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ORIGINAL PAPER

Inhibition of Brain Energy Metabolism by the Branched-chain Amino Acids Accumulating in Maple Syrup Urine Disease

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Abstract In the present work we investigated the in vitro effect of the branched-chain amino acids (BCAA) accumulating in maple syrup urine disease (MSUD) on some parameters of energy metabolism in cerebral cortex of rats. $^{14}CO_2$ production from [1-^{14}C]acetate, [1-5-^{14}C]citrate and [U-^{14}C]glucose, as well as glucose uptake by the brain were evaluated by incubating cortical prisms from 30-day-old rats in the absence (controls) or presence of leucine (Leu), valine (Val) or isoleucine (Ile). All amino acids significantly reduced $^{14}CO_2$ production by around 20–55%, in contrast to glucose utilization, which was significantly increased by up to 90%. Furthermore, Leu significantly inhibited the activity of the respiratory chain complex IV, whereas Val and Ile markedly inhibited complexes II–III, III and IV by up to 40%. We also observed that trolox (α-tocopherol) and creatine totally prevented the inhibitory effects provoked by the BCAA on the respiratory chain complex activities, suggesting that free radicals were involved in these effects. The results indicate that the major metabolites accumulating in MSUD disturb brain aerobic metabolism by compromising the citric acid cycle and the electron flow through the respiratory chain. We presume that these findings may be of relevance to the understanding of the pathophysiology of the neurological dysfunction of MSUD patients.

Keywords Maple syrup urine disease · Leucine · Valine · Isoleucine · Energy metabolism

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Introduction

Maple syrup urine disease (MSUD), or branched-chain ketoaciduria, is an inborn error of metabolism caused by a severe deficiency of the mitochondrial enzyme complex branched-chain L-2-keto acid dehydrogenase (BCKD) activity [1]. The metabolic defect leads to accumulation of the branched-chain amino acids (BCAA) isoleucine (Ile), leucine (Leu) and valine (Val), and the corresponding branched-chain 2-keto acids (BCKA) L-2-ketoisovaleric (KIV), L-2-ketoisocaproic (KIC) and L-2-keto-3-methylvaleric (KMV) acids [1, 2]. The hydroxyl derivatives L-2-hydroxyisovaleric, L-2-hydroxyisocaproic and L-2-hydroxy-2-methylvaleric acids, produced by the reduction of their respective L-2-keto acids, also accumulate, but to a lesser extent [3].

The clinical features of MSUD include ketoacidosis, failure to thrive, poor feeding, apnea, ataxia, seizures, coma, psychomotor delay and mental retardation [4]. Severe brain edema and cerebral atrophy are usually seen in MSUD patients. In this scenario, the pyramidal tracts of the spinal cord and the myelin content around the dentate nuclei, the corpus callosum and the cerebral hemispheres are most affected [1].

The severity of the disease ranges from the severe classical form to mild variants including the thiamine-responsive patients [1, 5]. Neurological sequelae are

present in most patients, but the mechanisms underlying the brain damage are not well established. However, Leu and KIC are considered the main neurotoxins in this disorder since increased plasma concentrations of these compounds (up to 5.0 mM) are associated with the appearance of neurological symptoms [1, 6–8]. In addition, it has been postulated that demyelination [3, 9, 10], neurotransmitter disturbances [11–14], reduced brain uptake of essential amino acids [15], induction of oxidative stress [16–18], apoptosis [19] and energetic deficit [8, 20–24] may be related to the brain injury of MSUD.

We have previously found that the BCKA accumulating in MSUD inhibit brain energy metabolism [23–24]. Thus, the present study was undertaken to investigate the in vitro influence of the BCAA which accumulate in MSUD on important parameters of energy metabolism, such as glucose uptake, $^{14}CO_2$ production from [1-14C] acetate, [1,5-^{14}C] citrate and [U-^{14}C] glucose, as well as on the activities of the respiratory chain complexes I–IV, citric acid cycle (CAC) and pyruvate dehydrogenase in cerebral cortex of young rats. We also tested the effects of α-tocopherol and creatine on the inhibitory effects provoked by the BCAA on the respiratory chain complexes. The main objective of the present investigation was to contribute to the understanding of the mechanisms underlying the neurological symptoms and cortical atrophy present in MSUD patients.

Experimental procedure

Reagents

All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA, except for the radio labeled compounds [1-^{14}C] acetate, [1,5-^{14}C]citrate, [U-^{14}C] glucose and [1-^{14}C] pyruvate, which were purchased from Amersham International, UK.

Animals

A total of 94 thirty-day-old Wistar rats obtained from the Central Animal House of the Departamento de Bioquímica, ICBS, UFRGS, were used. Rats were kept with dams until weaning at 21 days of age. The animals had free access to water and to a standard commercial chow and were maintained on a 12:12 h light/dark cycle in an air-conditioned constant temperature (22 ± 1°C) colony room. The "Principles of Laboratory Animal Care" (NIH publication 85-23, revised 1985) were followed in all experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

Tissue preparation

Thirty-day-old rats were sacrificed by decapitation, the brain was rapidly removed and the cerebral cortex was isolated and cut into two perpendicular directions to produce 400 μm wide prisms using a McIlwain chopper. Prisms were pooled, weighed and used for $^{14}CO_2$ production, glucose uptake and for the determination of the respiratory chain enzyme activities. Cortical prisms were exposed at 37°C to the BCAA, after which the assays were carried out. We also measured the activities of the respiratory chain complexes, as well as of some enzymes of the citric acid cycle and pyruvate dehydrogenase in homogenates (1:10, w/v) from rat cerebral cortex prepared in SETH buffer, pH 7.4 (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 UI/ml heparin). The homogenates were centrifuged at 800 × *g* for 10 min and the supernatants kept at –70°C until used for enzyme activity determination. Finally, mitochondrial fractions prepared according to Cassina and Radi [25] were used for the determination of complex I activity. The period between homogenate preparation and enzymatic analysis was always less than 5 days, whereas complex I activity was measured on the day of the mitochondria preparation. The various parameters of energy metabolism were determined after pre-incubation and in the presence of 1–5 mM of Ile, Leu or Val according to standard methods. Control groups did not contain the BCAA in the incubation medium.

