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Cholesterol metabolites exported from human brain

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ABSTRACT

The human brain contains approximately 25% of the body's cholesterol. The brain is separated from the circulation by the blood brain barrier. While cholesterol will not passes this barrier, oxygenated forms of cholesterol can cross the barrier. Here by measuring the difference in the oxysterol content of blood plasma in the jugular vein and in a forearm vein by mass spectrometry (MS) we were able to determine the flux of more than 20 cholesterol metabolites between brain and the circulation. We confirm that 24S-hydroxycholesterol is exported from brain at a rate of about 2–3 mg/24 h. Gas chromatography (GC)–MS data shows that the cholesterol metabolites 5α-hydroxy-6-oxocholesterol (3β,5α-dihydroxycholestan-6-one), 7β-hydroxycholesterol and 7-oxocholesterol, generally considered to be formed through reactive oxygen species, are similarly exported from brain at rates of about 0.1, 2 and 2 mg/24 h, respectively. Although not to statistical significance both GC–MS and liquid chromatography (LC)–MS methods indicate that (25R)26-hydroxycholesterol is imported to brain, while LC–MS indicates that 7α-hydroxy-3-oxocholest-4-enoic acid is exported from brain.

1. Introduction

The human brain contains about 25% of the body's cholesterol, and cholesterol makes up about 2% of brain [1]. The brain is isolated from the circulation by the blood brain barrier (BBB) which is impermeable to cholesterol. The consequence of this is that essentially all brain cholesterol is synthesised from acetyl CoA in brain itself. The half life of cholesterol in human brain is about 5 years [2]. It is metabolised in brain by the cytochrome P450 (CYP) 46A1 enzyme to 24S-hydroxycholesterol (24S-HC, cholest-5-en-3β,24S-diol), which by virtue of the added hydroxy group to the cholesterol side-chain can pass the BBB and be exported from brain at a reported rate of about 4–7 mg/24 h [3–5]. It is believed that export of 24S-HC correspond to about 2/3 of cholesterol turn-over in brain of rodents, the origin of the remaining 1/3 has yet to be established [1]. In contrast to 24S-HC, (25R)26-hydroxycholesterol (26-HC) is reported to be imported to human brain at a rate of about 4–5 mg/24 h [5,6]. Note, we use the systematic nomenclature where addition of a hydroxy group to a terminal carbon atom of the cholesterol side-chain introducing R stereochemistry at C-25 results in the oxysterol named (25R)26-hydroxycholesterol [7]. The commonly used, but systematically incorrect, name for this compound is 27-hydroxycholesterol. Despite the high rate of import of 26-HC into brain the level of 26-HC in human brain (1–2 ng/mg) is much lower than that of 24S-HC (20 ng/mg) [8]. Interestingly, Meaney et al. have reported that the 26-HC metabolite 7α-hydroxy-3-oxocholest-4-enoic acid (7αH,3O-CA) is exported from brain at a rate of about 2 mg/24 h, accounting for much of the consumption of 26-HC in brain [9].

Abbreviations: MS, mass spectrometry; GC, gas chromatography; LC, liquid chromatography; BBB, blood brain barrier; CYP, cytochrome P450; 24S-HC, 24S-hydroxycholesterol; 26-HC, (25R)26-hydroxycholesterol; 7αH,3O-CA, 7α-hydroxy-3-oxocholest-4-enoic acid; 3β,5α-diHC-6O, 3β,5α-dihydroxycholestan-6-one; C-triol, cholestane-3β,5α,6β-triol; 4α-HC, 4α-hydroxycholesterol; 4β-HC, 4β-hydroxycholesterol; GP, Girard P; 7β-HC, 7β-hydroxycholesterol; 7O-C, 7-oxocholesterol; 25-D_3, 25-hydroxyvitamin D_3; 7α,25-diHCO, 7α,25-dihydroxycholest-4-en-3-one; 7α,26-diHCO, 7α,(25R)26-hydroxycholest-4-en-3-one; ROS, reactive oxygen species; 7α,26-diHC, 7α,(25R)26-dihydroxycholesterol; 7α,25-diHC, 7α,25-dihydroxycholesterol; HSD3B7, hydroxysteroid dehydrogenase 3B7; 3β-HCA, 3β-hydroxycholest-5-en-(25R)26-oic acid; 3β,7α-diHCA, 3β,7α-dihydroxycholest-5-enoic acid; CHD, coronary heart disease.

