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ORIGINAL ARTICLE

Creatine plus pyruvate supplementation prevents oxidative stress and phosphotransfer network disturbances in the brain of rats subjected to chemically-induced phenylketonuria

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Abstract

Phenylketonuria (PKU) is the most common inborn error of amino acid metabolism. Usually diagnosed within the first month of birth, it is essential that the patient strictly follow the dietary restriction of natural protein intake. Otherwise, PKU impacts the development of the brain severely and may result in microcephaly, epilepsy, motor deficits, intellectual disability, and psychiatric and behavioral disorders. The neuropathology associated with PKU includes defects of myelination, insufficient synthesis of monoamine neurotransmitters, amino acid imbalance across the blood-brain barrier, and involves intermediary metabolic pathways supporting energy homeostasis and antioxidant defenses in the brain. Considering that the production of reactive oxygen species (ROS) is inherent to energy metabolism, we investigated the association of creatine+pyruvate (Cr + Pyr), both energy substrates with antioxidants properties, as a possible treatment to mitigate oxidative stress and phosphotransfer network impairment elicited in the brain of young Wistar rats by chemically-induced PKU. We induced PKU through the administration of α-methyl-L-phenylalanine and phenylalanine for 7 days, with and without Cr + Pyr supplementation, until postpartum day 14. The cotreatment with Cr + Pyr administered concurrently with PKU induction prevented ROS formation and part of the alterations observed in antioxidants defenses and phosphotransfer network enzymes in the cerebral cortex, hippocampus, and cerebellum. If such prevention also occurs in PKU patients, supplementing the phenylalanine-restricted diet with antioxidants and energetic substrates might be beneficial to these patients.

Keywords Phenylketonuria · Oxidative stress · Brain energy metabolism · Phosphotransfer network · Creatine and pyruvate · Inborn error of metabolism

Abbreviations

PKU	Phenylketonuria
IEM	Inborn error of metabolism
PAH	Phenylalanine hydroxylase
Phe	Phenylalanine
HPA	Hyperphenylalaninemia
ROS	Reactive oxygen species
Cr	Creatine
Pyr	Pyruvate
αMePhe	α-methyl-L-phenylalanine
DCFH	2′,7′-dihydrodichlorofluorescein
SH	Sulfhydryl groups
GSH	Reduced glutathione
CAT	Catalase
SOD	Superoxide dismutase
GPx	Glutathione peroxidase
CytCK	Cytosolic creatine kinase
MtCK	Mitochondrial creatine kinase
PCr	Phosphocreatine
PK	Pyruvate kinase
HK	Hexokinase
AK	Adenylate kinase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
OXPHOS	Oxidative phosphorylation

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Introduction

Phenylketonuria (PKU) is an inborn error of metabolism (IEM) caused by mutations in the gene that encodes the enzyme phenylalanine hydroxylase (PAH) (Blau 2016). The enzyme synthesized, then, is unable to hydroxylate phenylalanine (Phe) into tyrosine, leading to Phe accumulation in blood and other tissues, impairing the overall development of the patient, remarkably the development of the brain (Scriver 1995; Kayaalp et al. 1997). The most common clinical finds include intellectual disability, epilepsy, motor deficits, and behavioral disturbances, among other neurological and psychiatric symptoms (Gonzalez et al. 2011; Bilder et al. 2013; Bilder et al. 2016). The defects of myelination are a striking feature observed in the central nervous system of PKU patients (Anderson and Leuzzi 2010), alongside with reduced synthesis of monoamine neurotransmitters (Surtees and Blau 2000), amino acid imbalance across the blood-brain barrier (de Groot et al. 2013), and reduced glutamatergic transmission (Martynyuk et al. 2005). Moreover, PKU disturbs metabolic pathways involved in energy homeostasis and oxidative balance (Schuck et al. 2015).

Oxidative stress has gained acceptance as a disturbance implicated in the pathogenesis of PKU; there is evidence of oxidative damage in blood cells of patients, in the brain of animal models of PKU, and from in vitro experiments (Sirtori et al. 2005; Ribas et al. 2011; Sanayama et al. 2011; Veyrat-Durebex et al. 2017; Rausell et al. 2019). Consistently, in previous work we observed oxidative stress in the brain of rats subjected to a hyperphenylalaninemia (HPA) model (Kienzle Hagen et al. 2002; Moraes et al. 2013), and that Phe induced enhancement in catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) activities in cortical astrocytes, as well as decreased the reduced glutathione (GSH) content and increased the production of reactive oxygen species (ROS) (Preissler et al. 2016). HPA may also impair the activity of the mitochondrial electron transport chain (Stepien et al. 2017). In a chronic model, HPA decreased succinate dehydrogenase and respiratory chain complexes I + III activities in rat cerebral cortex (Rech et al. 2002). Further, in a recent study, Dimmer et al. showed decreased complexes I + III and IV activities in cerebral cortex and hippocampus of rats subjected to acute HPA (Dimer et al. 2018), suggesting mitochondrial dysfunction might occur even in isolated episodes of Phe elevation.

While mitochondria provide high-energy phosphates, the phosphotransfer network allows the communication between cellular sites of ATP consumption and production, being functionally coupled to oxidative phosphorylation (OXPHOS) (Dzeja and Terzic 2003; Schlattner et al. 2016). Phosphotransfer enzymes also play an antioxidant role by recycling ADP through the inner mitochondrial membrane. As the physiological rate of mitochondrial ROS production is inversely proportional to the availability of cytosolic ADP (Cadenas and Davies 2000), the ADP/ATP ratio is fundamental to keep low mitochondrial membrane potential ($\Delta\psi m$) (100–150 mV) and maintains the proton gradient across the membrane ($\Delta\mu H+$). We have reported that Phe disturbs the activity of enzymes involved in keeping the ADP/ATP homeostasis, such as creatine kinase (CK) and pyruvate kinase (PK), in vitro and in the cerebral cortex of rats subjected to PKU (Feksa et al. 2002; Costabeber et al. 2003; Feksa et al. 2003).

