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ORIGINAL ARTICLE

Creatine supplementation reduces oxidative stress biomarkers after acute exercise in rats

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Abstract The objective of this study was to evaluate the effect of creatine supplementation on muscle and plasma markers of oxidative stress after acute aerobic exercise. A total of 64 Wistar rats were divided into two groups: control group ($n = 32$) and creatine-supplemented group ($n = 32$). Creatine supplementation consisted of the addition of 2% creatine monohydrate to the diet. After 28 days, the rats performed an acute moderate aerobic exercise bout (1-h swimming with 4% of total body weight load). The animals were killed before (pre) and at 0, 2 and 6 h ($n = 8$) after acute exercise. As expected, plasma and total muscle creatine concentrations were significantly higher ($P < 0.05$) in the creatine-supplemented group compared to control. Acute exercise increased plasma thiobarbituric acid reactive species (TBARS) and total lipid hydroperoxide. The same was observed in the soleus and gastrocnemius muscles. Creatine supplementation decreased these markers in plasma (TBARS: pre 6%, 0 h 25%, 2 h 27% and 6 h 20%; plasma total lipid hydroperoxide: pre 38%, 0 h 24%, 2 h 12% and 6 h 20%, % decrease). Also, acute exercise decreased the GSH/GSSG ratio in soleus muscle, which was prevented by creatine supplementation (soleus: pre 8%, 0 h 29%, 2 h 30% and 6 h 44%, % prevention). The results show that creatine supplementation inhibits increased oxidative stress markers in plasma and muscle induced by acute exercise.

Keywords Creatine supplementation · Acute exercise · Oxidative stress markers · Antioxidant

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Introduction

It is now well established that an acute session of eccentric exercise promotes reactive oxygen species generation, which may lead to oxidative stress (Finaud et al. 2006; Bloomer 2008). Several studies have shown increased oxidative stress biomarkers after acute exercise in rats (Bachur et al. 2007) and humans (Michailidis et al. 2007; Deminice et al. 2011). The extent of oxidation is dependent on the exercise mode, intensity, and duration (Bloomer 2008).

Since Harris et al. (1992) demonstrated that creatine (Cr) supplementation increases muscle Cr and phosphorylcreatine (PCr) content, creatine has became the most popular supplement proposed as an ergogenic aid. This is due to the fact that Cr plays an important role in rapid energy provision during muscle contraction through the ATP–CP system (Wyss and Schulze 2002). Over the last few years, creatine supplementation has been extended to the medical field to treat a number of muscular, neurological and cardiovascular diseases such as gyrate atrophy (Sipilä et al. 1981; Heinänen et al. 1999), McArdle disease (Vorgerd et al. 2000), Duchenne dystrophy (Felber et al. 2000; Tarnopolsky et al. 2004), myasthenia gravis (Stout et al. 2007), amyotrophic lateral sclerosis (Mazzini et al. 2001), and Parkinson's disease (Matthews et al. 1999). A reader interested in the therapeutic role of creatine supplementation is encouraged to consult the recent review of Gualano et al. (2010).

Over the past decade, creatine has been shown to exert antioxidant effects (Sestili et al. 2011). Lawler et al. (2002) first demonstrated that Cr acts as an antioxidant scavenger primarily against radical species. Later studies have shown protective effects against oxidative stress in cultured cells (Lenz et al. 2005; Sestili et al. 2006), DNA and RNA damage (Guidi et al. 2008; Fimognari et al. 2009); and in vivo experiments on rats (Deminice et al. 2009). Creatine has also been shown to have anti-inflammatory activity in endothelial cells (Nomura et al. 2003). Thus, there are some in vitro data available showing that creatine may act as a scavenger of radical species. However, few research articles exist demonstrating the in vivo antioxidant capacity of Cr against oxidative stress induced by acute exercise. The present study aims to test Cr antioxidant activity in rats exposed to acute aerobic exercise of moderate intensity.