$^{14}CO_2$ production

Cortical prisms (50 mg) were added to small flasks (11 cm^3) containing 0.5 ml Krebs–Ringer bicarbonate buffer, pH 7.4. Flasks were pre-incubated in a metabolic shaker at 37°C for 15 min (90 oscillations. min^{-1}). After pre-incubation, 0.2 μCi [1-^{14}C] acetate and 1.0 mM of unlabeled acetate were added to the incubation medium. In some experiments, we added 1 mM coenzyme A to the incubation medium. We also measured CO_2 production from [U-^{14}C]glucose (0.2 μCi) or [1,5-^{14}C] citrate (0.2 μCi) in the presence of 5.0 mM unlabeled glucose or 1.0 mM unlabeled citrate, respectively. Ile, Leu or Val was added to the incubation medium at final concentrations of 1.0–5.0 mM. The flasks were gassed with a O_2/CO_2 (95:5%) mixture and sealed with rubber stoppers and Parafilm M. Glass center wells containing a folded 65 mm/5 mm piece of Whatman 3 filter paper were hung from the stoppers. After 60 min of incubation at 37°C in a metabolic shaker (90 oscillations. min^{-1}), 0.1 ml of 50% trichloroacetic acid was added to the medium and 0.1 ml of benzethonium hydroxide was added to the center of the wells with needles introduced through the rubber stopper. The flasks were left to stand for 30 min to complete $^{14}CO_2$

trapping and then opened. The filter papers were removed and added to vials containing scintillation fluid, and radioactivity was counted [26]. Results were expressed as percentage of controls.

Pyruvate dehydrogenase (PDH) activity

Homogenates (1:10, w/v) prepared in Krebs-Ringer bicarbonate buffer, pH 7.4, were added to small flasks (11 cm^3) in a volume of 425 µl. Flasks were pre-incubated at 35°C for 15 min in the absence or presence of 5 mM Leu and 5 mM KIC in a metabolic shaker (90 oscillations. min^{-1}) with 625 µM *n*-dodecyl-*β*-D-maltoside in order to permeabilize the mitochondrial membranes. After pre-incubation, [1-^{14}C] pyruvate (0.065 µCi) plus 1.0 mM unlabeled pyruvate were added to the incubation medium. The total volume of incubation was 500 µl. The flasks were gassed with a O_2/CO_2 (95:5) mixture and sealed with rubber stoppers and Parafilm M. Glass center wells containing a folded 60 mm/4 mm piece of Whatman 3 filter paper were hung from the stoppers. After 60 min incubation at 35°C in a metabolic shaker (90 oscillations. min^{-1}), 0.2 ml of 50% trichloroacetic acid was supplemented to the medium and 0.1 ml of benzethonium hydroxide was added to the centre of the wells with needles introduced through the rubber stopper. The flasks were left to stand for 30 min to complete $^{14}CO_2$ trapping and then opened. The filter papers were removed and added to vials containing scintillation fluid, and radioactivity was counted [26]. Results were expressed as percentage of controls.

Citric acid cycle (CAC) enzyme activities

Maximal activities of the CAC enzymes were achieved by freeze-thawing three times the homogenates. The activity of succinate: phenazine oxireductase (soluble succinate dehydrogenase-SDH) was determined as described by Sorensen and Mahler [27], whereas the activities of NAD-specific isocitrate dehydrogenase (isocitric acid dehydrogenase, ICDH), citrate synthase (CS) and α-ketoglutarate dehydrogenase were accessed by the methods of Plaut [28], Shepherd and Garland [29] and Humphries and Szweda [30], respectively. These activities were measured in cortical homogenates in the presence or absence of 5 mM Leu, 1.0 mM Ile or 1.0 mM Val and calculated as nmol min^{-1} mg $protein^{-1}$.

Respiratory chain enzyme activities

Maximal activities of the respiratory chain complexes I, II and II–III were obtained by freeze-thawing three times the homogenates or mitochondrial preparations, whereas maximum complex IV activity occurred in the presence of lauryl maltoside according to standard methods. The activities of succinate-2,6-dichloroindophenol (DCIP)-oxidoreductase (complex II) and succinate:cytochrome *c* oxidoreductase (complex II–III) were determined in homogenates from cerebral cortex according to Fischer et al. [31]. The activity of ubiquinol:cytochrome *c* oxidoreductase (complex III) and of cytochrome *c* oxidase (complex IV) were assayed in cortical homogenates according to the method described by Birch-Machin et al. [32] and Rustin et al. [33], respectively. NADH dehydrogenase (complex I) activity was measured in mitochondrial preparations from cerebral cortex. Complex I was determined by the rate of NADH-dependent ferricyanide reduction at λ = 420 nm (ε = 1 mM^{-1} cm^{-1}) according to Cassina and Radi [25]. The methods described to measure these activities were slightly modified, as described in details in a previous report [34]. The BCAA Leu, Ile or Val, at 1.0–5.0 mM concentrations, were initially exposed to cortical prisms for 1 h at 37°C after which the homogenates were prepared and the techniques carried out. In some experiments, 1 mM α-tocopherol or creatine were co-incubated with the BCAA. We also measured the activities of the various respiratory chain complexes in the presence of the BCAA added at the beginning of the enzymatical assays without previous preincubation. The activities of the respiratory chain complexes were calculated as nmol min^{-1} mg $protein^{-1}$ or µmol min^{-1} mg $protein^{-1}$. Some results were expressed as percentage of controls.