The treatment of PKU consists of a Phe-restricted diet, accompanied by a Phe-free medical formula, to prevent Phe accumulation in blood (van Wegberg et al. 2017). Although efficient in lowering Phe levels, this diet allows very restrict intake of natural protein, compromising the patient's compliance with the treatment, especially during adolescence when parental control over the diet weakens (MacDonald et al. 2010). Considering the interplay between energy conversions and the generation of ROS, we propose an approach with creatine and pyruvate (Cr + Pyr), substrates that offer both energy support and antioxidant protection, as possible adjuvant treatment. Cr + Pyr prevented lipid peroxidation and damage to sulfhydryl groups elicited by intra-hippocampal administration of Phe, ameliorating total radical-trapping antioxidant potential and total antioxidant reactivity (Berti et al. 2012), and prevented disturbances in the activity of enzymes from the phosphotransfer network in cerebral cortex and hippocampus in the offspring of rats subjected to a maternal model of PKU (Bortoluzzi et al. 2014). Further, the treatment with Cr + Pyr was able to prevent the reduction in dendritic spine density in the stratum radiatum of the CA1 hippocampal field and the posterodorsal medial amygdala of PKU rats (Dos Reis et al. 2013).

In the present study, we aimed to evaluate the concomitance of oxidative stress and disturbs of energy homeostasis in the brain of young Wistar rats subjected to a chemical model of PKU, and the effect of a cotreatment with Cr + Pyr concurrently with PKU induction. Tested parameters include enzymatic and no enzymatic antioxidant defenses, as well as ROS production, and the activity of phosphotransfer enzymes, in the cerebral cortex, hippocampus, and cerebellum of the pups. Furthermore, we investigated if the treatments affected brain mass and if there is a relation between oxidative stress and the activity of enzymes from the phosphotransfer network.

Material & methods

Chemicals

All reagents used were purchased from Sigma Chemical Co. (St. Louis, MO, USA), except salts for buffer solutions, which were of analytical grade and were purchased from local suppliers.

Animals

Forty-eight Wistar rats obtained from the Department of Biochemistry of Federal University Federal of Rio Grande do Sul were used in the experiments (twenty-four rats for each set of parameters analyzed, oxidative stress and phosphoryl transfer network). Pups were breed with their mothers, housed as 6 litters with 8 pups each, and kept in the department's vivarium on a 12–12 h light/dark cycle in a room acclimatized at constant temperature (22 ± 1 °C), with water and commercial chow (Supra, Porto Alegre, RS, Brazil) ad libitum. The Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil, approved the experimental protocol (project number 29388). The "Principles of laboratory animal care" (Guide for the Care and Use of Laboratory Animals, NIH publication n°. 80–23, revised 1996; http://www.nap.edu/readingroom/books/labrats/) were followed. All efforts were made to minimize animal suffering, using only the number of animals necessary to produce reliable scientific data.

Treatments

Pups were divided into four groups with 6 animals each, in a manner that each pup in a given group was from a distinct litter. Groups were: 1) saline (Sal); 2) phenylketonuria (PKU): phenylalanine 5,2 μmol per g of body weight (Feksa et al. 2002) + α-methyl-L-phenylalanine (αMePhe) 1.6 μmol per g of body weight (Kienzle Hagen et al. 2002); 3) creatine 0.4 mg per g of body weight (Stockler et al. 1994) + pyruvate 0.2 mg per g of body weight (Ryu et al. 2006); and 4) PKU + Cr + Pyr. Saline was the vehicle for all the solutions. The volumes used were 10 μl per g of body weight for Cr + Pyr and the PAH inhibitor αMePhe, and 30 μl per g of body weight for L-Phe. Pups received twice a day at 10–12 h interval subcutaneous administration of L-Phe or saline and intraperitoneal administration of Cr + Pyr or saline. αMePhe was administered intraperitoneally once a day to PKU and PKU + Cr + Pyr groups, by the morning. L-Phe, αMePhe, Cr + Pyr, and saline solutions were buffered to pH 7.4 immediately before the administration. Animals were treated from postpartum day 7 (P7) until postpartum day 14 (P14), and sacrificed without anesthesia 12 h after the last routine of treatment, on postpartum day 15 (P15).

Tissue dissection

Brains were removed from the skull using the foramen magnum as the lower limit and rapidly dissected free of meninges and superficial blood vessels. Brains were dissected in 3 regions of interest: cerebellum, cerebral cortex, and hippocampus, using consistent anatomical landmarks as criteria for dissection. The cerebellum was dissected by cutting the cerebellar peduncles at the surface of the brainstem; the cerebral cortex comprised all regions from dorsolateral to the olfactory tract, excluding the hippocampus, and was dissected from each hemisphere by peeling it away from the striatum and other subcortical structures under a stereomicroscope. The weight of the whole brain and each structure of interest were recorded for subsequent homogenization.

Tissue homogenization

Tissues were homogenized in 10 volumes (1:10 *w*/*v*) of buffer solution phosphate-KCl (20/40 mM) pH 7.4 for analysis of oxidative stress parameters, and SET buffer (0.32 M sucrose/1 mM EGTA/10 mM Tris–HCl) pH 7.4 for analysis of energy metabolism parameters. Homogenates were centrifuged at 800×g for 10 min at 4 °C for evaluation of oxidative stress parameters and hexokinase (HK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and adenylate kinase (AK) activities. Pellets were discarded, and part of the supernatant from samples homogenized in the SET buffer went through second centrifugation at 10,000×g for 15 min at 4 °C, resulting in a supernatant containing cytosol and other cellular components, such as endoplasmic reticulum. This supernatant was collected for determination of pyruvate kinase (PK) and cytosolic creatine kinase (CytCK) activity, and the pellet containing mitochondria, myelin, synaptosomes, and membrane fragments, was resuspended in the same SET buffer, centrifugated at 10,000×g for 15 min at 4 °C, and resuspended in 100 mM Trizma/ 15 Mm MgSO4 buffer, pH 7.5, for determination of mitochondrial creatine kinase (MtCK) activity. All the supernatants were stored for no more than one week at −80 °C when assays were not carried out immediately.

