* synthesis and salvage of ceramides. SPT: serine palmitoyl transferase, KDSR: 3-ketodihydrosphingosine reductase, CerS: ceramide synthases, DES: dihydroceramide desaturase.

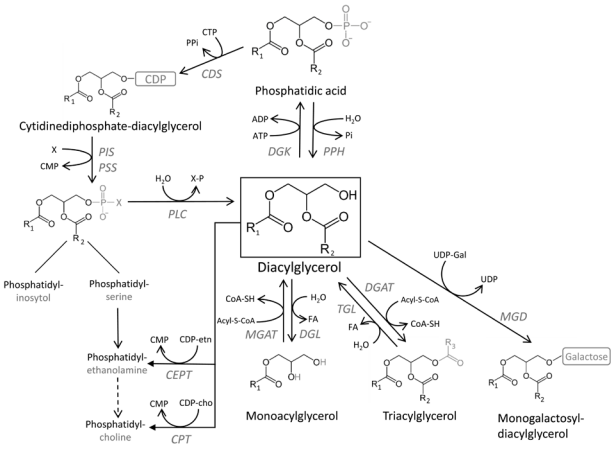

Fig. 2 Metabolism of diacylglycerols. CDS: cytidinediphosphate-diacylglycerol synthase, CEPT: choline/ethanolamine phosphotransferase, CPT: choline phosphotransferase, DGK: diacylglycerol kinase, PPH: phosphatidate phosphohydrolase PI PLC: phosphoinositide-phospholipase C, MGAT: monoacylglycerol acyltransferase, DGAT: diacylglycerol acyltransferase, DGL: diacylglycerol lipase, TGL: triacylglycerol lipase, MGD: monogalactosyl-diacyclglycerol synthase.

activation of protein kinase C isoenzymes [11]. DAGs also have an effect on insulin secretion in case of oversupply of FAs [12]. Since DAGs are central intermediates of phospholipid metabolism, any alteration in their structure would largely affect the composition of cellular membranes as well.

It has become evident that FA surplus exerts many of its pathologically relevant deleterious effects in the cells through the accumulation of ceramides and DAGs. Therefore, biological and medical investigations on lipotoxicity, could growingly exploit the accurate and sensitive analytical methods which are suitable for a simultaneous assessment of these two groups of lipid species.

Ceramides can be measured by different techniques, such as enzymatic measurements involving diacyglycerol kinase assay [13, 14], or chromatographic methods, such as thin-layer chromatography [15, 16] or high-performance liquid chromatography [17-19]. However, these methods may have limitations regarding the quantification of individual ceramide species. Tandem mass spectrometry can be a useful method to quantify different forms of ceramide simultaneously. In case of multiple reaction monitoring mode, a prior separation step is not essential as the different transitions are specific to the molecules [20-23]. However, if tandem mass spectrometry is coupled to high-performance liquid chromatography, the selectivity improves and the method is more robust since the components are separated from the impurities. An HPLC-MS/MS method was developed and used to quantify various ceramides in biological samples [23-26].

Measurement of DAGs was carried out by DAG kinase assay [12], capillary gas chromatography [27] and liquid chromatography coupled to mass spectrometry [28-30]. Leiker et al. used the 2,4-difluorophenyl urethane derivatives of DAGs in a normal phase separation [28] to separate 1,2-diacylglycerols and 1,3-diacylglycerols.

The main problem to be faced in case of mass spectrometry is the poor ionization ability of DAGs. To avoid this problem, derivatizing compounds, such as *N*-chlorobetainyl chloride [30] are used in the sample preparation step, alternatively ammonium acetate [28] or sodium acetate [29] buffers are added to the eluent.

We sought to develop a sensitive and reproducible HPLC-MS/MS method with a simple, chloroform-free sample preparation which is suitable for the quantitation of ceramides and DAGs in a single run. Here we demonstrate a 14-min run to separate 12 individual lipid intermediates. Our method permits the use of pure methanol as an extracting agent instead of a chloroform/methanol mixture. This method was tested analyzing the effects of palmitate treatment on ceramide and DAG levels in HepG2 human hepatocarcinoma cells.

