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Interictal ghrelin levels in adult patients with epilepsy

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ABSTRACT

Purpose: In vitro or in animal models of epilepsy, ghrelin showed a clear anticonvulsant action, whose mechanisms are somewhat obscure. In humans however, a controversial relation exists between ghrelin and epilepsy. Yet most studies investigated just total ghrelin levels, without a proper distinction between acylated (AG) or unacylated ghrelin (UAG). We thus evaluated separately AG and UAG interictal levels in adult patients with epilepsy, and their relation to clinical features.
Method: Cross-sectional study in a tertiary referral centre. Fifty-six patients were recruited: 19 with idiopathic generalized epilepsy, 18 with cryptogenic focal epilepsy and 19 with symptomatic focal epilepsy. Twenty-six healthy subjects of similar age, sex and body mass index (BMI) acted as controls. AG and UAG levels were measured following an overnight fasting and contrasted to the clinical and biometric features.
Results: AG and UAG levels were similar between patients and controls. The AG/UAG ratio was higher in patients, also when weighted for covariates (age, BMI, gender, and drugs). Splitting patients according to their epileptic syndrome, drug-resistance or antiepileptic drug number/type resulted in no significant difference in AG, UAG or their ratio. Yet, AG and UAG levels were positively predicted by disease duration, independently by confounders.
Conclusion: In adult patients with epilepsy, interictal ghrelin levels did not differ from controls, though the AG/UAG ratio was imbalanced. Interpretation of the latter phenomenon is uncertain. Further, levels of AG and UAG were in direct proportion to disease duration, which may represent a long-term compensatory mechanism, antagonistic to the epileptic process.

1. Introduction

Ghrelin is a 28-amino acid neuropeptide discovered as the endogenous ligand of the growth hormone secretagogue receptor type 1a (GHS-R1a), which stimulates the release of growth hormone by the pituitary gland.[1–3] It acts as an orexigenic peptide promoting appetite, food intake, body weight gain and adipogenesis.[4,5] Ghrelin is produced mainly by the X/A-like cells of the oxyntic stomach mucosa and circulates in the bloodstream in two forms. Acylated ghrelin (AG) bears an acylation on the Ser3 residue, that is essential for its endocrine activities, particularly its activation of the GHS-R1a.[6] Unacylated (often termed desacylated) ghrelin (UAG) was thought not to bind to the GHSR-1a. Still it exerts metabolic, cardiovascular, and antiproliferative effects, probably by binding to different GHS-R subtypes.[6,7] However, some recent studies suggest that high doses of UAG are agonist to the GHS-R1a, at least at central nervous system (CNS) sites.[8] The discovery of the enzyme which octanoylates ghrelin, i.e. ghrelin O-acyltransferase (GOAT), has made the system more intriguing.[9]

In addition to the pituitary gland, the GHSR-1a can be found in various CNS regions, especially the hippocampus and the hypothalamus.[10,11] Probably, this widespread localization accounts for the multiple functions of ghrelin. In fact, apart from its endocrine aspects, ghrelin is also implied in memory, learning and sleep. Studies performed in vitro or in animal models of epilepsy ascribed to ghrelin clear antiepileptic properties, though the mechanisms would be somewhat obscure.[12–15] This triggered investigations on ghrelin in human epilepsy, but, to date, findings are rather contradictory. Several studies observed higher ghrelin levels, but others showed lower levels in patient groups differing

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for age, disease or treatment duration.[16] To our knowledge, the correlation with drug-resistant epilepsy has never been reported. However, most studies investigated just total ghrelin in the interictal period, which may not reflect the actual circulating distribution of AG and UAG.[16]

The primary aim of this study was to evaluate separately AG and UAG interictal levels in a cohort of adult patients with epilepsy treated with antiepileptic drugs (AEDs) compared with healthy subjects. The secondary aim was to identify if those levels were associated with any clinical features of the epileptic syndrome.

