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Argininic acid alters markers of cellular oxidative damage *in vitro*: Protective role of antioxidants

Daniela Delwing-de Lima[a,b,*], Simone Sasso[b], Leticia Dalmedico[c], Débora Delwing-Dal Magro[d], Eduardo Manoel Pereira[c], Angela T.S. Wyse[e]

[a] *Departamento de Medicina, Universidade da Região de Joinville – UNIVILLE, Rua Paulo Malschitzki, 10 – Zona Industrial Norte, CEP 89201-972, Joinville, SC, Brazil*
[b] *Programa de Pós-Graduação em Saúde e Meio Ambiente, Universidade da Região de Joinville – UNIVILLE, Rua Paulo Malschitzki, 10 – Zona Industrial Norte, CEP 89201-972, Joinville, SC, Brazil*
[c] *Departamento de Farmácia, Universidade da Região de Joinville – UNIVILLE, Rua Paulo Malschitzki, 10 – Zona Industrial Norte, CEP 89201-972, Joinville, SC, Brazil*
[d] *Departamento de Ciências Naturais, Centro de Ciências Exatas e Naturais, Universidade Regional de Blumenau, Rua Antônio da Veiga, 140, CEP 89012-900, Blumenau, SC, Brazil*
[e] *Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, Porto Alegre, RS, Brazil*

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ABSTRACT

We, herein, investigated the *in vitro* effects of argininic acid on thiobarbituric acid-reactive substances (TBA-RS), total sulfhydryl content and on the activities of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in the blood, kidney and liver of 60-day-old rats. We also verified the influence of the antioxidants (each at 1.0 mM) trolox and ascorbic acid, as well as of N^G-nitro-L-arginine methyl ester (L-NAME) at 1.0 mM, a nitric oxide synthase inhibitor, on the effects elicited by argininic acid on the parameters tested. The liver, renal cortex and renal medulla were homogenized in 10 vol (1:10w/v) of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl; and erythrocytes and plasma were prepared from whole blood samples obtained from rats. For *in vitro* experiments, the samples were pre-incubated for 1 h at 37 °C in the presence of argininic acid at final concentrations of 0.1, 1.0 and 5.0 μM. Control experiments were performed without the addition of argininic acid. Results showed that argininic acid (5.0 μM) enhanced CAT and SOD activities and decreased GSH-Px activity in the erythrocytes, increased CAT and decreased GSH-Px activities in the renal cortex and decreased CAT and SOD activities in the renal medulla of 60-day-old rats, as compared to the control group. Antioxidants and/or L-NAME prevented most of the alterations caused by argininic acid on the oxidative stress parameters evaluated. Data suggest that argininic acid alters antioxidant defenses in the blood and kidney of rats; however, in the presence of antioxidants and L-NAME, most of these alterations in oxidative stress were prevented. These findings suggest that oxidative stress may be make an important contribution to the damage caused by argininic acid in hyperargininemic patients and that treatment with antioxidants may be beneficial in this pathology.

1. Introduction

Hyperargininemia (OMIM 207800) is caused by a deficiency of the enzyme, arginase 1 (ARG1, EC 3.5.3.1), which catalyses the last step in the urea cycle, the hydrolysis of arginine to ornithine and urea (Scaglia and Lee, 2006). It is caused by mutations in the 8 exons of the ARG1 gene located on chromosome 6q23 (Sparkes et al., 1986). According to Scaglia and Lee (2006) the main biochemical abnormality of hyperargininemia involves increased arginine levels in the blood and other fluids. This disease usually appears in infants and toddlers and, rarely, during the neonatal period (De Deyn et al., 1997). It manifests as progressive spastic paraparesis (with a smaller effect on the upper extremities) with loss of developmental milestones, which gradually evolves into severe mental retardation, poor growth with consequent short stature, and seizures; some patients experience episodes of irritability, nausea, poor appetite, and lethargy (Crombez and Cederbaum, 2005). In neonates, hyperargininemia has been reported to present with cirrhosis (Braga et al., 1997), cholestasis (Gomes Martins et al., 2011), and cerebral edema (Picker et al., 2003).