Oxidative stress parameters

2′,7′-Dihydrodichlorofluorescein oxidation assay

ROS production was measured using LeBel's method (LeBel et al. 1992). Samples were incubated for 30 min at 37 °C in the dark with 20 mM sodium phosphate buffer with 140 mM KCl pH 7.4 and 100 μM reduced 2′7′- dichlorofluorescein diacetate (H2DCF-DA) solution. H2DCF-DA is cleaved by cellular esterases to H2DCF, which is oxidized to DCF by reactive oxygen species (ROS) present in samples. DCF fluorescence intensity parallels to the number of reactive species formed. Fluorescence was measured using excitation of 480 nm and emission of 535 nm wavelengths. A calibration curve was prepared with standard DCF (0.25–10 μM), and the levels of reactive species were expressed as nanomoles of DCF formed per milligram of protein.

Total sulfhydryl content

Sulfhydryl content (SH) was assessed through Aksenov's method (Aksenov and Markesbery 2001), which consists in the reduction of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) by thiols, producing a yellow derivative (TNB) with absorption measured spectrophotometrically at 412 nm. Briefly, 0.1 mM DTNB was added to 120 μl of cortex cerebral, hippocampus and cerebellum supernatants, followed by a 30-min incubation at room temperature, protected from light. Sulfhydryl content inversely correlates to oxidative damage to proteins but also reflects reduced glutathione (GSH) and other thiols levels. Results were expressed as nanomoles of TNB per milligram of protein.

Reduced glutathione content

GSH content was evaluated according to Browne and Armstrong (Browne and Armstrong 1998), whose method consists of the reaction of GSH with the fluorophore o-phthalaldeyde (OPT) after the deproteinization of the sample with metaphosphoric acid. Then, 150 μL of the samples were incubated with an equal volume of o-phthaldialdehyde (1 mg/ml of methanol) for 15 min at 37 °C. Immediately following the incubation, fluorescence was measured using excitation of 350 nm and emission of 420 nm wavelengths. A calibration curve was prepared with standard GSH (1 mM), and results were expressed as nmol of GSH per mg of protein.

Catalase

CAT activity was assayed according to Aebi (Aebi 1984) by measuring the absorbance decrease of H_2O_2 at 240 nm. Reaction medium contained 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0 and 10 μl of the supernatants. CAT is responsible for the transformation of H_2O_2 in H_2O. Hydrogen peroxide can react with thiol and methionyl groups of enzymes and other proteins, and form high reactive hydroxyl radicals. One CAT unit is defined as 1 μmol of hydrogen peroxide consumed/min, and the specific activity was calculated as CAT units per mg of protein.

Superoxide dismutase

SOD is involved in transforming superoxide free radicals in hydrogen peroxide, a less reactive substance. SOD activity was evaluated using Marklund's method (Marklund and Marklund 1974). Pyrogallol is highly sensitive to the superoxide radical, and the activity of SOD is directly proportional to the inhibition of the pyrogallol autoxidation. SOD activity was indirectly assessed spectrophotometrically at 420 nm. A calibration curve with purified SOD was used as standard. One unit of SOD corresponds to 50% inhibition of pyrogallol autoxidation. The specific activity of SOD was expressed as units per milligram of protein.

Glutathione peroxidase

GPx activity was measured following Wendel's method (Wendel 1981), which uses tert-butyl hydroperoxide as substrate. NADPH disappearance was continuously monitored in a spectrophotometer at 340 nm for 4 min. One GPx unit is defined as 1 mmol of NADPH consumed per minute, and the specific activity was reported as units of GPx per milligram of protein.

Phosphoryl transfer network

Mitochondrial and cytosolic Creatine kinase

Mitochondrial (MtCK) and cytosolic (CytCK) CK activity were assayed in a mixture containing the following final concentrations: 65 mM Tris–HCl buffer, pH 7.5, 7 mM phosphocreatine, 9 mM MgSO4, and 1 μg of protein (cytosolic or mitochondrial-rich fraction) in a final volume of 0.1 ml. After 10 min of pre-incubation at 37 °C, the reaction was started by with 0.3 μmol of ADP, and the addition of 1 μmol of p-hydroxymercuribenzoic acid stopped it after 10 min. Reagent concentrations and the incubation time were chosen to assure linearity of the enzymatic activity. Appropriate controls were carried out to measure the chemical hydrolysis of phosphocreatine. The creatine formed was estimated according to the colorimetric method of Hughes (Hughes 1962). The color develops by the addition of 0.1 mL 2% α-naphthol and 0.1 mL 0.05% diacetyl in a final volume of 1 mL and read after 20 min at 540 nm in a spectrophotometer. Results were expressed as μmol of creatine formed per min per mg of protein.

Pyruvate kinase

PK activity was assayed as described by Leong (Leong et al. 1981). The incubation medium consists of 0.1 M Tris/HCl buffer, pH 7.5, 10 mM MgCl2, 0.16 mM NADH, 75 mM KCl, 5.0 mM ADP, 7 U of L-lactate dehydrogenase, 0.1% (*v*/v) Triton X-100, and 10 μL of the mitochondria-free supernatant in a final volume of 0.5 ml. After 10 min of pre-incubation at 37 °C, the addition of 1 mM phosphoenolpyruvate started the reaction, which was measured at 340 nm for 2 min in a spectrophotometer. Results were expressed as μmol of pyruvate formed per min per mg of protein.