Materials and methods

Animals and treatment

Sixty-four male Wistar rats (initial weight approximately 120 g) were obtained from the Animal Care Unit at the Faculty of Medicine of Ribeirão Preto. All procedures were approved by the Animal Care Committee of the same institution and were in accordance with the Guidelines of the Brazilian Council on Animal Care. The animals were kept in individual cages on a 12/12-h light/dark cycle at a mean temperature of 22°C and were divided at random into two groups: C, control group ($n = 32$) and CrS, creatine-supplemented group ($n = 32$). Creatine supplementation was performed by adding 2% monohydrate creatine (Acros Organics, New Jersey, NY, USA) to the control diet (AIN-93, Reeves et al. 1993) for 28 days. A diet supplemented with 2% Cr was chosen because it has been previously shown to increase plasma and total muscle Cr content (Deminice et al. 2009). The animals had free access to food and water throughout the experiment and received fresh food and water every 2 days. Food intake and body weight were measured weekly throughout the study period (Table 1).

After 4 weeks of Cr supplementation the animals were submitted to an acute bout of exercise. Ten microliter blood samples were taken from a tail vein for lactate assay with a portable Accusport Accutrend Lactate analyzer (Boehringer Mannheim, Castle Hill, Australia). All rat groups were killed by decapitation before ($n = 8$) and at 0 ($n = 8$), 2 ($n = 8$) and 6 h ($n = 8$) after acute exercise. Blood was collected into heparinized tubes and centrifuged and plasma was stored at −80°C. The gastrocnemius (red portion, type I fibers) and soleus (type I fibers) muscles were freeze-clamped with aluminum tongs pre-cooled in liquid nitrogen, weighed and stored at −80°C.

Table 1 Final weight, weight gain, diet and creatine intake for the various groups after 4 weeks of creatine supplementation

	C	CrS
Initial weight (g)	120.5 ± 5.2	119.6 ± 4.8
Final weight (g)	281.8 ± 7.4	276.7 ± 6.2
Weight gain (g/4 weeks)	161.2 ± 7.1	157.1 ± 5.7
Diet intake (g/day)	20.0 ± 1.1	20.4 ± 1.2
Creatine intake (g/day)	–	0.41 ± 0.02

Values are mean ± SEM, $n = 32$

Acute aerobic exercise protocol

After 1 week of adaptation (keeping the animal in shallow water at 31 ± 1°C for 20 min, 5 days/week for 1 week), the animals were adapted to the exercise protocols. The purpose of the adaptation periods was to reduce stress without promoting exercise training adaptations. The adaptation to exercise consisted of 15 min of swimming 3 days/week for 2 weeks. The acute exercise test consisted of 1 h of swimming carrying a load of 4% of body weight (adapted elastic backpack attached to the chest) in a 50-cm deep tank. According to Voltarelli et al. (2002), a load of 4% of body weight corresponds to an under-lactate threshold swimming intensity.

Biochemical analyses

Freeze-clamped muscle samples were homogenized in ice-cold 50 mM sodium phosphate, pH 7.0, and the homogenates were centrifuged at 13,000*g* for 10 min at 4°C. The supernatants were utilized for the determination of muscle thiobarbituric acid reactive species (TBARS) and total lipid hydroperoxide as parameters of lipid peroxidation by the method of Costa et al. (2006).

Reduced (GSH) and oxidized glutathione (GSSG) were determined in muscle by the method of Rahman et al. (2006) using a 5% phosphoric acid muscle homogenate. Ferric reducing antioxidant power (FRAP) was assayed in plasma by the method of Costa et al. (2006). Plasma α-tocopherol was determined by HPLC (LC-20A Shimadzu®, Kyoto, Japan) as described by Jordão et al. (2004). Plasma catalase activity was determined by measuring the decomposition of hydrogen peroxide at 230 nm, as proposed by Aebi (1984).

Plasma and total muscle Cr was assayed by the Jaffe reaction using a method described by Deminice et al. (2009).

Total blood hematocrit and hemoglobin were measured to correct plasma volume shifts (adapted from Dill and

Costill 1974). Assays were carried out in duplicate. The coefficient of variation for each measurement was less than 6% for all assays.

