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Plasma endocannabinoid levels in multiple sclerosis

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ABSTRACT

Background: Multiple sclerosis (MS) is a chronic inflammatory disease of the CNS. Therapies that affect the endocannabinoid (EC) system may have immunomodulatory, symptomatic and neuroprotective effects.
Aim: The aim of this study was to determine how levels of EC and related compounds are altered in MS.
Methods: Plasma and whole blood were collected from 24 MS patients (10 relapsing–remitting (RR); 8 secondary-progressive (SP); 6 primary-progressive (PP); 19 females; 25–66 years) and 17 controls (10 females; 22–62 years). Plasma EC and related compounds were quantified by liquid chromatography–tandem mass spectrometry. Fatty acid amide hydrolase (FAAH), cannabinoid receptors CB_1 and CB_2 mRNA were measured by quantitative reverse transcriptase-polymerase chain reaction.
Results: Anandamide (AEA) and palmitoylethanolamide (PEA) were higher in RRMS compared to controls ($p=0.001$ and $p=0.027$). AEA, PEA and oleoylethanolamide were also increased in SPMS plasma ($p=0.001$, $p=0.004$, and $p=0.005$). PPMS patients had higher AEA plasma levels compared to controls ($p=0.009$). FAAH mRNA was decreased in SPMS ($p=0.04$) but not in RRMS or PPMS blood. CB_1 ($p=0.012$) and CB_2 mRNA ($p=0.003$) were increased in the PPMS.
Conclusion: The EC system is altered in MS. It may be dynamically modulated depending on the subtype of the disease, but further studies with larger subgroups are needed to confirm this.

1. Introduction

Multiple sclerosis (MS), a chronic inflammatory disease of the central nervous system (CNS), is characterized by autoimmune responses against myelin proteins that lead to impairment of normal neural function. MS clinical subtypes include relapsing–remitting (RRMS), secondary-progressive (SPMS) and primary-progressive (PPMS) [1]. Cannabinoid drugs such as delta-9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol have shown therapeutic potential in controlling MS-associated neurological disturbances including pain, spasticity, and mobility impairment [2] [3]. On the other hand, cannabis-based medications may also have a negative effect on symptoms associated with MS [4]. The effects or cannabis-based medicines are largely mediated through the activation of the cannabinoid receptors, CB_1 and CB_2. These receptors are also activated by elements of the endocannabinoid (EC) system, which includes endocannabinoids (EC) anandamide (AEA) and 2-arachidonylglycerol (2-AG), EC-related compounds including palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) and EC catabolic enzymes such as fatty acid amide hydrolase (FAAH).

ECs are crucial in a variety of physiological functions [5] including metabolic processes, regulation of pain, and regulation of glutamate effects in neuronal cells [6,7]. Genetic deletion of FAAH improves motor deficits in an MS model [8]. Changes in cerebrospinal fluid (CSF) EC levels of MS patients are related to disease-induced dysregulation of EC signalling [9,10]. There is controversy about the impact of MS on levels of ECs in CSF. Increased levels of AEA but not 2-AG have been reported in MS patients and in animal model [9]. On the other hand, it was recently reported that ECs are reduced in MS and that levels of AEA and PEA, whilst still below control levels, were increased during relapse [10]. The differences may reflect disease-related level of disability. Therefore, here we measured levels of EC and EC-related compounds in plasma from MS patients and correlated these levels with disability status scores and mRNA levels of FAAH and CB_1 and CB_2 receptors.