Hexokinase

Mitochondrial bound hexokinase (HK) activity was determined based on a previous method with minor modifications

(da Silva et al. 2004). Briefly, enzyme's activity was determined by NADH formation in a medium containing 10 mM Tris–HCl, pH 7.4, 0.1 mM Ap5A AK inhibitor, 5 mM D-glucose, 1 mM ATP, 10 mM $MgCl_2$ and 1 unit/ml Glc-6-P dehydrogenase (*Leuconostoc mesenteroides*). The reaction was started by addition of 1 mM NAD+ after pre-incubation for 10 min at 37 °C, and the absorbance at 340 nm was monitored for 2 min.

Adenylate kinase

Adenylate kinase (AK) activity was measured by a coupled enzyme assay with HK and glucose 6-phosphate dehydrogenase (G6PD), according to Dzeja et al. (Dzeja et al. 1985). The reaction mixture contained 100 mM KCl, 20 mM HEPES, 20 mM glucose, 4 mM $MgCl_2$, 2 mM NADP+, 1 mM EDTA, 4.5 U/ml of HK, 2 U/ml of G6PD, and 1 μg of protein homogenate. The addition of 2 mM ADP started the reaction, and the reduction of NADP+ was followed at 340 nm for 3 min in a spectrophotometer. ADP, NADP+, G6PD, and HK were dissolved in water. The results were expressed in μmol of ATP formed per min per mg of protein.

Glyceraldehyde-3-phosphate dehydrogenase

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity was measured as described by Mazzola and Sirover (Mazzola and Sirover 2005) with minor modifications. Reaction mixtures contained 0.25 mM NAD+ and buffer medium, pH 8.9, (100 mM glycine, 100 mM potassium phosphate monobasic and 5 mM EDTA). Samples were mixed and the reaction started by 2.2 mM glyceraldehyde-3-phosphate. The activity of GAPDH was determined by NADH formation following absorbance at 340 nm for 2 min and were expressed as μmol of NADH formed per min per mg of protein.

Protein determination

The protein content was determined as described by Lowry (Lowry et al. 1951) in each centrifugation fraction of the cerebral cortex, hippocampus, and cerebellum, using serum bovine albumin as standard.

Statistical analysis

Data were expressed as mean ± SD and analyzed by one-way ANOVA for comparison of means (Supplemental Tables 1 and 3), followed by Tukey test (Supplemental Tables 2, 4, and 5). Interactions between the treatments were verified using two-way ANOVA (PKU and Cr + Pyr as factors). Comparisons between sulfhydryl content and the activities of the enzymes of the phosphotransfer network were performed through Spearman's correlation method. Data were analyzed in the Statistical Package for the Social Sciences software (SPSS 20.0 for Windows), and graphics were produced in GraphPad Prism (version 7.0 for Windows). p values lower than 0.05 were considered significant.

Results

We assessed the effect of the treatments on the generation of ROS through the DCFH oxidation. Phe + αMePhe administration (PKU group) increased the formation of ROS in the cerebral cortex and hippocampus, but not in the cerebellum (Fig. 1a). The DCF levels found in PKU pups treated with Cr + Pyr (PKU + Cr + Pyr group) shows that Cr + Pyr restrained ROS formation in the cerebral cortex [$F(1,20) = 7{,}16$; $p = 0{,}015$] and hippocampus [$F(1,20) = 8{,}96$; $p = 0{,}007$], keeping DCF levels comparable to those from pups which received only vehicle (Sal group). Although DCF formation was significantly higher in the cerebellum of PKU + Cr + Pyr pups than in Sal pups, such effect was not due to the synergy between the two treatments [$F(1,20) = 2{,}07$; $p = 0{,}166$].

The induction of PKU compromised the reduced power and protein integrity, significantly decreasing SH content in the cerebral cortex, hippocampus, and cerebellum (Fig. 1b). The coadministration of Cr + Pyr prevented the degradation of SH groups in the three cerebral structures under study [cortex $F(1,20) = 5{,}57$; $p = 0{,}027$; hippocampus $F(1,20) = 8{,}21$; $p = 0{,}010$; cerebellum $F(1,20) = 5{,}46$; $p = 0{,}03$]. PKU did not alter the GSH content in the cerebral cortex, hippocampus nor cerebellum (Fig. 1c), neither did the treatment with Cr + Pyr [cerebral cortex $F(1,20) = 1{,}04$; $p = 0{,}319$; hippocampus $F(1,20) = 0{,}10$; $p = 0{,}753$; cerebellum $F(1,20) = 0{,}9$; $p = 0{,}765$].

CAT, SOD and GPx provide the first line of antioxidant defense against ROS through enzyme-catalyzed dismutation of O_2 to H_2O_2, which is further reduced to oxygen and water (Salminen and Paul 2014). In our study, pups subjected to PKU presented decreased CAT activity in cerebral cortex, hippocampus, and cerebellum (Fig. 1d), an effect prevented by cotreatment with Cr + Pyr [cerebral cortex $F(1,20) = 11{,}68$; $p = 0{,}003$; hippocampus $F(1,20) = 16{,}35$; $p = 0{,}001$; cerebellum $F(1,20) = 11{,}83$; $p = 0{,}003$]. Phe + αMePhe treatment affected SOD activity only in the hippocampus, decreasing it, which Cr + Pyr coadministration avoided [$F(1,20) = 23{,}29$; $p < 0{,}001$]. The treatments did not affect SOD activity neither in cerebral cortex [$F(1,20) = 0{,}94$; $p = 0{,}344$] nor cerebellum [$F(1,20) = 0{,}12$ $p = 0{,}738$] (Fig. 1e). Phe + αMePhe decreased GPx activity (Fig. 1f) in the cerebral cortex, and Cr + Pyr administration prevented it [$F(1,20) = 7{,}88$; $p < 0{,}011$]. We verified no alteration in GPx activity in hippocampus, nor effect of Cr + Pyr on PKU condition [$F(1,20) = 0{,}13$; $p = 0{,}723$]. PKU induction slightly enhanced GPx activity in the

