Title:

Anticonvulsant properties of an oral ketone ester in a pentylenetetrazole-model of seizure

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Abstract

The ketogenic diet is known to have an anti-epileptic effect; in fact it is currently used to treat drug resistant epilepsies. The efficacy of this diet is thought to be correlated to the elevation of blood ketone bodies. Because of problems with compliance to this diet, there is an interest in evaluating alternative pharmacological treatments that can have anti-seizure effects by elevating ketone bodies. In the present experiment, an orally administered synthetic ketone ester (R,S – 1,3 – butanediol acetoacetate diester, or BD-AcAc2) was evaluated for its anti-seizure efficacy in a rat model.

The threshold for seizure induction with progressive intravenous infusion of pentylenetetrazole (PTZ) was evaluated in anesthetized Wistar rats two hours after a single 1 ml intragastric administration of BD-AcAc2 (i.e. 4 g/Kg b.w., treated group) or water (control group). After correction for the dose of anesthetic, the results showed that the administration of BD-AcAc2 induced an elevation of the PTZ threshold (140 ± 11 mg/Kg for the treated group, 122 ± 6 mg/Kg for the control group), along with an increased level of blood β -hydroxybutyrate (2.7 ± 0.3 mM for the treated group, 1.4 ± 0.1 mM for the control group).

This result suggests that ketone esters may pave the road towards the establishment of a “ketogenic diet in a pill”.

Key-words

ketone ester; ketogenic diet; seizure threshold; pentylenetetrazole.

Abbreviations

ANCOVA, analysis of covariance; KD, ketogenic diet; BD-AcAc2, 1,3-butanediol acetoacetate diester; CNS-OT, central nervous system oxygen toxicity; ATA, atmospheres absolute; PTZ, pentylenetetrazole; β HB, β -hydroxybutyrate; cEEG, cortical-electroencephalogram; AcAc, acetoacetate; ROS, reactive oxygen species; RNS, reactive nitrogen species

1. Introduction

The ketogenic diet (KD) is a high fat (about 90% of total dietary kcal ingested), adequate protein, and low-carbohydrate diet that is rigidly calculated and tailored to the needs of each patient. It is primarily used for the treatment of drug-resistant epilepsies in childhood and is often only implemented as a last resort. Currently, the diet is constrained to some specific foods; this leads to problems with compliance, as a consequence of unpleasant palatability.

Recently, a number of authors have proposed new formulations of KD in “tablets”, using synthetic compounds to induce the same pharmacological effects of a KD, without following its dietary restrictions (Rho and Sankar, 2008). It is widely believed that the anti-seizure effects associated with the KD are principally produced as a consequence of the metabolism of ketone bodies (notably β -hydroxybutyrate, acetoacetate and acetone). A direct anticonvulsant action has been recognized in particular for the administration of acetoacetate and acetone, as reported in some experimental models (Bough and Rho, 2007; Masino et al., 2012).

Recently, D’Agostino et al. (2013) have employed a synthetic precursor of ketone bodies in the form of a ketone diester, specifically 1,3-butanediol acetoacetate diester (BD-AcAc₂), to prevent the grand mal-like seizures induced by central nervous system oxygen toxicity (CNS-OT). These authors demonstrated a significant increase in the seizurogenic threshold of $574 \pm 116\%$ in animals treated with BD-AcAc₂ compared to water-treated controls.

In order to further evaluate the anti-seizure properties of orally administered ketone bodies, in the present study we tested BD-AcAc₂ in an adult Wistar rat model of seizures induced by progressive e.v. injection of pentylenetetrazole (PTZ).

