Chemistry and Physics of Lipids 175–176 (2013) 33–40

Contents lists available at ScienceDirect

Chemistry and Physics of Lipids

journal homepage: www.elsevier.com/locate/chemphyslip

Lipid profiles in brains from sheep with natural scrapie

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ARTICLE INFO

Article history:
Received 3 July 2013
Accepted 1 August 2013
Available online 16 August 2013

Keywords:
Scrapie
Prion disease
Brain lipid composition
Fatty acid profile

ABSTRACT

Prion diseases are fatal neurodegenerative disorders affecting many mammals, ovine scrapie being the archetypal prion disease. Several independent studies in murine and cell-based models of scrapie have highlighted the presence of a link between prion generation and lipid alterations; yet, no data on natural disease are available. In this study we investigated levels of total lipids and cholesterol as well as profiles of fatty acids in brain homogenates from symptomatic and asymptomatic scrapie-infected sheep vs. healthy sheep, all belonging to the same flock. Lipid extracts were analyzed by means of gas chromatography and high performance liquid chromatography. Data of fatty acids were submitted to multivariate statistical analysis to give a picture of the brain lipid profiles of sheep. Interestingly, results revealed abnormalities in the brain fatty acid unsaturation of infected/symptomatic animals. Significant reduction of monoene 18:1 n-9 was detected in brain lipids from infected/symptomatic sheep, as compared to healthy and infected/asymptomatic animals, and this alteration occurred in combination with a significant increase in 18:0 level. The unsupervised Principal Component Analysis showed that infected/symptomatic and healthy sheep samples lie in two different regions of the plot, infected/asymptomatic lie mostly next to healthy. The increase of cerebral saturated fatty acids provides a rough indication of presumed alterations in lipid raft domains of nervous cells during scrapie, suggesting that they may exist in a notable viscous liquid-ordered state. Such physicochemical alteration would have a profound impact on the raft thermodynamic properties, its spatial organization, and signal transduction, all potentially relevant for prion generation.

1. Introduction

Prion diseases or Transmissible Spongiform Encephalopathy (TSE) diseases are fatal neurodegenerative disorders affecting man and many mammals (Prusiner, 1998; Aguzzi and Calella, 2009; Schneider et al., 2008). Human prion disorders are Creutzfeldt–Jakob disease, fatal familial insomnia, Gerstmann–Sträussler–Scheinker syndrome, and Kuru (Prusiner, 1998; Aguzzi and Calella, 2009), while animal diseases include scrapie of sheep and goats, transmissible mink encephalopathy in mink, chronic wasting disease in elk, and bovine spongiform encephalopathy in cattle and exotic ungulates (Prusiner, 1998; Schneider et al., 2008; Aguzzi and Calella, 2009).

Prions, commonly considered to be the causative agents of TSEs, are infectious proteins consisting of abnormally folded, partially protease resistant isoforms of the normal cellular prion protein (PrP^{C}), generally denoted as prion protein-scrapie (PrP^{Sc}) (Aguzzi and Calella, 2009; Smith et al., 2012; Pinheiro, 2006). The key molecular event is the structural conversion of PrP^{C} into PrP^{Sc}, a self-propagating process in which PrP^{Sc} acts as a conformational template recruiting PrP^{C} for further conversion (Verity and Mallucci, 2011). The misfolding process takes place in particular cell membrane domains named rafts (Pinheiro, 2006; Simons and Ehehalt, 2002; Michel and Bakovic, 2007; Fantini et al., 2002). Lipid rafts are specialized membrane microdomains characterized by high content of sphingolipids, cholesterol, and saturated fatty acids, that serve as organizing centers for different cellular processes, including trafficking of membrane and membrane-proteins (Fabelo et al., 2011; Korade and Kenworthy, 2008). Interestingly, the misfolding of the prion protein has been reported to affect the raft structure and to alter the lipid–protein and protein–protein interactions (Bate et al., 2008).