2 Materials and methods

2.1 Chemicals and reagents

C16:0, C17:0, C18:0, C18:1(9Z) (>99 %) ceramides were purchased from Avanti Polar Lipids Inc., 1,2-dipalmitoyl-*rac*-glycerol (>99 %), 1-palmitoyl-2-oleoyl-*sn*-glycerol (>99 %), 1,2-dioleoyl-*sn*glycerol (>97 %), 1-octadecanoyl-2hexandecanoyl*sn*glycerol (>99 %), palmitic acid (>99 %), ammonium acetate (>98 %) and bovine serum albumin (BSA) were from Sigma-Aldrich (St. Louis, MO). Methanol (gradient grade) and acetonitrile (gradient grade) were obtained from Merck. Chloroform (99.8 %) for lipid extraction was from Panreac and isopropanol was purchased from Molar Chemicals. All experiments and measurements were carried out by using Millipore ultrapure water.

2.2 Standard solutions

Standard stock solutions of C16:0, C18:0 and C18:1 ceramides and all DAG standards were prepared in methanol in a concentration of 5 µg/ml each. C17:0 ceramide was used as an internal standard in a concentration of 50 ng/ml. The solutions were kept in glass vials at 20 °C.

2.3 Cell cultures

HepG2 human hepatocarcinoma cells were purchased from Sigma-Aldrich (St. Louis, MO) (cat. number: 85011430-1VL) and cultured in Minimum Essential Medium Eagle containing Earle's Salts and sodium bicarbonate, supplemented with 2 mM L-glutamine, 1 % non-essential amino acids, 10 % FBS and 1 % antibiotics (Thermo Scientific), at 37 °C in humidified atmosphere, containing 5 % CO_2.

2.4 Cell treatment with fatty acids

Treatment of the cells with albumin-conjugated palmitate was based on our previous studies [31]. Briefly, before treatment, the culture medium was changed for FBS and antibiotic free medium for 1 hour. Palmitate was diluted in isopropanol to a concentration of 50 mM, conjugated with 4.16 mM FA free BSA in 1:4 ratios, on 37 °C for 1 hour. The working solution was always freshly prepared with FBS free and antibiotic free medium, and it contained palmitic acid at a final concentration of 250 µM. Cells were treated for 8 hours at 70-80 % confluence in 6-well plates.

2.5 Sample preparation–Extraction

At 70-80 % confluence, cells were washed once with phosphate buffer (PBS), then harvested in 200 µl PBS by scraping. The samples were then centrifuged in a benchtop centrifuge (10 min, 1000 rpm, 4 °C). The supernatant was removed, and the cells were suspended in 115 µl PBS. 15 µl of each cell suspension was removed for determination of protein concentration, and the rest was centrifuged (10 min, 1000 rpm, 4 °C). The supernatant was removed and the remaining sample was suspended in 200 µl of isopropanol/methanol (50/50 v/v%), which already contained the C17:0 ceramide (50 ng/ml). After this procedure, the samples were immediately frozen at 20 °C and stored at 20 °C until further processing.

Before the analysis, the samples were thawed at room temperature. The samples were sonicated for 15-20 sec using an ultrasonic sonotrode, then centrifuged in a benchtop centrifuge (10 min, 13400 rpm, 24 °C). The supernatants were transferred to vials for immediate liquid chromatography tandem mass spectrometry analysis.

2.6 Measurement of protein concentration

Cells were washed twice with PBS, then harvested in 100 µl lysis buffer by scraping. The lysis buffer contained 0.1 % SDS, 5 mM EDTA, 150 mM NaCl, 50 mM Tris, 1 % Tween 20, 1 mM Na_3VO_4, 1 mM PMSF, 10 mM benzamidine, 20 mM NaF, 1 mM pNPP and protease inhibitor cocktail. The lysates were centrifuged with benchtop centrifuge (10 min, 10000 rpm, 4 °C). Protein concentration of the supernatant was measured with Pierce BCA Protein Kit Assay (Thermo Scientific) according to the manufacturer's recommendation, then stored at 20 °C until use.

2.7 Chromatographic separation

Compounds were separated by high performance liquid chromatography using a Perkin Elmer series 200 high pressure gradient pump, autosampler, online degasser and a thermostat. A Kinetex® 5 µm, C8 100 Å, LC Column, 100 x 3 mm column was used at 0.5 ml/min flow rate with a gradient elution of methanol (mobile phase A) and 10 mM ammonium-acetate (mobile phase B): 0 min at 90 % A; 1 min at 90 % A; 9 min at 95 % A; 10.5 min at 98 % A; 11.5 min at 98 % A; 12 min at 90 % A; 14 min at 90 % A.

