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Response to creatine analogs in fibroblasts and patients with creatine transporter deficiency

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ABSTRACT

Creatine transporter (CRTR) deficiency is one of the most frequent causes of X-linked mental retardation. The lack of an effective treatment for this disease, in contrast to creatine (Cr) biosynthesis disorders that respond to Cr monohydrate (CM), led us to analyze the efficacy of a lipophilic molecule derived from Cr, creatine ethyl ester (CEE), in fibroblasts and patients with CRTR deficiency. CM and CEE uptake studies were performed in six controls and four fibroblast cell lines from patients. We found a significant increase in Cr uptake after 72 h of incubation with CEE (500 μmol/L) in patients and control fibroblasts compared to incubation with CM. Subsequently, we assayed the clinical effect of CEE administration in four patients with CRTR deficiency. After 1 year of treatment, a lack of significant improvement in neuropsychological assessment or changes in Cr level in brain ^{1}H MRS was observed, and CEE was discontinued. In conclusion, this 12-month trial with CEE did not increase the brain concentration of Cr. Our *in vitro* data lend support to the idea of a certain passive transport of CEE in both pathological and control cells, although more lipophilic molecules or other cell systems that mimic the BBB should be used for a better approach to the *in vivo* system.

Introduction

Creatine (Cr) is an essential molecule for the central nervous system that comes from dietary sources and endogenous biosynthesis, mainly in liver and pancreas [1]. Cr is transported from blood to the central nervous system by a Cr transporter protein (CRTR) that depends on Na^+ and Cl^- [2].

In humans, two inborn errors of Cr biosynthesis, arginine–glycine amidinotransferase (AGAT) [OMIM 612718] and guanidinoacetate methyltransferase (GAMT) deficiencies [OMIM 612736], and one of transport, the X-linked CRTR defect [OMIM 300352], are known. All these defects are characterized by the absence or decrease of Cr in the brain measured by *in vivo* proton magnetic resonance spectroscopy (^{1}H MRS) [3]. The main clinical features of these disorders include mild or severe mental retardation, autistic behavior, language delay, movement disorders and epilepsy [4]. CRTR deficiency is the most frequent of these; its biochemical hallmark is an increased urinary Cr/creatinine ratio, and the final diagnosis is achieved by the study of Cr uptake in fibroblasts and by the mutational analysis of *SLC6A8* gene.

Treatment of Cr synthesis defects consists of oral administration with Cr monohydrate (CM) at high doses. Supplementation with CM leads to partial restoration of cerebral Cr concentration and clinical progress in the acquisition of motor skills together with slight improvement of some cognitive functions [5]. However, CRTR deficiency is unresponsive to this treatment [3,6]. CM is a polar hydrophilic molecule that probably is not able to cross the lipid membrane of the blood–brain barrier (BBB) without the action of CRTR [7]. Therefore, other therapeutic strategies, such as administration of L-arginine [8,9], have been proposed, with contradictory effects.

Recent *in vitro* studies showed an increased content of Cr when CRTR-blocked mouse hippocampus cells were incubated with lipophilic Cr-derived compounds. These results indicate that Cr-lipophilic analogs could cross lipid membranes independently of the CRTR [10].

Abbreviations: Cr, creatine; CRTR, creatine transporter; AGAT, arginine–glycine amidinotransferase; GAMT, guanidinoacetate methyltransferase; GA, guanidinoacetate; ^{1}H MRS, proton magnetic resonance spectroscopy; CM, creatine monohydrate; CEE, creatine ethyl ester; MEM, minimal essential medium; SS, saline solution; WISC-R, Weschler Intelligence Scale for Children-Revised; VABS, Vineland Adaptive Behavior Scale; BBB, blood–brain barrier .

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Creatine ethyl ester (CEE), which is a lipophilic molecule, has been used by many athletes for enhanced performance without secondary effects [11], but the clinical effectiveness of CEE has not yet been studied. Therefore, we decided to evaluate the efficacy of CEE supplementation both in fibroblasts and in patients with CRTR deficiency.