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Gas chromatography (GC)–mass spectrometry (MS) and liquid chromatography (LC)–MS have been exploited widely for measurement of plasma or serum oxysterols [10–15]. In this work we have utilised both these methods measuring the levels of cholesterol metabolites in the jugular vein and a vein within the arm allowing us to measure the rate of flux of cholesterol metabolites out from, and into, brain.

2. Methods

2.1. Patient samples

Plasma was from Policlinico Umberto I, Rome, provided with written informed consent, institutional review board and ethical approval, and collected according to the principles of the Declaration of Helsinki. Sixteen males and two females were enrolled in the study. Sixty percent were current smokers, and 45% were also affected by type 2 diabetes and under oral antidiabetic medications. Patients were also taking medications which included antiplatelets, betablockers, angiotensin converting enzyme inhibitors, and statins. Export of cerebral oxysterols from brain was studied by measuring the gradient of concentration between the jugular vein (blood exiting the brain) and a forearm vein sample taken from 18 coronary heart disease patients undergoing on-pump myocardial revascularization surgery. Blood from the jugular vein was drawn in the intensive care unit through a 4F catheter, which was placed in the right jugular bulb via echoscan guide and position confirmed by neck X-ray.

Blood was collected in EDTA tubes; plasma was separated within 2 h of collection and stored at −80 °C until assay.

2.2. GC–MS analysis

Oxysterols were determined by GC–MS using deuterium-labelled internal standards as described by Dzeletovic et al. and Iuliano et al. [14,15]. In brief, 10 μL BHT in ethanol (5 mg/mL) and 50 μL EDTA (10 mg/mL) were added to a solution of 1 mL of plasma and 10 μL of ethanol containing deuterium labelled internal standards. Samples were subjected to alkaline hydrolysis (600 μL ethanolic KOH 5.9 M) for 2 h at room temperature with stirring. At the end of incubation, the solution was neutralised with 200 μL phosphoric acid and sterols extracted in chloroform:methanol (2:1, v/v). Solvent was evaporated under a stream of nitrogen, the residue dissolved in 1 mL of toluene, and oxysterols separated from cholesterol by solid phase extraction. Silica cartridges (100 mg), previously equilibrated with n-hexane, were loaded with toluene-dissolved samples. Cholesterol and non-cholesterol neutral sterols were eluted with 1% propan-2-ol in hexane before eluting oxysterol with 10% propan-2-ol in n-hexane. After removal of solvent, samples were converted to trimethylsilyl ethers by treatment with 130 μL Sylon HTP (hexamethyldisilylazane:trimethylchlorosilane:pyridine, 3:1:9) (Supelco, Bellafonte, PA) at 60 °C for 30 min. After incubation, the solution was evaporated under a stream of nitrogen, and the residue dissolved in n-hexane and transferred to an autosampler vial. Analyses were performed on an Agilent 6890N GC equipped with a 7683 series automatic liquid sampler, and interfaced with an Agilent 5973 Mass Spectrometer (Agilent Technologies; Palo Alto, CA). Separation was carried out on a 30 m capillary column (HP-5MS 30 × 0.25 mm ID, 0.25 μm thickness). Quantification of oxysterols was made by the isotope dilution method.

