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Impairment of energy metabolism in hippocampus of rats subjected to chemically-induced hyperhomocysteinemia

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Abstract

Homocystinuria is an inherited metabolic disease biochemically characterized by tissue accumulation of homocysteine (Hcy). Mental retardation, ischemia and other neurological features, whose mechanisms are still obscure are common symptoms in homocystinuric patients. In this work, we investigated the effect of Hcy administration in Wistar rats on some parameters of energy metabolism in the hippocampus, a cerebral structure directly involved with cognition. The parameters utilized were $^{14}CO_2$ production, glucose uptake, lactate release and the activities of succinate dehydrogenase and cytochrome *c* oxidase (COX). Chronic hyperhomocysteinemia was induced by subcutaneous administration of Hcy twice a day from the 6th to the 28th day of life in doses previously determined in our laboratory. Control rats received saline in the same volumes. Rats were killed 12 h after the last injection. Results showed that Hcy administration significantly diminished $^{14}CO_2$ production and glucose uptake, as well as succinate dehydrogenase and COX activities. It is suggested that impairment of brain energy metabolism may be related to the neurological symptoms present in homocystinuric patients.

Keywords: Homocysteine; Homocystinuria; Brain energy metabolism; Experimental model

1. Introduction

Hyperhomocysteinemia is the biochemical hallmark of homocystinuria (HCU), an inborn error of metabolism caused by severe deficiency of cystathionine β-synthase (CBS, EC 4.2.1.22) activity. Affected patients usually present a variable degree of neurological dysfunction, ocular and vascular complications [1,2]. Although the thromboembolic features of HCU are thought to be due to vascular lesions induced by homocysteine (Hcy), the mechanisms underlying the variable degree of mental retardation occurring in the affected patients are not yet established.

There is a considerable body of evidence showing that increased plasma Hcy level is a strong and independent risk factor for the development of some neurodegenerative diseases such as dementia, Alzheimer's disease and Parkinson's disease [3–7], in which cellular energy depletion was found to be associated [8–12]. Furthermore, mitochondrial abnormalities that reflect compromised energy metabolism have been identified in hepatocytes of patients with hyperhomocysteinemia and in endothelial cells from the aorta of hypertensive rats with diet-induced hyperhomocysteinemia [13,14].

Animal models are useful to better understand the pathophysiology of diseases. We have recently developed in our laboratory a chemical experimental model of hyperhomocysteinemia [15], in which the Hcy levels were similar to those found in human HCU [1]. In the present study, we used this model to investigate the effect of high sustained Hcy levels on some parameters of energy metabolism, namely $^{14}CO_2$ production, glucose uptake and the activities of succinate dehydrogenase and cytochrome *c* oxidase (COX) in rat hippocampus, in the hope of better understanding the mechanisms underlying the neurological dysfunction found in homocystinuric patients.

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2. Materials and methods

2.1. Reagents

All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA, except for the radiolabeled compound [U-^{14}C] acetate which was purchased from Amersham International PLC, UK.

2.2. Animals

Wistar rats obtained from the Central Animal House of the Departamento de Bioquímica, ICBS, UFRGS were used. Rats were kept with dams while receiving the drugs 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 the experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Pôrto Alegre.

2.3. Chemically-induced hyperhomocysteinemia

Hcy was dissolved in 0.9% NaCl solution and buffered to pH 7.4. Hcy solution was administered subcutaneously twice a day at 8-h intervals from 6 to 28 days of age. Control animals received saline solution in the same volumes as those applied to Hcy-treated rats. Hcy doses were calculated from pharmacokinetic parameters previously determined in our laboratory [15]. During the first week of treatment, animals received 0.3 μmol Hcy/g body weight. In the second week, 0.4 μmol Hcy/g body weight was administered to the animals, and in the last week rats received 0.6 μmol Hcy/g body weight. Rats subjected to this treatment achieved plasma Hcy levels similar to those found in homocystinuric patients. Treated animals achieved maximal plasma Hcy levels 15 min after subcutaneous injection of Hcy (0.40–0.50 mmol/l). Maximal brain levels were achieved 15 min after Hcy administration (0.04–0.06 mmol/kg wet weight tissue). Twelve hours after treatment, plasma Hcy concentrations returned to normal levels (0.01 mmol/l) and brain Hcy levels were not detected.