Glucose uptake

Cortical prisms (50 mg) were first pre-incubated at 37°C for 15 min in the presence of 5 mM Leu, 1 mM Ile or 1 mM Val and then incubated under a O_2/CO_2 (95:5) mixture at 37°C for 60 min in Krebs–Ringer bicarbonate buffer, pH 7.0 containing 5.0 mM glucose (in a total volume of 0.5 ml) in a metabolic shaker (90 oscillations min^{-1}). Glucose was measured in the medium before and after incubation by the glucose oxidase method [35]. Glucose uptake was determined by subtracting the amount after incubation from the total amount measured before incubation [36]. In some experiments, the NMDA antagonist MK-801 (10 µM) was used alone or in the presence of 5 mM Leu. Results were calculated as µmol glucose h^{-1} g $tissue^{-1}$ and expressed as percentage of controls.

Protein determination

Protein was measured by the method of Lowry et al. [37] using bovine serum albumin as standard.

Statistical analysis

All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software using a PC-compatible computer. Unless otherwise stated, results are presented as means ± standard error of the mean. All assays were performed in duplicate or quadruplicate and the mean was used for statistical analysis. Data were analyzed using the one-way analysis of variance (ANOVA) followed by the post-hoc Duncan multiple range test when F was significant. For analysis of dose-dependent effect, linear regression was used. The Studenťs t-test for paired samples was also used for comparison of two means. Only significant values are shown in the text. Differences between the groups were rated significant at $P < 0.05$.

Results

BCAA inhibit $^{14}CO_2$ production from acetate, citrate and glucose in rat cortical prisms

We tested the influence of the BCAA on $^{14}CO_2$ production from different labelled substrates in cortical homogenates. Twenty rats were used in these experiments. Figure 1A shows that all BCAA significantly inhibited CO_2 production from [1-^{14}C] acetate at doses of 1 mM and higher with a maximal inhibition of around 55% [$F(6,35) = 9.374$, $P < 0.001$]. These effects were dose-dependent for Leu ($P < 0.001$), Ile ($P < 0.05$) and Val ($P < 0.05$). Furthermore, the addition of 1 mM coenzyme A in the medium did not prevent Leu inhibitory effect on acetate oxidation, suggesting that shortage of coenzyme A was not responsible for Leu-induced inhibitory action (results not shown). We also verified that Leu (5 mM) significantly inhibited CO_2 formation from [1,5-^{14}C] citrate (30% inhibition) [$t(6) = 4.136$, $P < 0.01$] (Fig. 1B). These data suggest that the citric acid cycle activity was reduced by the BCAA and that these compounds do not compete with acetate for the monocarboxylic mitochondrial membrane carrier to enter mitochondria. Finally, CO_2 generation from glucose was inhibited (25% inhibition) by 5 mM Leu [$t(8) = 2.805$, $P < 0.05$] (Fig. 1C).

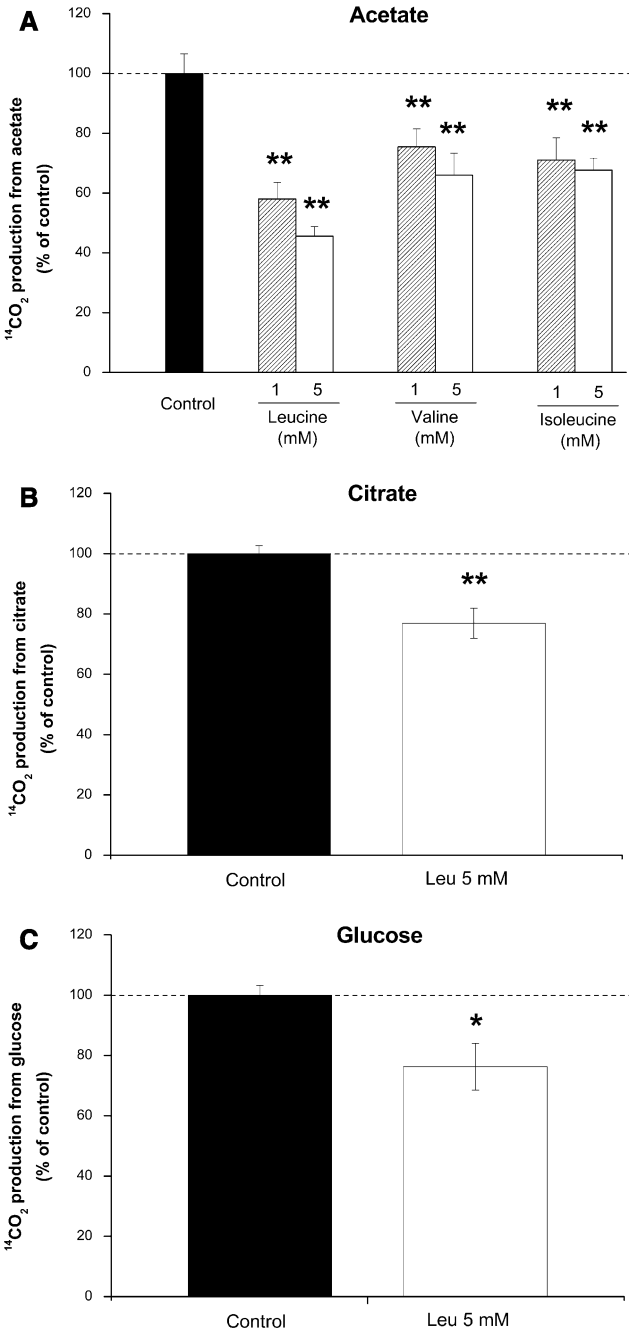

Fig. 1 In vitro effect of leucine, valine and isoleucine on $^{14}CO_2$ production from acetate (**A**) in cerebral cortex from young rats. The effect of leucine on $^{14}CO_2$ production from citrate (**B**) and glucose (**C**) are also shown. Data represent means ± S.E.M. for six independent experiments (animals) performed in duplicate and are expressed as percentage of control. Control values ranged from 299 to 499 nmol CO_2/h/g tissue. *P<0.05, **$P < 0.01$ compared to control (Duncan multiple range test)

BCAA do not alter pyruvate dehydrogenase and key citric acid cycle (CAC) enzyme activities

Cerebral cortex was obtained from 28 rats in order to determine the activities of pyruvate dehydrogenase, citrate synthase, isocitrate dehydrogenase and succinate dehydrogenase and α-ketoglutarate dehydrogenase.

