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Glycosphingolipids and oxidative stress: Evaluation of hydroxyl radical oxidation of galactosyl and lactosylceramides using mass spectrometry

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ABSTRACT

Galactosylceramide (GalCer) and lactosylceramide (LacCer) are structural and signaling lipids, playing important roles in signal transduction and cell adhesion. They are especially abundant in the nervous system and in important components of the myelin sheath. Although neurodegenerative disorders are associated with increased oxidative stress and lipid oxidation, the connection between oxidative stress and glycosphingolipid modification has been scarcely addressed.

In this study, we aimed to characterize the structural changes caused by the hydroxyl radical to GalCer and LacCer molecular species using electrospray ionization mass spectrometry (ESI–MS and MS/MS) and high performance liquid chromatography–tandem mass spectrometry (HPLC-MS^n). ESI–MS and LC–MS spectra of 24:1GalCer and 24:1LacCer after free radical oxidation showed the formation of new species, which were identified as keto, hydroxyl and hydroperoxy derivatives, arising from modification in the mono unsaturated fatty acyl chain. Formation of ceramide and oxidized ceramides was also observed as a result of 24:1GalCer and 24:1LacCer radical oxidation. 24:1GlcCer (glucosylceramide) was detected after LacCer oxidation, probably due to oxidative cleavage of lactosyl moiety. This study shows that glycosphingolipids are prone to radical induced oxidation, which can be one of the causes of the increased ceramides content and pro apoptotic events during oxidative conditions and neurodegeneration. This MS study will support the future identification of oxidized galactosyl- and lactosylceramide species in sphingolipidomic studies applied to biological samples related with oxidative conditions.

1. Introduction

Oxidative stress is a major feature underlying several pathologies, including neurodegenerative disorders. It is caused by an imbalance in the redox state of the cell, causing an increase of reactive oxygen species (ROS) production and oxidative damage to biomolecules (Gandhi and Abramov, 2012). Several studies documented an elevation in protein and lipid peroxidation markers in the cortex and hippocampus of Alzheimer's disease patients, prior to senile plaques and neurofibrillary tangles detection (Lovell et al., 1995; Hensley et al., 1995). Despite the evidences supporting the role of oxidative stress in neurodegenerative disorders (Gandhi and Abramov, 2012; Kovacic and Somanathan, 2012), it is unclear if oxidative stress is just an epiphenomenon or a real causative trigger of neural dysfunction (Fang et al., 2012). Antioxidant therapies were largely unsuccessful in neurodegenerative disorders prevention, raising questions about the absolute requirement of oxidative stress in the neurodegenerative processes (Gandhi and Abramov, 2012; Gong et al., 1991; Dumont et al., 2010). In order to investigate the role of oxidative stress in neurodegeneration, efforts have been made to identify specific situations in which ROS action is able to induce

Abbreviations: Cer, ceramide; CID, collision-induced dissociation; CNS, central nervous system; ESI, electrospray ionization; GalCer, galactosylceramide; GSLs, glycosphingolipids; HPLC, high performance liquid chromatography; LacCer, lactosylceramide; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PNS, peripheral nervous system; Q-TOF, quadrupole time-of-flight; RICs, reconstructed ion chromatograms; ROS, reactive oxygen species; TEMPO, (2,2,6,6-tetramethylpiperidin-1-yl)oxyl.

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selective alterations in neuronal signaling cascades. For instance, recent evidences have shown that, upon exposure to hydrogen peroxide, primary cultured hippocampal neurons suffer axonal transport inhibition for several hours, before any sign of axonal degeneration (Fang et al., 2012). Moreover, it was also reported that ROS directly induce sphingomyelin degradation to ceramide via neutral sphingomyelinase activation in human primary oligodendrocytes supporting the involvement of oxidative stress in triggering selective cell damage (Jana and Pahan, 2007). Glycosphingolipids (GSLs) are amphipathic molecules composed of a hydrophilic carbohydrate chain and a hydrophobic ceramide moiety. These class of sphingolipids are major signaling lipids, especially abundant in the nervous system, where they contribute to plasma membrane structure, signal transduction and cell adhesion (Zheng et al., 2006). As GSLs are primarily sorted in lipid rafts in the plasma membrane where they may interact with many signaling molecules (Zheng et al., 2006). Also, GSL are major constituents of central nervous system and myelin and many studies have demonstrated that GSLs are central for brain development, maturation, maintenance and degeneration (reviewed in (Xu et al., 2010; Yu et al., 2009)). Interestingly, neurodegenerative disorders have been related both to oxidative stress and lipid oxidation (Gandhi and Abramov, 2012) and distortions in the cell sphingolipidome (Xu et al., 2010), but no link has ever been found involving glycolipid oxidation. However, we have recently shown that, upon UVA-induced photooxidation, GalCer and LacCer were oxidized to hydroperoxy derivatives and LacCer suffered oxidative cleavage, being converted to GlcCer (Santinha et al., 2014). Few other publications identified oxidative modifications of glucosylceramide (GlcCer) and galactosylceramide (GalCer) induced by gamma radiation and TEMPO ((2,2,6,6-tetramethylpiperidin-1-yl)oxyl), with formation of ceramides and glucuronylceramide, respectively (Santinha et al., 2014; Hsu and Turk, 2004).

