Glycoconj J (2015) 32:615–623
DOI 10.1007/s10719-015-9607-5

ORIGINAL ARTICLE

Hypoxia remodels the composition of the constituent ceramide species of HexCer and Hex2Cer with phytosphingosine and hydroxy fatty acids in human colon cancer LS174T cells

Kouji Tanaka[1] · **Keiko Tamiya-Koizumi**[1] · **Masaki Yamada**[2] · **Takashi Murate**[1] · **Reiji Kannagi**[3] · **Mamoru Kyogashima**[4]

Received: 4 April 2015 / Revised: 9 June 2015 / Accepted: 25 June 2015 / Published online: 22 July 2015

Abstract Oxygen-requiring enzymes, such as Δ4-desaturase (dihydroceramide desaturase), sphingolipid Δ4-desaturase/C-4-hydroxylase, and fatty acid 2-hydroxylase are involved in ceramide synthesis. We prepared free ceramides, sphingomyelins and glycosphingolipids (GSLs) from cancer cells cultivated under conditions of normoxia and hypoxia, and analyzed these compounds using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Human colon cancer LS174T cells were employed because these cells highly express hydroxyl fatty acids and phytosphingosine (t18:0) which are expected to be greatly influenced by changes in oxygen levels. As expected, the populations of dihydro-species of free ceramide and sphingomyelin with C16:0 non-hydroxy fatty acid were elevated, and the populations of HexCers and Hex2Cers, composed of C16:0 or C16:0 hydroxy fatty acid (C16:0h), and sphingosine (d18:1) or t18:0, were decreased under hypoxia. However, appreciable populations of HexCer and Hex2Cer species of C24:0 or C24:0h and t18:0 remained. These results suggest that the individual species of GSLs with fatty acids possessing different alkyl chain lengths, either non-hydroxy fatty acids or hydroxyl fatty acids, may be metabolized individually.

Keywords Ceramides · Glycosphingolipids · Hypoxia · Sphingolipid-species · Phytosphingosine · Hydroxy-fatty acid

Introduction

We have previously investigated the influence of oxygen content on free ceramide (hereinafter, free ceramide is abbreviated as Cer and constitutional ceramide is described as ceramide) and the constituent ceramide species of sphingomyelin and neutral glycosphingolipids (GSLs) from human colon cancer Caco-2 cells using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Unexpectedly, even under normoxia, the species of Cers and sphingomyelins largely consisted of dihydrosphingosine (d18:0) with non-hydroxy fatty acids (NFAs), that is dihydroceramide species (d18:0-NFAs), and all of them were completely converted to d18:0-NFAs under hypoxia. On the contrary, under normoxia, the major species of GSLs were composed of sphingosine (d18:1) with hydroxy fatty acids (HFAs), that is d18:1-HFAs. When the cells were cultured under hypoxia, the major species of GSLs altered to be d18:0-NFAs, though appreciable amount of d18:1-HFAs were conserved. Thus, the constituent ceramide species of the GSLs were distinctly different from those of the Cers and sphingomyelins in Caco2 cells, and they changed individually according to changes in oxygen levels. Usually intestinal cells abundantly express HFAs and/or phytosphingosine (t18:0) [1, 2]. However, Caco2-cells do not express t18:0 in their constitutional ceramide of GSLs; therefore, we employed another human colon cancer cell line LS174T, which expresses large amounts of t18:0 and HFAs, to investigate the influence of hypoxia on the constituent ceramide composition of the GSLs with t18:0.

✉ Mamoru Kyogashima
mkyogashi@nichiyaku.ac.jp

[1] Department of Medical Technology, Nagoya University Graduate School of Health Sciences, Daiko-Minami 1-1-20, Higashi-ku, Nagoya 461-8673, Japan

[2] Shimadzu Corporation, Nishinokyo-Kuwabaracho 1, Nakagyo-ku, Kyoto 604-8511, Japan

[3] Research Complex for the Medicine Frontiers, Aichi Medical University, Yazako, Nagakute, Aichi 480-1195, Japan

[4] Department of Microbiology and Molecular Cell Biology, Nihon Pharmaceutical University, Komuro 10281, Inamachi, Saitama 362-0806, Japan

Materials and methods

Cells and hypoxic culture

LS174T cells were from Tohoku University. Cells were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10 % fetal calf serum at 37 °C with CO_2 (5 %) and O_2 (20 %) for 3 days (normoxia conditions). For hypoxia experiment, 1 day after being cultured under normoxia conditions, the cells were transferred to conditions of 37 °C with CO_2 (5 %) and O_2 (0.5 %) in a multi-gas incubator (Juji Field, Inc., Tokyo, Japan) for 3 days.

Preparations of Cers, sphingomyelins and GSLs

Sphingolipids were purified from 5×10^7 cells as previously described [3, 4]. Briefly, lipids were extracted with a chloroform–methanol mixture. Using Florisil (Wako, Osaka, Japan) column chromatography, GSLs in Folch lower phase were completely separated by acetylation/deacetylation method from phospholipids such as alkali resistant ether glycerophospholipids and sphingomyelin [5]. Cers and sphingomyelins were prepared by preparative thin-layer chromatography (TLC).

MALDI-TOF MS, MS/MS

Mass spectrometry was performed as previously described [3, 4]. Lipids were prepared as 10–350 pmol/μl solutions in chloroform:methanol (2:1). One microliter each of matrix (10 mg/ml 2,5-dihydroxybenzoic acid in chloroform:methanol (2:1) with or without 0.5 % trifluoroacetic acid and lipid solution were mixed vigorously and 1 μl of the resultant solution was applied onto the surface of a stainless steel MALDI-TOF plate. Analyses were performed in positive ion mode using an AXIMA-Performance mass spectrometer (Shimadzu/Kratos, UK) equipped with a nitrogen UV laser (337 nm). The instrument was operated at an acceleration voltage of 20 keV and a pulsed extraction function to improve mass resolution was carefully applied to the *m/z* range 400–1600, based on the sizes of the target molecules. The TOF analyzer was calibrated using the following external calibrants: a dimer of a-cyano-4-hydroxycinnamic acid ($[2\ M + H]^+$; 379.09), human angiotensin II ($[M + H]^+$; 1046.54), and ACTH 8–39 ($[M + H]^+$; 2465.20). Helium gas was used for high-energy CID (20 keV) fragmentation for MS/MS analysis. All mass spectrometric data were acquired and analyzed using MALDI-MS software (Shimadzu/Kratos, UK). The compositions of molecular species from each class of lipid were calculated from the peak areas obtained from spectra as previously described [3, 4].