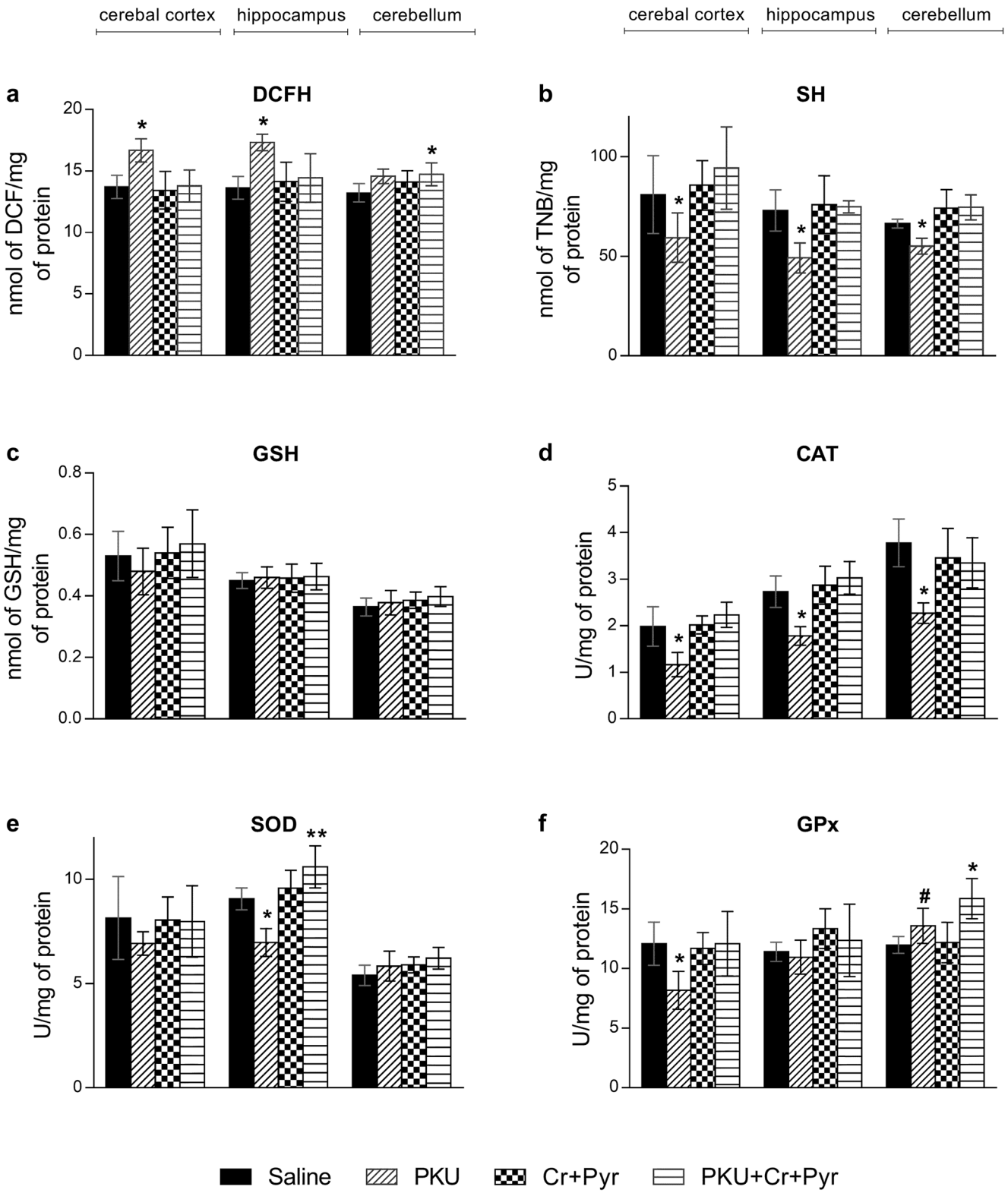


Fig. 1 Effects of PKU induction and/or Cr + Pyr administration on parameters of oxidative stress. Data are expressed as mean ± SD for 6 animals in each group. DCFH (2`7`-dihydrodichloro fluorescein oxidation), expressed as nmol of DCF per mg of protein; SH (sulfhydryl content), expressed as nmol TNB/mg protein; GSH (reduced glutathione), expressed as nmol of GSH per mg of protein; GPx (glutathione peroxidase), expressed as μmol of NADPH per min per mg of protein; SOD (superoxide dismutase), expressed as SOD U per mg of protein; CAT (catalase), expressed as CAT U per mg of protein. *$p < 0.005$ compared to the other groups; **$p < 0.05$ compared to saline and PKU; #equal to Cr + Pyr + PKU (one-way ANOVA followed by the Tukey test)

cerebellum, not above Sal levels, while in the PKU + Cr + Pyr group GPx activity was significantly enhanced, which would possibly be a consequence of Cr + Pyr supplementation, but we found no interaction between treatments [F(1,20) = 3,12; p = 0,093].

Oxidative stress may compromise the phosphotransfer network efficiency, since these enzymes contain thiol groups susceptible to ROS that can trigger conformational modifications, leading to enzyme inhibition or loss of function. Pups subjected to PKU presented lower MtCK, PK and HK activities in the three cerebral structures under study (Fig. 2a, b, c). The cotreatment with Cr + Pyr prevented such effect on MtCK activity in cerebral cortex [F(1,20) = 5,81; p = 0,5026], hippocampus [F(1,20) = 6,00; p = 0,024], and cerebellum [F(1,20) = 4,75; p = 0,041], and on HK activity in cerebral cortex [F(1,20) = 5,72; p = 0,027], hippocampus [F(1,20) = 9,88; p = 0,005], and cerebellum [F(1,20) = 8,79 p = 0,008]. Cr + Pyr also preserved PK activity in cerebral cortex [F(1,20) = 4,55; p = 0,045] and hippocampus [F(1,20) = 15,96; p = 0,001], but not in cerebellum [F(1,20) = 3,97; p = 0,06].