We tested the effect of Leu on pyruvate dehydrogenase (PDH) activity in order to evaluate whether Leu-induced-inhibition of glucose oxidation could be due to inhibition of this critical enzyme. The Leu derivative keto acid KIC was also added to the assays. We observed that 5 mM Leu did not affect PDH activity, whereas 5 mM KIC significantly

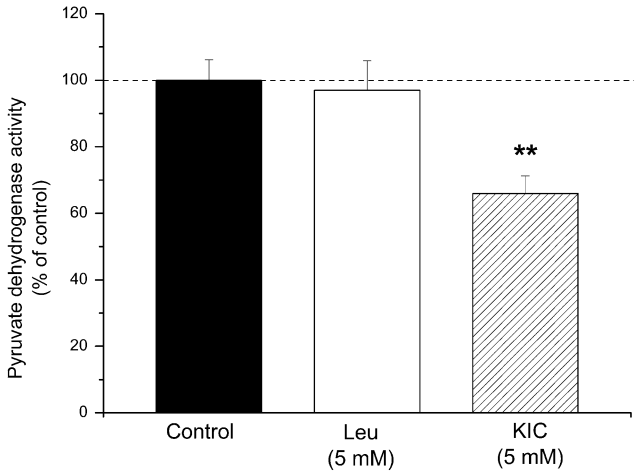

Fig. 2 In vitro effect of leucine (Leu) and -ketoisocaproic acid (KIC) on pyruvate dehydrogenase activity in cerebral cortex from young rats. Data represent means ± S.E.M. for five independent experiments (animals) performed in duplicate and are expressed as percentage of control. Control values ranged from 470 to 680 nmol CO_2/h/g tissue. $^{**}P < 0.01$ compared to control (Duncan multiple range test)

reduced (35% inhibition) this activity [$F(2,12) = 7.458$, $P < 0.01$] (Fig. 2). Furthermore, we verified that the activities of citrate synthase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase and succinate dehydrogenase were not altered by 5 mM Leu, 1 mM Ile or 1 mM Val (Table 1).

BCAA inhibition of the respiratory chain in rat cerebral cortex homogenates is probably mediated by reactive oxygen species

The next set of experiments were performed to evaluate the effect of Leu, Val and Ile on the activities of the respiratory chain complexes I–IV since the inhibitory effects of these amino acids on $^{14}CO_2$ production could be secondary to an impaired mitochondrial electron transport. Cerebral cortex prisms from 31 animals were exposed to 1–5 mM of the BCAA for 1 h and the respiratory chain complex activities measured afterwards. It can be seen in Fig. 3 that Val and Ile significantly inhibited the activities of complexes II–III, III and IV, whereas Leu significantly inhibited the activity of complex IV (complex II–III: $F(6,55) = 3.456$, $P < 0.01$); complex III: $F(6,35) = 3.733$, $P < 0.01$; complex IV: $F(6,50) = 3.519$, $P < 0.01$). Complex I activity measured in mitochondrial preparations was not altered by the presence of the BCAA. Interestingly, when these amino acids were added at the beginning of the assays no alteration of the respiratory chain activities was detected in the brain homogenates and mitochondrial preparations (Table 2).

Previous reports have shown that BCAA elicit oxidative stress in the brain [17]. Therefore, in order to evaluate whether reactive species were involved in the inhibitory effects elicited by these compounds on the activities of respiratory chain complexes II–III, III and IV, we co-incubated cerebral cortex prisms in the presence of the BCAA (5 mM) and trolox (1 mM, soluble vitamin E) or creatine (1 mM). We observed that trolox (Fig. 4) and creatine (Fig. 5) were able to fully prevent the inhibitory effects induced by the BCAA, indicating that they were probably mediated by the generation of reactive species.

BCAA stimulate glucose uptake by rat cortical prisms

We also investigated the in vitro effect of Ile, Leu and Val on glucose uptake by rat cerebral cortex since glucose is the main substrate for brain metabolism. For these experiments a total of 12 rats were used. As can be observed in Fig. 6A, Ile, Leu and Val, at the maximal plasma concentrations found in MSUD patients (5 mM Leu and 1 mM Val and 1 mM Ile), significantly increased glucose uptake by cortical prisms with an average stimulation around 90% [$F(3,20) = 3.28$, $P < 0.05$], as compared to controls. These results indicate that the major metabolites that accumulate in MSUD activate glucose utilization by the brain. Since glutamate stimulates cerebral glucose utilization [38, 39] and the nitrogen of the BCAA is used to form glutamate by

Table 1 In vitro effect of isoleucine (Ile), leucine (Leu) and valine (Val) on key enzyme activities of the citric acid cycle in rat cerebral cortex homogenates