TLC solvents

The solvent mixture used for sphingomyelins or GSLs was chloroform:methanol:H_2O (65:25:4 by volume) and the solvent mixture for Cers was chloroform:methanol:acetic acid (95:1:4). Cers including d18:1-NFAs, t18:0-NFAs, d18:1-HFAs, and t18:0-HFAs were separated completely by the solvent [6].

Reverse transcription polymerase chain reaction (RT-PCR)

RNA was isolated using an RNeasy Mini kit (QIAGEN, Hilden, Germany), and first strand cDNA was prepared with 5 μg of RNA using a Super Script III First-Strand system (Invitrogen). Semi-quantitative RT-PCR (sqRT-PCR) was performed with Ampli Taq Gold 360 Master Mix (Applied Biosystems, Foster City, CA). The primers used are shown in Table 1.

Results and discussion

Linear mode and MS/MS analyses of Cers and sphingomyelins

Figure 1 shows the linear mode mass spectra of Cers (left column) and sphingomyelins (right column) from LS174T cells cultured under normoxia (upper row), and hypoxia (lower row). We have previously reported that in MALDI-TOF MS analysis, the major molecular ions of typical Cers of d18:1-FAs were detected as $[M + H - H_2O]^+$ and $[M + Na]^+$, and ions of dihydro-Cers of d18:0-FAs were detected as $[M + H]^+$ and $[M + Na]^+$, because of the high-sensitivity to dehydration of the trans-double bond in the long-chain base (LCB) [4]. Under normoxia, $[M + H - H_2O]^+$ ions at *m/z* 520.7, 630.8 and 632.8 and $[M + Na]^+$ ions at *m/z* 560.7, 648.8 and 650.8,

Table 1 Primer sequence for semi-quantitative RT-PCR

Gene	Forward	Reverse
G3PDH	AAGGTCATCCATGACAAC	CACCCTGTTGCTGTAGCCA
GLUT1	TCATCGTGGCTGAACTCTTCAG	TCACACTTGGGAATCAGCCCC
DES1	CCATGGGGAGCCGCGTCTCGCG	TGAAAGCGGTACAGAAGAACCAGC
DES2	ACACCCGCCCGCAAGCTGCTCTG	TGCCAGCCTGTACACCCGCTTC
FA2H	TGGAGAACGAGCCTGTAGC	AACAGGAAGCGGTGGATG

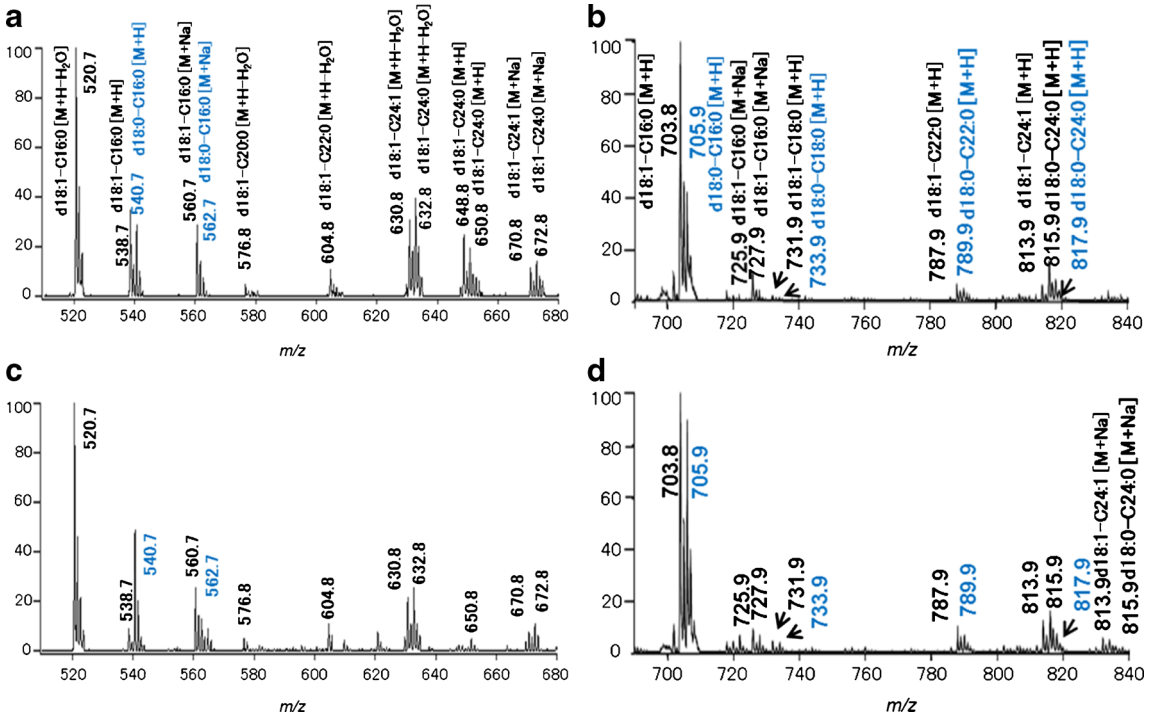


Fig. 1 Linear mode mass spectra of Cers (**a**) and (**c**), and sphingomyelins (**b**) and (**d**), under normoxia (**a**) and (**b**), and hypoxia (**c**) and (**d**). *Black and blue letters* indicate ions from the species with d18:1-NFA and d18:0-NFA, respectively

corresponding to Cers of d18:1-C16:0, d18:1-C24:1 and d18:1-C24:0 were detected as major ions (Fig. 1a). Accordingly major $[M + H]^+$ ion at *m/z* 703.8, and small ions at *m/z* 813.9 and 815.9 corresponding to sphingomyelins of d18:1-C16:0, d18:1-C24:1 and d18:1-C24:0 were detected (Fig. 1b). In addition, $[M + H]^+$ ion corresponding to dihydroceramides of d18:0-C16:0 was detected at *m/z* 540.7 (Fig. 1a) and ions corresponding to dihydro-sphingomyelins, d18:0-C16:0, d18:0-C22:0 and d18:0-C24:0 were detected at *m/z* 705.9, 789.9 and 817.9 (Fig. 1b). Under hypoxia, ions at *m/z* 540.7 (d18:0-C16:0) (Fig. 1c), and at *m/z* 705.9 (dihydro-sphingomyelin d18:0-C16:0) were more prominently detected (Fig. 1d). These findings agreed with the previous reports of tumor cells containing appreciable populations of dihydro-species of Cers and sphingomyelins [7–9] and their composition were elevated under hypoxia [3, 10].