CytCK, AK, and GAPDH activities responded distinctly to treatments in each cerebral structure. Phe + αMePhe administration impaired CytCk activity in the cerebral cortex, and Cr + Pyr prevented it [F(1,20) = 12,38; p = 0,002], but promoted no alteration on this enzyme activity in the hippocampus [F(1,20) = 0,87; p = 0,361] nor cerebellum [F(1,20) = 2,03; p = 0,170] (Fig. 2d). Phe + αMePhe did not affect AK activity in cerebral cortex [F(1,20) = 0,95; p = 0,340], but decreased it in hippocampus, which Cr + Pyr prevented [F(1,20) = 6,21; p = 0,022], and enhanced AK activity in the cerebellum, an effect that was mitigated by Cr + Pyr as seen in the PKU + Cr + Pyr group [F(1,20) = 14,18; p = 0,001] (Fig. 2e). PKU induction diminished GAPDH activity in cerebral cortex, and enhanced it in the cerebellum, both effects prevented by Cr + Pyr cotreatment [cerebral cortex F(1,20) = 5,89; p = 0,025; cerebellum F(1,20) = 12,26; p = 0,002]. Phe + αMePhe administration did not alter GAPDH activity in the hippocampus, while Cr + Pyr treatment increased it, and, although insignificant, this effect remained noticeable in PKU + Cr + Pyr group [F(1,20) = 0,06; p = 0,807] (Fig. 2f).

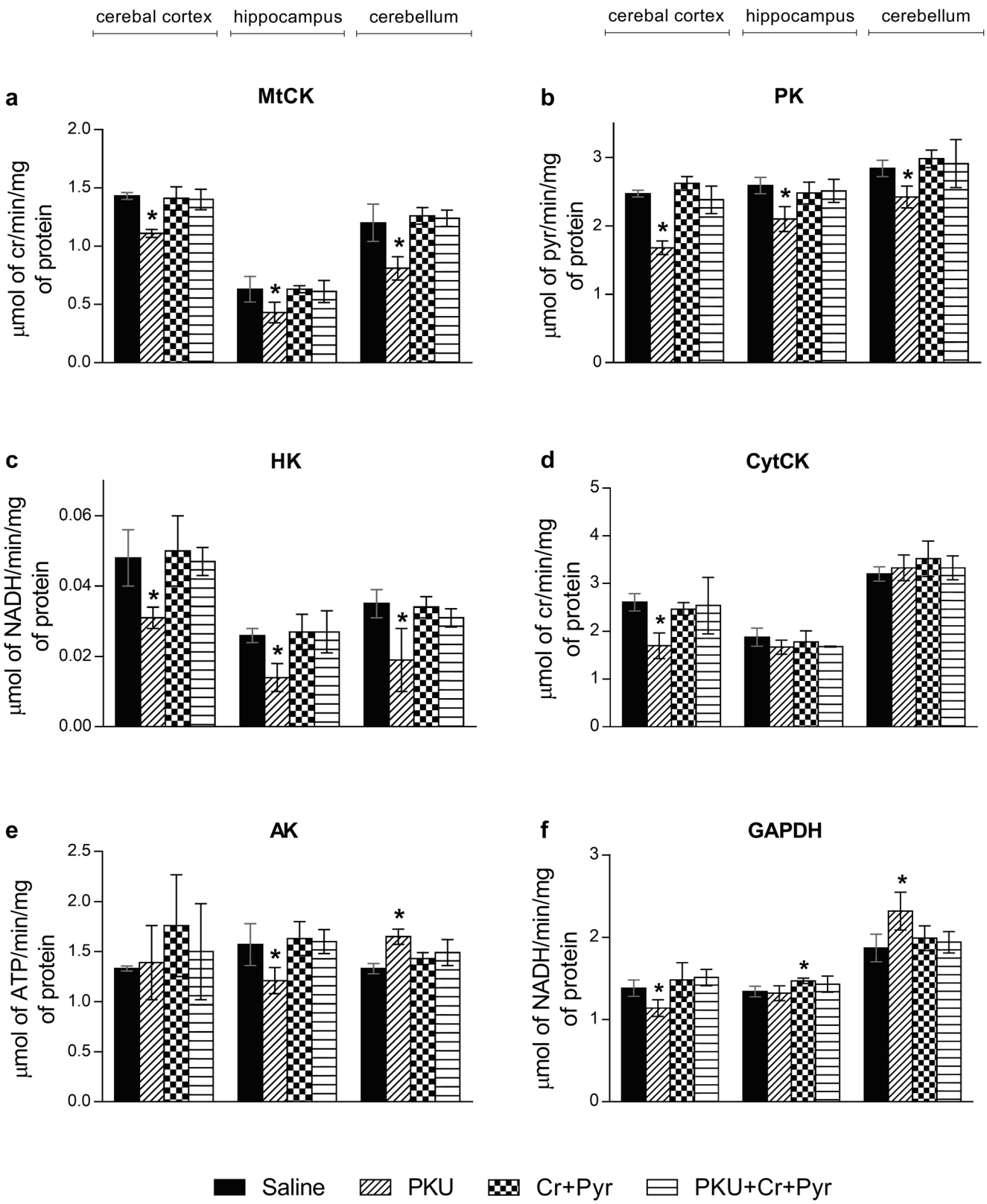
The brains from pups subjected to PKU were remarkably smaller, and indeed weighted about 20% less than the whole brain from animals treated with vehicle (Table 1). This effect was partially prevented by Cr + Pyr, as observed in the PKU + Cr + Pyr group [F(1,44) = 17,64 p < 0,001]. The cerebral cortex, hippocampus, and cerebellum suffered the same effect under PKU induction. Although the hippocampus and cerebral cortex of pups subjected to PKU and cotreated with Cr + Pyr presented a weight similar to Sal treated pups, it is unclear if Cr + Pyr is responsible for such effect [F(1,44) = 0,30; p = 0,586; hippocampus F(1,44) = 3,76; p = 0,059; cerebellum F(1,44) = 1,10; p = 0,30].