3β,5α-Dihydroxycholestan-6-one (3β,5α-diHC-6O), 3β,5α-[$^2H_6$]dihydroxycholestan-6-one ([$^2H_6$]-3β,5α-diHC-6O), cholestane-3β,5α,6β-triol (C-triol) and [$^2H_6$]cholestane-3β,5α,6β-triol ([$^2H_6$]-C-triol) were synthesised as previously described [16]. 4α- and 4β-hydroxyholesterols (4α-HC, 4β-HC), synthesised as described in [17], were kindly donated by G. Lizard, Université de Bourgogne. All other deuterated and non-deuterated oxysterols were from Avanti Polar Lipids (Alabaster, AL).

2.3. LC–MS analysis

Oxysterols were analysed as their Girard P (GP) derivatives using deuterium labelled internal standards as described in Griffiths et al. and Crick et al. [18,19]. In brief, 100 μL of plasma was added to 1.05 mL of ethanol containing deuterated internal standards 24S-[$^2H_7$]hydroxycholesterol, 22R-[$^2H_7$]hydroxycholesterol, 22R-[$^2H_7$]hydroxycholest-4-en-3-one, 7α-[$^2H_7$]hydroxycholesterol, 7α,25-[$^2H_6$]dihydroxycholesterol and [$^2H_7$]cholesterol (Avanti). The solution was diluted to 70% ethanol and centrifuged. The supernatant (1.5 mL 70% ethanol) was loaded on a Sep-Pak tC_{18} 200 mg cartridge (Waters, Elstree, UK) and the flow-through and a 5.5 mL wash with 70% ethanol combined. This fraction, the oxysterol fraction, was dried under reduced pressure, re-constituted in 100 μL of propan-2-ol and treated with KH_2PO_4 buffer (1 mL 50 mM, pH 7) containing 3 μL of cholesterol oxidase (2 mg/mL in H_2O, 44 units/mg protein) for 1 h at 37 °C. Methanol (2 mL), glacial acetic acid (150 μL) and GP reagent (150 mg, 0.8 mmole) were added and the mixture incubated at room temperature over night. To remove excess derivatisation agent the reaction mixture was applied to a 60 mg OASIS HLB cartridge (Waters). A re-cycling protocol was adopted where the eluate is diluted with an equal volume of water and re-cycled on the column until the eluate is 17.5% methanol (19 mL). After a wash with 10% methanol (6 mL) GP-derivatised oxysterols were eluted in methanol (2 mL).

In contrast to sample preparation for GC–MS analysis, hydrolysis was not performed for LC–MS, hence non-esterified oxysterols were measured by LC–MS while total oxysterols by GC–MS.

2.4. Statistics

Paired sample *t* tests were performed. $P < 0.05$ was considered statistically significant. $^*P < 0.05$; $^{**}P < 0.01$.

3. Results

3.1. GC–MS

Using GC–MS we measured the levels of 12 oxysterols in the jugular vein and in a vein in the forearm. Of these, we found statistical differences in the levels of 7β-hydroxycholesterol (7β-HC, $P < 0.05$), 7-oxocholesterol (7-OC, $P < 0.05$), 3β,5α-diHC-6O ($P < 0.01$) and 24S-HC ($P < 0.01$) corresponding to a flux of about 2, 2, 0.1 and 3 mg/24 h out from brain, assuming a flow of plasma of 450 mL/min through brain [6] (Fig. 1 and Supplementary data Table S1).

3.2. LC–MS

Using LC–MS we similarly measured the levels of 20 cholesterol metabolites and also 25-hydroxyvitamin D_3 (25-D_3). An important methodological difference between the LC–MS and the GC–MS analysis was that only non-esterified metabolites were measured by LC–MS. We found a statistical difference in the concentration of 24S-HC ($P < 0.01$) between the two veins, corresponding to a flux from brain of about 2 mg/24 h. In an earlier report we noted that 7α,25-dihydroxycholest-4-en-3-one (7α,25-diHCO, $P < 0.01$) and 7α,(25R)26-dihydroxycholest-4-en-3-one (7α,26-diHCO, $P < 0.01$) were similarly exported from brain at a rate of about 0.5 and 1 mg/24 h, respectively [19] (Fig. 2 and Supplementary data Table S2).

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