Linear mode and MS/MS analyses of HexCers and Hex2Cers

Figure 2a shows the linear mode mass spectra of HexCers and Hex2Cers from the LS174T cells cultured under normoxia. The ions detected were all sodiated. Major detected ions corresponding to HexCers were at *m/z* 722.8, (d18:1-C16:0), 724.9 (d18:0-C16:0), 738.9 (d18:1-C16:0h), 740.9 (t18:0-C16:0h), 754.8 (t18:0-C16:1h), 756.9 (t18:0-C16:0h), 824.9 (t18:0-C22:0), 840.9 (t18:0-C22:0h), 850.9 (d18:1-C24:0h), 852.9 (t18:0-C24:0), 866.9 (t18:0-C24:1h), 868.9 (t18:0-C24:0h) and 882.9 (t18:0-C25:0h), whereas those corresponding to Hex2Cers were at *m/z* 884.9, (d18:1-C16:0), 898.9 (d18:1-C16:1h), 918.9 (t18:0-C16:0h), 1002.9 (t18:0-C22:0h), and 1030.9 (t18:0-C24:0h). The profile of HexCer from LS174T is more complicated than that from Caco2 cells, which consisted mainly of d18:1-C16:0h, d18:1-C24:1h and d18:0-C24:0h. Figure 2b shows the linear mode mass spectra of HexCers and Hex2Cers from the LS174T cells cultured under hypoxia. Compared with normoxia, the ions at *m/z* 724.8 (d18:0-C16:0) almost disappeared, although the corresponding ions found in the species of Cer and sphingomyelin were increased (Fig. 1c, d). In addition, the ions at *m/z* 740.9 (t18:0-C16:0), 754.8 (t18:1-C16:1h) and 756.9 (t18:1-C16:0h) were markedly decreased. Conversely the ions at *m/z* 866.9 (t18:0-C24:1h) and 868.9 (t18:0-C24:0h) were less prominent compared with those observed under normoxia, but appreciable levels of the ions were still detected. Furthermore, the ions observed at *m/z* 850.9 (d18:1-C24:0h) and 852.9 (t18:0-C24:0) under normoxia remained under hypoxia. Similarly, the ions at *m/z* 884.9 (d18:1-C16:0) and 918.9 (t18:0-C16:0h) were decreased, but those at 1002.9 (t18:0-C22:0h) and 1030.9 (t18:0-C24:0h) remained relatively unchanged in Hex2Cers.

To confirm the molecular species, MS/MS analyses were performed. MS/MS analysis of the ion at *m/z* 738.9 from HexCer under normoxia shows characteristic ions at *m/z* 484.0, b2(d18:1) + H + Na derived from the constituent ceramide of d18:1 with HFA, and at *m/z* 319.8 from the ceramide of C16:0h [4] (Fig. 3a). Another LCB derived ion at *m/z* 556.6 from t18:0-C16:0 was also detected from the ion at *m/z* 740.9,