2.8 Mass spectrometry

Ceramide and DAG compounds were detected using a triple quadrupole mass spectrometer (Applied Biosystems MDS SCIEX 4000 Q TRAP).

The instrument was used in positive multiple reaction monitoring mode. The source parameters of the mass spectrometer are presented in Table 1. The transitions and retention times for each ceramide and DAG are shown in Table 2.

2.9 Quantification of ceramide and diacylglycerol levels

Ceramides and DAGs were quantified by the ratios of their peak areas and of the C17:0 ceramide. This compound is a non-naturally occurring ceramide, thus it can be used as an internal standard.

3 Results

3.1 Optimization of tandem mass spectrometry

Full scan mass spectra were recorded for each ceramide and DAG standard in positive ion mode during direct infusion. Ceramides gave protonated ion spectrum, however, DAGs showed poor ionization. Thus, we added ammonium-acetate in a concentration of 10 mM to the eluent. This way, the ammonium-adduct of the DAGs became the precursor ion for this compound. Each precursor ion was separated in the first quadrupole (Q1), fragmented in the second quadrupole (Q2), and the product ions were scanned in the third quadrupole (Q3). We found that, as reported before [20, 22, 25, 32], one typical fragment for ceramides is the m/z 264. This is generated with the loss of the acyl group and two molecules of water. (Fig. 3). The other chosen transition was produced with the loss of one molecule of water from the precursor ion.

Fatty acid synthase enzyme yields palmitic acid (16:0) as the major primary intermediate of *de novo* FA synthesis in human cells. Palmitic acid can be elongated by two-carbon units and/or desaturated by enzyme systems in the ER membrane, thus it serves as the precursor of all other endogenous FAs. We aimed to determine the levels of those DAGs that contain the most abundant endogenous FAs of human cells, such as palmitic (16:0), stearic (18:0), palmitoleic (16:1 cis Δ9) and oleic (18:1 cis Δ9) acids in various combinations. In case of DAGs, we decided to use the ammonium-adduct as a precursor ion for each DAG, and the two daughter ions were generated with the loss of the ammonia and one of the FA chain (Fig. 4). For those DAGs, which contained two identical FAs, the other daughter ion was produced from the precursor ion with the loss of the ammonia and one molecule of water. Among the biologically relevant DAGs having two identical FA chains, the

Table 1 Source parameters of mass spectrometer

Global source settings	
Collision gas (CAD)	Medium
Curtain gas (CUR)	30 psi
Nebulizer gas (GSI)	30 psi
Auxiliary gas (GS2)	20 psi
Ion spray voltage (IS)	5500 V
Temperature (TEM)	400 °C
Entrance potential (EP)	10 V

Table 2 Quantitative LC/MS/MS parameters for ceramide and diacylglycerol analysis

Compound	Symbol	Mass transition (m/z)	Retention time (min)
N-palmitoyl-sphingosine	C16:0	538.60–264.30 538.60–520.60	7.2
N-heptadecanoyl-sphingosine	C17:0	552.61–264.30 552.61–534.60	8.0
N-stearoyl-sphingosine	C18:0	566.52–264.30 566.52–548.60	8.8
N-oleoyl-sphingosine	C18:1	564.66–264.30 564.66–546.50	6.5
Dipalmitoyl-glycerol	16:0-16:0	586.50–313.30 586.50–551.50	10.2
Palmitoyl-palmitoleoyl-glycerol	16:0-16:1	584.50–311.30 584.50–313.30	9.1
Palmitoyl-stearoyl-glycerol	16:0-18:0	614.60–313.30 614.60–341.30	11.7
Palmitoyl-oleoyl-glycerol	16:0-18:1	612.50–313.30 612.50–339.30	10.7
Palmitoleoyl-oleoyl-glycerol	16:1-18:1	610.50–311.30 610.50–339.30	9.9
Distearoyl-glycerol	18:0-18:0	642.60–341.30 642.60–607.60	12.9
Stearoyl-oleoyl-glycerol	18:0-18:1	640.60–339.30 640.60–341.30	12.0
Dioleoyl-glycerol	18:1-18:1	638.80–339.30	11.4