Since the integrity of thiol-groups influence the ligand and substrate binding properties, as well as the conformational changes that result in the activation of enzymes, we choose SH content to prospect whether oxidative stress would be related to the alterations in the phosphotransfer network. Further, PKU induction decreased SH content uniformly in the cerebral cortex, hippocampus, and cerebellum, suggesting protein damage, and Cr + Pyr prevented it in the three cerebral structures. We found significant Spearman's correlations (Table 2), indicating dependence between alteration in the activity of enzymes in the phosphotransfer network and damage to sulfhydryl groups, possibly due to oxidative stress.

Discussion

Oxidative stress and energy metabolism are intimately related. OXPHOS is the main source of ROS, generated as by-products in the electron transport chain. Indeed, 1–2% of the O_2 consumed by the respiratory chain is diverted to generate ROS such as superoxide ($O^{\circ -}_2$) and hydrogen peroxide (H_2O_2), mainly through the respiratory complexes I and III (Cadenas and Davies 2000). Agreeing, we have previously reported that PKU impairs the activity of these complexes in the cerebral cortex of Wistar rats (Rech et al. 2002). In the present study, PKU induction increased ROS formation in the cerebral cortex and hippocampus. The cotreatment with Cr + Pyr prevented ROS increase in these cerebral structures, but in the cerebellum, it slightly enhanced the ROS production elicited by PKU. It is possible that the enhancement in the GPx activity observed in cerebellum accounted for ROS detoxification, along with unaltered SOD activity. Also, brain structures and regions present idiosyncratic susceptibility to ROS and antioxidant defenses, so it is reasonable that distinct structures present diverging responses to oxidative stress (Calabrese et al. 2002; Campese et al. 2007).

The brain is highly susceptible to redox imbalance, partly because of its high uptake of oxygen, accounting for up to 20% of basal O_2 consumption, partly a consequence of its modest antioxidant defenses – the levels of antioxidants enzymes such as GPx and CAT are low in most brain regions (Halliwell 1992; Cobley et al. 2018), while brain metabolism generates considerable amounts of H_2O_2 (Gal et al. 2005). Veyrat-Durebex and colleagues described that HPA correlates with a global decrease in the expression of 22 antioxidant genes in white blood cells of PKU patients (Veyrat-Durebex et al. 2017), and Zhang and Gu reported altered gene expression in embryonic rat neurons induced by Phe, notably mRNA up-regulation of manganese-containing SOD and copper-zinc containing SOD (Zhang and Gu 2005). Our PKU model disturbed the activity of CAT, SOD, and GPx, enzymes involved in H_2O_2 detoxification. Pups subjected to PKU presented lower CAT activities in the cerebral cortex (−41%), hippocampus



(−34–36%) and cerebellum (−37–42%). SOD was altered only in the hippocampus, as evidenced by the activity 20–26% lower than Sal in the PKU group. GPx activity was 31–35% inferior to Sal in the PKU group in the cerebral cortex, and 26–38% higher than Sal in the PKU + Cr + Pyr group in the cerebellum. The cotreatment with Cr + Pyr prevented most of the

Fig. 2 Effects of PKU induction and/or Cr + Pyr administration on enzymes of phosphoryl transfer network. Data are expressed as mean ± SD for 6 animals per group. MtCK (mitochondrial creatine kinase) and CytCK (cytosolic creatine kinase) are expressed as μmol of creatine per min per mg of protein; AK (adenylate kinase), expressed as μmol of ATP per min per mg of protein; PK (pyruvate kinase), expressed as μmol of pyruvate per min per mg of protein; HK (hexokinase), expressed as μmol of NADH per min per mg of protein; GAPDH (glyceraldehyde-3-phosphate dehydrogenase), expressed as μmol of NADH per min per mg of protein. *$p < 0.005$ compared to the other groups (one-way ANOVA followed by the Tukey test)

alterations induced by PKU on the antioxidant enzymes possibly acting as scavengers (Lawler et al. 2002; Long and Halliwell 2009; Kladna et al. 2015), which agrees with the prevention against ROS formation in cerebral cortex and hippocampus, and the protection of SH groups observed in the three brain structures.

The communication between intracellular compartments of ATP consumption and production performed by phosphotransfer enzymes also plays an antioxidant role. The ADP generated by HK activity reaches the mitochondrial matrix promptly, where it is used as a substrate by F_1F_0 ATP synthase to produce ATP at the expenses of mitochondrial membrane potential ($\Delta\Psi m$) (da Silva et al. 2004). This ATP is further exchanged with another external ADP molecule to be utilized again by HK, generating cycling of ADP/ATP that keeps the ADP at steady-state levels and low $\Delta\Psi m$ values (Korshunov et al. 1997; Zorova et al. 2018). CytCK and MtCK prevent ROS accumulation in a similar way, coupling OXPHOS and the phosphorylation of Cr into phosphocreatine (PCr) using intramitochondrially produced ATP. PCr is exported to the cytosol, whereas the produced ADP is pumped back to the mitochondrial matrix via adenine nucleotide translocator (ANT), thus stimulating OXPHOS (Meyer et al. 2006). AK present in mitochondrial compartments also supports the ADP/ATP cycling, enabling the transfer and making available the energy of two high-energy phosphoryls, the β- and the γ-phosphoryls of a single ATP molecule. The resulting AMP signals feedback to mitochondrial respiration amplified by the generation of two molecules of ADP at the mitochondrial intermembrane site (Dzeja and Terzic 2009).