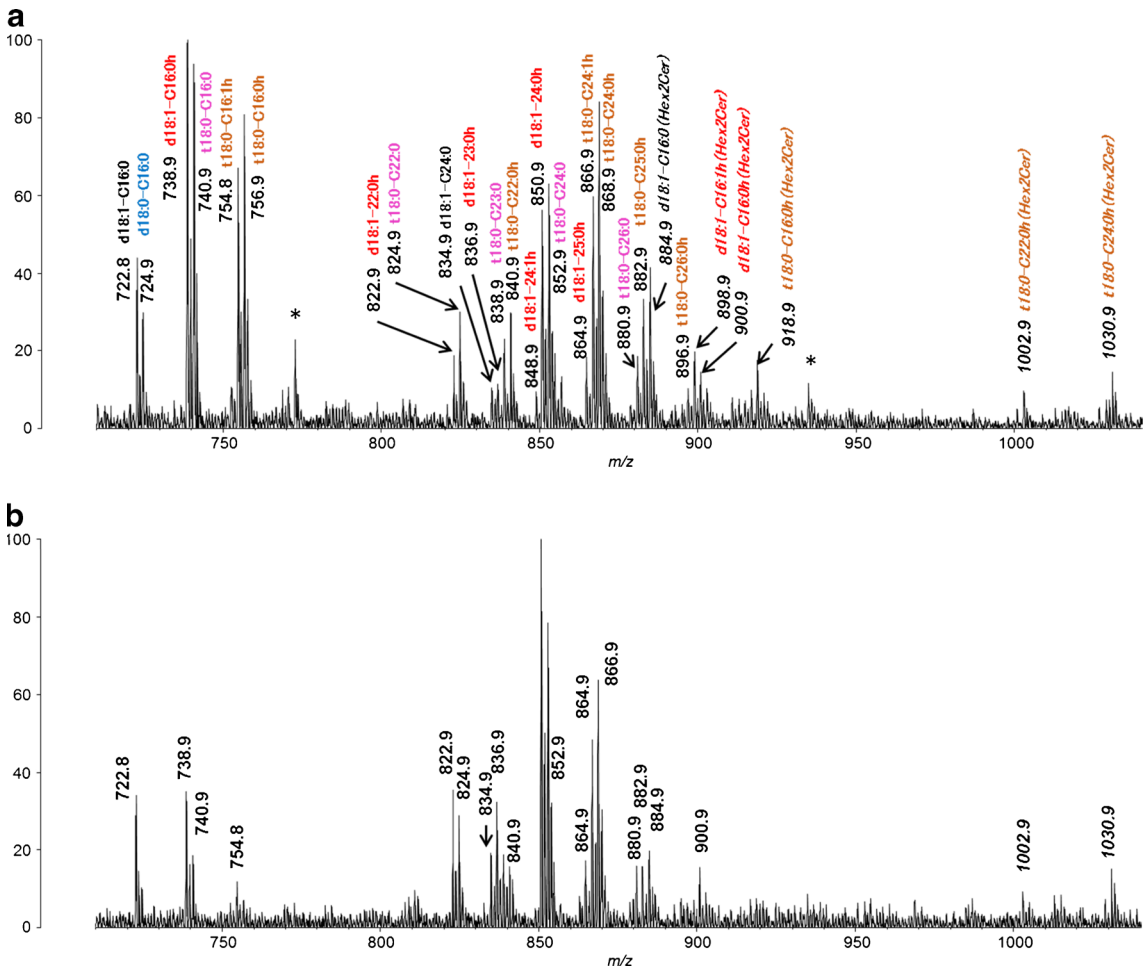


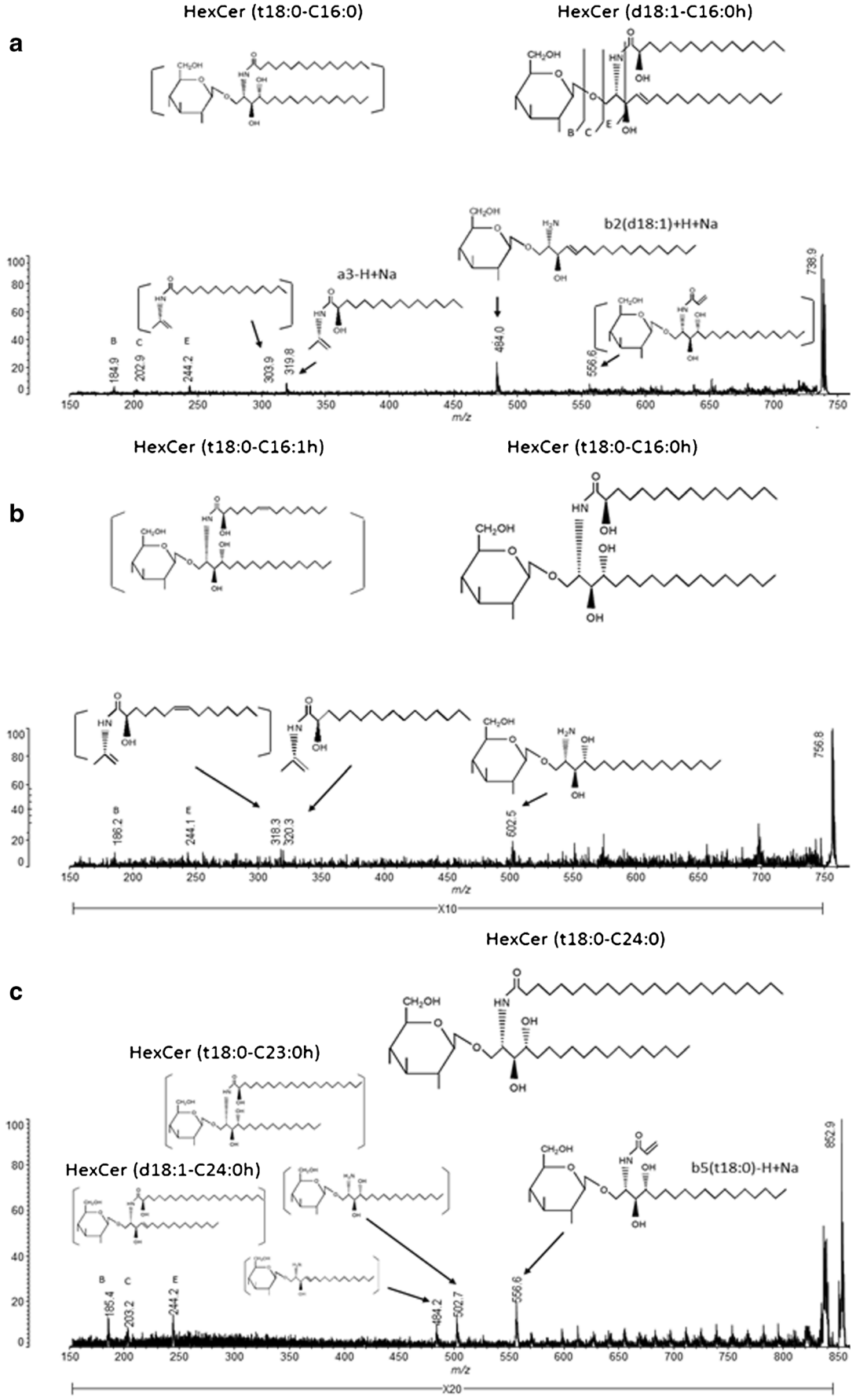
Fig. 2 Linear mode mass spectra of HexCers and Hex2Cer under normoxia (**a**) and hypoxia (**b**). *Black* (d18:1-NFA), *blue* (d18:0-NFA), *red* (d18:1-HFA), *pink* (t18:0-NFA), *brown* (t18:0-HFA). *Italic letters* indicate Hex2Cer species

which was leaked from the MS/MS gate of 738.9. Thus, the ions at *m/z* 738.9 and 740.9 from HexCers under normoxia were identified as HexCer (d18:1-C16:0h) and HexCer (t18:0-C16:0), respectively. Likewise ions at *m/z* 754.8 and 756.9 from the HexCers under normoxia, and ions at *m/z* 850.9, 852.9 and 854.9 from HexCers under hypoxia were identified as HexCer (t18:0-C16:1h) and HexCer (t18:0-C16:0h) and HexCer (d18:1-C24:0h), HexCer (t18:0-C24:0) and HexCer (t18:0-C23:0h), respectively, because the LCB-derived ions at *m/z* 502.5, and 484.2 and 556.6 were observed (see Fig. 3b, c and legends).

