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Creatine prevents the imbalance of redox homeostasis caused by homocysteine in skeletal muscle of rats

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ABSTRACT

Homocystinuria is a neurometabolic disease caused by severe deficiency of cystathionine beta-synthase activity, resulting in severe hyperhomocysteinemia. Affected patients present several symptoms including a variable degree of motor dysfunction, being that the pathomechanism is not fully understood. In the present study we investigated the effect of chronic hyperhomocysteinemia on some parameters of oxidative stress, namely 2′7′ dichlorofluorescein (DCFH) oxidation, levels of thiobarbituric acid-reactive substances (TBARS), antioxidant enzyme activities (SOD, CAT and GPx), reduced glutathione (GSH), total sulfhydryl and carbonyl content, as well as nitrite levels in soleus skeletal muscle of young rats subjected to model of severe hyperhomocysteinemia. We also evaluated the effect of creatine on biochemical alterations elicited by hyperhomocysteinemia. Wistar rats received daily subcutaneous injection of homocysteine (0.3–0.6 μmol/g body weight), and/or creatine (50 mg/kg body weight) from their 6th to the 28th days age. Controls and treated rats were decapitated at 12 h after the last injection. Chronic homocysteine administration increased 2′7′dichlorofluorescein (DCFH) oxidation, an index of production of reactive species and TBARS levels, an index of lipoperoxidation. Antioxidant enzyme activities, such as SOD and CAT were also increased, but GPx activity was not altered. The content of GSH, sulfhydril and carbonyl were decreased, as well as levels of nitrite. Creatine concurrent administration prevented some homocysteine effects probably by its antioxidant properties. Our data suggest that the oxidative insult elicited by chronic hyperhomocystenemia may provide insights into the mechanisms by which homocysteine exerts its effects on skeletal muscle function. Creatine prevents some alterations caused by homocysteine.

1. Introduction

Homocysteine (Hcy), a sulfur-containing amino acid, is metabolized by remethylation to methionine or by transsulfuration to cysteine via cystathionine (McCully, 2011; Williams and Schalinske, 2010). It is an intermediate for many key processes such as cellular methylation and cellular antioxidant potential. The normal plasma Hcy concentrations vary from 5 to 14 μmol/l. Hyperhomocysteinemia is a systemic condition and has been attributed to multi-organ pathologies (Veeranki and Tyagi, 2013). It may be classified as mild (15–30 μmol/l), moderate (31–100 μmol/l) and severe (>100 μmol/l) (Mudd et al., 2001; Stead et al., 2001). In some cases, Hcy concentrations can achieve above 100 μM, which is characteristic of classical homocystinuria (HCU), an inborn error of metabolism caused by a deficient of enzyme cystathionine β-synthase activity (CβS, EC 4.2.1.22) (Mudd et al., 2001). Affected patients exhibit plasma concentrations of Hcy that can reach up to 500 μmol/l and usually present clinical and pathologic manifestations that affect the motor system (Huang et al., 2011; Leishear et al., 2012), whose mechanisms are unknown. In addition, it has been observed that hyperhomocysteinemia is associated with muscle dysfunction (Veeranki and Tyagi, 2013).

Oxidative stress plays an important role in skeletal muscle damage in hyperhomocysteinemia (Swart et al., 2012). Free radicals are produced as a normal function of cell metabolism, such as mitochondrial bioenergetic, xanthine oxidase, peroxisomes, inflammation processes, phagocytosis, and arachidonate pathways. External factors that help to promote the production of free radicals are smoking, environmental

Abbreviations: DCFH, dichlorodihydrofluorescein diacetate; TBARS, thiobarbituric acid-reactive substances; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GSH, reduced glutathione; Hcy, homocysteine; HCU, homocystinuria; CβS, cystathionine β-synthase; RS, reactive species; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; NADPH, nicotinamide adenine dinucleotide phosphate; $O_2^{\cdot-}$, superoxide anion; NOS, nitric oxide synthase; GAA, guanidinoacetic acid; DTNB, 5,5′-dithio-bis (2-nitrobenzoic acid); EDTA, ethylenediamine tetraacetic acid; ANOVA, one-way analysis of variance.