2.4. Tissue and homogenate preparation

Twelve hours after the last injection, animals were sacrificed by decapitation, the brain was rapidly removed and the hippocampus was isolated. Hippocampus was 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, lactate release and glucose uptake assays. For succinate dehydrogenase and COX activities determination, hippocampus was homogenized (1:10, w/v) in SETH buffer, pH 7.4 (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 UI ml^{-1} heparin). The homogenates were centrifuged at $800 \times g$ for 10 min and the supernatants kept at −70 °C until used for enzyme activity determination. The maximal period between homogenate preparation and enzyme analysis was always less than 5 days.

2.5. $^{14}CO_2$ production

Hippocampus prisms (50 mg) were added to small flasks (11 cm^3) containing 0.5 ml Krebs–Ringer bicarbonate buffer, pH 7.4, 0.2 μCi [U-^{14}C] acetate and 0.5 mM of the unlabeled acetate. 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 $\times$ 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. The filter papers were removed and added to vials containing scintillation fluid, and radioactivity was counted [16].

2.6. Lactate release

Hippocampus prisms (50 mg) were incubated under an O_2/CO_2 (19:1) mixture at 37 °C for 60 min in Krebs–Ringer bicarbonate buffer, pH 7.0 (in a total volume of 1 ml) in a metabolic shaker (90 oscillations $\times$ min^{-1}) [17]. Two volumes of 0.6 N perchloric acid were immediately added to the prisms and the excess of perchloric acid was precipitated as a potassium salt by the addition of one volume of a solution containing 0.5 N KOH, 0.1 M imidazol, and 0.1 KCl. The solution was then centrifuged for 5 min at $800 \times g$. Lactate was measured in the medium before and after incubation by the lactase–peroxidase method [18]. Lactate release was calculated by subtracting lactate content found after incubation from the amount found before incubation. Lactate concentrations in the medium at the beginning of the incubation were practically nilled.

2.7. Glucose uptake

Hippocampus prisms (100 mg) were incubated in Krebs–Ringer bicarbonate buffer, pH 7.0 (in a total volume of 1 ml), containing 5.0 mM glucose in a O_2/CO_2 (19:1) mixture in a metabolic shaker (90 oscillations $\times$ min^{-1}) at 37 °C for 60 min [17]. Glucose was measured by the glucose oxidase method [19] and the

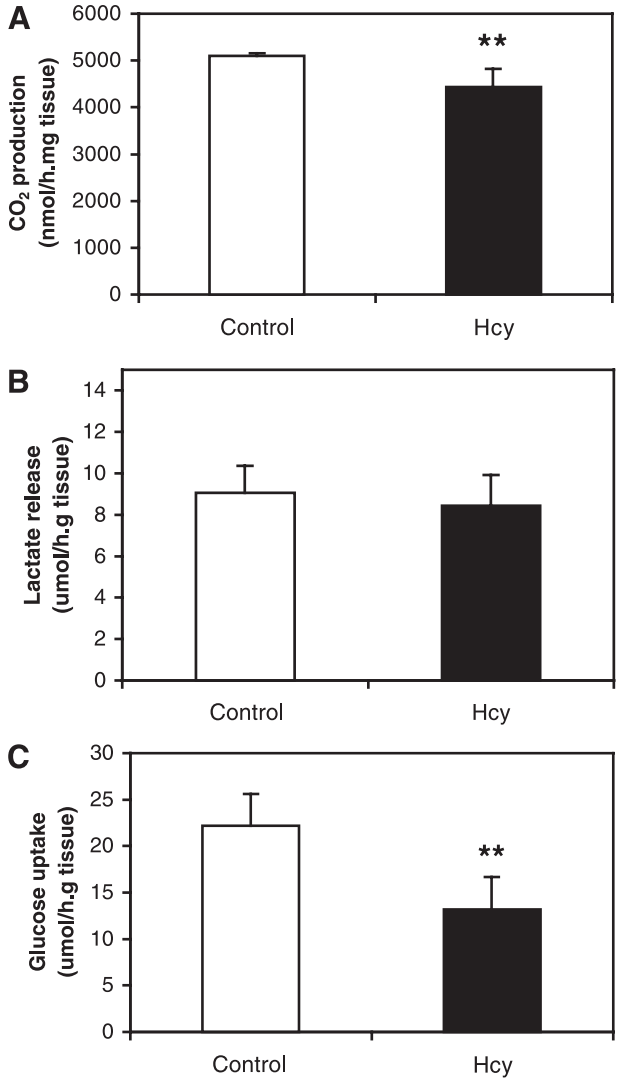

Fig. 1. Effect of chronic administration of homocysteine on $^{14}CO_2$ production (A), lactate release (B) and glucose utilization (C) in rat hippocampus. Data are expressed as mean ± S.D. for four to five independent experiments (animals) performed in duplicate. Different from control, $^{**}P < 0.01$ (Student's *t*-test).

uptake determined by subtracting the amount after incubation from the total amount measured before incubation.