Statistical analysis

Data are reported as mean ± standard error of mean. A linear mixed effects model was used to detect possible differences between groups at the time of killing and possible differences in relation to the time of killing (pre and 0, 2 and 6 h after exercise) in the same group. The level of significance was set at $P < 0.05$ in all analyses.

Results

No significant differences in final body weight, body weight gain or diet intake were observed between groups after 4 weeks of experimentation. As expected, the supplemented group ingested more Cr compared to the control group.

Blood lactate concentration was significantly higher ($P < 0.05$) immediately after acute exercise in both groups compared to other times of killing. Cr supplementation did not alter blood lactate concentration after exercise (Table 2). As expected, plasma and total muscle Cr concentrations were significantly higher ($P < 0.05$) in the creatine-supplemented group compared to control.

According to the plasma markers of oxidative stress, significant increases in TBARS (40.3% at 0 h, 43.1%, at 2 h, and 37.1% at 6 h), and lipid hydroperoxide (26.8% at 0 h, 22.1% at 2 h, and 35.1% at 6 h) were shown up to 6 h after exercise in the control group. Cr supplementation inhibited these increased lipid peroxidation markers induced by acute exercise (Fig. 1). Creatine-supplemented

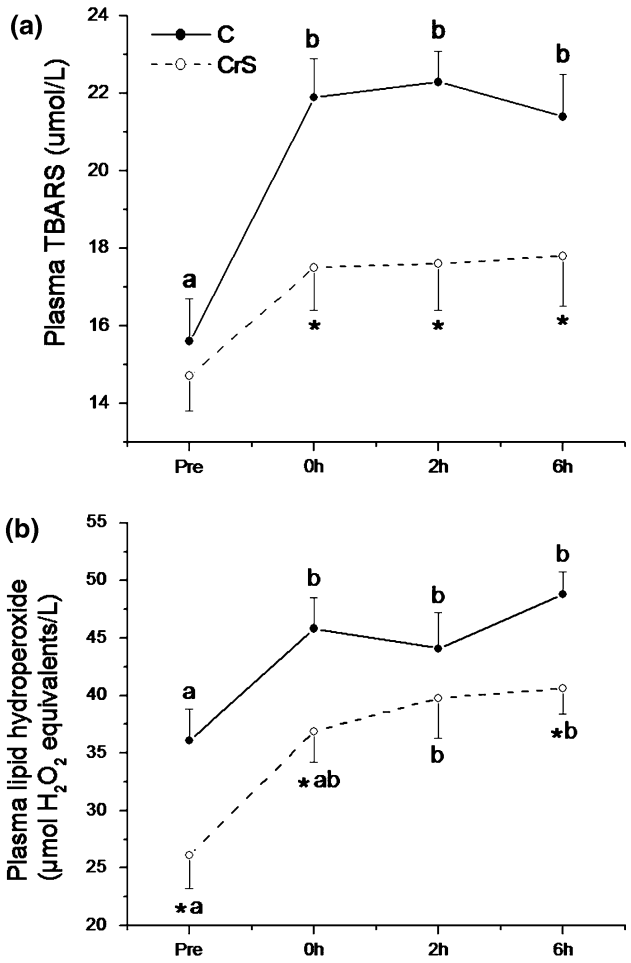

Fig. 1 Plasma TBARS (**a**) and lipid hydroperoxide (**b**) pre and 0, 2 and 6 h after acute exercise in the control (C) and creatine-supplemented (CrS) groups. Values are mean ± SEM, $n = 8$. [ab]Means in a row followed by different letters were significantly different; *indicates significant difference in relation to the control at the same time of killing ($P < 0.05$ by linear mixed effects model)

Table 2 Total blood lactate, plasma creatine and muscle free creatine, creatine phosphate and total muscle creatine, pre and 0, 2 and 6 h after acute exercise in the control (C) and creatine-supplemented (CrS) groups