Compositional comparison of ceramides species of Cers, sphingomyelins and HexCers and Hex2Cers between normoxia and hypoxia

For a comprehensive understanding of the metabolism of sphingolipids, we believe the importance of overviewing constituent ceramide species of GSLs together with those of Cers and sphingomyelins, though this task is laborious and some technical limitation remain. One of the hurdle is that no chemically defined molecular standards, exist to quantify individual GSL species even though they belong to the same class of GSLs, such as HexCer and Hex2Cer. It is well known the huge diversity of GSLs in terms of not only sugar portions

Fig. 3 Fragmentation scheme (*upper*) and MS/MS profile of the ion at *m/z* 738.9 (**a**), 756.8 (**b**), and 852.9 from HexCer species. The ion designations for ceramide and carbohydrate were asigned as previously described [4, 11]. In (**a**), the LCB derived ion at *m/z* 484.0 and FA derived ion at *m/z* 319.8 indicate molecular ion at *m/z* 738.9 is HexCer (d18:1-C16:0 h). The LCB ion at *m/z* 556.6 and FA ion at *m/z* 303.8 derived from an ion leaked from the selected ion gate of 738.9 indicate the molecular ion 736.9 is from HexCer (d18:1-C16:0h) illustrated in *brackets*. In (**b**), the LCB ion at *m/z* 602.5 and FA ion at *m/z* 320.3 indicate the molecular ion at *m/z* 756.8 is HexCer (t18:0-C16:0h). The FA ion at *m/z* 318.3 from an ion leaked from the selected ion gate of 756.8 indicate the molecular ion at *m/z* 754.8 is from HexCer (t18:0-C16:1h) illustrated in *brackets*. In (**c**), LCB ion at *m/z* 556.6 indicates molecular ion at *m/z* 852.9 is from HexCer (t18:0-C24:0). The LCB ions at *m/z* 484.2 and 502.7 from ions leaked from the selected ion gate of 852.9 indicate that both ions at *m/z* 850.9 and 854.9 are from HexCer (d18:1-C24:0h) and HexCer (t18:0-C23:0 h), respectively, illustrated in *brackets*. It is difficult to detect longer fatty acid-derived ions [4]


a
HexCer (t18:0-C16:0)
HexCer (d18:1-C16:0h)
b2(d18:1)+H+Na
a3-H+Na
184.9
202.9
244.2
303.9
319.8
484.0
556.6
738.9
m/z
b
HexCer (t18:0-C16:1h)
HexCer (t18:0-C16:0h)
186.2
244.1
318.3
320.3
602.5
756.8
m/z
X10
c
HexCer (t18:0-C24:0)
HexCer (t18:0-C23:0h)
HexCer (d18:1-C24:0h)
b5(t18:0)-H+Na
185.4
203.2
244.2
484.2
502.7
556.6
852.9
m/z
X20

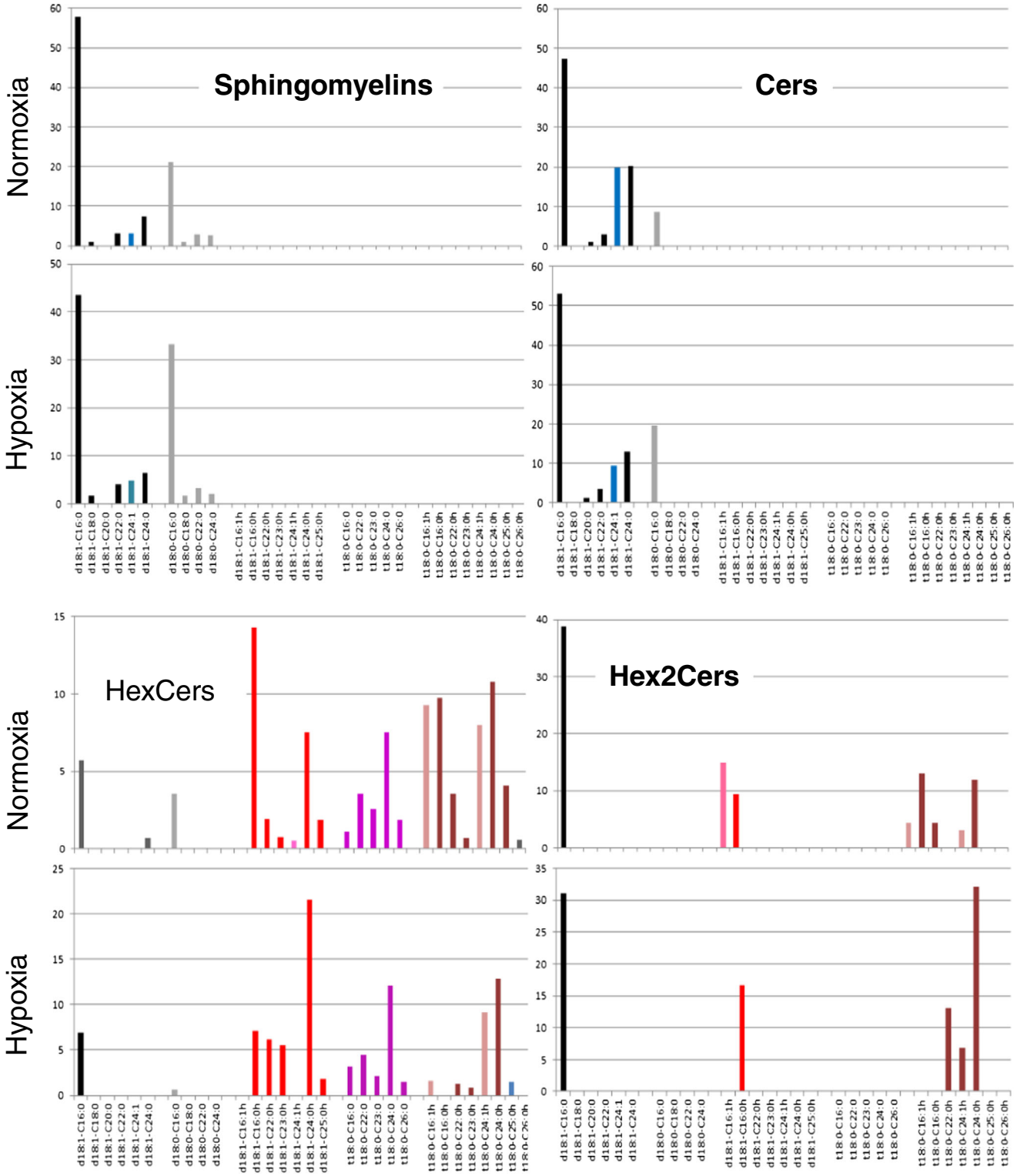


Fig. 4 The ceramide compositions of the sphingomyelins and Cers (*upper*), and HexCers and Hex2Cers (*lower*), under normoxia and hypoxia. Compositions were calculated based on the peak areas of the linear mass spectra

but also of the constituent ceramide species, composed of different lengths of FAs and LCBs; either or both of which are occasionally hydroxylated and with the presence or absence of unsaturation. Therefore, we provisionally compared the relative ceramide compositions of Cers, sphingomyelins, HexCers, and Hex2Cers, calculated based on the peak areas of the linear mass profiles (Fig. 4) for a quick overview of the sphingolipid metabolism [3, 4].