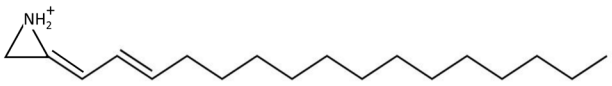

Fig. 3 Typical fragment ion of m/z = 264 of ceramides generated upon positive ESI ionization

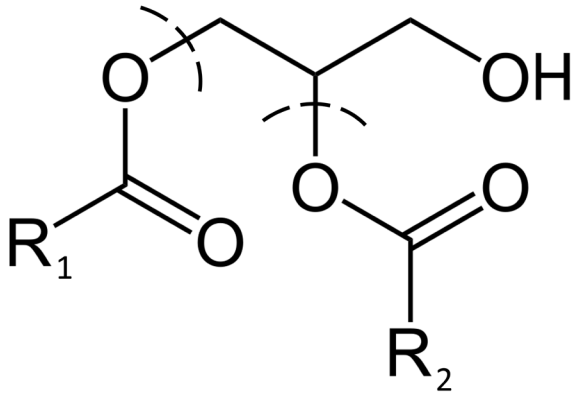

Fig. 4 Fragmentation of diacylglycerols with the loss of one fatty acid chain

second transition of only dioleoyl-glycerol (18:118:1) cannot be used because it yields ions of the same m/z as the ones produced from the 18:018:2 DAG, so in this case, only one transition, the 638.6 → 339.3 was used.

3.2 Liquid chromatography

Fig. 5 shows a typical chromatogram of ceramides and DAGs of a control sample of the cell culture. Although in some cases, there is no baseline separation between the investigated compounds, with the MRM mode we can perform the specific identification of coeluting ceramides and DAGs. By using this gradient method, we were able to separate four ceramides and eight DAGs during a 14 min run.

3.3 Sample preparation

A chloroform/methanol (1:2 v/v) mixture is often used for the extraction of ceramides. However, the loss during sample drying is intolerable when studying cultured cells, due to very small amount of the samples, and carcinogenic properties of chloroform are well-known, and hence, we decided to use chloroform-free methanol for extraction. Therefore, we studied the difference between the following two extraction methods using either pure methanol or a chloroform/methanol mixture. For extraction with methanol only, washed cells were suspended in methanol, sonicated for 15-20 sec, and then centrifuged at 13400 rpm for 10 minutes. The supernatant was directly injected from HPLC vials. For extraction with a chloroform/methanol mixture, 45 µl of chloroform was added to 90 µl of cell

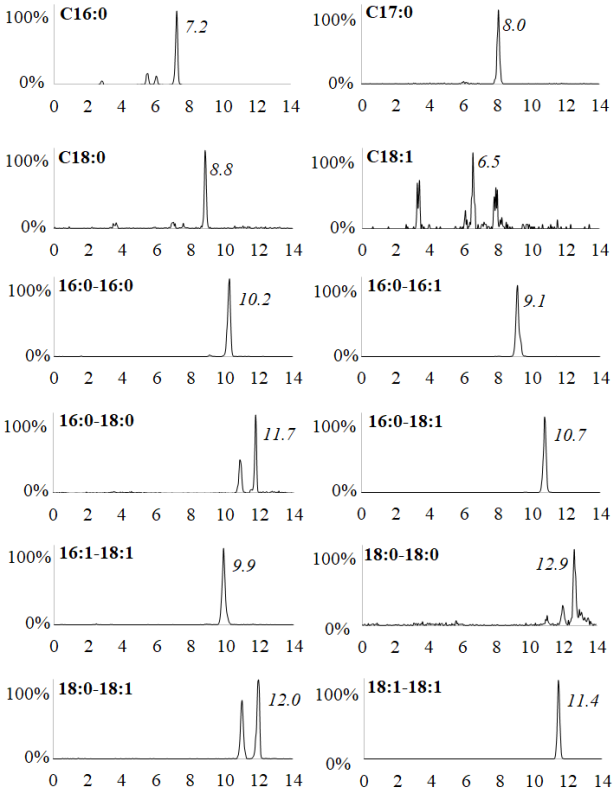

Fig. 5 Typical chromatograms of a control cell sample containing the measured ceramides and diacylglycerols. With the MRM mode, the specific identification of coeluting compounds can be achieved

suspension in methanol. The samples were sonicated and centrifuged as above. The supernatant was then dried and reconstituted in 90 µl of methanol. The solutions were sonicated for 15-20 sec and centrifuged again, and the supernatants were transferred to HPLC vials for measurement. We found no difference between the results obtained with two methods (Fig. 6); therefore, to save time and samples, ceramides and DAGs were extracted with methanol only in the subsequent measurements.