2. Results

2.1. General experimental variables

Table A shows the mean values of several variables recorded for each experimental group. Body weight, β -ketonemia, blood glucose, urethane dose and colonic temperature can all have an effect on the seizure threshold, but all these variables did not differ significantly between the two experimental groups, allowing the comparison of the results between the two groups and supporting the conclusion about the effect of the treatment. As expected, the BD-AcAc2-treated group had significantly higher blood levels of β -hydroxybutyrate (β HB) compared to the control group at the time of PTZ infusion start ($P < 0.05$). It can be noted that β HB was already high in both groups before the gavage treatment (normal values are expected to be lower than 1.0 mM); this finding was likely due to over-night fasting, which was necessary in the present model to assure an empty stomach at the time of the gavage and to have a reproducible time course of the effect. In addition, in the water-gavaged group, β HB at the time of starting the PTZ infusion was lower than before the gavage; this is a broadly discussed effect of urethane anesthesia, which also explains the increase in blood glucose levels observed at this time in both groups (Sánchez-Pozo et al., 1988).

2.2. Seizure PTZ-threshold.

A positive and significant correlation was observed between the dose of urethane injected (g/Kg b.w.) and the PTZ-threshold (mg/Kg b.w.); the slope of the linear regression between these two variables did not differ between the two experimental groups. These findings made it mandatory and allowed to consider the dose of urethane as a covariate and to perform an analysis of covariance (ANCOVA) to correct the PTZ-threshold for the dose of urethane. No other significant correlations were observed between the PTZ-threshold and the other experimental variables of table A. The mean values of the observed PTZ-thresholds were higher in the treated group compared to the control group (table A); the ANCOVA demonstrated a significant effect for the treatment taking the urethane dose as covariate ($F_{1,13} = 5.62$, $P < 0.05$).

3. Discussion

The main results from the present study showed that the oral administration of BD-AcAc2 in Wistar rats leads to a significant increase in blood β HB and an increased latency to seizure onset compared to the control group. These data are consistent with a previous work by D'Agostino et al. (2013) who demonstrated the ability of BD-AcAc2 to induce a significant increase in blood ketones, as well as in the epileptogenic threshold in the CNS-OT-seizure model.

The choice of PTZ as a pro-convulsant agent was based on the fact that this compound is one of the most commonly employed drugs in acute convulsive seizure models, which correlate to generalized seizures in humans. In addition, this model was already used by Bough et al. (1999) to demonstrate the efficacy of KD against seizure.

While decreases in chloride conductance may underlie increased neuronal activity, it has also been reported that voltage-dependant potassium channels may play a role in seizure induction in rat (Lasoń et al., 2013). Indeed, allosteric modulation of a variety of ion channels may underlie the dysregulations seen in epileptiform discharges (Meldrum et al., 2007). Ketone bodies have previously been suggested to have an inhibiting role by several researchers. Ma et al. (2007) found that β HB and acetoacetate (AcAc), at physiological concentrations, reduce spontaneous discharges of GABAergic neurons in the rat's substantia nigra - pars reticulata, through the opening of ATP-sensitive potassium channels. In addition, Juge et al. (2010) demonstrated that AcAc, at physiological levels, is a potent inhibitor of glutamate vesicular transporters, which are pivotal for the release of the excitatory neurotransmitter glutamate. From a neurochemistry perspective, it has been demonstrated that, under ketosis, less glutamate is converted to aspartate and relatively more glutamate is converted to glutamine and GABA (Yudkoff et al., 2001, 2005).

The model used in the present study, with the intravenous infusion of PTZ and the evaluation of the seizure onset by cortical-electroencephalogram (cEEG), was chosen because, compared to the intraperitoneal administration of PTZ, it results in a smaller variability of data, allowing the use of a smaller number of animals. Because this was a preliminary experiment aimed to reveal a possible acute anti-seizure effect of BD-AcAc2, it was chosen to perform all the study under general

anesthesia to assure the lowest possible level of suffering for the animals; future studies will evaluate the long-term efficacy and toxicity of BD-AcAc2 and will establish the dose-response relation and the possible therapeutic range of the dose in awake animals. In the present model the confounding effect of urethane on seizure threshold has been taken in account and controlled: i) by demonstrating that there was not a significant difference for the mean dose of urethane between the two experimental groups (table A), ii) by correcting the PTZ-threshold for the dose of urethane and iii) by evaluating the results with the ANCOVA. It should also be pointed out that the initial cEEG discharges were associated with twitching of rats' whiskers and/or ears and/or head, confirming the clinical meaning of the cEEG discharges. Nonetheless, only the latency of the discharge on cEEG was considered for the evaluation of the seizure threshold.

