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ORIGINAL ARTICLE

Intracerebroventricular administration of α-ketoisocaproic acid decreases brain-derived neurotrophic factor and nerve growth factor levels in brain of young rats

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Abstract Maple syrup urine disease (MSUD) is an inherited aminoacidopathy resulting from dysfunction of the branched-chain keto acid dehydrogenase complex, leading to accumulation of the branched-chain amino acids (BCAA) leucine, isoleucine and valine as well as their corresponding transaminated branched-chain α-ketoacids. This disorder is clinically characterized by ketoacidosis, seizures, coma, psychomotor delay and mental retardation whose pathophysiology is not completely understood. Recent studies have shown that oxidative stress may be involved in neuropathology of MSUD. However, the effect of accumulating α-ketoacids in MSUD on neurotrophic factors has not been investigated. Thus, the objective of the present study was to evaluate the effects of acute intracerebroventricular administration of α-ketoisocaproic acid (KIC) on brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) levels in the brains of young male rats. Ours results showed that intracerebroventricular administration of KIC decreased BDNF levels in hippocampus, striatum and cerebral cortex, without induce a detectable change in pro-BDNF levels. Moreover, NGF levels in the hippocampus were reduced after intracerebroventricular administration of KIC. In conclusion, these data suggest that the effects of KIC on demyelination and memory processes may be mediated by reduced trophic support of BDNF and NGF. Moreover, lower levels of BDNF and NGF are consistent with the hypothesis that a deficit in this neurotrophic factor may contribute to the structural and functional alterations of brain underlying the psychopathology of MSUD, supporting the hypothesis of a neurodegenerative process in MSUD.

Keywords Maple syrup urine disease · α-ketoisocaproic acid · Brain-derived neurotrophic factor · Nerve growth factor

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Introduction

Maple syrup urine disease (MSUD) or branched-chain ketoaciduria is an autosomal recessive metabolic disorder caused by a deficiency of the branched-chain α-keto acid dehydrogenase complex (BCKAD) activity, with a world frequency estimated in approximately 1 in 185,000 newborns (Chuang et al. 2008). The metabolic defect leads to accumulation of the branched-chain amino acids leucine, isoleucine and valine, as well as their corresponding transaminated branched-chain α-keto acids α-ketoisocaproic (KIC), α-ketoisovaleric (KIV) and α-keto-β-methylvaleric (KMV) acids in tissue and body fluids (Chuang and Shih 2001). The major clinical features presented by MSUD patients include convulsions, ketoacidosis, apnea, hypoglycemia, coma, ataxia, psychomotor delay, and mental retardation (Chuang and Shih 2001; Schonberger et al. 2004). Neuropathologic findings characteristic of the disease are cerebral edema, atrophy

of the cerebral hemispheres, spongy degeneration of the white matter, and delayed myelination (Chuang and Shih 2001; Jan et al. 2003; Klee et al. 2013; Treacy et al. 1992).

Although the mechanisms of brain damage in MSUD are still unclear, it appears that leucine and KIC are considered to be the main neurotoxic metabolites since increased plasma concentrations of these metabolites are associated with the appearance of neurological symptoms (Chuang and Shih 2001; Snyderman et al. 1964) and can reach concentrations as high as 5.0 mM in untreated patients (Zielke et al. 1996). In addition, it has been postulated that metabolites accumulation in MSUD causes brain energy deficit (Amaral et al. 2010; Howell and Lee 1963; Ribeiro et al. 2008; Sgaravatti et al. 2003), oxidative stress (Barschak et al. 2008; Barschak et al. 2009; Bridi et al. 2005; Mescka et al. 2011; Mescka et al. 2013; Scaini et al. 2012b), neuronal apoptosis (Jouvet et al. 2000a; Jouvet et al. 2000b), and increases acetylcholinesterase activity in brain (Scaini et al. 2012a). Furthermore, these metabolites are also reported to cause impairment of myelin development (Taketomi et al. 1983; Treacy et al. 1992; Tribble and Shapira 1983), reduced brain uptake of essential amino acids and subsequently disturbed neurotransmission (Araujo et al. 2001; Tavares et al. 2000; Wajner et al. 2000; Wajner and Vargas 1999; Zielke et al. 2002).

Brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) are members of the neurotrophin (NT) gene family, which play critical roles on growth, differentiation, maintenance, and synaptic plasticity in neuronal systems (Bibel and Barde 2000; Huang and Reichardt 2001). NGF serves as a neurotrophic factor for basal forebrain cholinergic neurons (Gnahn et al. 1983; Gu et al. 2009; Klein et al. 2000; Mendell et al. 2001; Nagahara et al. 2009; Sofroniew et al. 2001), while BDNF is associated with a broader spectrum of functions, promoting the functioning and survival of dopaminergic, GABAergic, noradrenergic, and serotonergic neurons (Lee et al. 2001; Lu et al. 2008; Murer et al. 2001; Soule et al. 2006; Tyler et al. 2002). Two different types of receptors expressed by responsive cells regulate the biological activity of NGF and BDNF: the specific Trk family of tyrosine protein kinases (NGF/TrkA and BDNF/TrkB) and p75 receptor that is a member of the tumor necrosis factor receptor superfamily (Lim et al. 2003; Lu et al. 2005; Meakin and Shooter 1992; Yoshii and Constantine-Paton 2010). Dysregulation of the levels of neurotrophins or their receptors, or alterations in neurotrophin function or trafficking, can damage neurons, leading to gradual neuronal degeneration (Mufson et al. 2003). Moreover, studies have shown a link between lower BDNF and NGF concentrations and neuronal and cognitive dysfunction (Belrose et al. 2014; Gelfo et al. 2011; Gu et al. 2009; Peng et al. 2004; Peng et al. 2005).

Accumulating evidence suggests BDNF and NGF as candidate molecules involved in the pathophysiology of MSUD. Scaini et al. (2013b) suggested that decreased NGF levels in hippocampus may play a role in cognitive dysfunction observed in MSUD. A recent study showed an increase in BDNF levels after chronic administration of H-BCAA, suggesting that the increase of BDNF levels had a correlation with the cognitive impairment (Scaini et al. 2013a). Moreover, it has been also shown that acute administration of H-BCAA causes an increase in the levels of pro-BDNF and a decrease tPA levels in brain of rats, suggesting that BCAA may play a role in BDNF post-translational processing (Scaini et al. 2015). However, the effect of the accumulating organic acids in MSUD on neurotrophic factors has not been so far investigated. Therefore, in order to determine the specific participation of KIC in neurotrophins levels in MSUD, the objective of the present study was to evaluate the effects of acute intracerebroventricular administration of KIC on BDNF and NGF levels in the brain of young male rats.

Materials and methods

Animals

Male Wistar rats at 30 days old (weighing 60–80 g) were obtained from the Central Animal House of the Universidade do Extremo Sul Catarinense. All rats were caged in groups of 5 with free access to food and water and were maintained on a 12-h light/dark cycle (lights on 7:00 am) at a temperature of 23 ± 1 °C. All experimental procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior recommendations for animal care, with the approval of the Ethics Committee of the Universidade do Extremo Sul Catarinense (protocol number 05/2014).

α-ketoisocaproic acid administration

Rats were anesthetized with an intraperitoneal (i.p.) injection of a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg) and thereafter placed on a stereotaxic apparatus. One small hole was drilled in the skull for microinjection, and 2 μL of a 0.8 μmol KIC solution dissolved in freshly prepared artificial CSF (aCSF) (147 mM NaCl; 2.9 mM KCl; 1.6 mM MgCl2; 1.7 mM CaCl2 and 2.2 mM dextrose) or ACFs (controls) at the same volume and concentration, was slowly injected bilaterally over 4 min into the lateral ventricle via needle connected by a polyethylene tube to a 10 μL Hamilton syringe (de Castro et al. 2004). The needle was left in place for another 1 min before being softly removed. The pH of each solution was previously adjusted to 7.4 with 0.1 N NaOH or 0.1 N HCl. The coordinates for injections were as follows: 0.6 mm posterior to bregma, 1.0 mm lateral to midline and 3.2 mm ventral from dura (Paxinos and Watson

1986). The rats were killed by decapitation without anesthesia 60 min after the intracerebroventricular administration of KIC or ACFs. The brain was rapidly excised on a Petri dish placed on an ice plate and the hippocampus, striatum and cerebral cortex were dissected.