	Control	Ile (1 mM)	Leu (5 mM)	Val (1 mM)
Citrate synthase	289 ± 15.1	304 ± 20.2	304 ± 14.8	303 ± 15.3
Isocitrate dehydrogenase	34.1 ± 4.02	35.6 ± 3.66	33.8 ± 2.77	33.8 ± 2.22
Succinate dehydrogenase	3.43 ± 0.27	3.57 ± 0.35	3.70 ± 0.44	3.74 ± 0.37
α-Ketoglutarate dehydrogenase	74.5 ± 9.93	66.8 ± 10.9	80.0 ± 12.5	85.0 ± 13.7

Values are expressed as nmol min^{-1} mg^{-1} protein and represent mean ± SEM for five independent experiments (animals) per group for citrate synthase, isocitrate dehydrogenase, succinate dehydrogenase and α-ketoglutarate dehydrogenase activities in cortical homogenates in the presence or absence (control) of the BCAA. There were no significant differences between the various groups for each enzyme activity (one-way ANOVA)

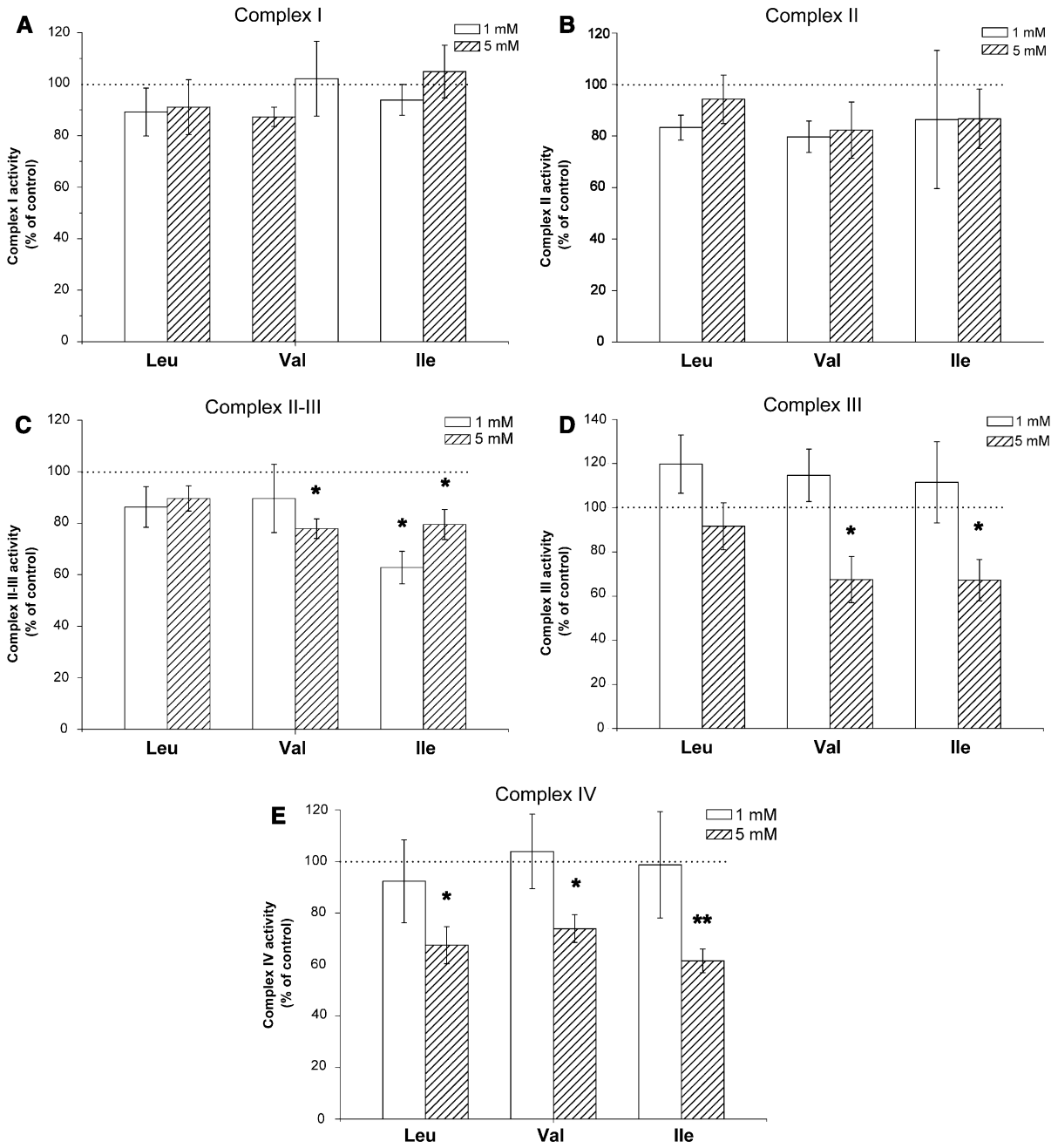

Fig. 3 In vitro effect of leucine (Leu), isoleucine (Ile) and valine (Val) on the respiratory chain complexes I (panel A), II (panel B), II–III (panel C), III (panel D) and IV (panel E) activities in cerebral cortex prisms pre-incubated at 37°C for 1 h. Data represent means ± S.E.M. for five to six independent experiments (animals) performed in duplicate and are expressed as percentage of control. $^{*}P < 0.05$, $^{**}P < 0.01$, compared to control (Duncan multiple range test)

transamination in this tissue [40, 41], we tested whether Leu-induced stimulatory effect on glucose uptake could be secondary to overstimulation of glutamate NMDA receptors. We observed that the NMDA antagonist MK-801 did not prevent Leu-elicited increase of glucose uptake by cortical slices, making this hypothesis unlikely [$F(3,20) = 38.6$, $P < 0.0001$] (Fig. 6B).