2.8. Respiratory chain enzyme activities

2.8.1. Succinate dehydrogenase activity

The activity of succinate: phenazine oxireductase (soluble SDH) was measured in hippocampus homogenates following the decrease in absorbance due to the reduction of 2,6-dichloroindophenol (DCIP) at 600 nm with 700 nm as reference wavelength ($\varepsilon = 19.1\ mM^{-1}\ cm^{-1}$) in the presence of phenazine methasulfate (PMS), according to Fischer et al. [20]. The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate and 8 μM DCIP was preincubated with 40–80 μg homogenate protein at 30 °C for 20 min. Subsequently, 4 mM sodium azide, 7 μM rotenone and 40 μM DCIP were added and the reaction was initiated by addition of 1 mM PMS and was monitored for 5 min.

2.8.2. Cytochrome c oxidase activity

COX activity was determined according to Rustin et al. [21]. Enzymatic activity was measured at 25 °C for 10 min by following the decrease in absorbance due to oxidation of previously reduced cytochrome *c* at 550 nm with 580 nm as reference wavelength ($\varepsilon = 19.1\ mM^{-1} \times cm^{-1}$). The reaction buffer contained 10 mM potassium phosphate, pH 7.0, 0.6 mM *n*-dodecyl-β-D-maltoside, 2–4 μg homogenate protein and the reaction was initiated by addition of 0.7 μg reduced cytochrome *c*. Reaction time and protein concentration were chosen to ensure the linearity of the reaction.

2.9. Protein determination

Protein was measured by the method of Lowry et al. [22] using bovine serum albumin as standard.

2.10. Statistical analysis

Data were analyzed by Student's *t*-test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software.

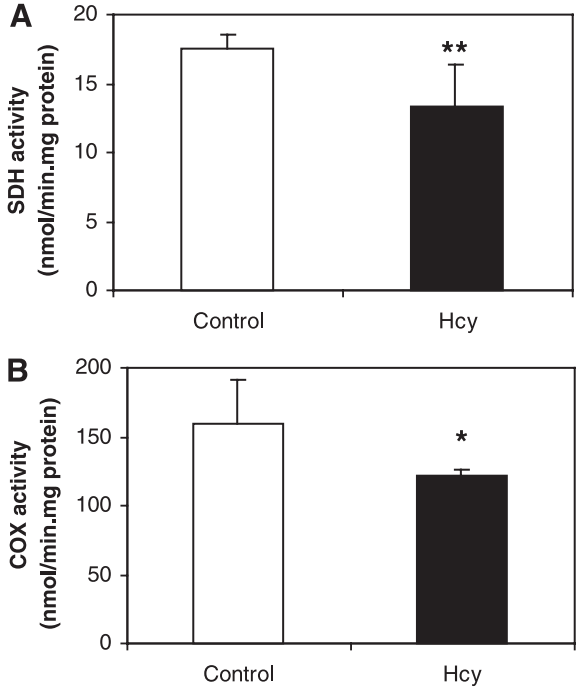

Fig. 2. Effect of chronic administration of homocysteine on succinate dehydrogenase (A) and cytochrome *c* oxidase (B) activities in rat hippocampus. Data are expressed as mean ± S.D. for five independent experiments (animals) performed in duplicate. Different from control, $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's *t*-test). SDH—succinate dehydrogenase; COX—cytochrome *c* oxidase.

3. Results

Fig. 1 shows that Hcy administration significantly inhibited $^{14}CO_2$ production [$t(8) = 3.82$; $P < 0.01$] and glucose uptake [$t(6) = 3.67$; $P < 0.01$] in rat hippocampus, when compared to the control group (saline). In contrast, lactate release was not affected by this treatment [$t(8) = 0.69$; $P > 0.05$].