BDNF and NGF protein levels

BDNF and NGF levels in the brain tissues [homogenized in phosphate buffer solution with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA)] were determined using a sandwich-ELISA assay with monoclonal antibodies specific for BDNF or NGF (Millipore, USA & Canada). Briefly, microtiter plates (96-well flat-bottom) were coated for 24 h with the samples (diluted 1:2 in sample diluent) and a standard curve (ranging from 7.8 to 500 pg/ml of BDNF or 15.6 to 1000 pg/ml of NGF). The plates were then washed four times with the sample diluent. After washing, a monoclonal anti-BDNF or anti-NGF rabbit antibody (diluted 1:1000) was added to each well and incubated for 3 h at room temperature. After washing again, a peroxidase-conjugated anti-rabbit antibody (diluted 1:1000) was added to each well and incubated at room temperature for 1 h. After the addition of the streptavidin-enzyme, substrate and stop solution, the amount of BDNF and NGF were determined by measuring the absorbance at 450 nm. The total protein was measured by Lowry et al. (1951) utilizing bovine serum albumin as a standard.

Immunoblotting

To perform the immunoblot experiments, the samples were first homogenized in Laemmli-sample buffer (62.5 mM Tris–HCl, pH 6.8, 1 % (*w*/*v*) SDS, 10 % (*v*/*v*) glycerol). Equal amounts of protein (30 μg/well) were fractionated by SDS-PAGE and electro-blotted onto nitrocellulose membranes. The protein loading and electro-blotting efficiency were verified with Ponceau S staining. The membranes were blocked in Tween-Tris-buffered saline (TTBS: 100 mM Tris-HCl, pH 7.5, containing 0.9 % NaCl and 0.1 % Tween-20) containing 5 % albumin. The membranes were incubated overnight at 4 °C with an antibody against pro-BDNF (Abcam - ab72440). The primary antibody was then removed, and the membranes were washed 4 times for 15 min. After washing, an anti-rabbit and anti-goat IgG peroxidase-linked secondary antibody was incubated with the membranes for 1 h (1:10,000 dilution) and the membranes were washed again. Finally, the immunoreactivity was detected using an enhanced chemiluminescence ECL Plus kit. After exposure, the membranes were stripped and incubated with a mouse monoclonal antibody to β-actin (Sigma - A2228) in the presence of 5 % milk. An anti-mouse IgG peroxidase-linked secondary antibody was incubated with the membranes for 1 h (1:10,000 dilution), and the membranes were washed again. The immunoreactivity was detected using an enhanced chemiluminescence ECL Plus kit. Densitometry was performed using the Image J v.1.34 software. SeeBlue ® Plus2 Prestained Standard (Invitrogen) was used as a molecular weight marker to make sure that the correct bands were analyzed for pro-BDNF and β-actin.

Statistical analysis

Results are presented as mean ± standard deviation. All assays were performed in duplicate, and mean was used for statistical analysis. Tests for determination of normality and equal variances were performed to examine whether our data qualified for parametric statistical tests. The data were normally distributed (Shapiro–Wilks, $p > 0.05$) with equal variances among samples (equal variances test, $p > 0.05$). Thus, Student's t test was used for the comparison of two means, and differences between the groups were considered to be significant at $p < 0.05$. All analyses were carried out on an IBM-compatible PC computer using the Statistical Package for the Social Sciences software (Armonk, New York, USA).