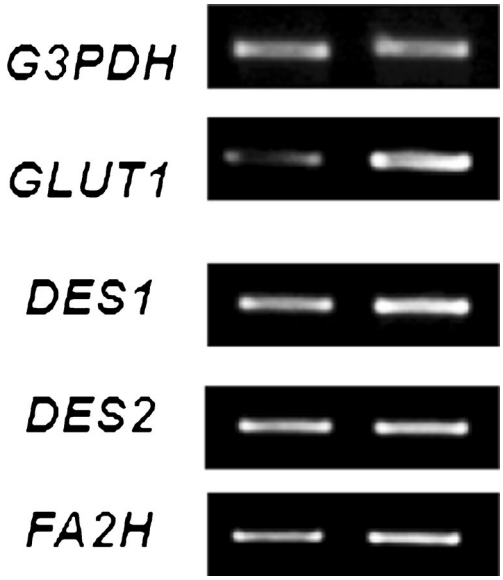


Fig. 5 mRNA levels of Glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*), *GLUT1*, *DES1*, *DES2*, *FA2H* examined using semi-quantitative RT-PCR in normoxia (*left*) and hypoxia (*right*)

As expected, both the major Cers and constituent ceramide of sphingomyelins were the usual species of d18:1-C16:0, d18:1-C24:1 and d18:1-C24:0 with substantial populations of dihydroceramide species which increase under hypoxia. Elevated populations of dihydro-species were previously reported in many cancer cells [7–9]. On the contrary, major components of the constituent ceramide of HexCers were d18:1-HFAs and t18:0-NFAs/HFAs, with a small population of the species d18:1-C16:0, under normoxia. In addition, species possessing odd number FAs such as C23:0, C23:0h and C25:0h were detected though these populations were small. These results are probably obtained because LS174T cells are derived from the intestine, not because they are cancer cell as mammalian intestinal epithelial cells abundantly express GSLs possessing t18:0-HFAs [1, 2]. However, under hypoxia, we observed unexpected outcomes. The populations of HexCers with d18:1-C16:0h, t18:0-C16:1h and t18:0-C16:0h were markedly decreased, which was expected, however, appreciable populations of HexCers with d18:1-C24:0h, t18:0-C24:0, t18:0-C24:1h and t18:0-C24:0h remained. The species of dihydro-GSLs (d18:0-NFAs), which is a major molecular species of GSLs observed from the Caco2 cells under hypoxia [3], were hardly detected in LS174T cells under hypoxia. Confirming that the LS174T cells were cultured under the intended hypoxic condition, we observed a marked elevation of mRNA *GLUT1* levels whose product is the glucose transporter well known to be induced by hypoxia [12] (Fig. 5). The mRNAs *DES1*, *DES2* and *FA2H*, that encode Δ4-desaturase [13, 14], sphingolipid Δ4-desaturase/C-4-hydroxylase [15, 16], and fatty acid 2-hydroxylase [17, 18] respectively, were slightly elevated, but the translated proteins were probably not active enough because they require oxygen. Furthermore, under a hypoxic condition, the species containing C24:1 or C24:1h were relatively sustained in Cer, sphingomyelin, HexCer and Hex2Cer, whereas the species containing C16:1h rapidly decreased in HexCer and Hex2Cer. These results suggest that C16 species including C16:0, C16:1, C16:0h and C16:1h in GSLs may be more rapidly metabolized than C24 species GSLs. This trend

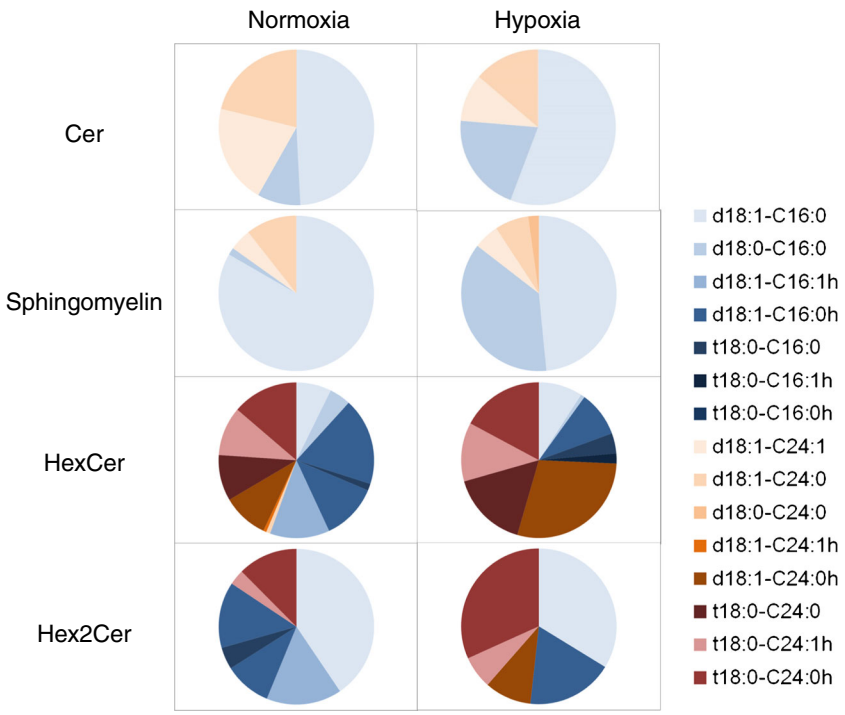


Fig. 6 Compositional comparison of the species with C16 fatty acids (*blue series*) and C24 fatty acids (*brown series*) between normoxia and hypoxia