Studies in scrapie-infected mice and cells revealed the presence of major molecular and biochemical modifications in the cellular cholesterol network (Bach et al., 2009; Bate et al., 2008; Pani et al., 2007; Vascellari et al., 2011). On the other hand, no major differences were reported in the content/composition of fatty acids, polar lipids, neutral lipids, phospholipids, and plasmalogens in the brain

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0009-3084/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.chemphyslip.2013.08.001

of scrapie-infected mice and hamsters (Pamplona et al., 2008; Guan et al., 1996; Dees et al., 1985; Heitzman and Skipworth, 1969).

To date, however, although ovine scrapie is the most studied prion disease (Schneider et al., 2008; Fitzmaurice et al., 2008), very few papers have been focused on the lipid alterations occurring during natural scrapie (Pani et al., 2007).

In an attempt to identify a lipid profile linked to prion ailments, we analyzed and compared the lipid composition of brains from uninfected (healthy) and scrapie-infected symptomatic (I/sympt) and asymptomatic (I/asympt) Sarda breed sheep coming from the same farm where a number of clinical scrapie cases have been diagnosed. We determined and compared levels of total lipids, total cholesterol, and fatty acids by means of gas chromatography (GC) and high performance liquid chromatography (HPLC) methods. Furthermore, GC fatty acid data were studied by means of multivariate statistical analysis. The unsupervised Principal Component Analysis (PCA) was applied to picture the fatty acid profiles of healthy and infected sheep. Malondialdehyde (MDA) levels were also measured in sheep brain homogenates as biochemical marker of lipid oxidative modification.

2. Materials and methods

2.1. Materials

Cholesterol, triolein, trilinolein, standards of fatty acids and fatty acid methyl esters, and Desferal (deferoxamine mesylate salt), were purchased from Sigma–Aldrich (Milan, Italy). All solvents used, of the highest available purity, were also from Sigma–Aldrich. The methanolic HCl (3 N) was purchased from Supelco (Bellefonte, PA). All of the other chemicals used in this study were of analytical grade.

2.2. Sheep selection and scrapie diagnosis

The study was carried out on 1 scrapie-affected Sarda sheep flocks located in Sardinia (Italy). The flock was randomly selected in Sardinia among those with a history of high clinical incidence of clinical cases of scrapie. Presence of the disease scrapie in the flock was confirmed after the notification of clinically suspected cases throughout the active surveillance for scrapie. In this flock, inside the framework of appropriate actions for eradicating scrapie, as is stipulated in EC regulations, all sheep carrying susceptible ARQ/ARQ genotypes must be sacrificed.

Thus, a number of the above susceptible animals, with or without neurological signs, were euthanized. At necroscopy, from each sheep the brains were collected and then divided into two hemispheres before being immediately frozen at −80 °C. One half was submitted to Western Blotting (WB) examination in order to define the scrapie status, as described elsewhere (Ligios et al., 2006). In the light of the WB results we selected 18 brains, 15 from ARQ/ARQ and 3 from AHQ/ARQ sheep. More in detail 7 of the brains were healthy (identified from 1H to 7H), 8 were PrP^{Sc} positive from sheep with clinical scrapie (I/sympt) (from 8IS to 15IS), 3 were PrP^{Sc} positive from sheep without clinical scrapie (I/asympt) (from 16IA to 18IA). For biochemical examination, the remaining half brain of these sheep was weighed and homogenized in ice for 2 min in saline solution, 1:1 weight:volume, using an Ultra Turrax blender. Aliquots of homogenates (500 mg of wet brain/mL of saline solution) were stored at −80 °C until lipid extraction. For further information see Table 1.