Results

We first investigated whether a single intracerebroventricular administration of KIC could induce changes in BDNF levels in hippocampus, striatum and cerebral cortex of young rats. We verified that the intracerebroventricular administration of KIC decreased the BDNF levels in hippocampus, striatum and cerebral cortex, when compared to the control group (Fig. 1). The assay does not allow us to distinguish between pro-BDNF and mature BDNF, as they are equally recognized by the antibody used in the ELISA. Due to the potential contrasting effects of pro-BDNF and mature BDNF, we felt that it was important to measure the level of the precursor protein in the brain. Immunoblot analysis detected a specific pro-BDNF signal (ab72440 antibody; Abcam) in the 28 kDa range.

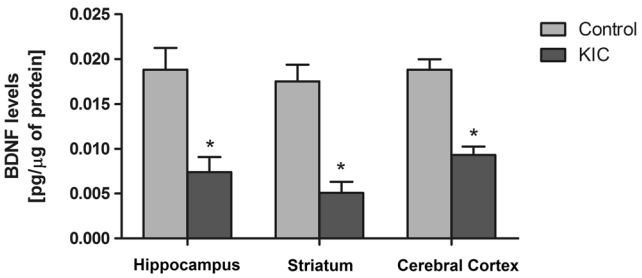

Fig. 1 The effect of acute administration of intracerebroventricular administration of α-ketoisocaproic acid (KIC) on brain-derived neurotrophic factor (BDNF) levels in the hippocampus, striatum and cerebral cortex of 30-day-old rats. Data are expressed as the mean ± standard deviation for five to six animals per group. *$p < 0.05$ compared to the control group (Student's t test)

Intracerebroventricular administration of KIC showed a trend towards a slight reduction in the protein levels of pro-BDNF in the hippocampus and cerebral cortex; however it did not reach significance (Fig. 2).

We also analyzed the effects of intracerebroventricular administration of KIC on NGF levels in the hippocampus, striatum and cerebral cortex. ELISA measurements demonstrated that NGF levels in the hippocampus were reduced after intracerebroventricular administration of KIC. On the other hand, no differences in NGF levels were observed in striatum or cerebral cortex when compared to the control group (Fig. 3).

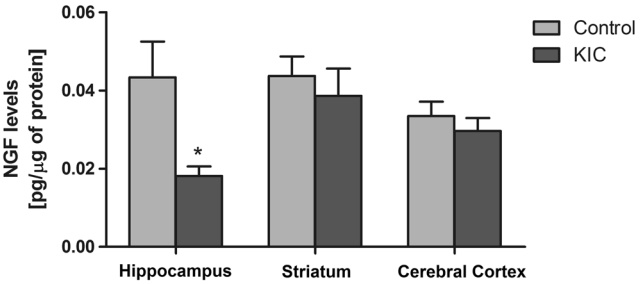

Fig. 3 The effect of acute administration of intracerebroventricular administration of α-ketoisocaproic acid (KIC) on nerve growth factor (NGF) levels in the hippocampus, striatum and cerebral cortex of 30-day-old rats. Data are expressed as the mean ± standard deviation for five to six animals per group. *$p < 0.05$ compared to the control group (Student's t test)

Discussion

Neurological sequelae are frequent in untreated patients affected by MSUD, but the mechanisms underlying the neurotoxicity in this disease are yet not well established. However, it is well known that KIC and its precursor leucine, which achieve 5.0 mM concentrations in tissues of MSUD patients, are the most toxic metabolites in this disease (Chuang and Shih 2001; Snyderman et al. 1964). We have previously demonstrated that the acute administration of BCAAs in young rats increased pro-BDNF, total-BDNF and tPA levels in striatum and decrease NGF levels in hippocampus. In the present report, we investigated the effects of the KIC, at concentrations similar to those found in MSUD patients on pro-BDNF, total-BDNF and NGF levels in hippocampus, striatum and cerebral cortex of young rats. Our present results showed that KIC provokes the most significant effects on BDNF levels in brain, since intracerebroventricular administration of KIC decrease BDNF levels in all analyzed structures, and decreased the NGF levels only in hippocampus.

