Steroids 99 (2015) 172–177

Contents lists available at ScienceDirect

Steroids

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

The oxysterol and cholestenoic acid profile of mouse cerebrospinal fluid

Peter J. Crick [a,1], Lien Beckers [b], Myriam Baes [b], Paul P. Van Veldhoven [c], Yuqin Wang [a,*], William J. Griffiths [a,*]

[a] College of Medicine, Grove Building, Swansea University, Singleton Park, Swansea SA2 8PP, UK
[b] Department of Pharmaceutical and Pharmacological Sciences, Laboratory of Cell Metabolism, Campus Gasthuisberg O/N2, B 3000 Leuven, Belgium
[c] Department of Cellular and Molecular Medicine, LIPIT, Campus Gasthuisberg O&N1, B3000 Leuven, Belgium

ARTICLE INFO

Article history:
Received 19 December 2014
Received in revised form 17 February 2015
Accepted 20 February 2015
Available online 7 March 2015

Keywords:
LC–MS
Derivatisation
Bile acid
Brain

ABSTRACT

Oxysterols and cholestenoic acids are oxidised forms of cholesterol with a host of biological functions. The possible roles of oxysterols in various neurological diseases makes the analysis of these metabolites in the central nervous system of particular interest. Here, we report the identification and quantification of a panel of twelve sterols in mouse cerebrospinal fluid (CSF) using liquid chromatography–mass spectrometry exploiting enzyme assisted derivatisation for sterol analysis technology. We found low levels of oxysterols and cholestenoic acids in CSF in the range of 5 pg/mL–2.6 ng/mL. As found in man, these concentrations are one to two orders of magnitude lower than in plasma.

1. Introduction

Oxysterols and cholestenoic acids are formed from cholesterol as intermediates in the synthesis of bile acids and steroid hormones. Oxidation of cholesterol is catalysed by numerous sterol hydroxylases, primarily members of the cytochrome P450 (CYP) family of enzymes [1]. Although the presence of oxysterols in mammalian circulation has been known for many years, recent studies have led to a renewed interest in these metabolites as they have been shown to possess potent biological activities. For example, 24S,25-epoxycholesterol is a ligand to the liver X receptors (LXRs) [2], 7α,25-dihydroxcholesterol (7α,25-diHC) plays a role in immune cell migration by binding to the Epstein-Barr virus induced gene 2 (EBI2) [3,4], and (25R)26-hydroxycholesterol (26-HC) has been shown to influence tumour growth by modulating the estrogen receptor β (ERβ) [5,6]. Note, here we use the systematic nomenclature where addition of a hydroxy group on the terminal carbon of the cholesterol side-chain introducing R stereochemistry at C-25, is at C-26 giving (25R)26-hydroxycholesterol. The common, but systematically incorrect, name for this molecule is 27-hydroxycholesterol [7,8].

The high level of cholesterol in the brain (approximately 2% wet weight of brain) has stimulated research investigating altered cholesterol metabolism as a causative factor in neurological diseases. For example, oxysterol levels have been shown to be perturbed in neurodegenerative diseases including Alzheimer's disease [9] and Parkinson's disease [10], while altered metabolism of 25-HC has been linked to neuroinflammation in mouse models [11]. Recently, it has been demonstrated that oxysterols promote midbrain neurogenesis [12] while cholestenoic acids regulate the survival of motor neurons both in *vitro* and in *vivo* [13].

Mouse models of neurological diseases are an important resource to investigate the causes and treatments of these conditions. Increasingly sophisticated mouse models for numerous diseases are becoming available, as well as knockout animals with mutations of specific enzymes involved in sterol homeostasis. To link observed biological phenomena to the underlying molecular causes it is useful to analyse the levels of oxysterols and cholestenoic acids in different tissues of the mouse. Blood plasma is often used for analysis as it is