We also examined the effect of hyperhomocysteinemia on the brain respiratory chain enzyme activities by measuring succinate dehydrogenase and COX activities in the rat hippocampus. As can be observed in Fig. 2, Hcy administration significantly inhibited succinate dehydrogenase [$t(8) = 2.93$; $P < 0.01$] and COX [$t(8) = 2.63$; $P < 0.05$] activities in this cerebral structure.

4. Discussion

HCU is an inborn error of metabolism caused by severe deficiency of CBS activity. Affected patients present tissue accumulation of Hcy, and clinically, mental retardation, brain ischemia, seizures and other neurological features. However, the mechanisms underlying the neurological dysfunction of HCU are still poorly known [1]. The understanding of the biochemical alterations in brain might possibly contribute to a better therapeutic management of homocystinuric patients.

The purpose of the present investigation was to evaluate some biochemical parameters of brain energy metabolism, namely $^{14}CO_2$ production, glucose uptake, lactate release, succinate dehydrogenase and COX activities in the hippocampus of rats subjected to chronic chemically-induced hyperhomocysteinemia. We produced high plasma sustained levels of Hcy in rats (up to 50-fold higher than in controls) similar to those found in homocystinuric patients [1]. Brain levels were also significantly increased [15].

First, we investigated the effect of hyperhomocysteinemia on the activity of the Krebs cycle by measuring $^{14}CO_2$ production from acetate. The anaerobic metabolism was evaluated by lactate release. We verified that Hcy administration significantly reduced $^{14}CO_2$ production and glucose uptake, but did not alter lactate production. Taken together, these findings suggest that chronic Hcy administration probably reduce aerobic glycolysis and glucose uptake. It is feasible that lactate production was not altered probably because brain glucose utilization was decreased. It can therefore be presumed that the reduction of Krebs cycle activity caused by Hcy administration might lead to a reduced energy production.

The next experiments were performed in order to evaluate the respiratory chain function by measuring succinate dehydrogenase and COX activities in the hippocampus of rats subjected to chronic administration of Hcy. We verified that Hcy markedly inhibited both activities. Therefore, it is possible that the reduction of these enzyme activities, which are considered markers of neuronal function, by Hcy administration might explain the reduction of glucose uptake and $^{14}CO_2$ production (Krebs cycle) observed. However, we cannot establish at the present whether the degree of inhibition of the electron transfer chain caused by Hcy would alter ATP biosynthesis, and this is presently being carried out in our laboratory.

COX is the terminal and rate-limiting enzyme of the mitochondrial respiratory chain, catalyzing the transfer of electrons from cytochrome *c* to molecular oxygen [23]. Therefore, an inhibition of this enzyme can potentially lead to incomplete reduction of oxygen, and consequently to increased free radical formation and reduced ATP synthesis [24–26]. On the other hand, since oxidative phosphorylation is very active in the brain [27,28], it is postulated that inhibition of the respiratory chain at two crucial complexes caused by high sustained levels of Hcy may be highly damaging to this tissue. Furthermore, considering previous findings from our and other laboratories demonstrating that Hcy impairs memory in rats [29], inhibits Na^+,K^+-ATPase in rat hippocampus [15,30–32], and induces neuronal death in culture of cerebellar granule cells [33], it is possible that the deficit of energy metabolism may underlie, at least partially, some of these observations, representing therefore an important mechanism responsible for the neurological damage in HCU.

On the other hand, it has been shown that reduction of brain energy production might compromise neurotransmitter (acetylcholine, glutamate, aspartate and GABA) and lipid synthesis, which might lead to neurological damage [34]. Lack of energy production may also cause a deficient glutamate uptake by glial cells and by neurons and even a reversion of the transporters, leaving excess of glutamate in the synaptic cleft and causing secondary excitotoxicity [5,35–40]. This is interesting in view of previous studies reporting that Hcy induces neuronal death mediated by activation of NMDA receptors [33]. These investigators also observed that Hcy enhanced free radical generation, which may in turn induce mitochondrial damage [41]. In this context, it is well known that mitochondria generate various reactive oxygen species, such as superoxide anions, hydroxyl radicals and hydrogen peroxide [42].

It is difficult to predict whether we can extrapolate our findings to the human condition. However, if the inhibition of energy metabolism caused by chronic Hcy treatment also occurs in humans, it may be presumed that impaired energy metabolism is associated with the neurological symptoms present in homocystinuric patients. Whether this or other abnormalities, such as oxidative stress or excitotoxicity, is mainly responsible for brain damage in homocystinuric patients is a matter for further investigation.

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