Levels of 7-oxocholesterol in cerebrospinal fluid are more than one thousand times lower than reported in multiple sclerosis

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Abstract  In a recent publication [Diestel et al. (1), high levels of the cholesterol oxidation product 7-oxocholesterol were reported in cerebrospinal fluid (CSF) of patients with multiple sclerosis (MS): ~7 mg/l. Unsurprisingly, such high levels were found to cause neuronal damage under in vitro conditions. The levels were considerably lower in CSF from control subjects: ~0.5 mg/l. In a previous work from this laboratory, we measured levels of 7-oxy-enated oxysterols in CSF from different groups of patients (2), and the levels were always found to be less than 0.01 mg/l (our unpublished observation). It is well established that the other oxysterols in CSF, 24S-hydroxycholesterol (24OHC) and 27-hydroxycholesterol (27OHC), are present in similarly low concentrations (3–6). In view of this, and in view of the suggestion by Diestel et al. (1) that 7-oxocholesterol could be an important link between demyelination and neuronal damage, we measured the levels of 7-oxocholesterol in CSF from 29 Swedish patients with MS and 24 control subjects. A highly accurate and sensitive method was used based on isotope dilution-mass spectrometry (ID-MS) using deuterium-labeled internal oxysterol standards. The results are discussed in relation to the fact that 7-oxy-enated steroids are easily artificially formed by autoxidation of cholesterol during workup procedures and analysis of sterols and oxysterols from biological samples.

MATERIALS AND METHODS

Patients

We retrospectively investigated CSF samples from 53 patients at the Division of Neurology and the Division of Geriatrics, Karolinska University Hospital in Huddinge. CSF was collected for

In a recent publication by Diestel et al. (1), high levels of the cholesterol oxidation product 7-oxocholesterol were reported in cerebrospinal fluid (CSF) of patients with multiple sclerosis (MS): ~7 mg/l. Unsurprisingly, such high levels were found to cause neuronal damage under in vitro conditions. The levels were considerably lower in CSF from control subjects: ~0.5 mg/l. In a previous work from this laboratory, we measured levels of 7-oxy-enated oxysterols in CSF from different groups of patients (2), and the levels were always found to be less than 0.01 mg/l (our unpublished observation). It is well established that the other oxysterols in CSF, 24S-hydroxycholesterol (24OHC) and 27-hydroxycholesterol (27OHC), are present in similarly low concentrations (3–6). In view of this, and in view of the suggestion by Diestel et al. (1) that 7-oxocholesterol could be an important link between demyelination and neuronal damage, we measured the levels of 7-oxocholesterol in CSF from 29 Swedish patients with MS and 24 control subjects. A highly accurate and sensitive method was used based on isotope dilution-mass spectrometry (ID-MS) using deuterium-labeled internal oxysterol standards. The results are discussed in relation to the fact that 7-oxy-enated steroids are easily artificially formed by autoxidation of cholesterol during workup procedures and analysis of sterols and oxysterols from biological samples.

Supplementary key words  27-hydroxycholesterol • 24S-hydroxycholesterol • neurological disease • lipid peroxidation • oxysterols

Abbreviations: ABAP, 2,2′-azobis-2-amidinopropane hydrochloride; BHT, 3,5-di-tert-butyl-4-hydroxytoluene; CSF, cerebrospinal fluid; ID-MS, isotope dilution-mass spectrometry; MS, multiple sclerosis; 24OHC, 24S-hydroxycholesterol; 27OHC, 27-hydroxycholesterol; SIM, selected ion monitoring; TMSi, trimethylsilyl.

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routine diagnostic purposes under varying conditions. These patients were not included in any of our previous studies. Aliquots of the CSF samples were frozen immediately at −80°C. All investigations on the patients with neurological diseases were approved by the Ethics Committee at Karolinska University Hospital in Huddinge, and each patient gave written informed consent. The subjects investigated were grouped and characterized as described below.

Control patients. This group consisted of 20 females and 4 males with a mean age of 43 ± 3 (SD) years. These subjects presented with headache of uncertain background without any clinical or laboratory signs of diseases of the central nervous system. The patients thus had normal CSF/serum albumin ratios and an absence of blood cells in the CSF (7, 8).

