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RESVERATROL DOES NOT PREVENT THE INHIBITORY EFFECT OF PHENYLALANINE ON PYRUVATE KINASE ACTIVITY IN THE CEREBRAL CORTEX OF MICE[1]

RESVERATROL NÃO PREVENIU O EFEITO INIBITÓRIO DA FENILALANINA SOBRE A ATIVIDADE DA PIRUVATOCINASE DE CÓRTEX CEREBRAL DE RATOS

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ABSTRACT

Phenylketonuria (PKU) is an autosomal recessive disorder caused by the deficiency of the hepatic enzyme phenylalanine hydroxylase (PAH), which leads to mental retardation in childhood by the brain's exposure to toxic levels of phenylalanine (Phe). The neuropathological mechanisms of this disease are not fully understood. Studies show that high levels of Phe in this disease alter parameters of oxidative stress and inhibit thiol enzymes involved in involved in the transfer network of phosphoryl groups as piruvatocinase (PK) and creatine kinase (CK). Resveratrol (RSV) is an antioxidant substance and it has been studied in the treatment for neurodegenerative diseases. The objective of this study was to evaluate, in vitro, the effects of RSV and Phe on PK activity in the cerebral cortex of mice at different incubation times (0, 30 and 60 minutes). Despite its potent antioxidant characteristic in vitro, resveratrol did not prevent the inhibition caused by Phe in the studied time intervals.

Keywords: hyperphenylalaninemia, phenylketonuria, energy metabolism, antioxidant.

RESUMO

Fenilcetonúria (PKU) é uma doença autossômica recessiva causada pela deficiência da enzima hepática fenilalanina hidroxilase (PAH), levando ao retardo mental na infância pela exposição do cérebro a níveis tóxicos de fenilalanina (Phe). Os mecanismos neuropatológicos desta doença ainda não são completamente conhecidos. Estudos mostram que níveis elevados de Phe nesta doença alteram parâmetros de estresse oxidativo e inibem enzimas tiólicas envolvidas na rede de transferência de grupos fosforil, como piruvatocinase (PK) e creatinacinase (CK). O resveratrol (RSV) é uma substância antioxidante e vem sendo estudado no tratamento de doenças neurodegenerativas. O objetivo deste trabalho foi avaliar, in vitro, os efeitos do RSV e da Phe sobre a atividade da PK em córtex cerebral de ratos em diferentes tempos de incubação (0, 30 e 60 minutos). Apesar de ser um potente antioxidante, in vitro, o resveratrol não preveniu a inibição causada pela Phe nos intervalos de tempos estudados.

Palavras-chave: *hiperfenilalaninemia, fenilcetonúria, metabolismo energético, antioxidante.*

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INTRODUCTION

Phenylketonuria (PKU, OMIM # 261600) is an inborn error of amino acid metabolism of autosomal recessive origin, caused by a mutation in the gene encoding the hepatic enzyme phenylalanine hydroxylase (PAH, EC 1.14.16.1), responsible for converting phenylalanine (Phe) to tyrosine (Tyr). In consequence of the deficiency or absence of the PAH activity, there is an accumulation of Phe levels and its metabolites phenylpyruvate, phenylacetate and phenyllactate in blood and other tissues from patients (SCRIVER; KAUFMAN, 2001).

According to data from ANVISA (2012), the incidence of PKU in Brazil is around a case to 22,000 live births. Once detected early and treated with a diet of low protein intake and supplementation with Phe free formulas, the severe intellectual disability that characterizes the progression of this disease is inhibited (SHARMAN et al., 2012). However, many adolescents and adults with PKU are unable to adhere fully to the restricted Phe diet for life, with plasma Phe levels above the desired range: 600-1300 mmol/L for adults (SANAYAMA et al., 2011) resulting in loss of brain function characterized by deficits of its operation, processing and motor control (BURTON; LEVITON, 2010). So even with early treatment, several studies have reported an increase in psychiatric, neurocognitive and behavioral problems in children and adults with PKU (BILDER et al., 2013).

The excess of Phe is toxic to the brain causing progressive impairment of their functions by interfering with the synthesis of myelin and neurotransmitters, in the somatic functions and of the central nervous system (GROOT et al., 2010; MONTEIRO; CÂNDIDO, 2006). Although dietary treatment in individuals with PKU change the main neurological symptoms some neuropsychological disorders still remain, studies are needed to unravel the pathogenic mechanisms that lead to cognitive impairment in these patients (SPRONSEN et al., 2001).