	Pre	0 h	2 h	6 h
Blood lactate (mmol/L)				
C	3.1 ± 0.1[a]	6.7 ± 0.6[b]	3.1 ± 0.1[a]	3.1 ± 0.2[a]
CrS	2.9 ± 0.3[a]	4.9 ± 0.5[b]	3.6 ± 0.1[a]	2.1 ± 0.2[a]
Plasma creatine (µmol/L)				
C	82.7 ± 3.8[a]	109.2 ± 4.6[b]	105.8 ± 5.5[ab]	89.2 ± 6.1[a]
CrS	246.6 ± 10.1*[a]	288.6 ± 14.3*[b]	232.9 ± 12.1*[a]	236.5 ± 6.4*[a]
Free muscle creatine (µmol/g tissue)				
C	17.5 ± 0.3	17.0 ± 0.4	16.8 ± 0.5	17.3 ± 0.6
CrS	18.8 ± 0.6	17.6 ± 0.5	18.3 ± 0.4	18.6 ± 0.3
Muscle PCr (µmol/g tissue)				
C	5.7 ± 0.3	6.5 ± 0.6	5.3 ± 0.7	4.6 ± 0.4
CrS	6.5 ± 0.5	7.3 ± 0.2	6.6 ± 0.6	5.4 ± 0.4
Total muscle creatine (µmol/g tissue)				
C	23.2 ± 0.6	22.6 ± 0.9	22.0 ± 0.9	21.9 ± 0.9
CrS	25.3 ± 0.6*	24.9 ± 0.6*	24.9 ± 0.7*	23.9 ± 0.3*

Values are mean ± SEM, $n = 8$

* Indicates a significant difference in relation to the control group at the same time of killing ($P < 0.05$ by linear mixed effects model)

[ab] Means in a row followed by different letters were significantly different

Table 3 Plasma markers of oxidative stress pre and 0, 2 and 6 h after acute exercise in the control (C) and creatine-supplemented (CrS) groups

	Pre	0 h	2 h	6 h
FRAP (μmo/L)				
C	325.2 ± 23.1	319.7 ± 21.3	295.9 ± 18.9	325.6 ± 34.4
CrS	409.8 ± 15.9*[a]	391.6 ± 30.6*[a]	302.1 ± 29.8[b]	346.5 ± 20.1[ab]
α-Tocopherol (μmol/L)				
C	0.99 ± 0.1	1.10 ± 0.1	1.29 ± 0.1	0.88 ± 0.1
CrS	1.34 ± 0.1	1.02 ± 0.1	0.95 ± 0.1	0.88 ± 0.1
Catalase (U/L)				
C	39.96 ± 4.9[a]	56.64 ± 6.5[b]	43.11 ± 5.1[ab]	62.70 ± 7.7[b]
CrS	42.06 ± 5.1[a]	57.94 ± 6.3[b]	38.72 ± 4.3[a]	37.19 ± 5.1*[a]
Uric acid (mg/dL)				
C	1.05 ± 0.1[a]	1.59 ± 0.2[b]	0.98 ± 0.1[a]	1.08 ± 0.1[a]
CrS	1.10 ± 0.1[a]	1.28 ± 0.2[ab]	0.96 ± 0.1[c]	0.99 ± 0.1
Creatine kinase (U/L)				
C	2,974.9 ± 368.4[a]	5,615.5 ± 654.3[b]	4,318.8 ± 472.3[ab]	2,734.8 ± 351.3[a]
CrS	3,815.4 ± 367.8*[a]	4,970.0 ± 588.2[ab]	3,763.9 ± 602.3[a]	2,745.7 ± 153.4[ac]

Values are mean ± SEM, $n = 8$

* Indicates a significant difference in relation to the control group at the same time of killing ($P < 0.05$ by linear mixed effects model)

[ab] Means in a row followed by different letters were significantly different

rats also showed a significant increase in total antioxidant capacity (FRAP) at times pre and 0 h compared to control. Increases in catalase activity, creatine kinase and uric acid were also induced by exercise. However, Cr supplementation had no effect on these markers (Table 3).