Since it is difficult to correlate ammonemia with the symptomatology seen in hyperargininemia, it has been suggested that accumulated catabolites of arginine in the physiological fluids could act as candidate

* Corresponding author at: Departamento de Medicina, Universidade da Região de Joinville, Rua Paulo Malschitzki, 10 – Zona Industrial Norte, CEP 89201-972, Joinville, SC, Brazil.
E-mail address: daniela.delwing@univille.br (D. Delwing-de Lima).

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neurotoxins that could perhaps be associated with the epilepsy and/or spasticity specifically seen in the disease (Joshua et al., 2010). Moreover, intravenous loading of arginine has been shown to induce a pronounced increase in the formation of α-keto-δ-guanidinovaleric acid, argininic acid and *N*-α-acetylarginine in patients with arginase I deficiency (Terheggen et al., 1972; Wiechert et al., 1976). In addition, hyperargininemic patients, especially when untreated, and also protein-restricted patients, are known to exhibit increased plasma α-keto-δ-guanidinovaleric, *N*-α-acetylarginine, homoarginine and argininic acid (Marescau et al., 1985, 1990; Mizutani et al., 1987).

Furthermore, increases in arginine levels may result in increased nitric oxide (NO) production, and a possible role for NO in the pathophysiology of hyperargininemia has been suggested (Buchmann et al., 1996; Wyse et al., 2001b; Reis et al., 2002; Delwing et al., 2003; Scaglia et al., 2004). In this context, chemical reactions of NO with the superoxide anion (O_2^-) and with other free radicals lead to the production of highly reactive intermediates (Turpaev, 2002). Moreover, changes in nucleotide hydrolysis (Balz et al., 2003), membrane fluidity (Silva et al., 1999) or oxidative stress (Wyse et al., 2001a) may further contribute to the pathophysiology of the disease.

It is known that high concentrations of free radicals induce oxidative stress and promote cellular injury by lipid peroxidation, enzyme inactivation, DNA damage and degradation of structural proteins (Halliwell and Gutteridge, 2007; Saugstad, 2001). Antioxidant mechanisms that can be either enzymatic (including catalases, dismutases, and peroxidases) or non-enzymatic (such as vitamin A, C, or E) are critical for protecting cells against ROS-induced damage both at steady state and upon acute oxidative stress (Oakley et al., 2009). With regard to antioxidant enzymes, CAT catalyzes the decomposition of H_2O_2 to yield oxygen and water. Glutathione peroxidase catalyzes the decomposition of H_2O_2 or organic peroxides and reduces glutathione (GSH), which forms oxidized glutathione (GSSG). GSSG is again reduced to GSH by glutathione reductase, thus forming the redox cycle. These enzymes are found in both cytosol and mitochondria and SOD catalyses the dismutation of the superoxide radical (Ichikawa et al., 1994; Halliwell and Gutteridge, 1985).

Previous studies have shown that guanidine compounds (GCs) induce oxidative stress in the rat brain, due to increased chemiluminescence and decreased enzymatic and non-enzymatic antioxidant defenses (Wyse et al., 2001a). As such, we investigated the *in vitro* effects of different concentrations of argininic acid (increased in the plasma and cerebrospinal fluid [CSF] of hyperargininemic patients) on an important parameter of lipoperoxidation, namely TBA-RS, and on an important parameter of protein damage, namely total sulfhydryl content, and on the activities of antioxidant enzymes catalase (CAT), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) in the blood, kidney and liver of rats. Furthermore, we also tested the influence of the antioxidants, α-tocopherol (trolox) and ascorbic acid and of N^G-nitro-L-arginine methyl ester (L-NAME) on the effects elicited by argininic acid, in order to investigate the possible participation of NO and/or its derivatives $ONOO^-$ and other free radicals on the effects of argininic acid on these parameters. Our working hypothesis is that high levels of argininic acid *in vitro* is able to induce oxidative damage to biomolecules and that the antioxidants, vitamins E and C, and L-NAME may provide protection against this damage.

