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Urinary biomarkers of oxidative stress and plasmatic inflammatory profile in phenylketonuric treated patients

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ABSTRACT

Oxidative stress has been proposed as an important pathophysiologic feature of various inborn errors of metabolism, including phenylketonuria (PKU). Considering that there are few studies relating oxidative stress and inflammation directly in PKU disease, the aim of this study was to evaluate and correlate oxidative damage to biomolecules, antioxidant defenses, pro-inflammatory cytokines, phenylalanine (Phe) and its metabolites (phenyllactic acid—PLA and phenylacetic acid—PAA) levels in urine and plasma from patients with PKU under dietary treatment. We observed a marked increase of isoprostanes, which is a lipid peroxidation biomarker, in urine from these treated patients. Next, we demonstrated that protein oxidative damage, measured by di-tyrosine formation, was significantly increased in urine from PKU treated patients and that decreased urinary antioxidant capacity was also observed. Our findings concerning to the inflammatory cytokines interleukin-6 and interleukin-1β, both significantly increased in these patients, provide evidence that the pro-inflammatory state occurs. Besides, interleukin-1β was positively correlated with isoprostanes. We observed a negative correlation between interleukin-6 and interleukin-10, an anti-inflammatory cytokine. Di-tyrosine was positively correlated with Phe, which indicates oxidative damage to proteins, as well as with PAA. These findings may suggest that the protein damage may be induced by Phe and its metabolite PAA in PKU. Our results indicate that pro-oxidant and pro-inflammatory states occur and are, in part, correlated and protein oxidation seems to be induced by Phe and PPA in PKU patients.

1. Introduction

Phenylketonuria (PKU) is the most frequent inherited disorder of amino acid metabolism. The incidence of this autosomal recessive disorder is between 1 in 10,000 and 1 in 15,000 people (Vockley et al., 2014). PKU is caused by deficiency of phenylalanine-4-hydroxylase (PAH), which is a liver-specific enzyme that catalyzes the conversion of L-phenylalanine (Phe) to L-tyrosine (Tyr). Failure of this conversion results in elevated concentrations of Phe and its metabolites, phenyllactic (PLA), phenylacetic (PAA) and phenylpyruvic (PPA) acids in blood and tissues of affected patients. These elevated concentrations interfere with normal development of the central nervous system, leading to severe mental retardation (Scriver and Kaufman, 2001; Casey, 2013; Vockley et al., 2014).

Phe alterations in PKU patients are conditioned by the severity of metabolic disruption and the amount of dietary Phe ingested. Increased concentrations of Phe have a neurotoxic effect, contributing to the structural brain damage, severe mental retardation, and psychiatric disturbances present in untreated patients with PKU. Increased cerebral phenylalanine reduces protein and cholesterol synthesis, impair synaptogenesis and alter glutamatergic transmission (de Groot et al., 2010; van Spronsen et al., 2009). Altered

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cholesterol and protein synthesis particularly affect myelination, as evidenced by human post-mortem and *in vivo* MRI studies (Bauman and Kemper, 1982; Bick et al.,1991). Hypomyelination occurs in untreated and in early-treated PKU patients; which appears to relate to intramyelinic edema (Anderson and Leuzzi, 2010). PKU is an inborn error of metabolism associated with diffuse brain pathology. The outcomes are usually severe when this metabolic disease is left untreated; however, white matter pathology is common even in early diagnosed and treated individuals. Multiple mechanisms by which Phe induces its deleterious effects on brain include impairment of large neutral amino acids (LNAA) uptake into brain, reduction of the availability for neurotransmitters synthesis, inhibition of key enzymatic activities, reduction of the activity of monoamine oxidase B as modifying gene and alteration on myelin metabolism (Ghozlan et al., 2004; Anderson et al., 2007; Blau et al., 2010).

There is a clear link between metabolic control and the outcome of individuals with PKU. Therefore, for most individuals with PKU, the control of Phe levels requires a golden standard therapy that is based on lifelong Phe-restricted diet. Patients with PKU have to eliminate high-proteins foods and have to accept Phe-free amino acid formula enriched with trace elements, vitamins, and minerals (Przyrembel and Bremer, 2000; Giovannini et al., 2007; Poustie and Wildgoose, 2010; Scriver and Kaufman, 2001). . Recently, newer treatments for PKU are emerging and some are current available such as administration of tetrahydrobiopterin, glycomacropeptide, LNAA, phenylalanine ammonia lyase, *etc*., (Blau et al., 2010).

Oxidative stress is a pathological process that has been described in an increasing number of neurodegenerative diseases. Excessive production of reactive oxygen species (ROS) by mitochondrial respiratory chain and NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) is usually thought to be responsible for tissue damage associated with a range of brain injury, inflammation, and degenerative diseases (Halliwell, 2006; Melo et al., 2011). The CNS (central nervous system) is very vulnerable to oxidative injury due to its high oxygen demand, high level of polyunsaturated fatty acids (PUFAs), and weak antioxidant defenses. Brain cells, especially neuroglial cells, are susceptible to the injurious effects of oxidative stress. Several studies have shown that brain cells like microglia and astrocytes induce and release diverse inflammatory mediators in response to oxidative stress. In addition, ROS act as a critical signaling molecule to trigger inflammatory responses in CNS through the activation of the redox sensitive transcription factors, including nuclear factor-κB (NF-κB) and activator protein-1(AP-1) (Uttara et al., 2011; Halliwell and Gutteridge, 2007).