The present work demonstrates similarities between the effects of dietary synthetic ketone esters and a KD. Taken together, the results of the present work suggest that ketone esters may pave the road towards the establishment of a "ketogenic diet in a pill" (Rho and Sankar 2008).

4. Experimental Procedure

4.1. Animals

Fourteen male Wistar rats (Harlan Laboratories, Udine, Italy), weighting 250 ± 15 g (mean $\pm$ S.D.), were randomly divided into two groups. Rats were housed in standard temperature and humidity conditions with a 12-h light–dark cycle (lights on from 07:00 a.m. to 07:00 p.m.). Animals were given free access to food and water until 04:00 p.m. on the day before the experiment; this assured that rats had an empty stomach at the time of the experiment, which was necessary to obtain a reproducible latency of the effect. All procedures fulfilled the requirements of the "Guide for the Care and Use of Laboratory Animals" by the National Research Council, implemented by EU and local rules; the research was approved by the local ethical committee of the University of Salerno and by the Ministry of Health of the Italian Government (prot. no. 38862, date 12-7-2013). All efforts were made to minimize suffering; rats were kept in standard housing conditions until the

moment of the experiment. All experimental procedures, including surgery and seizure-induction were performed under urethane general anesthesia; at the end of the experiments rats were still under general anesthesia and were sacrificed with overdose of anesthetic.

4.2. Surgery

On the day of the experiment, the following variables were measured and recorded for each rat: weight, blood glucose (GlucoMen LX plus, Menarini Diagnostics, Firenze, Italy) and blood β HB (Freestyle Optium, Abbott, Rome, Italy); blood drops were obtained by tail prick. Rats were then given, by gavage, 1 ml of water (group 1) or 1 ml of BD-AcAc2 (equivalent to a dose of 4g/Kg b.w.; group 2). Details on BD-AcAc2 synthesis are reported elsewhere (D'Agostino et al., 2013). Thirty min after the gavage, rats were anesthetized with 1 g/Kg b.w. of urethane (Sigma-Aldrich, Milano, Italy) with additional doses of urethane titrated until all pain reflexes were absent (tail flick, eye blink). From this time to the end of the experiment, body temperature was maintained at 36.5 ± 0.5 °C with a thermostated heating pad. The femoral vein was then accessed by inguinal incision and insertion of a polyethylene catheter (outer diameter 0.6 mm) to a depth of 6 cm. Placement of electrodes to record cEEG was achieved by percutaneous incision of the scalp and osteotomy of the calvarium. Two holes (1.4 mm in diameter) were drilled on the skull at the following coordinates: first hole at 2 mm lateral from midline and 2 mm anterior from bregma; second hole at 2 mm lateral from midline and 2 mm posterior from bregma. Two stainless steel screws (1.6 mm in diameter; Novara Metalli, Novara, Italy) were placed into these holes to a depth of 2 mm from the skull surface and served as recording electrodes. A small-needle, the reference electrode, was placed subcutaneously in the posterior of the neck.

4.3. Recording

The recording and reference electrodes were connected to a custom made digital amplifier (Viggiano and Coppola, 2014), which provided a gain of 5000 V/V, a band pass filter from 1.6 Hz

to 100 Hz and an analog-to-digital conversion with a resolution of 12 bits and a sampling frequency of 1000 samples/s. The amplifier sent the digital readings to a PC through a standard USB port; a custom software written with LabView (National Instruments, Austin, Texas, USA) provided real time visualization and recording of the cEEG for subsequent analysis and allowed manual marking of the events of interest (e.g. perfusion start and perfusion stop).