Animal model studies demonstrate that elevated Phe concentrations inhibit the activity of certain enzymes of phosphoryltransfer network such as the pyruvate kinase (FEKSA et al., 2002) and creatine kinase (COSTABEBER et al., 2003), and also the respiratory chain enzymes (RECH et al., 2002) besides altering oxidative stress parameters in rat brains (HAGEN et al., 2002). These data suggest that Phe induces oxidative stress and alters cellular energy status in the brain.

Pyruvate kinase (PK) is a crucial enzyme of the glycolytic pathway, involved in the maintenance of cell energy balance. PK catalyzes the formation of adenosine triphosphate (ATP) and pyruvate from the irreversible transfer of the phosphoryl group from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP) in the cell (JURICA et al., 1998).

Resveratrol (3,4,5-trihidroxistilbene) (RSV) is a polyphenol present in many foods, such as peanuts, walnuts, cranberry, grape skin. Grape juice and red wine, especially, have high concentrations of RSV (BHAT et al., 2001; STIVALA et al., 2001). To RSV are attributed antioxidant and neuroprotective properties (BHAT et al, 2001; DASGUPTA; MILBRANDT, 2007;

MINAKAWA et al., 2012; FONSECA-KELLY et al., 2012) in animal models of neurodegenerative diseases - namely, Alzheimer's disease (AD), Parkinson's disease, Huntington's disease and epilepsy (ALBANI et al., 2010). Considering that in PKU there is an accumulation of plasma Phe resulting in cognitive deficits, which may be partially explained by inhibition of the enzyme PK and thus decreased production of ATP, the present study aimed to evaluate, in vitro, if the antioxidant power of resveratrol would prevent the inhibitory effect on PK over Phe from cerebral cortex of rats at different times of preincubation.

METHODOLOGY

Animals and Reagents: 6 male Wistar rats of 30 days of life, from the Central Animal House of The University Federal of Santa Maria were used in this experiment. During the adaptation period, rats had free access to water and standard commercial feed. The temperature was maintained at 22 ± 1°C, 12/12 h light-dark cycle. Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1996) were followed in all experiments and the experimental protocol was approved by the Ethics Committee on Animal Use of Franciscan University Center (CEUA/UNIFRA), Santa Maria, RS, Brazil, under number 0131/2012. All efforts were made to minimize the number of animals used and their suffering. All chemical reagents were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Preparation of Brain Tissue: the animals were killed by decapitation, the brain was rapidly dissected, on a plate of Petry kept chilled. The cerebral cortex was rapidly isolated, weighed and homogenized 1:20 (m/v) in Tris-sucrose buffer (0.32 M sucrose, 1 mM EGTA, 10 mM Tris-HCl) pH 7.4 with a Potter-Elvehjem homogenizer glass. The homogenized was centrifuged at 10,000 *x g* for 15 min at 4 ° C in a refrigerated Eppendorf centrifuge. The sediment was discarded and the supernatant used to determine the protein concentration and the PK activity.

Protein determination: Protein content was determined by the method of Lowry et al (1951) using serum bovine albumin as standard.

Enzyme Assay: PK activity was measured as Leong et al (1981). The incubation medium contains 0.1 M Tris / HCl, pH 7.5; 10 mM $MgCl_{2;}$ 0.16 mM NADH; 75 mM KCl; 5.0 mM ADP; 7 units of L-lactate dehydrogenase; 0.1% (v/v) Triton X-100; and 10 μL of mitochondria-free supernatant in a final volume of 0.5 mL. The reaction was initiated by addition of 1.0 mM phosphoenolpyruvate. The experiment was performed in duplicate at 37°C. The results were expressed as μmol pyruvate formed per minute per mg of protein. When present in the incubation medium, the final concentration was 3 mM Phe and RSV was 1 μM. Phe and RSV in the concentrations mentioned above, do not alter the reading of the blank sample. Phe was dissolved in the same Tris-sucrose buffer (0.32 M sucrose, 1 mM EGTA, 10 mM Tris-HCl) pH 7.4 and resveratrol was initially dissolved in

DMSO, whose final concentration in the assay was 0.008 %, and previous tests, DMSO did not alter the PK activity and did not affect the inhibition caused by Phe on the enzyme and did not interfere with the effect of resveratrol. The dose of 3 mM Phe used in this experiment is similar to that found in the blood of untreated PKU patients (SCRIVER; KAUFMAN, 2001).