Oxidative inactivation of glycolytic enzymes is observed in organisms ranging from *E. coli* to mammalian cells (Reichmann et al. 2018). PK, which catalyzes the irreversible conversion of phosphoenolpyruvate to pyruvate, can be inactivated by H_2O_2 (Halliwell 1992). Moreover, in vitro (Feksa et al. 2003; Horster et al. 2006; Yuan et al. 2018) and in vivo (Weber 1969; Miller et al. 1973; Feksa et al. 2002; Bortoluzzi et al. 2014) studies describe the inhibitory effect of Phe over PK activity, and so we observed in the present study. Oxidative and allosteric inhibition of brain PK activity may be related to the reduction of glucose metabolism observed in the brain of the phenylketonuric patients (Pietz et al. 2003; Wasserstein et al. 2006; Ficicioglu et al. 2013). GAPDH also suffers oxidative inactivation, being able to sense redox changes through a cysteine residue in the catalytic domain. This cysteine is particularly sensitive to H_2O_2-induced oxidation, which leads to the formation of a cysteine sulfenic acid, a reaction that inactivates GAPDH, and acts as a metabolic switch for rerouting the carbohydrate flux from glycolysis to pentose phosphate pathway, aiming to reestablish the redox equilibrium of the cytoplasmic NADP(H) (Hildebrandt et al. 2015). More studies are required to clarify the consequences of oxidative stress over energy pathways of the brain in PKU.

The effects of Cr + Pyr we observed in oxidative stress parameters and phosphotransfer network may rely on Cr and Pyr properties to act both as antioxidants and energy substrates. Studies report that cotreatment with Cr ameliorates conditions like Alzheimer disease, and other age-related neurodegenerative diseases presenting energy failure and oxidative stress among their pathophysiological mechanisms (Smith et al. 2014; Rae and Broer 2015). Pyr can increase neuronal survivance after events such as excitotoxic cascades triggered by ischemic injury suppressing ROS generation and preventing ATP depletion (Suh et al. 2005; Yu et al. 2005; Moro et al. 2016). The phosphotransfer network is remarkably plastic – remodeling the cellular energy network compensates deficiency in a specific enzyme – but the disturbance in the activity of two or more enzymes can lead to energy inefficiency (Dzeja et al. 2011). Because of poor antioxidant defenses in the brain, it is conceivable that the mechanisms recycling

Table 1 Effects of Phe + αMePhe load (PKU) and/or Cr + Pyr administration on the weight of the whole brain and cerebral structures

	Saline	PKU	Cr + Pyr	PKU + Cr + Pyr
Whole brain	1.25 ± 0.049	1.02 ± 0.05*	1.2 ± 0.065	1.11 ± 0.048$^{\#}$
Cerebral cortex	0.494 ± 0.025	0.456 ± 0.023$^{\#}$	0.517 ± 0.021*	0.473 ± 0.015$^{\#\#}$
Hippocampus	0.0811 ± 0.004	0.0712 ± 0.006*	0.0821 ± 0.006	0.078 ± 0.005
Cerebellum	0.152 ± 0.011	0.117 ± 0.012*	0.146 ± 0.014	0.119 ± 0.013$^{\#}$*

Data are expressed in grams as mean ± SD for 12 animals in each group. *$p < 0.005$ compared to the other groups; $^{\#}p < 0.005$ compared to saline; $^{\#\#}$ equal to saline but not different from PKU; $^{\#}$*equal to PKU, $p < 0.005$ from saline (one-way ANOVA followed by the Tukey test)

Table 2 Spearman's correlation coefficient between total sulfhydryl content and thiol-containing enzymes activities of phosphoryl transfer network in the cerebral structures

Enzyme	Cerebral Cortex	Hippocampus	Cerebellum
MtCK	0.625**	0.394	0.526*
PK	0.753**	0.589*	0.741*
HK	0.389*	0.467*	0.307
CytCK	0.528**	0.424*	0.150
AK	0.178	0.449*	−0.389
GAPDH	0.796**	0.508*	−0.610**

* $\rho < 0.05$ and ** $p < 0.01$

MtCK (mitochondrial creatine kinase); PK (pyruvate kinase); HK (hexokinase); CytCK (cytosolic creatine kinase); AK (adenylate kinase); GAPDH (glyceraldehyde-3-phosphate dehydrogenase)

ADP play a role in counteracting oxidative stress. Cr and Pyr have antioxidant and energy properties, being able to scavenge ROS and to favor the ADP/ATP ratio for normal mitochondrial function.

It is well-described that PKU affects brain mass and volume (Pearsen et al. 1990; Pfaendner et al. 2005; Perez-Duenas et al. 2006; Bodner et al. 2012), and our model reproduced it. The rat brain increases more than 6× in mass from birth to adulthood. Cerebral cortex and hippocampus follow this pattern, but cerebellum goes through a much superior relative gain in mass, by a factor of 20.7, which agrees with its predominantly postnatal development (Altman 1969; Bandeira et al. 2009). These particularities of postnatal cerebral development may be relevant to understand and further investigate the response of each cerebral structure to PKU induction and Cr + Pyr supplementation.

In conclusion, we observed oxidative stress and energy metabolism alterations occurring concomitantly, also that there is a correlation between damage to sulfhydryl groups and disturbance of energy homeostasis in the cerebral cortex, hippocampus, and cerebellum of young Wistar rats subjected to PKU. Further, we demonstrated that the supplementation with a combination of energetic and antioxidant substrates, creatine and pyruvate, was able to prevent most of the alterations on both sets of parameters evaluated. Future research might elucidate the linkage between oxidative stress and disturbances of the phosphotransfer network, clarifying how these processes contribute to the pathophysiology underlying cerebral underdevelopment in PKU. Although more experiments should be carried out, the results open a new possible approach for nutritional management of PKU. We suggest that creatine and pyruvate supplementation, in addition to Phe restrictive diets, may offer clinical benefits for PKU children by preventing redox imbalance and energy inefficiency throughout brain development and maturation in early ages.

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Compliance with ethical standards

Conflict of interest We have no conflict of interest to declare.

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