Two different approaches were used to normalize the measured amount of ceramides and DAGs. One was based on the measurement of wet cell mass, i.e. all of the supernatant was removed from the washed cell pellets, and the volume of methanol in which the cells were suspended was proportional to the pellets' weight. The other approach was based on the measurement of protein content, i.e. 15 µl of each cell suspension in PBS was withdrawn before pelleting the cells to determine the protein concentration, and the amount of methanol for suspension of the pellet was constant. The lipid concentrations were normalized to the protein concentration of the same sample. Since the first method did not yield precise and reliable results, all data were normalized to protein contents in the subsequent experiments.

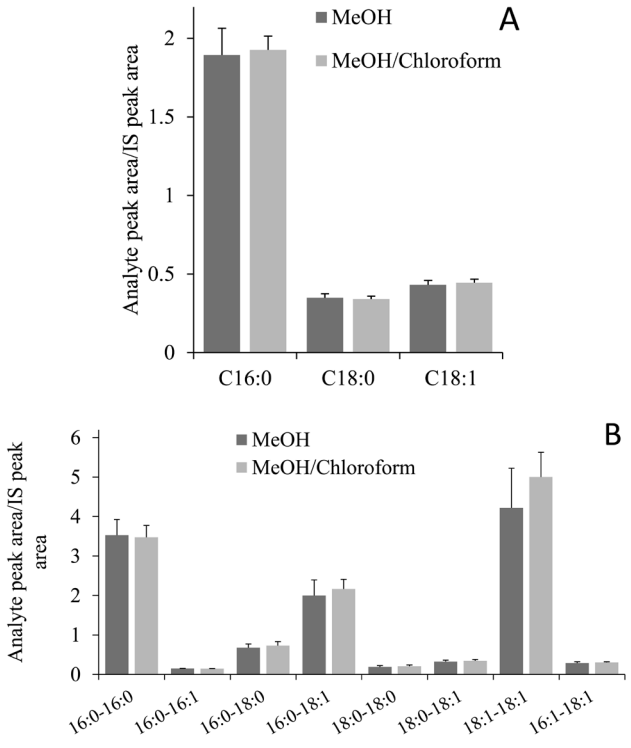

Fig. 6 Comparison of the different extraction methods. MeOH: Washed cells were suspended in methanol, sonicated for 15-20 sec, and then centrifuged at 13400 rpm for 10 minutes. The supernatant was directly injected from HPLC vials. MeOH/chloroform: 45 µl chloroform was added to 90 µl cell suspension in methanol. The samples were sonicated and centrifuged as above. The supernatant was then dried, reconstituted in 90 µl methanol, sonicated, centrifuged, and the supernatants were transferred to vials for HPLC analysis. Relative ceramide (A) and DAG (B) peak areas compared to the internal standard peak area are presented as mean values ± S.D.; n=10.

The percentage of ceramide and DAG recovery was measured by adding known amount of ceramides and DAGs to pooled cell suspension. Recovery was between 79.1 % and 86.4 %.

3.4 Effect of palmitate treatment on ceramide and DAG levels in HepG2 cells

After optimizing the sample preparation steps, we treated the cells with BSA-conjugated palmitate at a concentration of 250 µM for 8 hours, and prepared samples from the collected cells as described.

Our results show an obvious accumulation of several palmitate derivatives. We observed an approximately 5-fold elevation in the level of C16:0 ceramide (0.192 ± 0.026 vs 0.976 ± 0.232 µg/mg protein in control vs palmitate-treated, respectively) and a 5.5-fold elevation in the level of C18:0 ceramide (0.0153 ± 0.004 vs 0.086 ± 0.015 µg/mg protein in control vs palmitate-treated, respectively) (Fig. 7). The C18:0 ceramide contains a stearoyl chain, which is produced in the cells by the elongation of palmitate.