Abbreviations: CSF, cerebrospinal fluid; CYP, cytochrome P450; LXRs, liver X receptors; 7α,25-diHC, 7α,25-dihydroxycholesterol (cholest-5-ene-3β,7α,25-triol); 26-HC, (25R)26-hydroxcholesterol,(cholest-5-en-3β,(25R)26-diol); BBB, blood brain barrier; CNS, central nervous system; EADSA, enzyme assisted derivatisation for sterol analysis; LC–MS^n, liquid chromatography–tandem mass spectrometry; 22R-HCO, 22R-hydroxycholest-4-en-3-one; GP, Girard P; SPE, solid phase extraction; LIT, linear ion trap; RIC, reconstructed ion chromatograms; 7-OC, 7-oxocholesterol (3β-hydroxycholest-5-en-7-one); 3β-HCA, 3β-hydroxycholest-5-enoic acid; 3β,7α-diHCA, 3β,7α-dihydroxycholest-5-enoic acid; 7αH,3O-CA, 7α-hydroxy-3-oxocholest-4-enoic acid; 7α,24-diHCO, 7α,24-dihydroxycholest-4-en-3-one.

* Corresponding authors. Tel.: +44 1792 602730 (Y. Wang). Tel.: +44 1792 295562, mobile: +44 7796 776363 (W.J. Griffiths).

E-mail addresses: y.wang@swansea.ac.uk (Y. Wang), w.j.griffiths@swansea.ac.uk (W.J. Griffiths).

[1] *Present address:* Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, Socinstrasse 57, 4002 Basel, Switzerland.

http://dx.doi.org/10.1016/j.steroids.2015.02.021
0039-128X/

straightforward to collect and gives a "snapshot" of oxysterol levels throughout the body. However, the presence of the blood brain barrier (BBB) raises questions of the relevance of plasma measurements in the context of neurological diseases. Previously we have analysed brain tissue which circumvents this problem and allows direct analysis of metabolites that may play a role in disease [14]. While this is a powerful approach, translation to human studies is only possible for post mortem analysis.

An alternative is to measure oxysterols in cerebrospinal fluid (CSF). This has the advantage of relatively straightforward sample collection in humans while representing conditions in the central nervous system (CNS) more faithfully than blood plasma. We have previously reported the identification and quantitation of about 25 oxysterols and cholestenoic acids in human CSF [15] and shown that inborn errors of cholesterol metabolism affect the levels of the metabolites present [13]. However, to the best of our knowledge, there have been no reports of the oxysterol profile of mouse CSF which makes comparisons between human diseases and mouse models difficult.

Here, we present a robust method for the identification and quantification of cholesterol metabolites in mouse CSF that opens new possibilities for biomarker discovery and investigations into the underlying causes of neurological diseases. We compare this data with concentrations of oxysterols and cholestenoic acids found in plasma to give an overview of cholesterol metabolism in mouse.

2. Experimental

2.1. Sample collection

CSF was from male (~40 g) and female (~34 g) Swiss/Webster mice (4–6 months), sedated with an overdose of Nembutal (150 μg/g body weight). CSF was collected from the cisterna magna as described by Liu and Duff [16] but only a single sampling of 5–10 μL was performed and the mouse was subsequently sacrificed. Care was taken to avoid blood vessels when penetrating the dura mater. Pools of 100 μL CSF for male mice and 54 μL CSF for female mice were made. Mice were bred in the animal housing facility of the KU Leuven, had ad libitum access to water and standard rodent food, and were kept on a 12 h light and dark cycle. All animal experiments were performed in accordance with the "Guidelines for Care and Use of Experimental Animals" and fully approved by the Research Advisory Committee (Research Ethical committee) of the KU Leuven. Male mouse (B6, 3 months of age) plasma was purchased from Jackson Laboratories (Maine, USA).

2.2. Analysis of oxysterols and cholestenoic acids

Oxysterols and cholestenoic acids from mouse plasma were analysed using enzyme assisted derivatisation for sterol analysis (EADSA) and liquid chromatography–tandem mass spectrometry (LC–MS^n) by the method previously described [17]. Metabolites from mouse CSF were analysed using a similar method, but with the modifications described below.