Materials and methods

Creatine uptake assay

CM and CEE uptake studies were performed in six controls and four fibroblast cell lines from patients previously diagnosed with CRTR deficiency. Fibroblasts were cultured in minimal essential medium (MEM) supplemented with fetal bovine serum. Cr uptake was carried out following the method of Salomons et al. [12], with some modifications. Briefly, CEE and CM were dissolved in MEM to reach a final concentration of 50 and 1000 μM; these solutions were filtered with 0.22 μm Millipore and then analyzed by MS/MS to quantify the exact concentration of CM and CEE after filtering. The solutions were diluted to obtain a final concentration of 25 and 500 μM in the culture medium. Cells were incubated with 25 and 500 μM of CM and CEE for 24 and 72 h. Prior to Cr measurement, cells were washed twice with Hanks balanced salt solution (Gibco–BRL, Germany). The pellet was washed with saline solution (SS) twice and suspended in 300 μL SS and then sonicated (three times, 10 s) on ice; then the pellet was lysed by ultrasonic disruption and centrifuged for 5 min at 4 °C, 8800*g*. An aliquot of the homogenate was taken for protein measurement. Cr uptake was measured in total cell lysates by HPLC-MS/MS (Waters-Micromass Manchester, UK, model Quatro micro™ API) with a method previously described [13]. Results were expressed as pmol Cr/μg protein.

Subjects

The patients were four boys previously reported by Fons et al. [8] with genetically confirmed CRTR deficiency. Clinical data are summarized in Table 1. Six control fibroblast cell lines were obtained from our repository cell bank.

Supplementation with CEE

CEE (CEE-PRO®, Muscle-tech Laboratory, USA) was analyzed by MS/MS; the aqueous extract of the preparation showed the following composition: 74% CEE, 15% creatinine, 7% CM and 4% other minor compounds (Fig. 1). Then it was orally supplemented (0.4 g/kg/day, administered in two divided doses) for 1 year. Total daily dose was from 9 to 18 g; the dosage was chosen on the basis of the amount of Cr supplementation for AGAT- and GAMT-deficient patients, which is about 0.4 g/kg/day.

The following variables were studied twice (before and after 1 year of treatment): physical exam including weight; seizure control; Video-EEG assessment; Cr peak in brain ^{1}H MRS; neuropsychological evaluation using the Weschler Intelligence Scale for Children-Revised (WISC-R) in one of the patients presenting with the mildest phenotype; and Vineland Adaptive Behavior Scale (VABS) in the other three severely handicapped patients. All were assessed by the same pediatric neuropsychologist.

Urinary guanidinoacetate (GA), creatine/creatinine ratio and creatinine were monitored every 3 months.

Informed consent was obtained from the patients' parents. The study was approved by the Ethics Committee of the Hospital San Joan de Deu, and samples from patients and controls were obtained in accord with the Helsinki Declaration of 1964, as revised in 2001.

Statistical analysis

Statistical analysis was performed using SPSS® software (version 14.0 for Windows®) from SPSS, Inc. Student *t*-test for paired and non-paired data comparison was used to compare results obtained in fibroblast patients under different Cr incubation conditions.

Results

CM and CEE uptake in fibroblasts

Results of CM and CEE uptake in fibroblasts are shown in Table 2. Baseline values are those expected for patients with this deficiency. When control fibroblasts were incubated with different CM and CEE concentrations and temperatures, significant differences versus baseline, in almost all the situations, were observed. Nevertheless,

Table 1
Summary of clinical, neuroradiological and biochemical data prior to and after 1 year of treatment with CEE.

Data	Patient 1		Patient 2		Patient 3		Patient 4	
Age (years)	17		14		10		11	
Neurological symptoms	Severe MR, severe language disorder, autistic behavior, well-controlled epilepsy		Severe MR, severe language disorder, autistic behavior, well-controlled epilepsy		Mild MR, expressive language disorder, mild central hypotonia		Severe MR, severe language disorder, autistic behavior, refractory epilepsy	
Creatine uptake in fibroblasts % compared to control	2.5		2.6		13.3		6	
SLC6A8 mutational analysis	c.1222_1224delTTC		c.878_879delTC		c.1631C>T		c.942_944delCTT	
Effect on protein	p.Phe408del		p.Lys293fsx3		p.Pro544Leu		p.Phe315del	
	Basal	After CEE	Basal	After CEE	Basal	After CEE	Basal	After CEE
^{1}H MRS (creatine peak)	Low-near absence		Absence		Low		Low-near absence	
Neuropsychological assessment	No changes in VABS		No changes in VABS		No changes in WISC-R		No changes in VABS	
	Basal	After CEE[a]	Basal	After CEE[a]	Basal	After CEE[a]	Basal	After CEE [a]
Urinary GA (mmol/mol creatinine) RV: 26–167	56	51	80	35	120	36.7	69	42.5
Urinary creatine/creatinine RV: 0.05–1.9	2.9	8.8	3.4	5.5	4.6	8.3	3.7	7.8
Urinary creatinine (mg/dl)	50.5	64.1	49.6	106.3	52.1	106.3	63.2	118.2

MR, mental retardation; GA, guanidinoacetate; RV, reference values.
[a] Mean of values obtained every 3 months during the year of treatment.