A significant elevation was found in the amount of different DAGs as well (Fig. 8), especially in the dipalmitoyl-glycerol (16:0-16:0) (18.5-fold, 0.71 ± 0.233 vs 13.08 ± 2.914 µg/mg protein in control vs palmitate-treated, respectively) the palmitoyl-palmitoleoyl-glycerol (16:0-16:1) (8-fold, 0.36 ± 0.059 vs 2.96 ± 0.389 µg/mg protein in control and palmitate-treated, respectively) and palmitoyl-stearoyl-glycerol (16:0-18:0) (6.5-fold, 0.12 ± 0.054 vs 0.77 ± 0.214 µg/mg protein in control vs palmitate-treated, respectively) levels.

The ratio of fully saturated DAGs (i.e. containing saturated FAs only) and partly or fully unsaturated ones (i.e. having at least one unsaturated FA chain) changed remarkably, from 0.11 to 0.82 upon palmitate treatment (Fig. 9).

4 Discussion

The role of ceramides and DAGs in a variety of pathological conditions has attracted a growing attention in the last few years. Excessive FA supply, which occurs in high-fat diet and in obesity, causes an elevation of intracellular acyl-CoA levels and thus stimulates several acylation reactions involved in the biosynthesis of complex lipids. Acceleration of sphingosine *N*-acylation produces ceramides, which are known to trigger ER stress, insulin resistance and apoptosis in various cell lines. Enhanced acylations also lead to

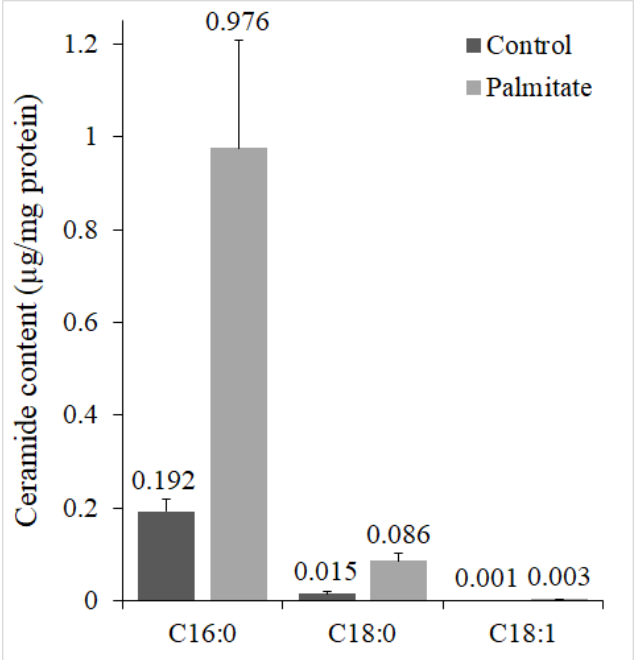

Fig. 7 Effect of palmitate treatment on ceramide levels. Cells were treated with BSA (control) or BSA-conjugated palmitate (250 µM) at 70-80 % confluence for 8 h. Ceramide levels were measured by LC-MS/MS and normalized to 1 mg protein. Data are shown as means ± S.D.; n=10.

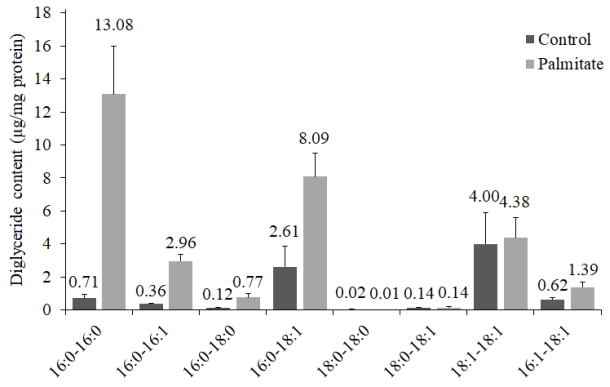

Fig. 8 Effect of palmitate treatment on diacylglycerol levels. Cells were treated with BSA (control) or BSA-conjugated palmitate (250 µM) at 70-80 % confluence for 8 h. Diacylglycerol levels were measured by LC-MS/MS normalized to 1 mg protein. Data are mean values ± S.D.; n=10.