Statistical analysis: Data were analyzed by two-way ANOVA followed by Tukey test when F values were significant. All data were analyzed using *Statistical* Package for Social Sciences - SPSS.

RESULTS

The results were expressed as a mean ± standard deviation (SD). The PK activity of the cerebral cortex of rats was measured in the presence and absence of Phe and RSV and at different times of pre-incubation of the cerebral cortex homogenate with these compounds.

Two-ways ANOVA showed a significant main effect of Phe ($F(1,21)= 19.19; p<0.001$) but RSV main effect and interaction between Phe and RSV were not significant at zero time of pre-incubation (fig 1). Similar results occurred at 30 and 60 min of pre-incubation: main effect of Phe were ($F(1,21)= 31.55; p<0.001$) and ($F(1,21)= 42.75; p<0.001$) respectively (Fig 2 and Fig 3). No significant effects were found for RSV main effect or interaction effect at 30 and 60 min of pre-incubation times.

Figure 1 - In vitro effect of 3 mM Phe and 1µM RSV on the pyruvate kinase activity of the cerebral cortex without pre-incubation: time = 0. Data are expressed as average ± SD for six independent experiments (animals) performed in duplicate. *p < 0.05 compared with control group (Two ways ANOVA).

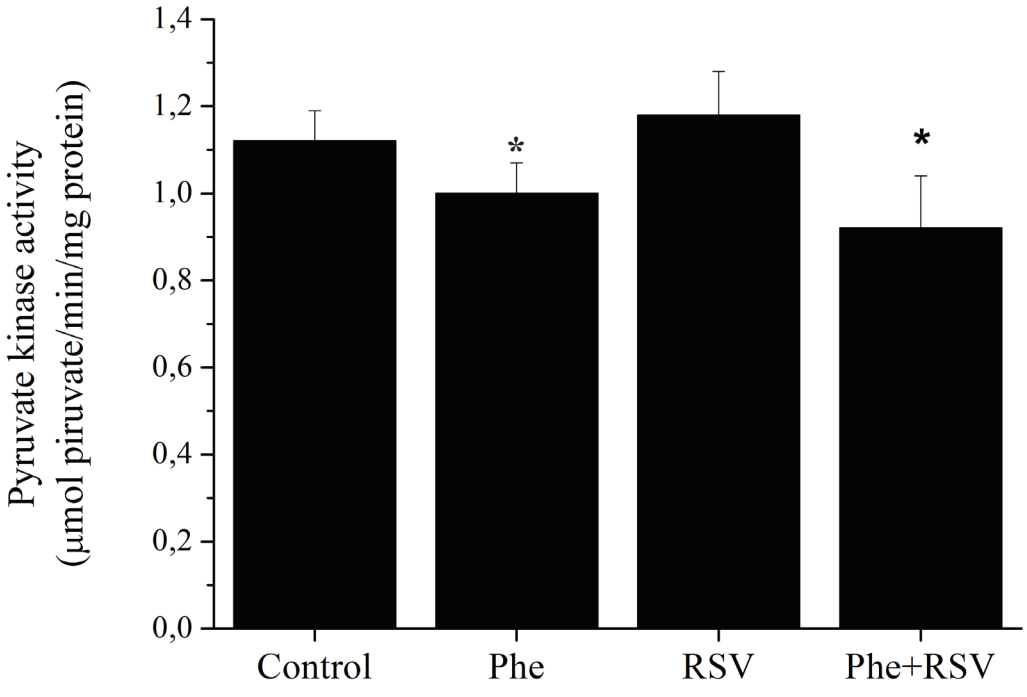


Figure 2 - In vitro effect of 3 mM Phe and 1µM RSV on the pyruvate kinase activity of the cerebral cortex with pre-incubation = 30 min. Data are expressed as average ± SD for six independent experiments (animals) performed in duplicate. *p < 0.05; **p < 0.01 compared with control group (Two ways ANOVA followed by Tukey test).

