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FULL-LENGTH ORIGINAL RESEARCH

Cerebrospinal fluid levels of the endocannabinoid anandamide are reduced in patients with untreated newly diagnosed temporal lobe epilepsy

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Summary

Purpose: The endocannabinoid system is involved in excitatory/inhibitory balance mechanisms within the central nervous system (CNS). Growing evidence shows that its perturbation leads to development of epileptic seizures in experimental models, thus indicating that endocannabinoids play an intrinsic protective role in suppressing pathologic neuronal excitability. Experimental data also demonstrate that the endocannabinoid anandamide (AEA) can antagonize epileptic discharges in hippocampal tissue. The objective of our study was to measure endocannabinoids levels in the cerebrospinal fluid (CSF) of drug-naive patients affected by temporal lobe epilepsy (TLE).

Methods: We measured the levels of both AEA and the other endocannabinoid, 2-arachidonoylglycerol (2-AG), in the CSF of drug-naive patients with TLE.

Results: A significant reduction of AEA was found in the CSF of patients with compared with healthy controls (epileptic patients = 2.55 ± 1.78 pmol/ml; healthy controls = 11.65 ± 7.53 pmol/ml; n = 9 for both groups, $p < 0.01$). 2-AG levels, however, were not affected (epileptic patients = 209.5 ± 146.56; healthy controls = 159.6 ± 110.2) (n = 6 for both groups, $p = 0.48$).

Discussion: Our findings seem to be consistent with experimental evidence demonstrating a significant prevention of epileptic seizures induced by endocannabinoids in models of epilepsy. Furthermore, they support the hypothesis that AEA may be involved in its pathogenesis, suggesting a hypothetical primary impairment of the endocannabinoid system in untreated TLE. The actual role of this in vivo dysregulation still remains unclear.

KEY WORDS: Endocannabinoids, Anandamide, 2-Arachidonoylglycerol, Temporal lobe epilepsy.

Epilepsy is related to pathologic hyperexcitability and hypersynchronous activity in large neural networks. Seizure seems to be the result of an imbalance between the two basic and antagonist neuronal properties—excitation and inhibition—toward excitation. The role of excitatory versus inhibitory mechanisms, electrical gap junctions, neuronal network oscillations, and rewiring of neuronal circuits in the pathogenesis of epilepsy is still unclear. Endogenous cannabinoids (endocannabinoids) are lipid mediators, mainly *amides* and *esters* of long-chain polyunsaturated fatty acids. They act as endogenous agonists for type-1 and type-2 cannabinoid receptors (CB1R and CB2R, respectively), thus mimicking in central and peripheral tissues the pharmacologic effects of delta-9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive ingredient of *Cannabis sativa* extracts such as hashish and marijuana (Maccarrone et al., 2007; Di Marzo, 2008). Arachidonyl ethanolamide (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) are the most studied endocannabinoids.

The endocannabinoid system is constituted of endocannabinoids, their target receptors, and the associated enzymes involved in their synthesis, transport, and degradation (Di Marzo, 2008). CB1R is the most expressed cannabinoid receptor subtype in the central nervous system (CNS), located in or near synaptic terminals (Chevaleyre et al.,

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2006). Its activation inhibits synaptic transmitter release by means of Ca^{2+}or K^+ channel modulation, and the inhibition of adenylyl cyclase (Maccarrone et al., 2007; Di Marzo, 2008). It is now widely accepted that the activation of a postsynaptic cell leads to the production of endocannabinoids, which spread in a retrograde direction across the postsynaptic membrane and the synaptic cleft. This allows the binding to and the activation of CB1Rs on presynaptic terminals. The final outcome is the inhibition of neurotransmitter release (Chevaleyre et al., 2006).

Endocannabinoids are involved in an ''on demand'' protection against seizures, as demonstrated in experimental murine models of epilepsy (Monory et al., 2006). The anticonvulsant effects of cannabinoids are mediated primarily by the CB1R activation (Wallace et al., 2001). Whether marijuana and other cannabis derivatives could have anticonvulsant properties in humans is still controversial (Consroe et al., 1975; Cunha et al., 1980; Gross et al., 2004; Mortati et al., 2007). Several cannabinoids have demonstrated anticonvulsant effects in animal models; however, proconvulsant effects have also been reported. In addition, CB1R activation in in vivo animal models by means of AEA, 2-AG, and selective synthetic agonists demonstrated significant prevention of epileptic seizure (Panikashvili et al., 2001; van der Stelt et al., 2001a,b; Wallace et al., 2001, 2002).