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pollutants, radiation, drugs, pesticides, industrial solvents and ozone (Carocho and Ferreira, 2013; Lobo et al., 2010). The balance between the production and neutralization of reactive species (RS) by antioxidants is very delicate, and if this balance tends to the overproduction of the RS, the cells start to suffer the consequences of oxidative stress (Wiernsperger, 2003). The main targets of RS are proteins, DNA (deoxyribonucleic acid) and RNA (ribonucleic acid) molecules, sugars and lipids (Craft et al., 2012; Halliwell, 2011). It has been suggested that oxidative stress and its consequent oxidative damage may be involved in muscular diseases (Hammouda et al., 2012; Swart et al., 2012). Supporting this notion, a reduction in antioxidant bioavailability along with increased oxidative stress has been reported in both experimental and human conditions.

Regarding enzymatic antioxidants, they are divided into primary and secondary enzymatic defenses. The primary defense, is composed of three important enzymes that prevent the formation or neutralize free radicals: superoxide dismutase (SOD) that converts superoxide anions, a highly deleterious radical for all types of cells and tissues, into hydrogen peroxide (H_2O_2) as a subtract for catalase and molecular oxygen (O_2); catalase (CAT) that converts hydrogen peroxide into water and molecular oxygen and has one of the biggest turnover rates known to man, allowing just one molecule of catalase to convert 6 billion molecules of hydrogen peroxide and glutathione peroxidase (GPx), which donates two electrons to reduce peroxides by forming selenoles and also eliminates peroxides as potential substrate for the Fenton reaction (Rahman, 2007). The secondary enzymatic defense includes glutathione reductase and glucose-6-phosphate dehydrogenase. Glutathione reductase reduces glutathione (GSH-antioxidant) from its oxidized to its reduced form, thus recycling it to continue neutralizing more free radicals. Glucose-6-phosphate regenerates NADPH (nicotinamide adenine dinucleotide phosphate) creating a reducing environment (Gamble and Burke, 1984; Ratnam et al., 2006). These two enzymes do not neutralize free radicals directly, but may act through of other endogenous antioxidants.

Studies using experimental models and endothelial cells incubated with elevated homocysteine levels indicate that Hcy may promote the formation of reactive oxygen species, especially the superoxide anion (O_2^-) (Weiss, 2005) by self-oxidation of homocysteine and/or cysteine (Hogg et al., 2006). This increase in intracellular production of free radicals can cause cellular damage (Streck et al., 2003). Moreover, investigators have reported that Hcy can cause changes in the defense system antioxidant (Blundell et al., 1996). Furthermore, studies in our group showed that severe hyperhomocysteinemia causes oxidative stress in other systems, such as cardiovascular (Kolling et al., 2011), hepatic (Matté et al., 2009) and cerebral (Streck et al., 2003).

Nitric oxide (NO) is synthesized in the thin endothelial lining of blood vessels by endothelial NO synthase (NOS) (Liu and Fung, 1998). The hallmark of endothelial dysfunction is a reduction in the bioavailability of NO and several studies have demonstrated that the Hcy reduces the availability of endothelial-derived NO (Mujumdar et al., 2001). A decrease in the bioavailability of NO is associated with an increase in leukocyte–endothelial cell interactions (Davenpeck et al., 1994), enhanced platelet adherence and aggregation (Radomski et al., 1990) and with the proliferation of smooth muscle cells (Liu and Fung, 1998). When NO is released by the endothelium, it can modulate blood flow, inflammation and platelet aggregation. Although NO appears to have a number of important physiological roles, it is also a free radical and may be cytotoxic (Ekelund et al., 1999). In addition, it has been shown that exercise increases NO production by vascular endothelial cells of the skeletal muscle (Gielen et al., 2010).

