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Short Communication

Decreased ghrelin and des-acyl ghrelin plasma levels in patients affected by pharmacoresistant epilepsy and maintained on the ketogenic diet

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SUMMARY

Background & aims: The gastric hormones ghrelin and des-acyl ghrelin have been found to be altered in patients treated with antiepileptic drugs. However, it is unknown if these hormones could be modified by other antiepileptic treatments, such as the ketogenic diet. Especially, a reduction in ghrelin levels could be relevant in view of the growth retardation observed under ketogenic diet treatment. For this reason we aimed to determine the changes in ghrelin and des-acyl ghrelin plasma levels in children affected by refractory epilepsy and treated with the ketogenic diet up to 90 days.
Methods: Both peptides were measured by immunoassays in plasma obtained from 16 children.
Results: Ghrelin plasma levels were progressively reduced by the ketogenic diet, reaching a minimum corresponding to 42% of basal levels after 90 days of ketogenic diet ($P < 0.05$, Duncan's test). Des-acyl ghrelin plasma levels were similarly affected, reaching minimal levels at 30 days (65% of basal levels), and maintaining a significant reduction until 90 days after the onset of ketogenic diet ($P < 0.01$ for both time intervals). No significant changes in growth were observed during the monitored period of ketogenic diet administration.
Conclusions: Ghrelin and des-acyl ghrelin are downregulated by the ketogenic diet in children affected by refractory epilepsy. Although no significant changes in growth were observed during the short time period of our investigation, the reduction in ghrelin availability may explain the reported growth retardation found in children treated with the ketogenic diet in the long-term.

1. Introduction

The peptide hormones ghrelin and des-acyl ghrelin are prevalently released by gastric P/D1 cells to modulate a variety of physiological functions, including growth, food intake and metabolism [1]. In relation to their roles, ghrelin and des-acyl ghrelin have been evaluated in metabolic diseases, or in disorders in which metabolic alterations are anyway found. To this regard, dysregulated ghrelin plasma levels were observed in patients affected by epilepsy and, for this reason, treated with antiepileptic drugs (AEDs). When considering children with epilepsy positively responding to valproate, ghrelin plasma levels were found to be reduced in association with significant body weight gain [2]. On the other hand, adults affected by epilepsy and treated with various AEDs, including valproate, presented increased ghrelin levels which were not associated with changes in body weight [3]. Other, different studies led to controversial results, as reviewed by Giordano and colleagues [4]. However, all these investigations were characterized by lack of determination of des-acyl ghrelin plasma levels. As ghrelin is rapidly converted to des-acyl ghrelin, a careful evaluation of both peptides in the same patients is required to properly understand their role [1,5].

Abbreviations: AEDs, antiepileptic drugs.

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Ghrelin and des-acyl ghrelin could also play a role in patients affected by epilepsy for their ability to modulate neuronal excitability. These peptides were tested in different seizure models and in presence of *status epilepticus* [6]. Des-acyl ghrelin was shown to increase the latency to *status epilepticus* following kainic acid treatment, and it was also able to lessen the seizure severity in the pilocarpine model. Furthermore, ghrelin was found to antagonize seizures induced by pentylenetetrazole or penicillin, as well as those scored in the kainic acid and pilocarpine models. Additionally, it has also to be considered that ghrelin is able to protect neurons from damage, an effect especially important when addressing *status epilepticus* [4,6].

Although ghrelin and des-acyl ghrelin are of utmost interest for patients affected by epilepsy, these peptides have not been evaluated in patients treated with the ketogenic diet for refractoriness to AEDs. For this reason, we evaluated ghrelin and des-acyl ghrelin blood levels in children treated with the ketogenic diet for three months.

2. Materials and methods

2.1. Experimental design

2.1.1. Patients affected by epilepsy

This observational investigation was conducted on a cohort of 16 subjects with diagnosis of refractory epilepsy [7], studied between November 2012 and June 2016. The inclusion criteria were: confirmed diagnosis of epilepsy; refractoriness to AEDs; age ranging from 0 to 14 years; informed consent signed by parents. Exclusion criteria were: acute or chronic metabolic diseases unrelated to epilepsy; lack of signed informed consent; lack of adherence to the nutritional protocol for at least 90 days.

The ketogenic diet was administered as previously described [8]. Total daily caloric intake fully corresponded to the recommended allowance of calories for child desirable weight-for-height (50th percentile) and was based on specific pathology formulas. The caloric intake was based on food diary annotated in the week preceding the ketogenic diet, adapted to specific requirements for age, sex, disease, and motor impairments. Ketosis was induced by gradually increasing the ketogenic ratio until reaching blood values above 3 mmol/L. Then, ketonemia was further increased until an optimal range for seizure control was obtained (Table 1). When weight was lower than the expected weight-for-height, the caloric need was adjusted. As the ketogenic diet is imbalanced for vitamins and minerals, a sugar-free preparation of multivitamins, iron and other oligominerals was provided to each patient. When required, supplementation with potassium citrate was also used.