The anticonvulsant effects of the endocannabinoid system in experimental models may bring up some therapeutic issues. Although *Cannabis sativa* was used to treat epilepsy since antiquity (Mechoulam & Lichtman, 2003), its anticonvulsant effect was not yet demonstrated; however, although the selective enhancement of endocannabinoid levels may induce an anticonvulsant effect, the therapeutic exploitation of CB1R agonists is not viable because of their negative psychotropic effects.

Furthermore, a marked downregulation of CB1R expression and glutamatergic axon terminals equipped with this receptor in human hippocampus affected by severe refractory epilepsy has recently been reported (Ludányi et al., 2008). The downregulation was also associated with a decreased expression of a CB1R-interacting protein and the 2-AG synthesizing enzyme.

In order to determine whether the dysregulation of endocannabinoid signaling is involved in the pathogenesis of temporal lobe epilepsy (TLE) in humans, we evaluated AEA and 2-AG levels in the cerebrospinal fluid (CSF) of drug-naive patients with TLE.

Materials and Methods

Patients and controls

Peripheral blood and CSF were collected from 12 untreated inpatients (9 F, 3 M, mean age 46 ± 15.58 years, range 27–72 years) affected by partial epilepsy. Patients gave their written informed consent and were admitted to the Neurological Clinic of the University of Rome Tor Vergata to participate in the diagnostic study. On the basis of clinical, neuroradiologic [1,5 or 3 Tesla brain magnetic resonance imaging (MRI)] and electroencephalography (EEG) characteristics (interictal and ictal EEG, when available), all patients were diagnosed as affected by cryptogenic (probably symptomatic) TLE, according to International League Against Epilepsy (ILAE) criteria (Engel, 2001). Patients were included after the second or third seizure, and the delay between the first seizure and CSF collection was determined. Patients were also completely seizure-free for >24 h before CSF collection. The following exclusion criteria were applied: (1) major psychiatric or medical illnesses, (2) migraine history, (3) intake of drugs affecting the CNS, and (4) CNS disorders other than epilepsy.

Twelve control subjects (9 F, 3 M, mean age 42.71 ± 15.18 years; range 17–82 years), matched for age and sex, were enrolled; all of them were inpatients at the same clinic and underwent lumbar puncture for diagnostic purposes. In all these subjects, clinical and instrumental data excluded CNS or systemic diseases. In particular, seven patients were admitted for suspected multiple sclerosis (five with paresthesias and two with dizziness); two patients were admitted for suspected subarachnoid hemorrhage (headache), and three patients for loss of consciousness due to vasovagal (one patient) or cardiogenic syncope (two patients).

Controls were drug-free for at least 3 months and none took any medication at the time of CSF collection or had personal or family history of epilepsy. Routine determinations in patients and controls included total cell count and measurement of the concentration of total proteins and albumin, both in CSF and serum. Samples were stored at −80°C until analysis.

Participants were informed about the study purpose and procedures, and written informed consent was obtained from all subjects. The study was approved by the institutional ethics committee.

CSF determination of endocannabinoids

Human blind specimens were sent to the biochemistry laboratory. Lipids were extracted from CSF, and the organic phase was dried under nitrogen in order to evaluate AEA or 2-AG endogenous levels. The dry pellet was resuspended in 20 μl methanol; it was processed and analyzed by high performance liquid chromatography (HPLC) with fluorometric detection, as reported (Centonze et al., 2007). It should be noted that AEA and 2-AG were detected independently, using different aliquots of CSF. The amount and quality of CSF withdrawn from the both groups was suitable to detect AEA in nine samples, and 2-AG in six samples only.

Statistical analysis

Statistical analysis was performed by the nonparametric Mann-Whitney *U* test, elaborating experimental data by

means of the STATISTICA 7.0 program (StatSoft, Tulsa, OK, U.S.A.).

Results

AEA and 2-AG levels in the CSF were measured both in patients and controls. An approximately 5-fold AEA decrease was detected in the CSF of epileptic patients compared with healthy controls [mean ± standard deviation (SD) of epileptic patients = 2.55 ± 1.78 pmol/ml; mean ± SD of healthy controls = 11.65 ± 7.53 pmol/ml] ($n = 9$ for both groups, $p < 0.01$). Conversely, 2-AG was not affected (mean ± SD of epileptic patients = 209.5 ± 146.56; mean ± SD of healthy controls = 159.6 ± 110.2) ($n = 6$ for both groups, $p = 0.48$) (see Fig. 1). Demographic, clinical, EEG, and CSF data of each epileptic patient are summarized in Table 1.