2.3. Lipid extraction from brain homogenates

Total lipids were extracted from brain homogenates using the method described by Folch et al. (1957) by addition of 12 mL of $CHCl_3$/MeOH (2/1, v/v) solution. After addition of 3 mL H_2O and centrifugation at 900 × *g* for 1 h, the $CHCl_3$ fraction was separated from the MeOH/H_2O mixture. Total lipids in $CHCl_3$ fraction were quantified by the method of Chiang et al. (1957). Separation of cholesterol and fatty acids (FA) was obtained by mild saponification (Rosa et al., 2011) as follows: 1 mL of the $CHCl_3$ fraction, containing the lipids, from each brain sample, was dried down and dissolved in 5 mL of EtOH and 100 μL of Desferal solution (25 mg/mL of H_2O), 1 mL of a water solution of ascorbic acid (25% w/v), and 0.5 mL of 10 N KOH were added. The mixtures were left in the dark at room temperature for 14 h. After the addition of 10 mL of *n*-hexane and 7 mL of H_2O, samples were centrifuged for 1 h at 900 × *g*. The hexane phase containing the unsaponifiable fraction (cholesterol) was collected and the solvent was evaporated. A portion of the dried residue was dissolved in 1 mL of MeOH and injected into the high-performance liquid chromatography (HPLC) system. After the addition of a further 10 mL of *n*-hexane to the mixtures, samples were acidified with 37% HCl to pH 3–4 and then centrifuged for 1 h at 900 × *g*. The hexane phase (saponifiable fraction) with FA was collected, and the solvent was evaporated. A portion of the dried residue was dissolved in 1 mL of CH_3CN with 0.14% CH_3COOH (v/v), and aliquots of the samples were injected into the HPLC system. An aliquot of dried FA was methylated with 1 mL of methanolic HCl (3 N) (Rosa et al., 2011; Christie, 1993) for 30 min at room temperature. After the addition of 4 mL of *n*-hexane and 2 mL of H_2O, samples were centrifuged for 20 min at 900 × *g*. The hexane phase with FA methyl esters was collected, and the solvent was evaporated. The residue was dissolved in 100 μL of *n*-hexane, and aliquots of the samples were injected into the GC system. The recovery of FA and cholesterol during the saponification was calculated using an external standard mixture prepared by dissolving 1 mg of triolein, trilinolein, and cholesterol in 5 mL of EtOH and processed as samples. All solvent evaporation was performed under vacuum.

2.4. HPLC analysis

Analyses of cholesterol and unsaturated FA were carried out with an Agilent Technologies 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a diode array detector (HPLC-DAD). Cholesterol, detected at 203 nm, was measured with the use of a Chrompack column (Chrompack, Middelburg, The Netherlands), Inertsil 5 ODS-3, 150 mm × 3 mm, and MeOH as the mobile phase, at a flow rate of 0.4 mL/min (Rosa et al., 2011). Analyses of unsaturated FA, detected at 200 nm, was carried out with a XDB-C_{18} Eclipse (150 mm × 4.6 mm, 3.5 μm particle size) (Agilent Technologies) equipped with a Zorbax XDB-C_{18} Eclipse (12.5 mm × 4.6 mm, 5 μm particle size) guard column (Agilent Technologies), with a mobile phase of $CH_3CN/H_2O/CH_3COOH$ (75/25/0.12, v/v/v), at a flow rate of 2.3 mL/min (Rosa et al., 2011). The temperature of the column was maintained at 37 °C.

The identification of cholesterol and FA was made using standard compounds and conventional UV spectra, generated with the Agilent Chemstation A.10.02 software. Calibration curves of all of the compounds were constructed using standards and were found to be linear, with correlation coefficients >0.995.

2.5. GC analysis of FA methyl esters

FA methyl esters were measured on a gas chromatograph Hewlett–Packard HP-6890 (Hewlett–Packard, Palo Alto, CA) with a flame ionization detector and equipped with a cyanopropyl methylpolysiloxane HP-23 FAME column (30 m × 0.32 mm × 0.25 μm) (Hewlett–Packard). Nitrogen was used as a carrier gas at a flow rate of 2 mL/min. The oven temperature was set at 175 °C; the injector temperature was set at 250 °C; and the detector temperature was set at 300 °C. The FA methyl