Leu is not significantly converted to α-ketoisocaproic acid under our experimental conditions

We finally tested whether KIC could be generated from Leu in significant amounts using the same incubation system employed in the assays. We observed that 5 mM Leu incubated for 1 h under our experimental conditions

Table 2 In vitro effect of isoleucine (Ile), leucine (Leu) and valine (Val) on the activities of the respiratory chain complexes in cerebral cortex of young rats

Respiratory Chain Complexes	Control	Ile (1 mM)	Leu (5 mM)	Val (1 mM)
Complex I	0.71 ± 0.06	0.73 ± 0.08	0.67 ± 0.04	0.70 ± 0.03
Complex II	2.88 ± 0.23	3.34 ± 0.29	3.24 ± 0.43	3.07 ± 0.30
Complex II–III	12.3 ± 0.46	12.0 ± 0.32	12.5 ± 0.46	12.3 ± 0.73
Complex III	27.1 ± 1.43	24.7 ± 2.42	29.4 ± 2.09	29.3 ± 4.17
Complex IV	309.8 ± 28.2	329.6 ± 26.9	297.6 ± 31.8	314.4 ± 25.3

Data represent mean ± SE for four to five independent experiments (animals) performed in duplicate and are expressed as μmol min^{-1} mg^{-1} protein for Complex I and as nmol min^{-1} mg^{-1} protein for complexes II, II–III, III and IV. The enzymatic analyses were measured in the absence (control) or presence of the BCAA. Homogenates were used for determining the activities of complexes II, II–III, III and IV, whereas mitochondrial preparations were used to measure complex I activity. There was no significant difference between the respiratory chain activities of the various groups for each enzymatic complex (one-way ANOVA)

(10-fold diluted homogenates) did not give rise to significant amounts of KIC (less than 1% conversion was detected by gas chromatography/mass spectrometry) (results not shown), suggesting that the amount of BCAA transaminases in our system was low.

Discussion

The pathophysiology of the neurological dysfunction and brain atrophy of MSUD patients seems to be multiple and still poorly known [1, 4]. Although alterations of energy metabolism caused by the BCAA and particularly the BCKA accumulating in this disorder have been reported [20–24], the exact mechanisms underlying the bioenergetic dysfunction are poorly known. Therefore, in the present study we investigated the role of the BCAA, at similar concentrations as those found in serum and CSF of MSUD patients, on important parameters of energy metabolism in cerebral cortex of young rats.

We first investigated the activity of the CAC by measuring CO_2 generated from acetate. It was verified a significant and dose-dependent reduction of CO_2 formation by over 50% in cortical prisms incubated in the presence of the BCAA, being Leu the amino acid with the greatest inhibitory action. The addition of coenzyme A in the medium did not prevent Leu inhibitory effect on acetate oxidation (results not shown), indicating that shortage of coenzyme A due to a competition between acetate and leucine or one of its breakdown products for coenzyme A is unlikely. The inhibitory action of the BCAA on acetate utilization cannot also be explained by a competition between the BCAA or its keto acid byproducts for the mitochondrial monocarboxylic transporter, since a similar Leu-induced inhibition of CO_2 formation was achieved with citrate that crosses the mitochondrial membrane via the tricarboxylic carrier and generates CO_2 after only two reactions catalyzed by aconitase and isocitrate dehydrogenase. Thus the reduction of CO_2 formation caused by the BCAA probably represents a true inhibition of the CAC and indicates a blockage of the aerobic metabolism.

CO_2 generation from glucose was also significantly reduced by Leu, reflecting a compromised aerobic glycolysis in rat brain. Furthermore, the Leu-induced decrease of glucose oxidation was not due to an inhibition of the pyruvate dehydrogenase complex activity, although α-ketoisocaproic acid (KIC), the byproduct of Leu, significantly decreased (by 35%) this activity. We cannot however attribute Leu effect to a competition between KIC derived from Leu and pyruvate originated from glucose for the monocarboxylic acid transporter since in our experimental conditions insignificant amounts of Leu (less that 1%) are converted to KIC. These results are in accordance with previous data showing an inhibition of pyruvate dehydrogenase caused by KIC but not by Leu [42].

We also observed that the BCAA did not alter the activity of key enzymes that control CAC activity, namely citrate synthase, isocitrate dehydrogenase, succinate dehydrogenase and α-ketoglutarate dehydrogenase. However, we cannot rule out that other activities of the CAC not measured here, such as aconitase, fumarase or malate dehydrogenase, were inhibited by the BCAA.

On the other hand, blockage of the CAC could be also secondary to an inhibition of oxidative phosphorylation. Thus, we carried out experiments in order to evaluate the effect of the BCAA on the respiratory chain function by measuring the activities of complexes I to IV in cerebral cortex of rats. We verified that when cerebral cortex was exposed for 1 h to the BCAA, Leu significantly inhibited complex IV, whereas Val and Ile markedly inhibited complexes II–III, III and IV activities by up to 40%. In contrast, complex I and II activities were not altered by the BCAA. Furthermore, trolox (α-tocopherol) and creatine totally prevented the inhibitory effects on the respiratory chain complex activities when cortical prisms were simultaneously incubated with these antioxidants and with