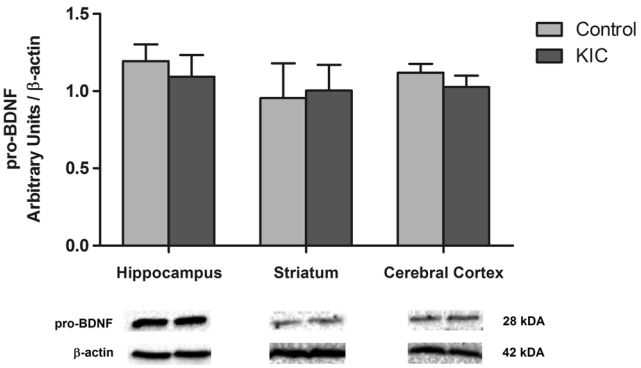

Fig. 2 The effect of acute administration of intracerebroventricular administration of α-ketoisocaproic acid (KIC) on pro-brain-derived neurotrophic factor (pro-BDNF) levels in the hippocampus, striatum and cerebral cortex of 30-day-old rats. Figure shows representative immunoblot bands in the shown groups. The ratio of the immunoblot integrated optical density (IOD) of the protein of interest to β-actin was used for statistical analysis. Data are expressed as the mean ± standard deviation for five to six animals per group

Although the exact mechanism through which KIC decreases the BDNF and NGF levels are still unknown, evidence from the literature shows that oxidative stress causes selective alterations in signalling cascades activated by BDNF and NGF, through several mechanisms, including p38 MAPK phosphorylation, decreased CREB phosphorylation or increased NF-kB DNA-binding activity (Haddad and Land 2002; Iwata et al. 1997; Zou and Crews 2006). Moreover, Jean et al. (2008) have demonstrated that glutamate, through the metabotropic glutamate receptors, also supports the production and release of BDNF from cultured basal forebrain astrocytes (Jean et al. 2008). Studies also showed that glutamate increases BDNF release in hippocampal neurons and in glial cells, such as Schwann cells and Muller cells (Canossa et al. 2001; Taylor et al. 2003; Verderio et al. 2006). Notably, oxidative stress and a decrease in glutamate levels have been implicated in the pathophysiology of MSUD, thus these findings may be closely related to the decrease in BDNF and NGF levels elicited by KIC in brain, observed in the present study.

Neurotrophins have several important effects in developing neurons, such as controlling neurite outgrowth, differentiation and cell survival, as well as synaptic plasticity and function (Huang and Reichardt 2001). Moreover, neurotrophins have been implicated in influencing the dynamic and complex signals that occur between neurons and glial cells that regulate myelination (Cellerino et al. 1997; Chan et al. 2004; Du et al. 2006; Vondran et al. 2010; Walsh et al. 1999; Xiao et al. 2010). Furthermore, studies demonstrate the importance of the reciprocal synergistic relationship between NGF and BDNF and acknowledge their potential implications in myelin formation and repair (Canossa et al. 1997; Michael et al. 1997; Reichardt 2006). Additionally, NGF and BDNF also play key roles in the fine-tuning of learning and memory performances (Mu et al. 1999; Rantamaki et al. 2013). Studies have shown that BDNF and NGF mRNA and protein are decreased in post-mortem brain tissue from subjects with AD, and these reduction correlates with the degree of cognitive impairment

(Garzon et al. 2002; Gelfo et al. 2011; Hock et al. 2000; Mufson et al. 2003; Peng et al. 2005).

It has been reported that BCAA can cause demyelination and impair memory performance (de Castro et al. 2004; Glaser et al. 2010; Scaini et al. 2012c; Taketomi et al. 1983; Treacy et al. 1992; Tribble and Shapira 1983; Vasques et al. 2005; Walsh and Scott 2010). Taking together these findings and the present results, we suggest that the effects of KIC on demyelination and memory processes may be mediated by reduced trophic support of BDNF and NGF. Moreover, lower levels of BDNF and NGF are consistent with the hypothesis that a deficit in this neurotrophic factor may contribute to the structural and functional alterations of brain underlying the psychopathology of MSUD, supporting the hypothesis of a neurodegenerative process in MSUD.

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