Caregivers were daily assisted by the dietician, also out of the hospital. Printed instructions, a list of approximately 40 recipes, and a specific diet calculator were all provided. Glucose and ketone levels were monitored during hospitalization and, at home, by parents for the whole period of observation, as previously described [8]. Ketosis and glycemia levels were communicated once a week to dietician and referring physician by caregivers. When ketonemia was above the limit of 5.0 mmol/L, fruit juice (approximately 30 mL) was immediately administered and the ketogenic ratio reduced to prevent further episodes. Height and weight were measured before starting the diet and at the end of the observation period. Information about demographic data, clinical features, diagnostic findings and therapeutic interventions were acquired for all patients (Table 1). The Ethics Committee of Modena and the hospital medical direction approved the research protocol according to local regulations.

2.2. Quantitative analysis of ghrelin and des-acyl ghrelin

2.2.1. Reagents and materials

To block conversion of ghrelin to des-acyl ghrelin [5], we used the protease inhibitor cocktail P2714 from Sigma–Aldrich (Milan, Italy). The enzyme-linked immunosorbent assay (ELISA) kit for ghrelin (EZGRA-88K) was obtained from Merck Millipore (Milan, Italy). For des-acyl ghrelin, we initially used the Ghrelin Unacylated Human ELISA kit (RA194063400R, BioVendor, Heidelberg, Germany). This last ELISA kit was then replaced by another from Bertin Pharma (Unacylated Ghrelin (human) Easy sampling ELISA kit A05319; Montigny-le-Bretonneux, France). Only results from this second kit were used for data analysis.

2.2.2. Sample processing

Fasting blood samples (8:00–9:00 a.m.) were coded to assure a blind processing for immunoassays. Approximately 2–3 mL per donor were obtained. Blood collected in tubes with dipotassium ethylenediaminetetraacetate dihydrate and 10% (v/v) P2714 was gently shaken and quickly placed on ice, then 15 min centrifuged (1800 g at 4 °C) to obtain plasma stored into sterile microtubes (200 μL) at −80 °C.

2.2.3. Immunoassays

Immunoassays were performed according to instructions. Standards, controls and coded samples were added to plates coated with the primary antibody and incubated at room temperature (RT) for 2 h. Then, plates were washed (300 μL to each well) five times and the enzyme added (100 μL) and incubated at RT for 2 h. After rewashing, substrate solution (200 μL) was dispensed to wells. Then, plates were gently shaken in the dark at RT for at least 30 min. Each well was mixed and absorbance measured at 414 nm using a microplate reader (DTX 880 multimode detector, Beckman Coulter, USA). A cubic polynomial fitting was used to determine concentrations from the calibration curves. The intra-assay coefficient of variation (%) for ghrelin and des-acyl ghrelin was, respectively, 3.8 and 6.8. For the same peptides, the inter-assay coefficient of variation was, respectively, 7.5 and 12.5.

2.3. Statistics

Data from immunoassays were analyzed using one-way analysis of variance with repeated measures followed by Duncan's test for comparisons. Height and body mass index values were compared by the Student's paired t-test. All statistical analyses were performed using Sigmaplot 13 (Systat Software, San Jose, CA). Data are presented as mean ± standard error of the mean (SEM) and regarded significantly different at $P < 0.05$.

3. Results

The examined cohort of patients affected by refractory epilepsy was composed by 5 males and 11 female children. The mean age was 7.0 ± 0.8 years (range 2.2–13.6). The demographic and clinical features of children are illustrated in Table 1. Apart from one case (F(8), in Table 1), all patients were on drug polytherapy. In response to diet, the patients' ketosis ranged from 2 to 5 mmol/L. The reduction in seizure frequency obtained by administering the ketogenic diet was satisfactory (>50%) in 87.5% of patients. No significant effects were observed on growth, as *z* scores were −0.826 ± 0.555 at basal levels, and −0.940 ± 0.651 90 days later. The calculated *z* scores for body mass index were also unmodified (0.153 ± 0.353 at basal levels, and 0.022 ± 0.314 90 days later).

Table 1
Demographic and clinical features of patients treated with the ketogenic diet. Ketosis levels were calculated by averaging all values obtained during each week.