2. Methods

2.1. Subjects

Fifty-six adult consecutive outpatients (Table 1) were recruited at the Epilepsy Clinic of the Department of Neurology of the University of Piemonte Orientale and participated in this cross-sectional study, over a 6-month period (January 2013–June 2013). The diagnosis of epilepsy was made on the basis of the clinical history, imaging, and electroencephalography (EEG) findings by two experienced epileptologists not involved in the present study, according to the ILAE criteria.[17] We included patients older than 18 years, with a definite diagnosis since at least one year, who had no AED change in the past month. Metabolic inclusion criteria were: a BMI <30 kg/m^2, total cholesterol <200 mg/dl, triglycerides <200 mg/dl, glucose level <126 mg/dl. Further criteria were no evidence of gastrointestinal, endocrine or chronic illness. Female patients were recruited if they had a normal menstrual cycle, and their blood samples were collected in the follicular phase. Females on oestrogen therapy were excluded. Written informed consent was obtained from all subjects. Twenty-six healthy volunteers of similar age, sex and metabolic profile were recruited as controls (Table 1). The experimental procedures were approved by the "Maggiore della Carità" Hospital Ethics Committee and were performed in accordance with the Declaration of Helsinki.

The subject height was measured by the Harpenden stadiometer, and weight by an electronic scale. BMI was calculated as body weight divided by squared height (kg/m^2). The lipid profile (cholesterol and triglycerides) and glucose levels were determined as well. Fasting AG and UAG levels in all subjects and circulating drug levels in patients were evaluated at 8.00–8.30 a.m. following an overnight fasting. Blood plasma samples for AG e UAG measures were collected in tubes containing EDTA and a protease inhibitor (pepstatin A 10 μM), centrifuged at 3000 rpm for 15 min (temperature = 4 °C), then supernatans were stored at −20 °C. Samples for AG determination were also added of 1 mM of p-hydroxymercuribenzoic acid (PHMB) to prevent AG degradation by protease, acidified by the addition of 1 N HCl,[18] then stored at −80 °C. AG and UAG levels, expressed in pg/ml, were measured separately by ELISA kits in according to the manufacturer's instructions (BioVendor – Laboratorni Medicina a.s., Brno, Czech Republic). Sensitivity was 0.8 pg/ml and 0.6 pg/ml for AG and UAG, respectively.

Table 1
Clinical and biometric data of patients and controls.

Epileptic syndrome	Patients			Controls
	IGE	CFE	SFE	
#	19	18	19	26
Gender (%)				
Men	63.2	50.0	52.6	53.8
Women	36.8	50.0	47.4	46.2
Age, years (SEM)	33.9 (2.6)	42.5 (2.5)	46.3 (3.5)	36.4 (2.2)
BMI (kg/m^2) (SEM)	24.9 (0.7)	24.6 (0.8)	22.2 (0.7)	22.3 (0.7)
Disease duration (SEM)	16.3 (2.9)	13.9 (2.7)	15.6 (3.6)	–
Seizure frequency (%)				
Seizure-free	94.7	83.3	26.3	–
<1/month	5.3	5.6	21.1	
≥1/month	–	11.1	52.6	
Treatment (%)				
1 drug	84.2	77.8	42.1	–
2 drugs	15.8	22.2	36.8	
3 drugs	–	–	21.1	

IGE, idiopathic generalized epilepsy; CFE, cryptogenic focal epilepsy; SFE, symptomatic focal epilepsy; BMI, body mass index.

2.2. Statistics

Biometric and biochemical data were expressed as mean value ± SEM. Initially, non-parametric Wilcoxon–Mann–Whitney tests were used to compare crude means. Correlation of AG and UAG levels with continuous clinical and biochemical measures was examined using Pearson coefficients. The distribution of continuous variables was then examined for skewness, and was logarithmically transformed where appropriate, to perform an analysis of covariance (ANCOVA). ANCOVA was used to determine differences among groups. Covariates were age, gender, and BMI. The stepwise linear regression model with two-tailed probability values and 95% confidence intervals finally measured the strength of the association between AG and UAG (dependent variables) and gender, age, BMI, disease duration or seizure frequency, number and type of AEDs (independent variables). A sample of 18 individuals for each group (patients and controls) was estimated to be sufficient to demonstrate a difference of 22 pg/ml in AG with a SD of 20 with 90% power and a significance level of 95%, using the Student's *t*-test. AG was used because it has the wider standard deviation in literature. Statistical significance was assumed as $p < 0.05$ and Bonferroni corrections for multiple comparisons were used. All statistical analyses were performed with the SPSS program for Windows, version 17.0 (SPSS Inc.; Chicago, IL, USA).