Creatine is endogenously synthesized by the liver, kidneys and pancreas from the amino acids glycine and arginine to form guanidinoacetic acid (GAA) and ornithine in a reaction catalyzed by the enzyme L-arginine: glycine amidinotransferase (Deminice et al., 2007; Xu et al., 2010). Next, the irreversible transfer of a methyl group from SAM to GAA is catalyzed by the enzyme SAM: guanidinoacetate N-methyltransferase (Wyss and Kaddurah-Daouk, 2000). Through this reaction, the endogenous synthesis of creatine consumes a considerable number of methyl groups. In humans, creatine synthesis has been reported to account for 70% of Hcy formation (Mudd and Poole, 1975). This amine is naturally found in food, especially meat and fish, although only half of the daily creatine requirement (about 1 g/d) comes from the diet (Persky and Brazeau, 2001). In skeletal muscle of vertebrates, the creatine participates in metabolic reactions within cells and is catabolized in the muscles generating creatinine which is then excreted by the kidney in urine (Terjung et al., 2000). Studies show that the creatine may have a protector and antioxidant role in certain neuromuscular and neurodegenerative diseases (Beal, 2011; Bender et al., 2006; Chung et al., 2007; Sestili et al., 2011).

Creatine supplementation has emerged as a promising adjunct therapy in several pathological conditions (Gualano et al., 2011), including muscular diseases (Deminice and Jordao, 2012; Wallimann et al., 1992). Interestingly, a growing body of experimental and clinical literature has suggested that creatine may exert protective effect in diseases, whose mechanisms seem to be associated with oxidative stress (Sestili et al., 2011). In fact, in vitro studies have revealed that the creatine may have antioxidant properties by acting as a scavenger of free radicals, such as superoxide anions and peroxynitrite (Lawler et al., 2002; Sestili et al., 2011). In addition, studies show that the creatine prevents oxidative stress parameter, such as lipid peroxidation, in heart of rats subjected to Hcy (Kolling et al., 2011).

In the present study we evaluate the effects of severe hyperhomocysteinemia on parameters namely 2′7′dichlorofluorescein (DCFH) oxidation, levels of thiobarbituric acid-reactive substances (TBARS), antioxidant enzyme activities (SOD, CAT and GPx), reduced glutathione (GSH), total sulfhydryl and carbonyl content, as well as nitrite levels in soleus skeletal muscle of young rats subjected to model of severe hyperhomocysteinemia. We also analyze the role of creatine on the possible biochemical changes observed in this model. Our hypothesis is that the alteration motors caused by Hcy may be associated with the oxidative insult and that creatine might improve such muscle damage since it has been postulated that creatine could act as an antioxidant agent preventing increased oxidative stress. Thus, we investigated the effects of creatine administration alone and in combination with Hcy.

2. Materials and methods

2.1. Ethical approval

Male or female Wistar rats (6-day-old) were obtained from the Central Animal House of the Department of Biochemistry, Institute of Basic Science of Health, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12/12 h light/dark cycle in an air-conditioned constant temperature (22 ± 1 °C) colony room. Rats had free access to a 20% (w/w) protein commercial chow and water. The NIH *Guide for the Care and Use of Laboratory Animals* (NIH publication no. 80-23, revised 1996) was followed in all experiments and was approved by the Ethical Committee of the Universidade Federal do Rio Grande do Sul, Brazil (#21847).

2.2. Subjects and reagents

2.2.1. Creatine and homocysteine chronic treatment

Creatine and D,L-Hcy were dissolved in 0.85% NaCl solution and buffered to pH 7.4. Hcy solution was administered subcutaneously twice a day at 8 h intervals from their 6th to the 28th days of age. Hcy doses were calculated from pharmacokinetic parameters previously determined in our laboratory (Streck et al., 2002). 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 the rats received 0.6 μmol Hcy/g body weight. Plasma