2.3. Extraction of oxysterols and cholestenoic acids from mouse CSF

CSF (54–100 μL) was added to ethanol (1.05 mL, Fisher Scientific) containing 24R/S-[25,26,26,26,27,27,27-$^2H_7$]hydroxycholesterol ([$^2H_7$]24R/S-HC) (2 ng), 22R-[25,26,26,26,27,27,27-$^2H_7$]hydroxycholest-4-en-3-one ([$^2H_7$]22R-HCO) (2 ng), 7α,25-[26,26,26,27,27,27-$^2H_6$]dihydroxycholesterol ([$^2H_6$]7α,25-diHC) (0.2 ng) and [25,26,26,26,27,27,27-$^2H_7$]cholesterol (20 μg) (all Avanti Polar Lipids) in an ultrasound bath. After 5 min water (Fisher Scientific) was added to give a final volume of 1.5 mL of 70% ethanol. The mixture was centrifuged for 30 min at 17,089×*g* at 4 °C. To remove cholesterol from the sample a solid phase extraction (SPE) step was used. A 200 mg tC_{18} Sep-Pak cartridge (Waters) was preconditioned with ethanol (4 mL) followed by 70% ethanol (6 mL) after which the sample was loaded. The flow-through was combined with a wash of 70% ethanol (5.5 mL) to give a 7 mL fraction containing oxysterols and cholestenoic acids. The solvent was evaporated in a vacuum centrifuge overnight.

2.4. Enzyme assisted derivatisation of oxysterols and cholestenoic acids

The dried sample was dissolved in propan-2-ol (50 μL, Fisher Scientific) and 50 mM KH_2PO_4 buffer (500 μL) then treated with 3 μL of cholesterol oxidase (2 mg/mL in H_2O, 44 units/mg protein, Sigma–Aldrich) and incubated for 1 h at 37 °C. The reaction was quenched by addition of methanol (1 mL) followed by glacial acetic acid (75 μL, VWR) and Girard P (GP, TCI Europe) reagent (75 mg). After vortexing, the mixture was incubated at room temperature in the dark overnight.

Excess reagent was removed by SPE using a 50 mg tC_{18} Sep-Pak cartridge (Waters) preconditioned with methanol (1.5 mL, Fisher Scientific), 10% methanol (1.5 mL) and 70% methanol (1 mL). The sample was loaded and allowed to flow through the cartridge. To ensure full recovery of all analytes of interest, a recycling procedure was used where the eluate was diluted with an equal volume of water and reapplied to the column. This procedure was repeated to give a final concentration of 17% methanol. The cartridge was then washed with 10% methanol (1.5 mL) and the analytes of interest eluted with methanol (3 × 250 μL to give Fr-1, Fr-2 and Fr-3) followed by ethanol (250 μL to give Fr-4). The solvent was evaporated from combined Fr-1 and Fr-2 using a vacuum centrifuge and the sample was re-suspended in 60% methanol (100 μL) for analysis by LC–MS^n.

2.5. LC–MS^n on the LTQ-Orbitrap

Oxysterols were separated using a RSLC nano Ultimate 3000 (Dionex) with a Hypersil Gold C_{18} column (1.9 μm particle size, 50 × 2.1 mm, Thermo Fisher). Mobile phase A consisted of 33.3% methanol, 16.7% acetonitrile (Fisher Scientific), 50% water, containing 0.1% formic acid (VWR) while mobile phase B was 63.3% methanol, 31.7% acetonitrile, 5% water, containing 0.1% formic acid. The gradient started at 20% mobile phase B for 1 min before increasing to 80% mobile phase B over 7 min. After holding for 5 min the gradient returned to 20% B over 6 s before re-equilibration for 3 min 54 s to give a total run time of 17 min. The flow rate was 200 μL/min and the eluent was directed to the atmospheric pressure ionisation source of an LTQ-Orbitrap Velos (Thermo Fisher). Eighty five microlitre of the derivatised mouse CSF was injected and a full scan was performed in the Orbitrap across the *m/z* range 400–610 at 30,000 resolution (full width at half maximum height). At the same time the linear ion trap (LIT) monitored MS^n transitions for GP tagged oxysterols and cholestenoic acids. Initial activation gave a characteristic $[M-79]^+$ fragment in MS^2 corresponding to the loss of pyridine, the $[M-79]^+$ ion was isolated and when activated further gave structurally informative MS^3 spectra.

3. Results and discussion

3.1. Analysis using EADSA reveals 12 oxysterols and cholestenoic acids in mouse CSF

We used EADSA to charge-tag sterols of interest with the GP reagent (Fig. 1). Enzymatic oxidation of the characteristic sterol