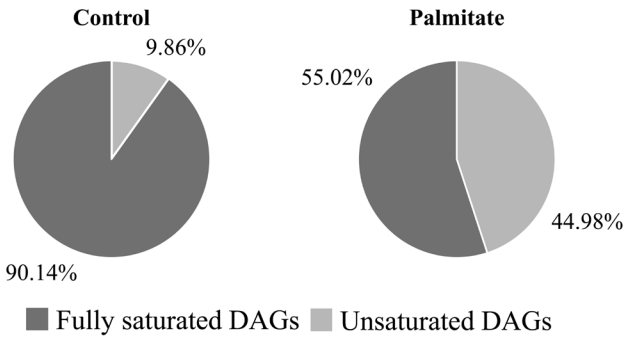

Fig. 9 Alteration in the ratio of fully saturated and at least partly unsaturated diacylglycerols upon palmitate treatment. Cells were treated with BSA (control) or BSA-conjugated palmitate (250 µM) at 70-80 % confluence for 8 h. The amounts of saturated (16:016:0, 16:018:0, 18:0-18:0) and at least partly unsaturated (16:016:1, 16:018:1, 16:118:1, 18:018:1, 18:118:1, 16:1-18:1) DAGs were measured by LC-MS/MS.

the production of triglycerides, and deposition of fat droplets is an obvious sign of lipotoxicity. It has been revealed, however, that obstruction of triglyceride synthesis before the final step, and the consequent accumulation of DAGs is especially deleterious because it activates different members of the protein kinase C (PKC) family, and hence contributes to stress and insulin resistance. Therefore, an increasing number of cellular and *in vivo* studies aim to monitor the intracellular levels of ceramides and DAGs along with alterations in cellular functions and development of characteristic pathological conditions.

Its high selectivity, sensitivity and accuracy make HPLC-ESI-MS/MS technique suitable for efficient and precise quantitative analysis of various cellular compounds of low molecular weight and of similar structures in biological matrices [33]. Here, we demonstrate the development of a reverse-phased HPLC-ESI-MS/MS method for the simultaneous determination of a wide range of ceramides and DAGs in cultured cells. A non-physiological C17:0 ceramide containing a fatty acyl chain of odd number of carbons, which cannot be produced in mammalian cells, was used as an internal standard in our methodology. The lipid species were separated from the impurities by liquid chromatography, and multiple reaction monitoring mode enabled the identification of coeluting components. Regarding the sample preparation, most of the experiments refer to the method reported by Bligh and Dyer [34] which uses a chloroform-methanol mixture to extract and purify lipids from biological samples. However, we demonstrate here, that the use of methanol alone provides the same yield in one step without a time-consuming extraction protocol in case of ceramides and DAGs.

Our experiments on palmitate-treated cultured cells clearly show the accumulation of lipid-intermediates. In case of ceramides, the increasing levels have two explanations. On one hand, the oversupply of palmitoyl-CoA induces the synthesis of sphinganine, which can react with another acyl-CoA in the acylation catalyzed by the ceramide synthase. Elongation of a palmitoyl-CoA yields stearoyl-CoA, which can give rise to the C18:0 ceramide. On the other hand, acylation of sphingosine, an intermediate of sphingolipid breakdown also produces ceramides in the salvage pathway. Manukyan et al. [35] used two different enzyme inhibitors, a serine palmitoyl transferase and a ceramide synthase inhibitor, to determine whether it is the *de novo* synthesis or the salvage pathway that plays the primary role in the accumulation of ceramides during prolonged exposure to FAs. Their results suggest that the *de novo* synthesis and the salvage pathway contributes equally to the increased ceramide levels.

In case of DAGs in palmitate treated cells, we found significantly elevated levels of all examined DAGs except the palmitoleoyl-oleoyl-glycerol and dioleoyl-glycerol. Interestingly, we observed a 17-fold elevation in the total level of DAGs containing two saturated fatty acyl chains. The synthesis of triglycerides follows a genetically determined pattern. Normally, a saturated fatty acyl group occupies the first position of glycerol and an unsaturated fatty acyl group is attached in the second position [36]. This may suggest that the further acylation of the fully saturated DAGs may stall. Moreover, since DAGs are precursors of phospholipids, their structure has direct effects on the composition of the membrane lipids. The increased level of saturation of membrane lipids has been reported to trigger the ER stress [4, 5], which in turn may cause cell death, fibrosis and inflammation.

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