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SHORT COMMUNICATION

Creatine-induced glucose uptake in type 2 diabetes: a role for AMPK-α?

Christiano Robles Rodrigues Alves · Júlio César Ferreira · Mário Alves de Siqueira-Filho · Carla Roberta Carvalho · Antonio Herbert Lancha Jr. · Bruno Gualano

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Abstract This study focused on understanding the signaling mechanisms leading to GLUT-4 translocation and increased skeletal-muscle glucose uptake that follow creatine (Cr) supplementation in type 2 diabetes ($n = 10$). AMPK-α protein content presented a tendency to be higher ($p = 0.06$) after Cr supplementation (5 g/d for 12w). The changes in AMPK-α protein content significantly related ($p < 0.001$) to the changes in GLUT-4 translocation ($r = 0.78$) and Hb1Ac levels ($r = -0.68$), suggesting that AMPK signaling may be implicated in the effects of supplementation on glucose uptake in type 2 diabetes.

Keywords Creatine supplementation · Insulin resistance · Molecular pathways

Introduction

Creatine (Cr) supplementation has emerged as a promising adjunct treatment in a broad spectrum of diseases, including those characterized by muscle wasting (Tarnopolsky and Martin 1999), joint syndromes (Neves et al. 2011), central nervous disorders (Klivenyi et al. 1999), and metabolic disturbances (Gualano et al. 2011).

Recently, we observed that Cr supplementation can improve glucose tolerance and metabolic control in type 2 diabetic patients undergoing exercise training (Gualano et al. 2011). Interestingly, the improvement in glycemic control (as assessed by the glycated hemoglobin (Hb1Ac) levels) was paralleled by an increase in glucose transporter 4 (GLUT-4) translocation to the sarcolemma rather than in total muscle GLUT-4 content. The mechanisms by which insulin or muscle contraction mediate GLUT-4 translocation and glucose transport have been described in depth (Sakamoto and Holman 2008; Pereira and Lancha 2004). However, the role of Cr supplementation in stimulating GLUT-4 trafficking and glucose uptake remains unclear so far.

Thus, this study focused on understanding the signaling mechanisms leading to GLUT-4 translocation and the increased skeletal muscle glucose uptake that follow Cr supplementation. To that end, we investigated "master-regulators" involved in insulin and muscle contraction signal transduction (i.e., insulin receptor (IR-β), AMP-activated protein kinase alpha (AMPK-α), Akt/protein kinase B (AKT-1), and p42/44 mitogen-activated kinase (MAPK p42/44)) in skeletal muscle of Cr-supplemented type 2 diabetic patients.

Materials and methods

Experimental design and subjects

This is a double-blind, randomized, placebo-controlled study, registered at clinicaltrials.gov as NCT0099204. The protocol was approved by the Local Ethical Committee and all subjects signed the informed consent. The details regarding this study (e.g., inclusion/exclusion criteria, sample's characteristics, drug regime, supplementation procedure, exercise training program, nutritional

C. R. R. Alves · J. C. Ferreira · A. H. Lancha Jr. · B. Gualano (✉)
School of Physical Education and Sports, University of Sao Paulo, Av Mello de Moraes, 65–Butantã, Sao Paulo, SP 05508-030, Brazil
e-mail: gualano@usp.br

M. A. de Siqueira-Filho · C. R. Carvalho
Biosciences Institute, University of Sao Paulo, Sao Paulo, Brazil

assessment, and clinical findings) have been reported elsewhere (Gualano et al. 2011). In short, non-vegetarian type 2 diabetic patients ($n = 25$) were randomly assigned to receive either Cr (5 g/day) or placebo (Pl; dextrose). In addition, the patients undertook a twice-weekly exercise training program throughout the study. At baseline and after 12 weeks, a sub-sample of ten subjects (gender: Pl two females and two males, and Cr three females and three males; age: Pl 57.3 ± 3; Cr 59.1 ± 2 years; BMI: Pl 32.5 ± 0.9; Cr 32.6 ± 0.8 kg/m^2; Hb1Ac: Pl 7.6 ± 0.7; Cr 7.6 ± 0.8%; $p > 0.05$) were submitted to muscle biopsies.