Oxidative stress has been proposed as an important pathophysiologic feature of various inborn errors of metabolism (IEM), including phenylketonuria. In the last few years, mounting evidence obtained from humans and experimental animal PKU models indicate oxidative stress as a mechanism contributing to the pathogenesis of PKU. Data from PKU patients were obtained from peripheral tissues, especially blood, demonstrating a decrease of antioxidant defenses, possibly because of dietary restriction of micro or macronutrients with antioxidant properties, and an increase of lipid, protein, and DNA oxidative damage, probably secondary to increased formation of reactive species (Rocha and Martins, 2012; Ribas et al., 2011).

Considering that there are a few studies relating oxidative stress and inflammation directly in PKU disease, the aim of this study was to evaluate and correlate oxidative damage to biomolecules, antioxidant defenses, pro-inflammatory cytokines, Phe and its metabolites (PLA and PAA) in urine and plasma from patients with PKU under dietary treatment.

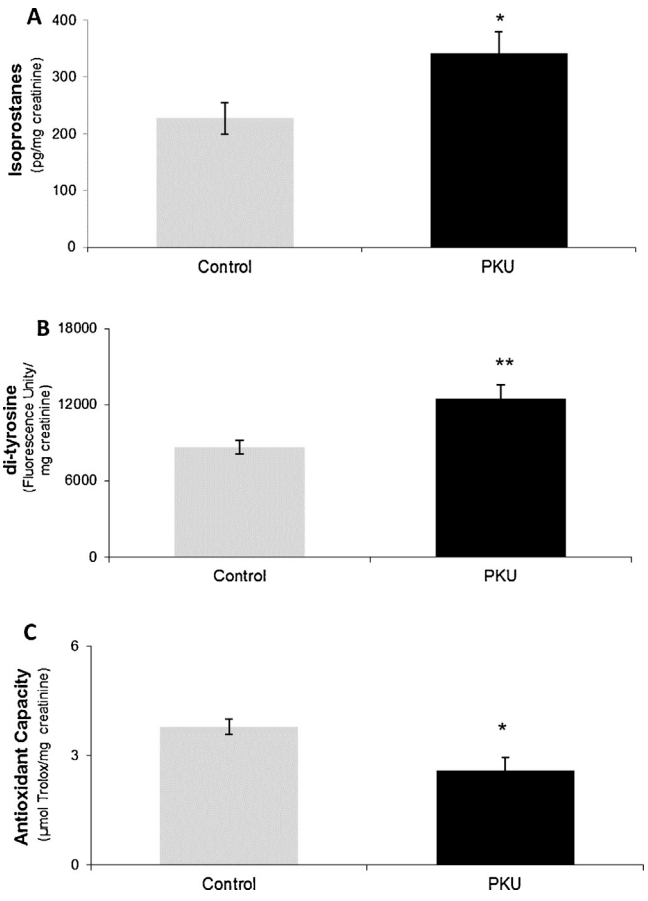

Fig. 1. Urinary isoprostanes (A) di-tyrosine (B) and antioxidant capacity (C) levels in PKU treated patients ($n = 8$) and controls ($n = 6$–8). Data represent the mean ± SEM. Difference from control, $^{*}p < 0.05$, $^{**}p < 0.01$ (unpaired Student's *t*-test).

2. Materials and methods

2.1. Subjects

For this study, we enrolled 10 patients with classical PKU (2 females and 8 males) under dietary treatment aged between 10 and 22 years old (16.6 ± 1.27 years old). PKU patients were submitted to a Phe-restricted diet with low consumption of protein supplemented with a semi synthetic amino acids formula (according to age: PKU 2 Secunda for individuals above 8 years old and PKU 3 Advanta for individuals above 15 years old—Support®). The diet contained 220–450 mg/(kg day) Phe and 2.55–4.00 g/(kg day) Tyr according to patients' age. All PKU patients, included in this study, were late diagnosed (over 3 months of age) ranging from 3 months to 5 years old. They presented at Medical Genetics Service of Hospital de Clínicas de Porto Alegre for precise evaluation of delayed mental and motor developmental. Immediately after diagnoses, all patients were placed on a phenylalanine restricted diet and presented mean annual blood Phe levels calculated around 847.95 ± 77.82 μmol/L. At the moment of the tests, the blood phenylalanine levels among the PKU patients enrolled in this study ranged from 490.37 to 1077.61 μmol/L (822.6 ± 75.11).

The control group ($n = 10$) consisted of healthy individuals with similar ages (17.14 ± 2.76 years old) and sex (4 females and 6 males) to the PKU treated patients. All participants or their legal guardians gave informed written consent for the present study which was approved by the Ethics in Research Committee of Hospital de Clínicas de Porto Alegre, RS, Brazil (projects no. 04-080 and 14-0180).