Sex	Age	Age at epilepsy onset	Seizure type	Etiology	Concomitant AEDs	Type of KD	Reduction in seizure frequency	β-hydroxybutyric acid (mmol/L, mean ± SEM)	Side effects
F(1)	2.2	2.2	Myocl	Genetic	LEV, TPM, CLB	Classic 2:1	<50%	3.5 ± 0.1	–
M(1)	2.6	2.5	Myocl, TC	Genetic	GVG, CBZ, NZP, CLB	Classic 3:1	>50%	4.6 ± 0.3	Hypercholesterolemia
M(2)	3	2.3	TC, drop attack	Unknown	VGB, VPA,TPX	Classic 4:1	>90%	4.5 ± 0.2	–
F(2)	9.6	8.0	Ab, spasm	Unknown	VPA, LTG, CLB	Classic 4:1	>90%	3.7 ± 0.2	Constipation
F(3)	4.2	4.2	Drop attack, Myocl, Ab	Structural	TPM, LEV	Classic 2:1	>90%	4.1 ± 0.1	Hypercholesterolemia
F(4)	5.2	5.2	TC, drop attack	Genetic	VPA, RFN, NZP	MCT 3.5:1	>90%	3.6 ± 0.1	Nausea, vomiting
M(3)	5.2	3.5	TC, drop attack	Unknown	LEV, TPM	Classic 3:1	>90%	3.3 ± 0.2	–
M(4)	5.8	5.7	TC, spasm	Metabolic	TPM, CZP	Classic 4:1	>90%	3.9 ± 0.2	Hyperphosphatemia
F(5)	6	5.7	Ab, TC	Structural	LEV, VPA	MCT 3:1	>50%	3.5 ± 0.2	Constipation
F(6)	7.2	2.6	Spasm	Metabolic	NZP, CBZ, LEV	Classic 2:1	>75%	3.9 ± 0.2	Hypercholesterolemia, hyperoxaluria
M(5)	7.3	7.3	Spasm, tremor	Genetic	CBZ, LEV	Classic 4:1	>75%	4.2 ± 0.2	–
F(7)	10.4	10.2	Spasm, drop attack, TC	Unknown	LEV, CBZ	Classic 4:1	>75%	3.7 ± 0.1	Vomiting, constipation
F(8)	9.2	9.2	Ab, spasm	Genetic	VPA	Classic 2.5:1	100%	3.6 ± 0.2	Hypercholesterolemia
F(9)	9.6	3.3	Ab	Unknown	VPA, LTG, CLB	Classic 4:1	<50%	3.9 ± 0.1	Hypertriglyceridemia, transient acidosis
F(10)	11.6	11	TC	Structural	LEV, ZSN	Classic 4:1	>50%	3.6 ± 0.1	Hypercholesterolemia, weight gain
F(11)	13.6	9.7	TC, Ab	Unknown	LEV, CZP, PB	Classic 3:1	>50%	3.3 ± 0.1	–

Abbreviations: Ab, absence; AEDs, antiepileptic drugs; CBZ, carbamazepine; CLB, clobazam; CZP, clonazepam; GVG, vigabatrin; KD, ketogenic diet; LEV, levetiracetam; LTG, lamotrigine; Myocl, myoclonus; NZP, nitrazepam; PB, phenobarbital; PCDC, pyruvate dehydrogenase complex deficiency; RFN, rufinamide; SEM, standard error of the mean; TC, tonic-clonic; TPM, topiramate; VPA, valproate; ZNS, zonisamide.

Figure 1 illustrates ghrelin and des-acyl ghrelin levels measured at basal levels and after the onset of the ketogenic diet. Specifically, we considered three time intervals by collecting samples after 15, 30 and 90 days of ketogenic diet administration. Differences in the number of the measured specimens were due to technical problems in collecting samples or to out-of-range values. Consistently, we observed a progressive decrease in ghrelin plasma levels becoming significant at the second time interval, i.e. 30 days after the beginning of the study (−49%, $P < 0.001$ vs basal levels, Duncan's test). At the end of the observation period, ghrelin plasma levels were further reduced to 42% of basal levels ($P < 0.05$ vs basal levels) (Fig. 1A).

In line with changes in ghrelin levels, des-acyl ghrelin was also reduced by the ketogenic diet, as illustrated in Fig. 1B. Overall, des-acyl ghrelin plasma levels were found to approximate 65% of control levels after 30 days of nutritional treatment ($P < 0.001$ vs basal levels). At the end of the observation period, des-acyl ghrelin levels were 80% of basal values ($P < 0.01$).

4. Discussion

We found that both ghrelin and des-acyl ghrelin were decreased by the ketogenic diet in children affected by refractory epilepsy. These changes became significant after 30 days and were maintained until the last time interval considered in our study. Indeed, acute reduction in ghrelin blood levels was recently described after the administration of ketone bodies in normal subjects [9]. Our results are in line with this observation and suggest that a more durable reduction in ghrelin production is observed with chronic ketosis.