MS patients. This group of patients consisted of 20 females and 9 males with a mean age of 41 ± 2 years. MS was diagnosed in accordance with the criteria of Poser et al. (9). On MRI immediately proceeding or after CSF collection, only five of the patients had positive gadolinium-enhancing lesions, whereas seven patients were without such lesions. For the other 17 patients, MRI scans had not been performed around the time of sampling.

Analysis of the oxysterols in CSF

The oxysterol levels both in CSF and in the oxidation induction experiments were determined by ID-MS essentially as previously described (10) with minor modifications to optimize the analysis for CSF.

To a screw-capped vial sealed with Teflon-lined septum, 500 μl of CSF was added together with 10 ng each of \( \text{[H}_4\text{]7α\text{-hydroxycholesterol, [H}_2\text{]7β\text{-hydroxycholesterol, [H}_3\text{]7α\text{-oxocholesterol, [H}_4\text{]24α\text{-hydroxycholesterol, and [H}_2\text{]27\text{-hydroxycholesterol [50 μl from a methanolic solution of 10 ng/50 μl (w/v) of each of the deuterated oxysterols] as internal standards. To perform autoxidation, 10 μl of 3,5-di-tet-butyl-4-hydroxytoluene (BHT; 5 mg/ml, w/v) and 20 μl of EDTA (10 mg/ml, w/v) were added to each vial, and argon was flushed through the vials to remove air. The alkaline hydrolysis was then allowed to proceed at room temperature (22°C) to mix with a magnetic stirring bar for 2 h in the presence of ethanol 0.35 M potassium hydroxide solution. The latter was prepared by diluting 6 ml of an aqueous 5.9 M KOH solution to 100 ml with ethanol.

The lipid fraction was obtained by chloroform (18 ml) extraction in separatory funnels after addition of 6 ml of 0.9% NaCl and 130 μl of phosphoric acid (85%) at pH 7. The chloroform phase was evaporated at room temperature under reduced pressure using a rotary evaporator. Cholesterol was separated from oxysterols by means of solid-phase extraction using a 100 mg Isolute Silica cartridge (International Sorbent Technology, Mid Glamorgan, UK) conditioned with hexane and eluted with 0.5% KOH solution to 100 ml with ethanol.

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SIM for \([\text{H}_4\text{]7\text{-oxocholesterol at m/z 478 (M}^+\text{)}, 18.83 \text{min and 7\text{-oxocholesterol at m/z 472 (M}^+\text{)}, 18.96 \text{min; [H}_2\text{]24\text{-hydroxycholesterol at m/z 416 (M}^+\text{-OTMSi-CD(CH}_3)_2\text{)}, 18.68 \text{min and 24\text{-hydroxycholesterol at m/z 413 (M}^+\text{-OTMSi-CH(CH}_3)_2\text{)}, 18.43 \text{min; [H}_2\text{]27\text{-hydroxycholesterol at m/z 462 (M}^+\text{-OTMSi)}, 19.64 \text{min and 27\text{-hydroxycholesterol at m/z 456 (M}^+\text{-OTMSi)}, 19.74 \text{min. Peak integration was performed manually, and sterols were quantified from SIM analyses using standard curves for the listed sterols.}

Analysis of cholesterol in CSF

Cholesterol levels in CSF samples were analyzed as described previously (11) with minor modifications. To each vial, 50 μl of CSF was added together with 1 μg of \([\text{H}_4\text{]7\text{-cholesterol (50 μl from a methanolic stock solution of 1 μg/50 μl) as an internal standard. To prevent autoxidation, 10 μl of BHT (5 mg/ml) and 20 μl of EDTA (10 mg/ml) were added to each vial, and argon was flushed through the vials to remove air. The alkaline hydrolysis was then allowed to proceed at room temperature (22°C) to mix with a magnetic stirring bar for 1 h in the presence of 1 ml of 99.9% ethanol and 0.25 ml of an aqueous 5.9 M KOH solution. After adding 1 ml of 0.9% NaCl and 50 μl of phosphoric acid (85%), a neutral pH (pH 7) was reached and the sterols and oxysterols were extracted by adding 2 ml of chloroform and gently shaking at room temperature. The chloroform phase was evaporated under argon, then suspended in 0.5 ml of a methanol-water solution (80/20, v/v). Cholesterol separated from other lipid compounds by means of solid-phase extraction using a 100 mg Isolute-MF C18 cartridge (International Sorbent Technology) and freshly conditioned consecutively first with 2 ml of methanol and thereafter with 2 ml of methanol-water (80/20, v/v). The cholesterol was finally eluted from the C18 cartridge with 2.5 ml of methanol.