2. Materials and methods

2.1. Subjects

Blood was collected from 24 MS patients attending the specialist MS outpatients clinic at the University Hospital Nottingham, Queen's Medical Centre (10 RRMS; 8 SPMS; 6 PPMS; 19 females and 5 males; mean age = 49, range, 25–66; mean expanded disability status scale — EDSS = 4.8; range, 1.5–6.5) and 17 healthy controls (10 females and 7 males; mean age = 43, range, 22–62) who were not significantly different in age and female:male ratio. At the time of blood withdrawal, patients were free of MS-specific immunomodulatory or immunosuppressive

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therapies including steroids. All RRMS patients were in clinical remission (no clinical relapse for >3 months prior to blood collection) and either awaiting initiation of disease modifying treatment or deemed not to be eligible for such treatment. Although patients were not tested for exogenous cannabinoid use, none of them reported the use of cannabis or cannabis-based medicine for at least 2 months prior to blood collection. Patients were also free of treatments known to have effects on the EC system for at least 2 months with the exception of 1 RRMS female (short-term course of phenytoin) and 1 SPMS male (indomethacin and rosuvastatin).

2.2. Sample preparation

Blood (4 ml) was collected in lithium heparin tubes (Becton-Dickinson, Oxford, UK) on ice, and centrifuged (10 min; 4 °C; 2000 ×*g*) within an hour of collection. 0.4 ml aliquots of the plasma were transferred into 1.5 ml tubes (Eppendorf, Hamburg, Germany) and immediately frozen by immersing into liquid nitrogen (−96 °C). Only samples without evidence of haemolysis were used. Internal standards 2-AG-d8 (1.0 nmol) and AEA-d8 (0.42 nmol) were added to thawed plasma or blank samples (0.4 ml) maintained at 4 °C. Ethyl acetate/hexane (9:1 v/v, 1.5 ml) was added to all samples that were then vortexed and centrifuged (10 min; 4 °C; 7000 ×*g*) for supernatant collection. This procedure was repeated three times and supernatants were pooled for EC recovery optimization and evaporated to dryness under nitrogen (35 °C) prior to analysis. Sample extracts reconstituted in 200 µl of acetonitrile were injected (10 µl) for analysis.

2.3. Mass spectrometry

A liquid chromatography–tandem-electrospray-ionization–mass spectrometry (LC-ESI-MS/MS) method based on the validated method of Richardson et al. [11] was used for analysis and quantification of AEA, PEA, OEA and 2-AG. Significance ($p<0.05$) in EC level differences between MS subgroups and controls was determined by unpaired *t*-test; a correction for multiple comparisons was made using false discovery rate (FDR). Relationships between EDSS scores and EC levels in all MS subgroups were assessed by bivariate Pearson's correlation and a multiple linear regression model.

2.4. Blood RNA extraction and reverse transcriptase reaction

Total RNA was extracted from the subjects' whole blood collected in Paxgene blood RNA tubes (PreAnalytix, Crawley, UK) following the manufacturer's instructions. RNA was quantified [average 97 ng/µl; range, 40–175 ng/µl] using a spectrophotometer (Nanodrop-1000 v3.3.0, USA). The concentration of RNA yielded for each sample was similar to that of previous studies [12]. Random primer (Promega, Southampton, UK), water and RNA were mixed (1:4:2.5) and incubated (70 °C, 5 min, Eppendorf). Buffer, nucleotides [10 mmol/L], ribonuclease inhibitor, reverse transcriptase (Promega), and water mixed with the annealed primers were incubated (42 °C, 1 h). cDNA was then used for qPCR assay.

2.5. Quantitative real-time polymerase chain reaction (qPCR)

cDNA in water (1:1), standards and non-template controls were added to forward and reverse primers (MWG Biotech, Ebersberg, Germany), Sybrgreen mastermix (Stratagene, La Jolla, CA, USA), and nuclease-free water. Cycling conditions were 10 min at 95 °C (denaturation), 15 s at 95 °C (annealing) and 60 s at 60 °C (extension) for 40 cycles (Mx4000, Stratagene). mRNA levels in each reaction (triplicates) were quantified using the following primers (for = forward; rev = reverse): *RPLPO for* 5′-CCA CGC TGC TGA ACA TGC T-3′; *RPLPO rev* 5′-TCG AAC ACC TGC TGG ATG AC-3′; *CB_1 for* 5′-CTG GAA CTG CGA GAA ACT G-3′; *CB_1 rev* 5′-CGC ATA CAC GAT GAA CAG AAG-3′; *CB_2 for* 5′-GCC TCT TCC CAA TTT AAA CAA C-3′; *CB_2 rev* 5′-AGT CAG TCC CAA CAC TCA TC-3′; *FAAH for* 5′-GCC TGG GAA GTG AAC AAA GGG ACC-3′; *FAAH rev* 5′-CCA CTA CGC TGT CGC ACT CCG CCG-3′. FAAH, CB_1 and CB_2 mRNA results were based on the normalised ratio to internal standard RPLPO (large ribosomal protein) and $p<0.05$ was considered significant using unpaired *t*-test.