We monitored ghrelin and des-acyl ghrelin levels for three months, so that we cannot establish if the reduction in their levels could cover the full length of the treatment protocol, usually of 2 years [10]. However, even transient changes in ghrelin levels raise important questions when occurring in children. The ketogenic diet is usually proposed to children and the reduction in ghrelin levels induced by this diet may affect the availability of growth hormone in a critical period of development. Although we did not identify significant changes in growth in the short period of observation considered in our study, growth retardation is a well-known phenomenon occurring in children maintained on the ketogenic diet [10].

A further question is the possible contribution of ghrelin and des-acyl ghrelin to the antiepileptic properties of the ketogenic diet. These neuroactive peptides were previously shown to acutely display anticonvulsant effects [4,11]. Although the role of ghrelin and cognate peptides has still to be established in the long-term, it appears improbable that a reduction in their levels could contribute to the therapeutic effects of the ketogenic diet. Other mechanisms, which include the increase in γ-aminobutyric acid (GABA) levels found in the brain under chronic ketosis [12,13], were possibly involved [4].

In conclusion, our investigation suggests that dysfunctions related to decreased ghrelin and des-acyl ghrelin availability could occur in children affected by refractory epilepsy and treated with the ketogenic diet. This possibility requires further investigations, also because our study was limited in several aspects. Indeed, we considered a small cohort with wide age range and, thus, different growth development. Polytherapy with different AEDs and sex imbalance were other sources of variability. These limitations could not be addressed because of the limited use of the ketogenic diet, generally considered as a last resource for difficult to treat epilepsy [14]. However, our data may provide an interpretation for the reported dysfunction of insulin-like growth factor-1 production in children maintained on the ketogenic diet [15]. Overall, when confirmed these findings indicate the need to address the growth hormone axis in children under nutritional treatment for epilepsy.

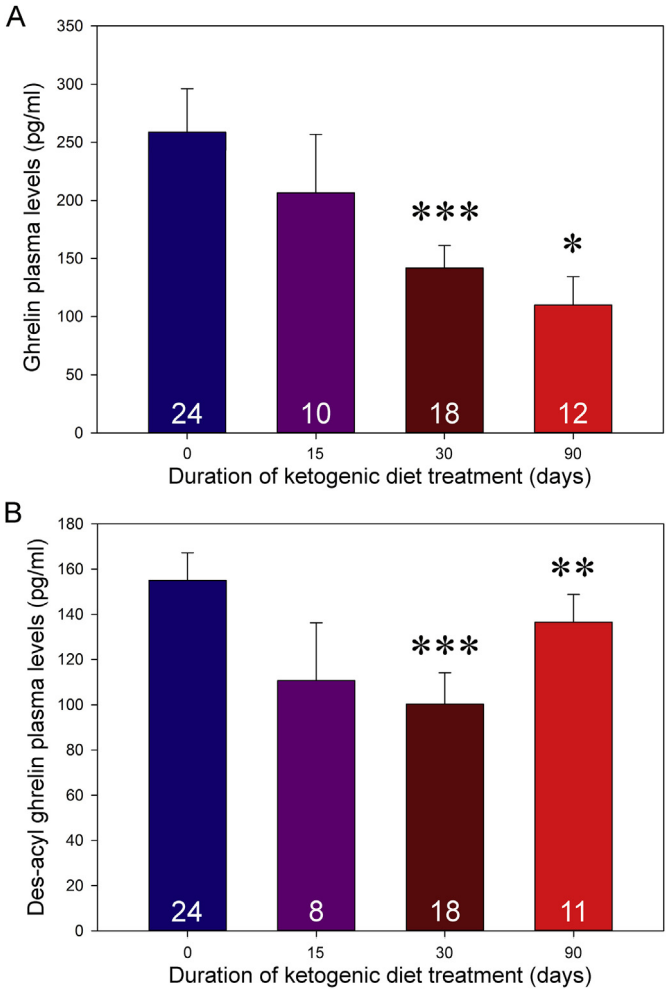

Fig. 1. Ghrelin and des-acyl ghrelin plasma levels measured in children affected by refractory epilepsy and maintained on the ketogenic diet for 90 days. (A), After 1 month of diet ghrelin levels were significantly decreased and the reduction was maintained for the remaining observation period ($^{*}P < 0.05$, $^{***}P < 0.001$ vs basal levels corresponding to 0 days, Duncan's test). (B), consistently, also des-acyl ghrelin levels were decreased in the same subjects ($^{**}P < 0.01$, $^{***}P < 0.001$ vs basal levels corresponding to 0 days. The numbers reported within each bar indicate the n of samples used for each time interval considered for immunoassays. Discrepancies in these numbers were due to technical problems or to out-of-range values.

Conflict of interest

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.

Author contribution

Concept and design of the study: G.B. Data acquisition and analysis: A.G., C.G., L.R., M.M., T.T. Drafting the manuscript and figures: G.B., M.M. All authors read and approved the final version of the manuscript.

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