At 1h:30min from the gavage, cEEG recording was started. At 2h:00min from the gavage, blood glucose and β HB were determined, the femoral catheter was connected to a syringe-pump prefilled with 20 mg/ml of PTZ (Sigma-Aldrich, Milano, Italy), and PTZ infusion was started at a rate of 0.5ml/min; this time was chosen because it was described that at this time there is the maximum effect of BD-AcAc2 gavage on blood β HB level (D'Agostino et al., 2013). When a sustained high frequency- and high amplitude-seizure discharge was evident (greater than 0.5 mV from the zero level of the signal and lasting more than 5 s), the infusion was interrupted and recording was maintained for further 30 min. The time sequence of the protocol is summarized in Figure 1-A. For each cEEG recording, the onset-time of the seizure discharge was measured, defined as the time from infusion start to the first of the above-threshold peaks; above-threshold peaks were defined as having an amplitude greater than 0.5 mV, with inter-peak latency less than 1 s (fig. 1-B). For each rat, the PTZ-threshold for seizure induction was defined as the injected dose of PTZ at the onset-time of the seizure discharge (mg/Kg b.w.).

4.4. Statistical analysis

Data are expressed as means $\pm$ S.E. Statistically significant differences between the two experimental groups were evaluated with the Student's t-test or the ANCOVA where appropriate. For the ANCOVA, the PTZ-threshold was corrected for the dose of urethane according to the formula: $Y'n = Yn + b * (x - Xn)$, where Yn was the raw PTZ-threshold value for the n -th rat, b was the regression coefficient between the dose of urethane and the PTZ-threshold estimated from all

experimental data, $\bar{x}$ was the mean dose of urethane among all rats of all experimental groups, X_n was the dose of urethane given to the n -th rat (Winer, 1971).

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Figure legends

Figure 1. A: scheme of the time sequence of the experimental protocol. Infusion was stopped when a sustained seizure discharge was present. B: example of cEEG recordings from a water-treated rat and from a BD-AcAc2-treated rat. The start of the PTZ injection is marked with the longer arrows; PTZ was injected with a constant rate of 10 mg/min. The PTZ-threshold was evaluated by the time between PTZ infusion start (longer arrows) and the time of the first spike of a seizure-like firing (short arrows); this spike was defined as having an amplitude greater than 0.5 mV (in both positive and negative phases) and with an inter-peak latency shorter than 1 s. In these particular examples, the dose of PTZ injected until the above defined PTZ-threshold were 138 mg/Kg b.w. for the water-treated rat and 207 mg/Kg b.w. for the BD-AcAc2-treated rat.

Table A. Main general variables. BD-AcAc2: group treated by gavage with the synthetic ketone ester before seizure induction. Water: group treated by gavage with water before seizure induction. The asterisk indicates statistical significant difference between the two groups ($P < 0.05$). The PTZ threshold was corrected for the dose of urethane considered as a covariate; the statistical comparison between the two groups for this variable was done by ANCOVA.

Figure 1

A

B

treatment by gavage	BD-AcAc₂	water
weight	248±6	236±4
β-ketonemia before gavage (mM)	2.1±0.2	2.3±0.2
blood glucose before gavage (mg/dL)	95±12	95±17
urethane dose (g/kg)	1.9±0.2	1.7±0.2
colonic temperature 2h after gavage (°C)	36.4±0.1	36.3±0.3
β-ketonemia 2h after gavage (mM)	2.7±0.3	1.4±0.1 *
blood glucose 2h after gavage (mg/dL)	132±19	166±40
PTZ threshold (mg/Kg)	147±18	121±15
PTZ threshold corrected by urethane dose as covariate (mg/Kg)	140±11	122±6 *

Table A. Main general variables. BD-AcAc₂: group treated by gavage with oral ketone ester before seizure induction. Water: group treated by gavage with water before seizure induction. The asterisk indicates statistical significant difference between the two groups ($P < 0.05$). The PTZ threshold was corrected for the dose of urethane considered as a covariate; the statistical comparison between the two groups for this variable was done by ANCOVA.