3. Results

Table 1 summarizes the subject clinical and biometric data. Patients with IGE were younger than the other groups ($p < 0.01$). Of the symptomatic focal epilepsy (SFE) patients, the majority (13/19) presented with temporal lobe epilepsy (TLE) and 7 were diagnosed with hippocampal sclerosis. Thirty-eight patients were seizure-free on a monotherapy, whereas 18 were drug-resistant according to the current definition.[19] The proportion of seizure-free patients was significantly ($p < 0.01$) smaller, and that of patients with monthly seizures was significantly ($p < 0.01$) larger in the SFE group as compared to the rest. Of the drug-resistant patients, 14 were on 2 AEDs, while 4 were on 3 or more AEDs. As to monotherapy, the most common drug was carbamazepine (CBZ) ($n = 14$), followed by valproate (VPA) ($n = 12$).

Overall, AG and UAG levels were similar between patients (AG = 12.4 ± 5.3 pg/ml; UAG = 231.6 ± 37.5 pg/ml) and controls (AG = 7.4 ± 2.1 pg/ml; UAG = 196.0 ± 37.3 pg/ml). The AG/UAG ratio was higher in patients than in controls (3.7 ± 0.8 vs 2.4 ± 0.4; $p < 0.02$). This finding remained significant even when weighted for BMI, age and gender (unstandard B: 0.200 ± 0.089, 95% CI 0.022–0.378; β: 0.287, $p < 0.02$).

Splitting patients according to their epilepsy diagnosis, drug-resistance (AG, $p = 0.405$; UAG, $p = 0.129$; AG/UAG, $p = 0.369$) and AED number (AG, $p = 0.133$; UAG, $p = 0.597$; AG/UAG, $p = 0.368$) resulted in no significant difference in the levels of AG, UAG or their ratio (Table 2). Considering monotherapy patients, values of AG and UAG were similar in both the CBZ (AG = 8.8 ± 2.2 and UAG = 241.7 ± 81.2 pg/ml) and the VPA subgroup (AG = 9.5 ± 3.0 and UAG = 186.4 ± 22.4 pg/ml).

Table 2
Acylated (AG) and unacylated (UAG) ghrelin levels in patients, according to the epileptic syndrome, drug resistance and number of antiepileptic drugs (AEDs).

	Epileptic syndrome			Drug resistance		# of AEDs		
	IGE	CFE	SFE	Yes	No	1	2	>2
#	19	18	19	18	38	38	14	4
Ghrelin (pg/ml)								
AG	8.2 ± 2.2	21.9 ± 13.9	6.8 ± 2.0	25.5 ± 18.6	7.9 ± 1.3	8.3 ± 1.4	31.6 ± 24.7	10.5 ± 3.4
UAG	250.8 ± 57.7	238.7 ± 95.3	207.4 ± 34.1	291.0 ± 94.6	204.4 ± 32.8	199.6 ± 31.4	356.0 ± 120.0	108.9 ± 34.3

No significant difference in the levels of AG, UAG or their ratio between these groups was detected. Data are expressed as mean ± standard error of the mean (SEM).

In patients, AG levels were predicted by disease duration independent from sex, age, and BMI (r: 0.351, r^2: 0.124; unstandard B: 0.015 ± 0.007, 95% CI 0.001–0.029; $p < 0.01$). The same was true for UAG levels (r: 0.433, r^2: 0.188; unstandard B: 0.008 ± 0.004, 95% IC 0.001–0.015; $p < 0.02$).