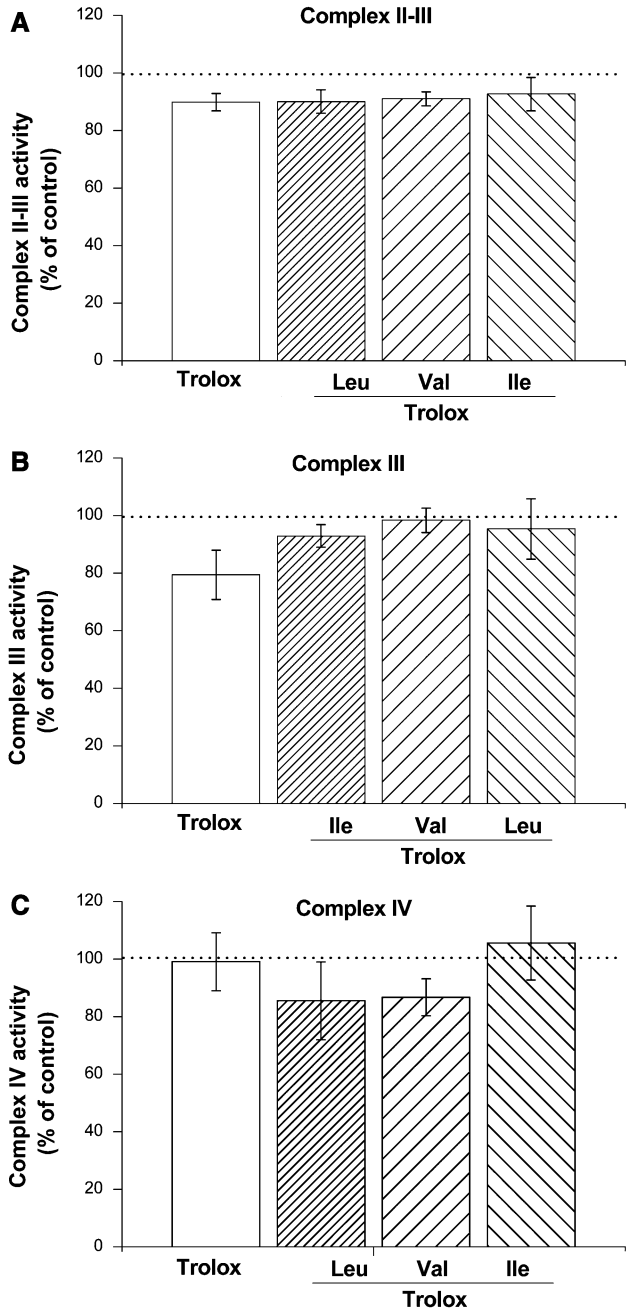

Fig. 4 In vitro effect of 1 mM trolox (soluble vitamin E) on the respiratory chain complexes II–III (panel A), III (panel B) and IV (panel C) activities in cerebral cortex homogenates pre-incubated at 37°C for 1 h in the presence of 5 mM of leucine (Leu), valine (Val) or isoleucine (Ile). Data represent means ± S.E.M. for four or five independent experiments (animals) performed in duplicate and are expressed as percentage of control. There were no significant differences between control and the other groups (ANOVA)

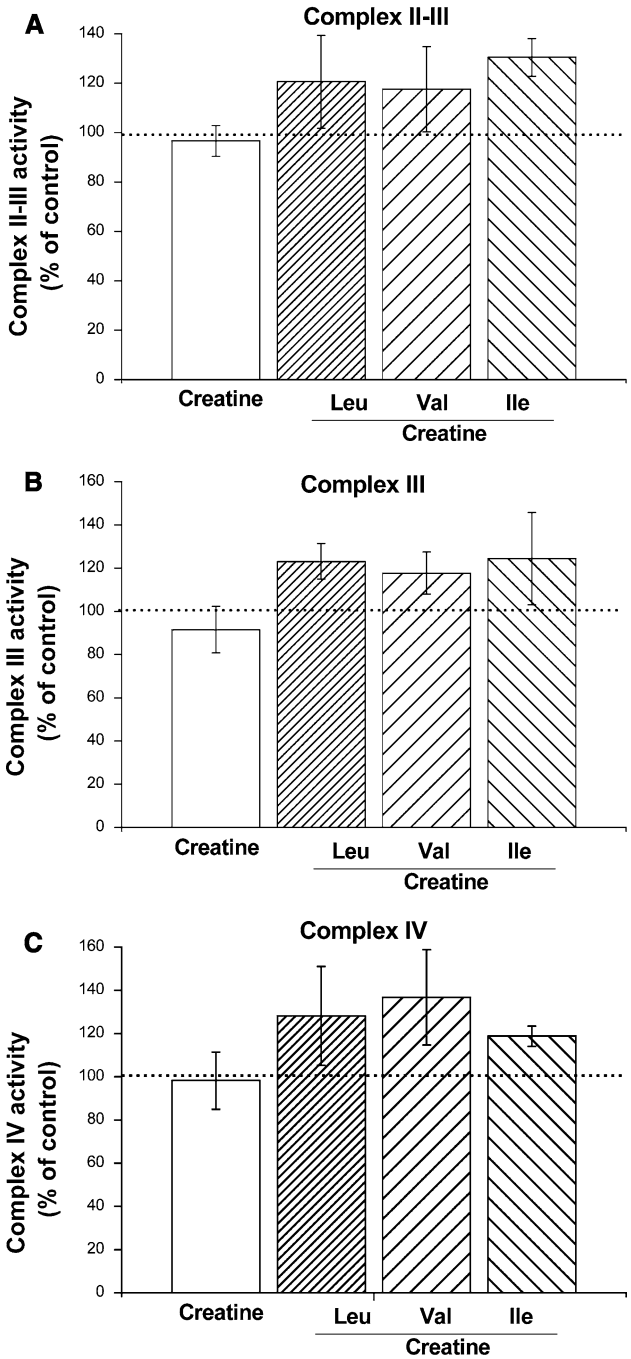

Fig. 5 In vitro effect of 1 mM creatine on the respiratory chain complexes II–III (panel A), III (panel B) and IV (panel C) activities in cerebral cortex homogenates pre-incubated at 37°C for 1 h in the presence of 5 mM of leucine (Leu), valine (Val) or isoleucine (Ile). Data represent means ± S.E.M. for four or five independent experiments (animals) performed in duplicate and are expressed as percentage of control. There were no significant differences between control and the other groups (ANOVA)

the BCAA, suggesting that free radicals were involved in these effects. This is in line with the observations that the mitochondrial electron transport chain and particularly complexes II, III and IV are vulnerable to oxidative insult [43–45] and that the BCAA induce oxidative stress in vitro [17]. Taken together, we cannot exclude the possibility that the inhibition of various steps of the respiratory chain by the BCAA resulted in an increased NADH/NAD^+ ratio,