Monitoring of oxysterol formation during lipid peroxidation in vitro

7-Oxogenated oxysterols in serum samples are formed from cholesterol in connection with lipid peroxidation (12). To evaluate the effect of autoxidation on the CSF concentrations of oxysterols, CSF samples collected for diagnostic analysis from patients with unspecified diagnoses were pooled to provide enough material to establish two aliquots of 750 μl each. One aliquot was immediately analyzed for cholesterol and the oxysterols, and the second was allowed to stand at room temperature for 24 h and analyzed thereafter. Cholesterol and oxysterols were analyzed as described above, and the experiment was conducted in triplicate.

Finally, to evaluate the effect of in vitro-induced lipid peroxi-
vation, CSF samples, freshly collected from 12 patients for diagnostic purposes, were pooled to reach a sufficient amount of sample. The CSF was then diluted (1:1, v/v) by addition of an aqueous NaCl solution (0.9%, w/v). Thereafter, 2,2′-azobis-2-aminopropionitrile (ABAP; Polyscience, Warrington, PA) was added to each test tube at baseline (time point 0) in a final concentration of 5.5 mM. The tubes were incubated for various periods of time in air at 37°C. The peroxidation was stopped after 2, 4, 8, 12, and 24 h by addition of 50 μl of a solution of BHT (5 mg/l) and 50 μl of EDTA (1 mg/ml) to the individual aliquots. The samples were capped, vortexed, and stored light-protected at −20°C before analysis.

Statistical analysis
Data are given as median, minimal to maximal range, and 25–75% confidence interval. Statistical comparisons were performed using the Mann-Whitney rank sum test, and P < 0.05 was considered significant.

RESULTS
Evaluation of the method
To evaluate the accuracy of the method, the recovery of the oxysterols from spiked CSF samples was measured. The mean recovery was found to be 98% for 7α-hydroxycholesterol, 105% for 7β-hydroxycholesterol, 97% for 7-oxocholesterol, 98% for 24OHC, and 97% for 27OHC.

Levels of cholesterol and oxysterols in CSF from MS patients and controls
The median CSF concentrations, ranges [minimum to maximum], and 25–75% confidence intervals of cholesterol, oxysterols, and the ratios of the oxysterol-to-cholesterol concentrations from controls and MS patients are shown in Table 1. There was no significant difference between CSF cholesterol levels in MS patients and controls. The levels of the 7-oxygenated oxysterols were of the same order of magnitude as those of 24OHC and 27OHC. In the case of 7-oxocholesterol, the levels were slightly but not significantly higher than those in controls (P = 0.002). In the case of 7α-hydroxycholesterol and 7β-hydroxycholesterol, the CSF levels in MS patients were slightly but not significantly higher than those in controls (P = 0.23 and P = 0.097, respectively).

The ratio of 7-oxocholesterol to cholesterol was significantly higher in MS patients than in controls (P = 0.018). No other cholesterol-corrected concentrations of the oxysterols measured here differed between the two groups (MS patients vs. controls).

Increase in levels of 7-oxygenated sterols after autodxidation at room temperature and after oxidation of cholesterol in CSF with use of a generator of hydroxyl radicals
Uncontrolled lipid peroxidation by incubation of a pool of CSF at room temperature for 24 h was found to increase the levels of 7α-hydroxycholesterol by 36% (P = 0.002), 7β-hydroxycholesterol by 102% (P = 0.002), and 7-oxocholesterol by 44% (P = 0.033) (Table 2). No significant changes in the concentrations of cholesterol, 24OHC, and 27OHC were found.