is more obvious in HexCers than in Hex2Cers. HexCers may consist of both glucosylceramide (GlcCer) and galactosylceramide (GalCer) that contain different constitutional Cers [4, 9, 19], reflecting this result. Such a compositional decrease was not seen in the C16 species of Cers and sphingomyelins. Before MS analysis, we screened for the occurrence of Cers, with t18:0 and/or HFAs using TLC. However, no substantial amount of d18:1-HFAs and t18:0-NFAs/HFAs were detected in any condition investigated, suggesting that Cers of d18:1-HFAs and t18:0-NFAs/HFAs were immediately converted into GSLs. This is consistent with previous result for Caco2 cells, though they lack t18:0 as a LCB [3]. A comparison of Cer, sphingomyelin, HexCer, and Hex2Cer which is probably lactosylceramide, species under normoxia and hypoxia is summarized in Fig. 6.

Ceramide synthase (CerS) 5 and CerS6 synthesize species with C16, and CerS2 synthesizes species with C24 [20, 21]. However, these enzymes may not be consequence in this experiment because they are not oxygen-requiring enzymes. Ceramide transfer protein (CERT) transport Cer from the endoplasmic reticulum (ER) to the Golgi apparatus in a non-vesicular manner [22], and sphingomyelin is synthesized by sphingomyelin synthase (SMS) 1, which transfers phosphocholine from phosphatidylcholine to Cers in the luminal side of the Golgi. Sphingomyelin is also synthesized by SMS2 localized in the plasma membrane and Golgi [23]. On the other hand, Cers for GlcCer synthesis are suggested to be transported from the ER to the Golgi in a vesicular-dependent manner [24], and GlcCer is synthesized on the cytosolic phase of the Golgi by UDP-glucose:ceramide glucosyltransferase [25], whereas GalCer is synthesized at the lumen of the ER by UDP-galactose:ceramide galactosyltransferase [26]. GlcCer is further transported to the Golgi either in a vesicular or non-vesicular manner [27]. These traffic and/or synthesis characteristics probably contribute to the distinct constitution of the ceramide-species of individual classes of sphingolipids and GSLs [28]. In addition, the individual species of GSLs with different length of FAs, regardless of either NFAs or HFAs, and/or saturated FAs or unsaturated FAs, may be differently metabolized in a manner dependent on the particular lengths of the FAs, when the cells are moved from normoxic to hypoxic conditions. Together with our previous report [3], we have demonstrated complicated remodeling of Cers and the ceramide of sphingomyelins and GSLs in human colon cancer cells, according to oxygen levels. The altered Cers and sphingomyelins composed of dihydro-species, comprising a lipid microdomain with GSLs, may modulate sphingolipid signaling [29]. The glycan sequence of GSLs expressed on the cell surface serve as cancer antigens [30] and for microbe recognitions [31]. It is evident that glycan expression efficiencies of GSLs are crucially influenced by their constituent ceramides [32–34]. Therefore, remodeling of the constituent ceramides of sphingolipids by hypoxia may alter cell behavior, and also may present the possibility for new therapeutic approaches based on sphingolipid biology.

References

1. Nishimura, K.: Phytosphingosine is a characteristic component of the glycolipids in the vertebrate intestine. Comp. Biochem. Physiol. B **86**, 149–154 (1987)
2. Yoneshige, A., Sasaki, A., Miyazaki, M., Kojima, N., Suzuki, A., Matsuda, J.: Developmental changes in glycolipids and synchronized expression of nutrient transporters in the mouse small intestine. J. Nutr. Biochem. **21**, 214–226 (2010)
3. Tanaka, K., Tamiya-Koizumi, K., Yamada, M., Murate, T., Kannagi, R., Kyogashima, M.: Individual profiles of free ceramide species and the constituent ceramide species of sphingomyelin and neutral glycosphingolipid and their alteration according to the sequential changes of environmental oxygen content in human colorectal cancer Caco-2 cells. Glycoconj. J. **31**, 209–219 (2014)
4. Tanaka, K., Yamada, M., Tamiya-Koizumi, K., Kannagi, R., Aoyama, T., Hara, A., Kyogashima, M.: Systematic analyses of free ceramide species and ceramide species comprising neutral glycosphingolipids by MALDI-TOF MS with high-energy CID. Glycoconj. J. **28**, 67–87 (2011)
5. Saito, T., Hakomori, S.I.: Quantitative isolation of total glycosphingolipids from animal cells. J. Lipid Res. **12**, 257–259 (1971)
6. Kyogashima, M., Tadano-Aritomi, K., Aoyama, T., Yusa, A., Goto, Y., Tamiya-Koizumi, K., Ito, H., Murate, T., Kannagi, R., Hara, A.: Chemical and apoptotic properties of hydroxy-ceramides containing long-chain bases with unusual alkyl chain lengths. J. Biochem. **144**, 95–106 (2008)
7. Rylova, S.N., Somova, O.G., Zubova, E.S., Dudnik, L.B., Kogtev, L.S., Kozlov, A.M., Alesenko, A.V., Dyatlovitskaya, E.V.: Content and structure of ceramide and sphingomyelin and sphingomyelinase activity in mouse hepatoma-22. Biochemistry (Mosc) **64**, 437–441 (1999)
8. Dyatlovitskaya, E.V., Kandyba, A.G., Kozlov, A.M., Somova, O.G.: Sphinganine in Sphingomyelins of tumors and mouse regenerating liver. Biochemistry (Mosc) **66**, 502–504 (2001)
9. Kyogashima, M., Taketomi, T.: Lipids from human platelets in primary thrombocythemia. Jpn. J. Exp. Med. **56**, 113–118 (1986)
10. Devlin, C.M., Lahm, T., Hubbard, W.C., Van Demark, M., Wang, K.C., Wu, X., Bielawska, A., Obeid, L.M., Ivan, M., Petrache, I.: Dihydroceramide-based response to hypoxia. J. Biol. Chem. **286**, 38069–38078 (2011)
11. Domonand, B., Costello, C.E.: A systematic nomenclature for carbohydrate fragmentations in FAB-MS/MS spectra of glycoconjugates. Glycoconj. J. **5**, 397–409 (1988)
12. Airley, R.E., Mobasheri, A.: Hypoxic regulation of glucose transport, anaerobic metabolism and angiogenesis in cancer: novel pathways and targets for anticancer therapeutics. Chemotherapy **53**, 233–256 (2007)
13. Michel, C., van Echten-Deckert, G., Rother, J., Sandhoff, K., Wang, E., Merrill Jr., A.H.: Characterization of ceramide synthesis. A dihydroceramide desaturase introduces the 4,5-trans-double bond of sphingosine at the level of dihydroceramide. J. Biol. Chem. **272**, 22432–22437 (1997)
14. Ternes, P., Franke, S., Zahringer, U., Sperling, P., Heinz, E.: Identification and characterization of a sphingolipid delta4-desaturase family. J. Biol. Chem. **277**, 25512–25518 (2002)