2. Materials and methods

2.1. Animals and reagents

Sixty-day-old male Wistar rats (180–200 g), obtained from the Tecpar Company, Curitiba, Brazil, were used in experiments. The animals from our own breeding stock were maintained on a 12 h light/12 h dark cycle at a constant temperature (22 ± 1 °C), with free access to water and commercial protein chow. 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 University of the Joinville Region, Joinville, Brazil, under the protocol number 019/2013 PRPPG/CEP. All chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA.

Preparation of erythrocytes, plasma and tissue samples: The animals were sacrificed by decapitation, in the absence of anesthesia, and then, the blood was collected and the liver and kidney removed for the evaluation of oxidative stress parameters.

Erythrocytes and plasma: were prepared from whole blood samples obtained from rats. Whole blood was collected and transferred to heparinized tubes for erythrocyte separation. Blood samples were centrifuged at 1000 × *g*, plasma was removed by aspiration and frozen at −80 °C until determination. Erythrocytes were washed three times with cold saline solution (0.153 mol/L sodium chloride). Lysates were prepared by the addition of 1 ml of distilled water to 100 µl of washed erythrocytes and frozen at −80 °C until determination of the antioxidant enzyme activities.

For the determination of antioxidant enzyme activities, erythrocytes were frozen and thawed three times, and centrifuged at 13 500 × *g* for 10 min. The supernatant was diluted in order to contain approximately 0.5 mg/mL of protein.

Liver and kidney: after decapitation, were removed, decapsulated and maintained in ice-cold buffered sodium phosphate (20 mM, pH 7.4, 140 mM KCl). The renal cortex was carefully separated from the renal medulla. The liver, renal cortex and renal medulla were homogenized in 10 vol (1:10w/v) of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. Homogenates were prepared using a Potter-Elvehejem homogenizer (Remi motors, Mumbai, India), employing 5 pulses, and centrifuged at 800°*g* for 10 min at 4 °C, to discard nuclei and cell debris. The pellet was discarded and the supernatant was saved in aliquots and stored at −20 °C for assaying the free-radical scavenging enzymes, total sulfhydryl content and for estimation of lipid peroxidation (Ferreira et al., 2012).

3. Experimental protocols

3.1. In vitro studies

For *in vitro* experiments, erythrocytes, plasma, liver and kidney supernatants were pre-incubated for 1 h at 37 °C in the presence of argininic acid at final concentrations of 0.1, 1.0 and 5.0 µM. Control experiments were performed without argininic acid addition. After incubation, aliquots were taken to measure TBA-RS, total sulfhydryl content and antioxidant enzymes.

3.2. Trolox (α-tocopherol), ascorbic acid and L-NAME administration

The assays were divided into eight groups: Group 1 (control-saline), group 2 (5.0 µM argininic acid), group 3 (control – 1.0 mM trolox), group 4 (5.0 µM argininic acid + 1.0 mM trolox), group 5 (control – 1.0 mM ascorbic acid), group 6 (5.0 µM argininic acid + 1.0 mM ascorbic acid), group 7 (control – 1.0 mM L-NAME) and group 8 (5.0 µM argininic acid + 1.0 mM L-NAME). The doses of trolox, ascorbic acid and L-NAME utilized were chosen according to Wyse et al. (2002), Silva et al. (2004) and Qi et al. (1995), respectively.

4. Assay of oxidative stress parameters

4.1. Thiobarbituric acid reactive substances (TBA-RS)

TBA-RS were determined according to the method described by Esterbauer and Cheeseman (1990). TBA-RS methodology measures malondialdehyde (MDA), a product of lipoperoxidation caused mainly by hydroxyl free radicals. For the measurements, plasma and tissues were mixed with 10% trichloroacetic acid and 0.67% thiobarbituric