Discussion

Despite the vast literature on the neuroprotective role of endocannabinoid pathways against neuronal hyperexcitability and epileptic seizures in experimental models, its failure has been poorly investigated in epileptic patients and ignored in drug-naive subjects. A growing body of evidence seems to demonstrate a significant alteration of the endocannabinoid AEA, mainly in animal model of mesial temporal epilepsy and, as a matter of fact, we found that AEA, but not 2-AG, is significantly reduced in the CSF of patients affected by TLE compared with healthy controls. This means that the two major endocannabinoids may be differentially engaged in TLE. Our narrow sample presumably encompassed both mesial and neocortical TLE. It is unfortunate that the small and heterogeneous sample did not allow us to detect any possible correlation between AEA levels and age and/or disease duration because of the low statistical power. Other parameters regarding disease severity, such as drug resistance and seizure frequency, could not be evaluated because patients were both drug-naive and newly diagnosed.

Our findings were obtained in untreated, newly diagnosed patients who were seizure-free for >24 h before CSF collection. In these patients, confounding factors such as antiepileptic drugs, drug-resistance, and seizures that may modulate ''endogenous'' antiepileptic mechanisms were excluded.

These data may be in agreement with the growing body of evidence that hypothesizes an experimental neuroprotective role of endocannabinoids (Marsicano et al., 2003) and their effective inhibition of refractory epilepsy in hippocampal neuronal culture models (Katona et al., 2006).

In addition, our findings may be consistent with the emerging concept that AEA and 2-AG have different regulatory mechanisms, and that AEA is preferentially involved in pathologic events (Marsicano et al., 2003; Monory et al., 2006; Centonze et al., 2007; Deshpande et al., 2007). Furthermore, Wallace et al. (2002) showed that AEA and its analog O-1812 act as anticonvulsants in the maximal electroshock seizure model, further implicating CB1R as a main site of seizure modulation. Similarly, Marsicano et al. (2003) confirmed the neuroprotective role of AEA, demonstrating that kainic acid treatment induced the increase of AEA in hippocampal neurons, without affecting 2-AG, thus providing ''on demand'' protection against acute excitotoxicity in CNS neurons.

On the basis of the available evidence, it can be inferred that the failure of AEA-mediated inhibition both via γ-aminobutyric acid (GABA)ergic and antiglutamatergic networks can contribute to the pathogenesis of untreated and newly diagnosed patients with TLE. In line with this, a previous study demonstrated that CB1R and AEA are involved in the control of neuronal excitability, thus reducing excitatory neurotransmission at a presynaptic site. This mechanism might be involved in the prevention of excessive excitability, leading to epileptiform activity; indeed the CB1R antagonist SR141716 blocked the inhibition evoked by exogenous cannabinoids (Ameri et al., 1999).

Because CB1R are expressed on both GABAergic interneurons (Katona et al., 1999; Chevaleyre et al., 2006) and glutamatergic hippocampal neurons (Marsicano & Lutz, 1999; Freund et al., 2003), a growing body of evidence

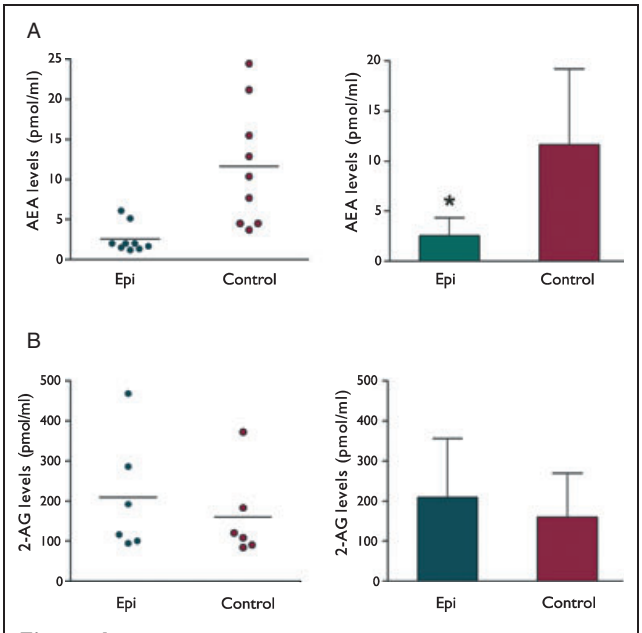

Figure 1.
AEA and 2-AG levels in cerebrospinal fluid (CSF) of epileptic and control patients. (**A**) The graphs show that AEA levels are significantly reduced in the CSF of epileptic versus control patients. (**B**) The graphs show that there is no significant difference in 2-AG levels in CSF of epileptic versus control patients.
Epilepsia © ILAE