15. Mizutani, Y., Kihara, A., Igarashi, Y.: Identification of the human sphingolipid C4-hydroxylase, hDES2, and its up-regulation during keratinocyte differentiation. FEBS Lett. **563**, 93–97 (2004)
16. Enomoto, A., Omae, F., Miyazaki, M., Kozutsumi, Y., Yubisui, T., Suzuki, A.: Dihydroceramide:sphinganine C-4-hydroxylation requires Des2 hydroxylase and the membrane form of cytochrome b5. Biochem. J. **397**, 289–295 (2006)
17. Alderson, N.L., Rembiesa, B.M., Walla, M.D., Bielawska, A., Bielawski, J., Hama, H.: The human FA2H gene encodes a fatty acid 2-hydroxylase. J. Biol. Chem. **279**, 48562–48568 (2004)
18. Kota, V., Hama, H.: 2′-Hydroxy ceramide in membrane homeostasis and cell signaling. Adv. Biol. Regul. **54**, 223–230 (2014)
19. Kyogashima, M., Tamiya-Koizumi, K., Ehara, T., Li, G., Hu, R., Hara, A., Aoyama, T., Kannagi, R.: Rapid demonstration of diversity of sulfatide molecular species from biological materials by MALDI-TOF MS. Glycobiology **16**, 719–728 (2006)
20. Tidhar, R., Futerman, A.H.: The complexity of sphingolipid biosynthesis in the endoplasmic reticulum. Biochim. Biophys. Acta **1833**, 2511–2518 (2013)
21. Mizutani, Y., Kihara, A., Chiba, H., Tojo, H., Igarashi, Y.: 2-Hydroxy-ceramide synthesis by ceramide synthase family: enzymatic basis for the preference of FA chain length. J. Lipid Res. **49**, 2356–2364 (2008)
22. Hanada, K.: Intracellular trafficking of ceramide by ceramide transfer protein. Proc. Jpn. Acad. Ser. B Phys. Biol. Sci. **86**, 426–437 (2010)
23. Taniguchi, M., Okazaki, T.: The role of sphingomyelin and sphingomyelin synthases in cell death, proliferation and migration-from cell and animal models to human disorders. Biochim. Biophys. Acta **1841**, 692–703 (2014)
24. Giussani, P., Colleoni, T., Brioschi, L., Bassi, R., Hanada, K., Tettamanti, G., Riboni, L., Viani, P.: Ceramide traffic in C6 glioma cells: evidence for CERT-dependent and independent transport from ER to the Golgi apparatus. Biochim. Biophys. Acta **1781**, 40–51 (2008)
25. Ishibashi, Y., Kohyama-Koganeya, A., Hirabayashi, Y.: New insights on glucosylated lipids: metabolism and functions. Biochim. Biophys. Acta **1831**, 1475–1485 (2013)
26. Honke, K.: UDP-Gal: ceramide galactosyltransferase (UGT8). In: Taniguchi, N., Honke, K., Fukuda, M., et al. (eds.) Handbook of glycosyltransferases and related genes, pp. 131–140. Springer, Tokyo (2014)
27. D'Angelo, G., Uemura, T., Chuang, C.C., Polishchuk, E., Santoro, M., Ohvo-Rekilä, H., Sato, T., Di Tullio, G., Varriale, A., D'Auria, S., Daniele, T., Capuani, F., Johannes, L., Mattjus, P., Monti, M., Pucci, P., Williams, R.L., Burke, J.E., Platt, F.M., Harada, A., De Matteis, M.A.: Vesicular and non-vesicular transport feed distinct glycosylation pathways in the Golgi. Nature **501**, 116–120 (2013)
28. Yamaji, T., Hanada, K.: Sphingolipid metabolism and interorganellar transport: localization of sphingolipid enzymes and lipid transfer proteins. Traffic **16**, 101–122 (2015)
29. Fabrias, G., Muñoz-Olaya, J., Cingolani, F., Signorelli, P., Casas, J., Gagliostro, V., Ghidoni, R.: Dihydroceramide desaturase and dihydrosphingolipids: debutant players in the sphingolipid arena. Prog. Lipid Res. **51**, 82–94 (2012)
30. Hakomori, S.I.: Structure and function of glycosphingolipids and sphingolipids: recollections and future trends. Biochim. Biophys. Acta **1780**, 325–346 (2008)
31. Karlsson, K.A.: Pathogen-host protein-carbohydrate interactions as the basis of important infections. Adv. Exp. Med. Biol. **491**, 431–443 (2001)
32. Kannagi, R., Stroup, R., Cochran, N.A., Urdal, D.L., Young Jr., W.W., Hakomori, S.: Factors affecting expression of glycolipid tumor antigens: influence of ceramide composition and coexisting glycolipid on the antigenicity of gangliotriaosylceramide in murine lymphoma cells. Cancer Res. **43**, 4997–5005 (1983)
33. Kyogashima, M., Ginsburg, V., Krivan, H.C.: Escherichia coli K99 binds to N-glycolylsialoparagloboside and N-glycolyl-GM3 found in piglet small intestine. Arch. Biochem. Biophys. **270**, 391–397 (1989)
34. Madar Johansson, M., Coddens, A., Benktander, J., Cox, E., Teneberg, S.: Porcine intestinal glycosphingolipids recognized by F6-fimbriated enterotoxigenic Escherichia coli. Microb. Pathog. **76**, 51–60 (2014)