Many studies suggest Fenton reaction as a source of hydroxyl radicals in neurodegenerative disorders, such as Parkinson's disease (Zecca et al., 2004) and Multiple Sclerosis (Hametner et al., 2013), due to increased iron deposition in brain cells. Despite the existing reports describing hydroxyl radical intervention in lipid peroxidation (Melo et al., 2012a; Reis et al., 2005a, 2007, 2003, 2004a, 2004b; Megli and Russo, 2008) and protein (Leeuwenburgh et al., 1998) and nucleic acids oxidation (Cooke et al., 2003), there is a lack of studies addressing the study of hydroxyl radical-induced modifications in GSLs. In the present study, the structural changes induced by the Fenton reaction in GalCer and LacCer are reported. GalCer and LacCer were chosen as they are present in signaling domains within the plasma membrane, performing key signaling functions in the nervous system. Additionally, as precursors of other sphingolipids, any alteration in GalCer and LacCer cell levels could induce imbalances affecting many other GSL classes (for review on GSLs synthesis see (Zheng et al., 2006)). Oxidative reactions were monitored by direct analysis using electrospray mass spectrometry (ESI–MS) and liquid electrospray mass spectrometry (LC–MS). The structural details of GSL oxidation products were assessed by tandem mass spectrometry (ESI–MS and LC–MS/MS). This work will allow the identification of the oxidative fingerprint of each GSL oxidation product, supporting their future identification in biological samples from animal models or patients suffering from neurodegenerative disorders, allowing future large-scale sphingolipidomic studies.

2. Materials and methods

2.1. Chemicals

D-galactosyl-ß-1,1′ *N*-palmitoyl-D-erythro-sphingosine (C16 Galactosyl(ß) Ceramide, d18:1/16:0), D-galactosyl-ß-1,1′ *N*-nervonoyl-D-erythro-sphingosine (C24:1 Galactosyl(ß) Ceramide, d18:1/24:1(15Z)) and D-lactosyl-ß1-1′-*N*-nervonoyl-D-erythro-sphingosine (C24:1 Lactosyl(ß) Ceramide, d18:1/24:1) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AI, USA) and used without further purification. Chloroform ($CHCl_3$) (HPLC grade) and methanol (MeOH) (HPLC grade) were used without further purification.

2.2. Glycosphingolipids oxidation by Fenton reaction

To oxidize the glycosphingolipids by the Fenton reaction, GalCer (d18:1/24:1), GalCer (d18:1/16:0), and LacCer (d18:1/24:1) were diluted to a final concentration of 1.00 mM in a mixture of $CHCl_3$/MeOH (1:1). In order to form the lipid vesicles, the solvent was evaporated to dryness. The resulting dry thin lipid film on the wall of the round-bottom flask was hydrated by the addition of ammonium hydrogenocarbonate buffer 5 mM (pH 7.4). Then, samples were vortexed vigorously for 5 min and sonicated for additional 5 min using a bath sonicator (Selecta Ultrasons); these steps were repeated twice. Suspensions were then incubated at 55–60 °C for 30 min (from each 5 min, suspensions were placed one minute in water bath ultrasons followed the one minute in vortex). Then, suspensions were vortexed vigorously for 5 min and sonicated for further 5 min; these steps were repeated twice. Under these experimental conditions, we obtained multilamellar liposomes. The Fenton oxidation of glycosphingolipids was initiated by adding to the lipid suspension $FeCl_2$ to a final concentration of 40 μM, H_2O_2 to a final concentration of 50 mM and EDTA to a final concentration of 40 μM and the mixture was incubated in the dark at 37 °C for up to 3 days. The oxidation products were then identified by ESI–MS, LC–MS and MS/MS. Controls were prepared by replacing H_2O_2, $FeCl_2$ and EDTA with ammonium hydrogenocarbonate buffer.

2.3. Mass spectrometry

The extent of oxidation and the identification of new products formed were monitored by ESI–MS in positive-ion mode, using a Q-TOF2 mass spectrometer (Micromass, Manchester, UK). Samples were introduced through direct infusion and the ESI conditions were as follows: a flow rate of 10 μL min^{-1}, voltage applied to the needle at 3 kV, a cone voltage at 30 V, source temperature of 80 °C and solvation temperature of 150 °C. The resolution was set to about 9000 (FWHM) and mass spectra were acquired for 1 min. Tandem mass spectra were acquired by collision-induced dissociation (CID), using argon as the collision gas (pressure measured as the setting in the collision cell 3.0×10^{-5} Torr). The collision energy used was between 25 and 30 eV. Tandem mass spectra were acquired for 1 min. Data acquisition was carried out with a MassLynx 4.0 data system. Exact mass measurements of all the new oxidation products were performed after lock mass for the ion $[M + Na]^+$ of each non-modified GSL observed in the ESI–MS spectra.

2.4. High performance liquid chromatography–tandem mass spectrometry

The oxidized mixture was separated by HPLC on a HPLC system (Waters Alliance 2690) coupled to a linear ion trap mass spectrometer LXQ (ThermoFinnigan, San Jose, CA, USA). 5 μL of the oxidized mixture, diluted in 70% of acetonitrile with 0.1% of formic acid, were introduced into a Supelco Bio Wide Pore C5 column (15 cm × 0.5 mm, 5 μm). The mobile phase A consisted of water with 5% acetonitrile and 0.1% of formic acid. The mobile phase B consisted of acetonitrile, with 0.1% of formic acid. The mobile phase gradient was programmed as follows: the initial