Table 1. Demographic, clinical features, and CSF dosages

Pt	Sex	Age (years)	Delay (months)	Interictal EEG	Ictal EEG	Seizure type	AEA pmol/ml	2-AG pmol/ml
1	F	58	48	Right FT Left FT	Right FT	CPS Verbal and oral automatisms; Loss of contact	N.A.	94.18
2	F	34	48	Left T	Left T	CPS, SG; Psychomotor arrest, speech arrest, jerks, and paresthesias of right face and arm	N.A.	468.7
3	F	33	36	Right FT	Right posterior T	CPS, SG Auditory aura	N.A.	286.26
4	F	28	12	Bilateral FT with right prevalence	N.A.	CPS Epigastric aura, psychomotor arrest;	2	192.15
5	F	30	12	Right FT	N.A.	CPS, SG Psychomotor arrest;	1.35	N.A
6	F	72	48	Left T	Left T	CPS, SG Confusion followed by GTC	1.5	N.A.
7	F	60	2	Right FCT	N.A.	CPS, SG Psychomotor arrest	1.67	N.A.
8	F	58	2	Bilateral FCT	N.A.	SPS, SG Epigastric aura	5.13	N.A.
9	M	55	12	Left FT	Left T	CPS, SG Orofacial automatism, confusion	2	N.A.
10	F	27	12	Left FT	N.A.	CPS, SG Speech arrest	6.1	N.A.
11	M	58	9	Right FT Left FT	Right FT Left FT	SPS, SG Psychomotor arrest, confusion, ictal tachycardia	1.2	100
12	F	54	12	Left T	N.A.	CPS Epigastric aura, speech arrest	2	116

F, female; M, male; SPS simple partial seizure; CPS complex partial seizure; SG secondarily generalized; TLE, temporal lobe epilepsy; N.A., not available; FT, frontotemporal; FCT, fronto-centro-temporal; T, temporal.

supports the role played by endocannabinoids in key epileptogenic circuits in the hippocampus, resulting in a protective action of CB1R stimulation against kainic acid–induced seizures (Monory et al., 2006). An important issue is whether the AEA reduction occurs in glutamatergic or GABAergic cell populations. Recently, Ludányi et al. (2008) observed a decreased ratio of CB1R positive excitatory axon terminals in the epileptic human hippocampus, as similarly observed in the rodent hippocampus (Katona et al., 2006; Kawamura et al., 2006; Monory et al., 2006). In addition, these authors (Ludányi et al., 2008) provided direct anatomic evidence that CB1Rs are also located presynaptically on glutamatergic axon terminals in human hippocampus but that they were severely reduced in the hippocampal formation of epileptic patients.

Consequently, the disruption of protective endocannabinoid signaling in patients with refractory TLE may be sustained by a reduction of CB1R density in human hippocampus (Ludányi et al., 2008). These authors evaluated patients affected by drug-refractory symptomatic TLE, hypothesizing that the lack of effective treatment may be due to their severe vulnerability to perturbations of network excitability. Our sample is different because it included drug-naive, newly diagnosed patients potentially treatable affected by mesial or lateral TLE. Nevertheless, very recently Kozan et al. (2009) demonstrated a CB1R role in regulating epileptiform activity in penicillin-induced epilepsy in rats, an experimental model that resembles human focal interictal discharges (Purpura et al., 1972). As a consequence, the neuroprotective endocannabinoid signaling may be diminished regardless of severity, disease duration, drug-resistance, or seizure frequency. Furthermore, epilepsy itself may be related to an increase of glutamatergic and/or a decrease of GABAergic currents, not sufficiently antagonized by AEA. The role of endocannabinoid signaling in human epileptogenesis has to be clarified by more extensive clinical studies regarding TLE.

Despite the small sample size of this study, our findings are particularly noteworthy being ''in vivo'' data obtained from untreated epileptic patients, which is difficult to reproduce in experimental models. It is difficult to determine if the AEA decrease may be a cause or a consequence of the seizures. However, the recent history of epilepsy, the narrow number of seizures, and the seizure-free delay before

CSF collection suggest a hypothetical primary impairment of the endocannabinoid system in untreated cryptogenic TLE.

The actual role of this ''in vivo'' dysregulation remains unclear. Further studies are necessary to clarify if this pathway is involved in each type of epilepsy (i.e., primary generalized or extra temporal lobe epilepsy) or whether it is typical of TLE.

However, the ineffectiveness of conventional antiepileptic drugs in one-third of patients affected by epilepsy (French, 2007) highlights the need to develop novel therapeutic targets, where endocannabinoid system could play an intriguing role.

Acknowledgments

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Disclosure: The authors have no conflicts of interest to report.

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