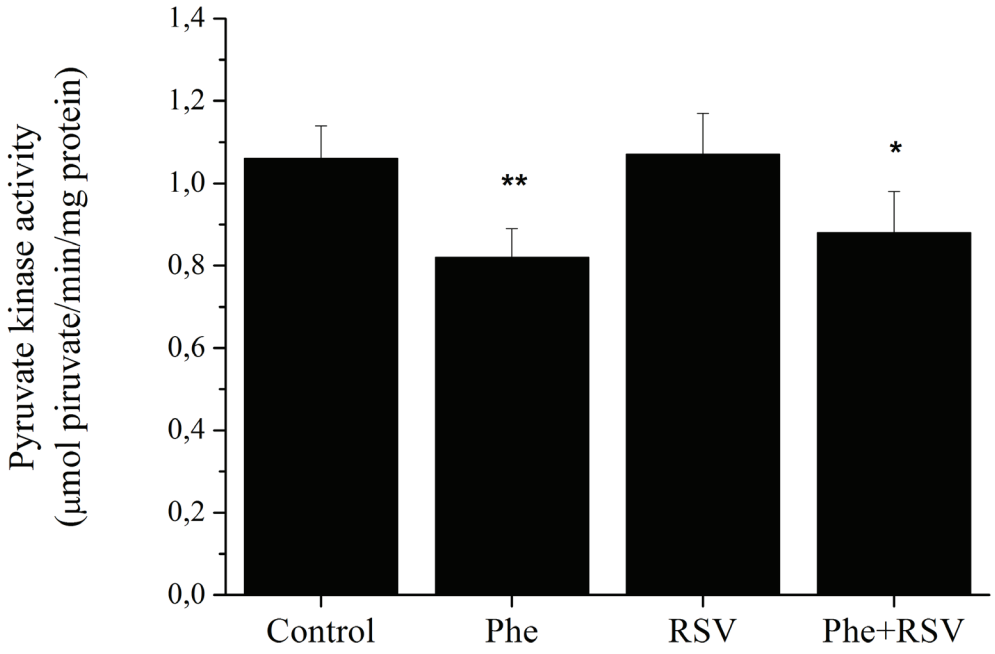
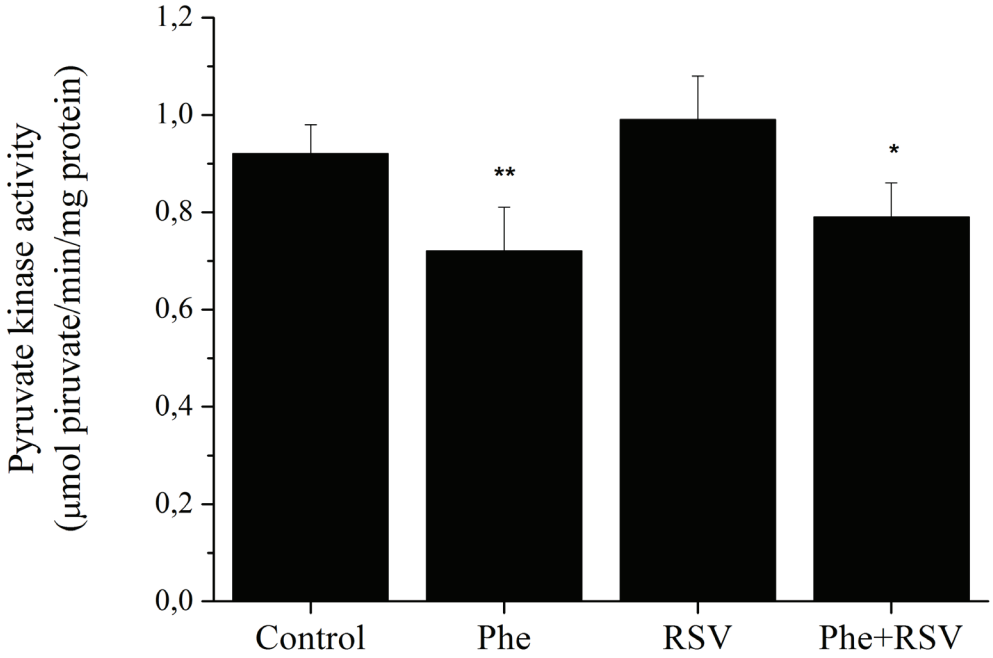


Figure 3 - In vitro effect of 3 mM Phe and 1µM RSV on the pyruvate kinase activity of the cerebral cortex with pre-incubation = 60 min. Data are expressed as mean ± SD for six independent experiments (animals) performed in duplicate. *p < 0.05; ** p < 0.01 compared with control group (Two ways ANOVA followed by Tukey test).



CONCLUSION

The in vitro results show that the PK activity in the cerebral cortex of rats is diminished at all the pre-incubation times tested. This result corroborates the findings of Feksa et al. (2002), who observed both in vitro and in vivo inhibition in the PK activity. Although RSV being considered an antioxidant substance, the concentration of RSV used was not able to prevent the reduction on the PK activity in the cerebral cortex of rats at all the times tested.

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