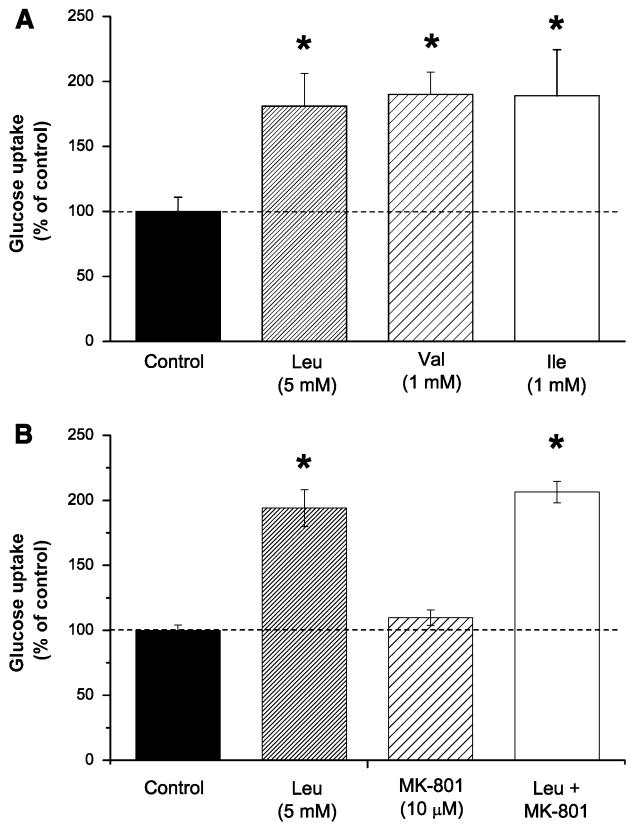

Fig. 6 In vitro effect of leucine, isoleucine and valine on glucose uptake by cerebral cortex from young rats (**A**). MK-801 was also used in some experiments in the absence or presence of Leu and glucose uptake measured (**B**). Data represent means ± S.E.M. for six independent experiments (animals) performed in duplicate and are expressed as percentage of control. Control values ranged from 153 to 288 μmol glucose/h/g tissue. *$P < 0.05$, compared to control (Duncan multiple range test)

leading secondarily to a reduced CO_2 formation since NADH is an alosteric inhibitor of key steps of the CAC.

We also observed that Leu, Val and Ile markedly increased glucose uptake (up to 90%) by rat cerebral cortex prisms *in vitro*, indicating that these metabolites stimulated the transport and/or utilization of this substrate by the brain. Since the BCAA and mainly Leu donates the nitrogen for glutamate synthesis in the CNS [40, 41], and glutamate activates brain glucose metabolism via NMDA receptor activation [38, 39], this effect could be indirectly mediated by glutamate formation. We observed that the blockage of NMDA receptors by MK-801 did not prevent the Leu-induced increase of glucose uptake by brain prisms, indicating that this effect was probably due to a distinct mechanism than NMDA receptor stimulation. Alternatively, enhanced glucose utilization by the brain could be due to stimulation of anaerobic glycolysis in which lower energy (ATP) outcome is achieved and more substrate (glucose) is necessary to compensate in order to keep cell homeostasis.

Furthermore, even though we did not measure lactate release by brain cortical prisms in the presence of the BCAA, the data on increased glucose uptake, decreased $^{14}CO_2$ production and reduced respiratory chain enzyme activities strongly suggest an activation of anaerobic glycolysis. Our present results are in agreement with previous in *vivo* studies demonstrating high concentrations of lactate and BCAA in brain of MSUD patients during acute metabolic attacks returning to normal values after clinical recovery, which is indicative of mitochondrial dysfunction during metabolic decompensation [46–48]. Other studies performed in diaphragm muscle rat cells demonstrating that Leu stimulates lactate and pyruvate release reinforce this hypothesis and suggest that stimulation of anaerobic glycolysis may indeed occur in the rat brain [42]. Finally, it was also demonstrated that the BCAA accumulating in MSUD stimulate insulin-independent glucose uptake in rat skeletal muscle [42, 49].

On the other hand, we cannot establish at the present whether the degree of inhibition of the CAC and the electron transfer chain caused by the BCAA would alter ATP synthesis. However, considering that oxidative phosphorylation is the main pathway responsible for ATP production, and since these metabolites cause apoptosis in glial and neuronal cells in vitro and in vivo in a dose- and time-dependent manner associated with a significant reduction in cell respiration [19] and also inhibit creatine kinase activity [23, 50], it is likely that the BCAA may provoke significant cellular energy deficit.

In conclusion, we report for the first time that the BCAA accumulating in MSUD inhibit the electron transport chain at various steps at the concentrations usually found in the affected individuals. This probably explains previous reports of impaired energy production caused by these metabolites, as identified by lower CO_2 production [22, 51–55] and provides a novel biochemical mechanism, i.e. a compromised oxidative phosphorylation, by which brain bioenergetics is affected by the BCAA [8, 20–23]. Although it is difficult to extrapolate our findings to the human condition, in case the in vitro inhibition of brain energy metabolism caused by the metabolites that most accumulate in MSUD also occurs under in vivo conditions, it is conceivable that lack of energy may be involved in the neurological symptoms present in MSUD patients. Interesting observations are that these patients present hypoglycemia, cerebral edema and high brain lactate levels, particularly during metabolic decompensation, when the levels of the BCAA dramatically increase [1, 46], reflecting a failure of the active ionic transport necessary to maintain the normal volume of neural cells.

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