Figure 1 shows the results of a kinetic experiment in which the changes in oxysterol concentrations were followed during 24 h after addition of a generator of hydroxyl radicals, ABAP, to a pool of CSF. The levels of the 7-oxygenated oxysterols were increased ~300-fold under the conditions used, with little or no effect on the levels of...

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**Table 1.** Absolute cholesterol and oxysterol levels and the ratios of oxysterol to cholesterol levels in CSF from controls and multiple sclerosis patients

| Variable                     | Controls (n = 24)          | Patients (n = 29)         |
|------------------------------|---------------------------|---------------------------|
| Cholesterol (mg/l)           | 4.50 [2.23–19.3] (3.86–5.93) | 4.29 [1.93–21.7] (3.04–5.14) |
| 7α-OHC (μg/l)                | 1.50 [0.67–4.43] (1.12–2.23) | 2.01 [0.65–6.68] (1.20–2.81) |
| 7β-OHC (μg/l)                | 1.11 [0.88–3.75] (0.79–1.24) | 1.43 [0.52–4.70] (0.98–2.26) |
| 7oxo (μg/l)                  | 0.86 [0.30–2.34] (0.69–1.05) | 1.24 [0.44–4.60] (0.97–1.72) |
| 24OHC (μg/l)                 | 1.54 [0.88–3.12] (1.20–2.29) | 1.52 [0.45–7.16] (1.06–2.09) |
| 27OHC (μg/l)                 | 0.80 [0.51–3.75] (0.65–0.95) | 0.97 [0.41–6.70] (0.71–1.35) |
| 7α-OHC/cholesterol (μg/mg)   | 0.29 [0.12–0.88] (0.21–0.52) | 0.51 [0.06–1.10] (0.21–0.82) |
| 7β-OHC/cholesterol (μg/mg)   | 0.19 [0.07–0.84] (0.17–0.28) | 0.39 [0.05–1.10] (0.21–0.54) |
| 7oxo/cholesterol (μg/mg)     | 0.20 [0.06–0.45] (0.16–0.45) | 0.32 [0.04–1.47] (0.19–0.52) |
| 24OHC/cholesterol (μg/mg)    | 0.35 [0.15–0.66] (0.25–0.51) | 0.33 [0.09–1.93] (0.26–0.52) |
| 27OHC/cholesterol (μg/mg)    | 0.20 [0.02–0.39] (0.14–0.25) | 0.23 [0.05–0.64] (0.15–0.32) |

Data are given as median, range [minimum to maximum], and 25–75% confidence intervals. CSF, cerebrospinal fluid; 7α-OHC, 7α-hydroxycholesterol; 7β-OHC, 7β-hydroxycholesterol; 7oxo, 7-oxocholesterol; 24OHC, 24-hydroxycholesterol; 27OHC, 27-hydroxycholesterol.

*Significantly different from controls (P = 0.002).
*Significantly different from controls (P = 0.018).
The 7-oxygenated sterols are increased by in vitro-induced lipid peroxidation. The cerebrospinal fluid was diluted 1:1 with an aqueous NaCl solution (0.9%, v/v). At time point 0, lipid peroxidation was induced by adding 2,2'-azobis-2-amidinopropane hydrochloride at a final concentration of 5 mM. The tubes were incubated for various periods of time at air at 37°C. The reaction in individual aliquots was stopped at 2, 4, 8, 12, and 24 h by the addition of 5,5-di-tert-butyl-4-hydroxytoluene and EDTA solutions. The samples were capped, vortexed, and placed in a −20°C freezer before analysis. The levels of 7-oxygenated steroids [7α-hydroxycholesterol (7αOHC), 7β-hydroxycholesterol (7βOHC), and 7-oxocholesterol (7oxo)] were increased up to 100-fold. In contrast, the levels of 24-hydroxycholesterol (24OHC) and 27-hydroxycholesterol (27OHC) were slightly but not significantly reduced, indicating the exclusively enzymatic origin of these side chain oxysterols.