As observed for plasma, acute exercise induced significant increases in TBARS and lipid hydroperoxide in soleus (24.3% at 0 h for TBARS and 37.5% at 0 h and 41.9% at 2 h % for lipid hydroperoxide) and red gastrocnemius muscles (12.1% at 0 h, 40.5%, at 2 h and 36.1% at 6 h for TBARS, and 23.2%, at 0 h, 20.9% at 2 h, and 32.3% at 6 h % for lipid hydroperoxide). Cr supplementation protected muscles against raised lipid hydroperoxide levels in red gastrocnemius muscle at 2 and 6 h (Fig. 2). In addition, the GSH/GSSG ratio decreased after exercise (26.3%, at 0 h and 13.6% at 6 h for the soleus, and red 20.3% at 0 h, 19.2% at 2 h and 14.7% at 6 h for the gastrocnemius). Conversely, Cr supplementation increased reduced GSH levels in both muscles and prevented the drop in GSH/GSSG ratio after acute exercise (Fig. 3).

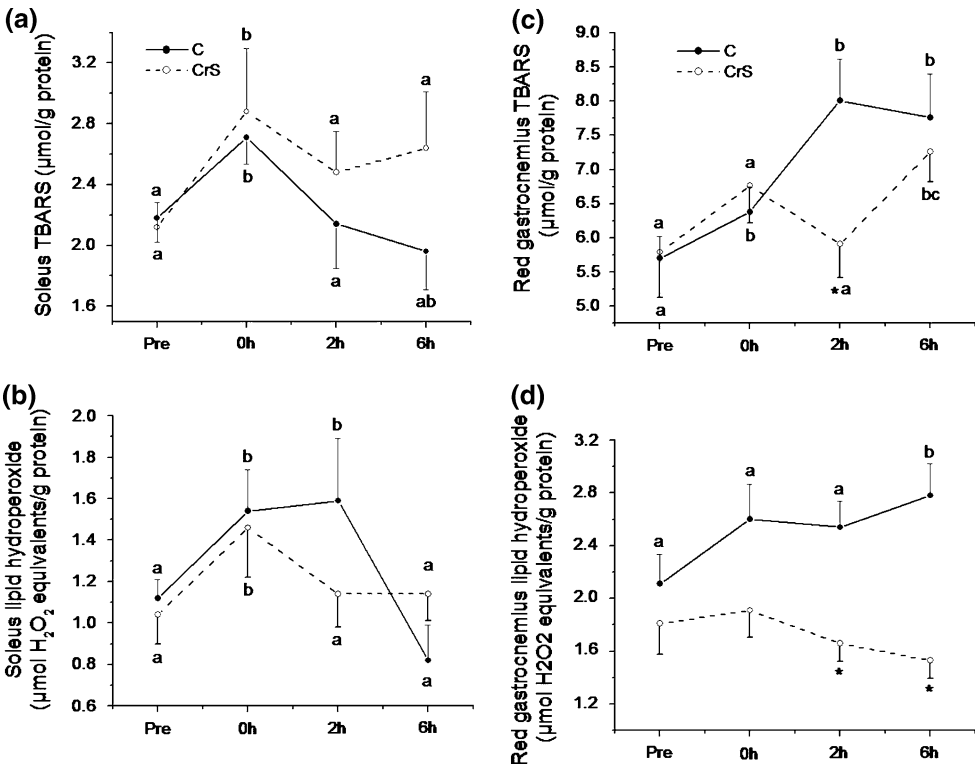

Fig. 2 Muscle markers of lipid peroxidation, TBARS (**a** and **c**) and total lipid hydroperoxide (**b** and **d**) pre and 0, 2 and 6 h after acute exercise in the control (C) and creatine-supplemented (CrS) groups. Values are mean ± SEM, $n = 8$. [ab]Means in a row followed by different letters were significantly different; *indicates significant difference in relation to the control at the same time of killing ($P < 0.05$ by linear mixed effects model)