This study was approved by the University of Nottingham Ethics Committee (healthy controls) and the Nottingham Research Ethics Committee (patients). All subjects gave informed consent to participate in the study.

3. Results

3.1. Plasma endocannabinoid levels in MS

Levels of AEA were significantly elevated in RRMS, SPMS and PPMS plasma compared to control plasma (Fig. 1A). In these relatively small clinical subgroups, levels of PEA were increased in RRMS ($p=0.027$) and SPMS plasma ($p=0.004$), but not significantly changed in PPMS plasma (Fig. 1B). Levels of OEA were only significantly elevated in SPMS plasma (Fig. 1C). 2-AG levels were unchanged in all of the MS subtype

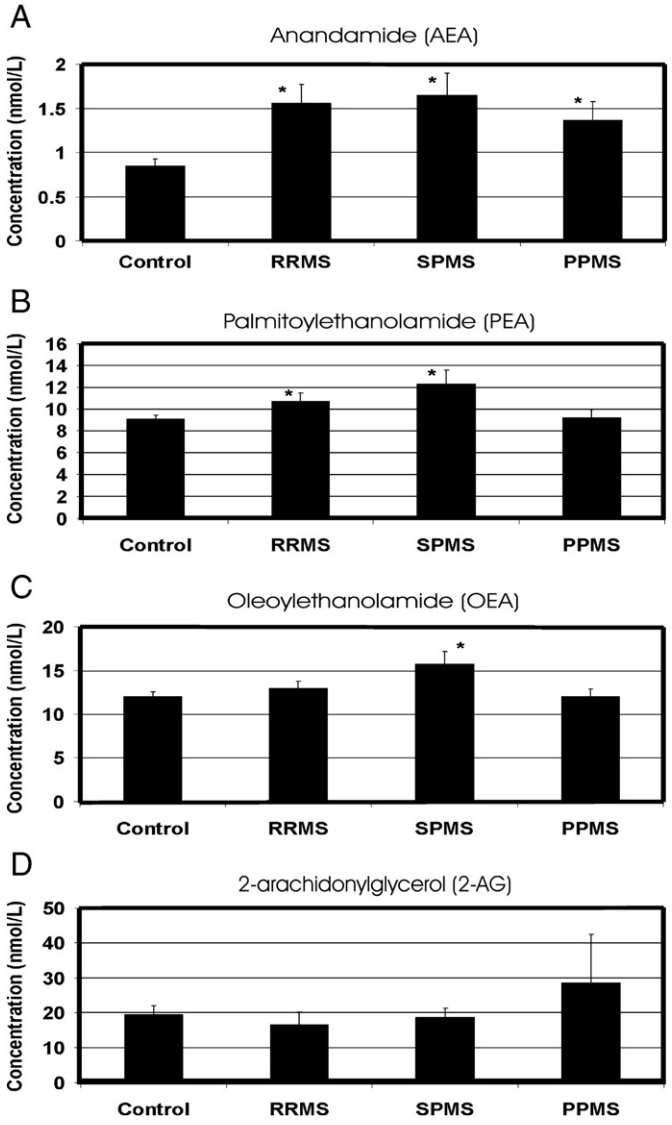

Fig. 1. A–D. Endocannabinoid levels in plasma from subgroups of MS patients versus control plasma. Data represent mean ± S.E.M. Statistical analysis used unpaired *t*-test, * = $p<0.05$.