24OHC and 27OHC. The baseline cholesterol concentration (time point 0) was 4.93 mg/l.

**DISCUSSION**

In the present study, the levels of 7-oxocholesterol measured in CSF appear to be more than one thousand times lower than those reported by Diestel et al. (1). In vitro-induced lipid peroxidation of CSF using a radical generator increased the levels ~300-fold, still considerably lower than the levels reported. The very much higher levels reported previously (1) cannot be solely interpreted on the basis of an uncontrolled lipid peroxidation or cholesterol autoxidation of the samples during sample handling. In our hands, incubation at room temperature in air was found to be responsible for an increase of the 7-oxygenated oxysterols ranging between a factor of 1.4 to 2.0.

In accordance with the findings by Diestel et al. (1), the absolute and cholesterol-corrected concentrations of CSF 7-oxocholesterol were significantly lower in the control population than in patients with MS. However, in Diestel’s report, this difference was 15-fold higher for the absolute concentrations of 7-oxocholesterol, whereas in our experiments, the difference was only a factor of 1.5. A similar but statistically not significant difference was also observed in the cases of 7α-hydroxycholesterol, an enzymatic and autodiduct metabolite of cholesterol, and 7β-hydroxycholesterol, mainly formed by autoxidation. The results of the mild as well as the aggressive attempts to oxidize cholesterol in CSF samples are consistent with the contention that the side chain-hydroxylated oxysterols 24OHC and 27OHC are endogenous products of Cyp46A1 and Cyp27A1, respectively, and are not formed by autoxidation.

The slight difference in 7-oxocholesterol concentrations between the Swedish patients with MS and controls may be attributable to increased oxidative stress in the MS patients as a consequence of demyelination. However, definitive evidence for this is lacking. No significant differences were found with respect to the CSF cholesterol levels in MS patients and controls. The observed differences in the 7-oxocholesterol concentrations are thus not attributable to variations in the cholesterol levels. In addition, our data suggest that the demyelination process is not associated with a significant increase in the levels of CSF cholesterol.

At this stage, our data do not allow a conclusion regarding whether or not the increased levels of 7-oxocholesterol previously found by Diestel et al. (1) in CSF from MS patients are the result of increased in vivo lipid peroxidation or a higher sensitivity toward such oxidation in the CSF of MS patients, which is responsible for ex vivo cholesterol autoxidation.

We can only speculate about the reason for the very marked difference between our results and those of Diestel et al. (1). Cholesterol is present in great excess in all biological fluids from mammals, and there is always a high risk that autoxidation and uncontrolled lipid peroxidation may result in falsely high levels of 7-oxygenated products.

The levels of 7-oxygenated steroids reported in plasma have been markedly decreased during the last two decades in parallel with the development of more accurate methodologies (13). The levels of 7-oxocholesterol in human plasma have been reported to vary by a factor of almost 200 in some previous publications (14). However, in a recent interlaboratory comparison study in which our laboratory participated together with seven others (14), the levels of 7-oxocholesterol varied by a factor of less than 3.

In this work, we applied a highly accurate GC-MS methodology using SIM detection of the oxysterols after derivatization. HPLC with classic detection systems is not sensitive enough for the determination of oxysterols in CSF, in which the levels are at least 20 times lower than in plasma. Other detection methods use flame ionization detection instead of SIM technologies by the use of mass selective detectors; however, the latter has a markedly higher specificity and sensitivity.

To prevent cholesterol autoxidation during our sample preparation, cholesterol was rapidly separated from oxysterols by solid-phase extraction using silica cartridges before derivatization and detection. Addition of a deuterium-labeled internal standard for each single compound at the very beginning of the workup procedure was performed to ensure optimal accuracy. An internal standard should by definition behave as similar as possible with respect to extraction and derivatization. A deuterium-labeled standard appears to be ideal from this point of view.

In view of the present findings, the hypothesis of Diestel et al. (1) that 7-oxocholesterol may represent a link between demyelination and progressive damage has to be reconsidered.
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