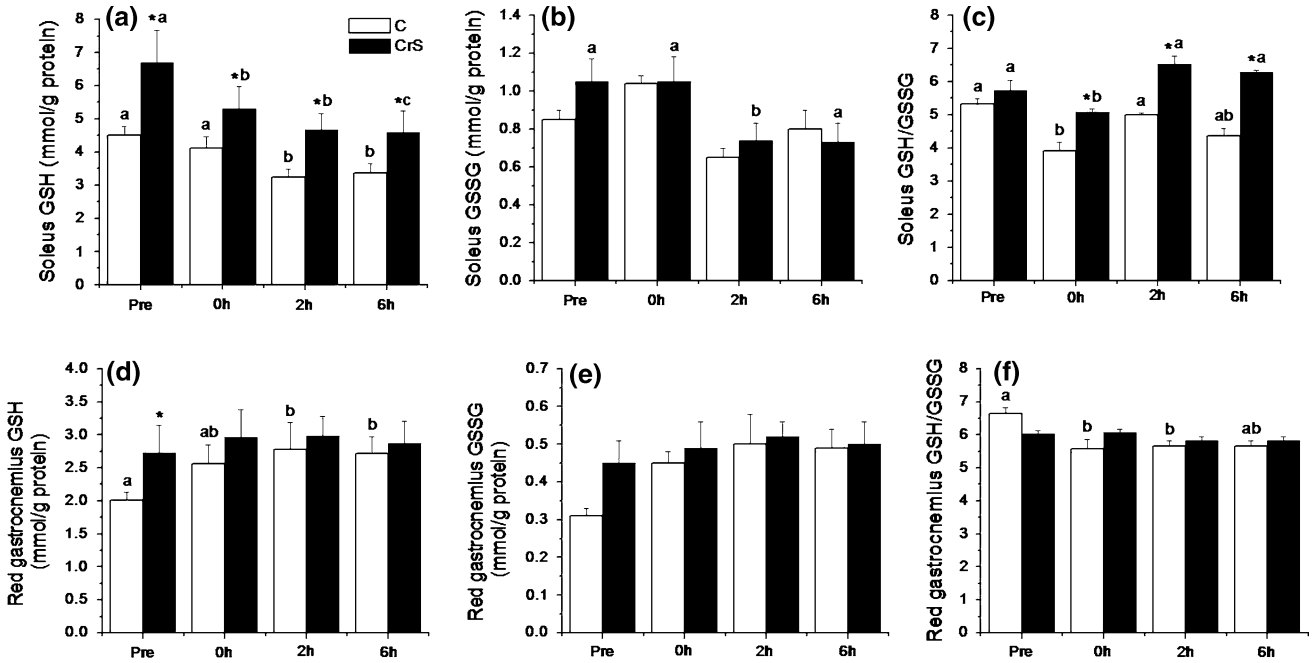

Fig. 3 Muscle reduced glutathione (GSH), oxidized glutathione (GSSG) and reduced/oxidized glutathione ratio (GSH/GSSG) pre and 0, 2 and 6 h after acute exercise in the control (C) and creatine-supplemented (CrS) groups. Values are mean ± SEM, $n = 8$. [ab]Means in a row followed by different letters were significantly different; *indicates significant difference in relation to the control at the same time of killing ($P < 0.05$ by linear mixed effects model)