4. Discussion

We report that interictal AG and UAG plasma levels in adult patients with various epilepsy forms were not different from those of healthy controls of similar age, sex and BMI. Still, the AG/UAG ratio was significantly ($p < 0.02$) higher in the population suffering from epilepsy. Finally, AG and UAG levels increased significantly ($p < 0.02$) in proportion to disease duration.

The recent literature on ghrelin and epilepsy is controversial. Berilgen et al.,[20] found higher levels of total ghrelin in patients, both with focal and generalized epilepsy. On the contrary, Dag et al.,[21] found reduced levels of total ghrelin in serum and saliva. Similarly, Aydin et al.,[22] reported on lower AG and UAG levels (in serum and saliva). However, UAG levels were calculated by subtracting AG from the total ghrelin, whereas in the present study the two forms were measured separately. Later, Aydin et al.,[23] confirmed their previous data in a cohort of patients in the immediate postictal period and after AED withdrawal. They found that changes in the two forms were time-dependent. These contrasting results could be based on heterogeneous patient features and antiepileptic treatment, different epilepsy phase (postictal vs interictal), or measurement techniques (e.g. saliva vs serum or calculated vs measured levels). Contradictory results were also obtained in paediatric populations, possibly because of additional confounding factors such as the modulation of ghrelin secretion by pubertal stage, age, weight gain or drug effects.[16]

At variance with other Authors we observed similar UAG and AG levels in patients and controls. Since we analyzed separately the two ghrelin species, we however might have depicted the (patho) physiology of the system more accurately than previous studies. Interestingly, we disclosed a higher AG/UAG ratio in patients, also when weighted for confounders. This suggests that the two forms may be imbalanced in epilepsy, as also shown in other pathologic circumstances such as the metabolic syndrome, type 1 diabetes, neonates and short post-feeding conditions.[24–26] Since both forms of ghrelin are biologically active and might interact, the AG/UAG ratio might represent a more reliable parameter than the respective levels on their own.[27] However, the biological significance of the ratio and its imbalance in epilepsy remains to be clarified.

The AED type did not influence ghrelin levels, at least in the two subgroups analyzed (VPA vs CBZ). This is somewhat in contrast to previous studies, since for instance ghrelin levels were increased in children treated with VPA in prepubertal (but not in pubertal) status.[28,29] Yet, Greco et al.,[30] found lower ghrelin levels in pubertal subjects who significantly gained weight during VPA treatment. However, if very young prepubertal children were considered separately, total ghrelin levels were decreased prior to/independent of a consistent weight gain while on CBZ and VPA.[31]

The present study has some limitations that need to be considered. First, all patients were treated with AEDs because of ethical constraints. This represents an important confounding factor and the ideal study should involve drug-naïve patients.[16] When ghrelin was measured in such patients, either with newly diagnosed IGE [22] or in the post-ictal period,[23] serum levels were lower compared with controls,[23] confirming evidence from animal models of epilepsy.[12] The second limitation was the sample size and the lack of statistical power with risk of false negative results, which possibly prevented detection of changes in the ghrelin levels among subgroups, especially between patients with and without drug-resistant epilepsy.

Still we consider it a valuable finding that disease duration significantly ($p < 0.02$) predicted UAG and AG levels. Pearson coefficients (UAG, $r = 0.433$; AG, $r = 0.351$) suggest a moderate positive association. Thus, ghrelin contribution to disease duration might not be direct and exclusive, but other factors with stronger association might be involved. However, we would speculate that disease duration basically represents the duration of the ongoing epileptogenic process, producing an imbalance between excitatory and inhibitory circuits, and possibly a longer exposure to drugs. If the anticonvulsant effects of ghrelin are mediated by GABA, the main inhibitory neurotransmitter,[13,32] higher levels of ghrelin would be required as a long-term compensatory mechanism, favouring neural inhibition.

Conflict of interest statement

None of the authors has any conflict of interest to disclose. 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.

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