Discussion

The main finding of the present study was that Cr supplementation reduced oxidative stress markers in plasma and oxidative muscle induced by a single bout of moderate aerobic exercise. It is been well established that acute exercise may increase lipid peroxidation markers and impair the antioxidant defense system. Our results agree with those recently reported in the literature (Bloomer 2008; Nikolaidis et al. 2008). The acute aerobic exercise proposed in the present study was able to increase plasma TBARS and total lipid hydroperoxide (Fig. 1). In soleus and red gastrocnemius muscles, in addition to the increased lipid peroxidation markers, a significantly decreased GSH/GSSG ratio was shown. Alternatively, Cr supplementation led to increased plasma and total muscle Cr content and consequently normalized these perturbations induced by exercise. These results are an in vivo confirmation of in vitro findings on the potential of Cr to remove reactive oxygen species (ROS) (Lawler et al. 2002; Sestili et al. 2006; Guidi et al. 2008; Fimognari et al. 2009). Lawler et al. (2002) first demonstrated direct scavenger effects of Cr on superoxide anion (O_2^-), peroxynitrite ($OONO^-$) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ASC) as determined by the rate of renewal of the $ABTS^+$ radicals, and concluded that Cr has a significant role as an antioxidant. Sestili et al. (2006) also demonstrated that Cr supplementation has a direct antioxidant activity via a scavenger mechanism on cultured cells exposed to different oxidative agents. These authors verified that Cr, at doses similar to those detected in plasma after supplementation, had an antioxidant cytoprotective activity on three cell lines against three different oxidative agents: H_2O_2, $OONO^-$ and tB-OOH. Recent investigations have also shown protective effects of Cr exposure on oxidatively injured mitochondrial DNA (Guidi et al. 2008) and against RNA-damaging agents (Fimognari et al. 2009). These authors also concluded that the protective activity of Cr against DNA- and RNA-damaging agents depends on its capacity to directly scavenge free radicals (Guidi et al. 2008; Fimognari et al. 2009). Taking all this into account, there is supporting in vitro evidence that Cr acts as a scavenger of radical species. The original contribution of the present study is the protective effect of Cr supplementation shown in vivo against oxidative stress induced by acute exercise. However, these results should only be extrapolated to humans with caution. Kingsley et al. (2009) in one of the few studies in humans shown short-term creatine supplementation ineffective in attenuating plasma oxidative stress induced by acute cycling exercise.

Although numerous studies have shown the protective effect of Cr against ROS generation and tissue damage, the exact mechanism by which Cr plays this protective role is still being debated (Sestili et al. 2011). In the present study, creatine-supplemented rats showed a significant increase in total plasma antioxidant capacity (FRAP) and muscle GSH,

suggesting that Cr supplementation up-regulates the antioxidant system, in addition to the direct scavenging capacity of this substance. In a recent study, Young et al. (2010) reported the capacity of Cr exposure to up-regulate the thiol redox system, of which GSH is an important component. In addition, several investigators have demonstrated other indirect antioxidant mechanisms such as hydration and membrane stabilization (Wyss and Schulze 2002). Increases and/or normalization of energy status of the cell have been shown to be promising indirect antioxidant mechanisms of action of Cr (Lenz et al. 2005; Berneburg et al. 2005). Meyer et al. (2006) demonstrated that increased Cr availability normalizes the PCr/Cr ratio and activates ADP recycling by mitochondrial creatine kinase (mt-CK), a mechanism that regulates oxygen consumption and reduces ROS generation in hyperglycemic conditions. Santiago et al. (2008), studying the modulation of mitochondrial kinases by ROS, concluded that mt-Ck may also participate as an active antioxidant system acting in coordination with classical oxidant scavenger enzymes against oxidative stress. The potential of creatine supplementation to reduce homocysteine levels and consequently cell toxicity has been demonstrated (Deminice et al. 2009) and may be also considered to be an indirect antioxidant action of creatine.

However, in the present study, the antioxidant effect on plasma was particularly different from that observed in muscle, suggesting that the antioxidant effects of Cr can be different in these tissues. This fact may be explained by the large difference of creatine retention between the two tissues (a 198% increase in plasma vs. a 10% increase in muscle). In addition, different forms of Cr can also determine the antioxidant effects since these effects are related to the availability of free Cr rather than of its phosphorylated form (Sestili et al. 2006).

Conclusion

In conclusion, Cr supplementation decreased oxidative stress markers in plasma and muscle induced by a single bout of moderate aerobic exercise. These results are an in vivo confirmation of in vitro findings about the potential of creatine to remove ROS. The antioxidant effect shown in plasma was partially observed in muscle, suggesting that the antioxidant effects of Cr can be different in these tissues. Although some studies have shown the protective effect of Cr against ROS generation and tissue damage, the exact mechanism by which Cr promotes such protective effects is unknown. Further experiments are necessary to determine the protective effect of Cr supplementation after different schemes and intensity of exercise. In addition, this effect is little known in humans.

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