Muscle biopsies, immunoblotting, GLUT-4 translocation, and Hb1Ac levels assessment

Muscle samples were obtained from the midportion of the *vastus lateralis* using the percutaneous needle biopsy technique with suction, according to a previous description (Gualano et al. 2011). The post intervention biopsies were done 72 h after the last training session. All biopsies were carried out after an 8-h overnight fast, and the last meal was a standard dinner.

Protein expression was measured through immunoblotting. In brief, the samples were subjected to SDS-PAGE in polyacrylamide gel (10%), as described elsewhere (Roschel et al. 2011). The blotted membrane was then blocked (5% non-fat dry milk, 10 mM Tris–HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween 20) for 1 h and then incubated overnight at 4°C with antibodies against IR-β, AKT-1 (Santa Cruz Biotechnology, CA), AMPK-α (Cell Signalling, Beverly, MA), and MAPK p42/44 (Millipore, MA).

Binding of the primary antibody was detected using peroxidase-conjugated secondary antibody for 1 h and developed using enhanced chemiluminescence (Amersham Biosciences, NJ), detected by autoradiography. Quantification analysis of blots was performed using the Scion Image software. The proteins were normalized for loading against Ponceau S stained membranes (Romero-Calvo et al. 2010).

To assess the GLUT-4 translocation, cellular fractionation was performed to obtain the nuclear pellet and the membrane fraction (supernatant), according to a previous description (Gualano et al. 2011). Total and membrane fractions were measured through immunoblotting, following the above-mentioned methods.

HbA1c was measured using the BioRad Variant HI automated analyzer (BioRad, Irvine, CA).

Statistical analyses

Normal distribution of data was confirmed by the Shapiro–Wilk test. Changes in protein expression were tested by unpaired Student's t test. Pearson correlations were performed between changes in AMPK-α protein expression and changes in GLUT-4 translocation and Hb1Ac levels for both groups. Data are expressed as mean ± SD. The level of significance to reject the null hypothesis was previously set at $p < 0.05$.

Results

Macronutrients and energy intake were comparable between groups (Pl group—carbohydrate: Pre 213 ± 43, Post 179 ± 44 g/d; lipid: Pre 54 ± 17, Post 50 ± 17 g/d; protein: Pre 82 ± 25, Post 64 ± 13 g/d; total energy: Pre 1,669 ± 248, Post 1,430 ± 355 kcal/d. Cr group—carbohydrate: Pre 180 ± 26, Post 182 ± 28 g/d; lipid: Pre 61 ± 17; Post 57 ± 18 g/d; protein: Pre 73 ± 25, Post 63 ± 22 g/d; total energy: Pre 1,590 ± 234, Post: 1,517 ± 204 kcal/d).

Figure 1 shows the protein expression data. IR-β, AKT-1 and MAPK p42/44 expression did not differ between groups (Panels a, b, and c, respectively). In contrast, AMPK-α expression presented a tendency to be higher in the Cr group when compared with the placebo group ($p = 0.06$, Panel d). Supporting this result, there was a positive relationship between changes in AMPK-α levels and changes in GLUT-4 translocation ($r = 0.71$; $p < 0.001$; Fig. 2a). In addition, an inverse correlation between changes in AMPK-α expression and changes in Hb1Ac levels was found ($r = -0.68$; $p < 0.001$; Fig. 2b). Finally, as expected, changes in GLUT-4 translocation inversely related to changes in Hb1Ac levels ($r = -0.89$; $p < 0.001$; Fig. 2c).

Discussion

In this study, we aimed to gather knowledge on the mechanisms underlying the beneficial effects of Cr supplementation combined with exercise training on glycemic control in type 2 diabetic patients. Using muscle samples from diabetic patients who were given Cr supplementation (5 g/d) for 12 weeks, we investigated candidate proteins (i.e., IR-β, AKT-1, MAPK p42/44, and AMPK-α) involved in either insulin or muscle contraction signaling leading to the GLUT-4 trafficking. The main finding of this study was that the increased AMPK-α protein expression was significantly related to the decreased Hb1aC levels and increased GLUT-4 translocation.

The insulin-stimulated translocation of GLUT-4 to the sarcolemma is initiated by the binding of insulin to its receptor (i.e., IR), resulting in a rapid autophosphorylation of the receptor as well as in the phosphorylation of insulin receptor substrates (IRSs), which link the initial event of insulin receptor signaling cascade to downstream events. IRSs, in turn, associate with phosphatidylinositol 3-kinase

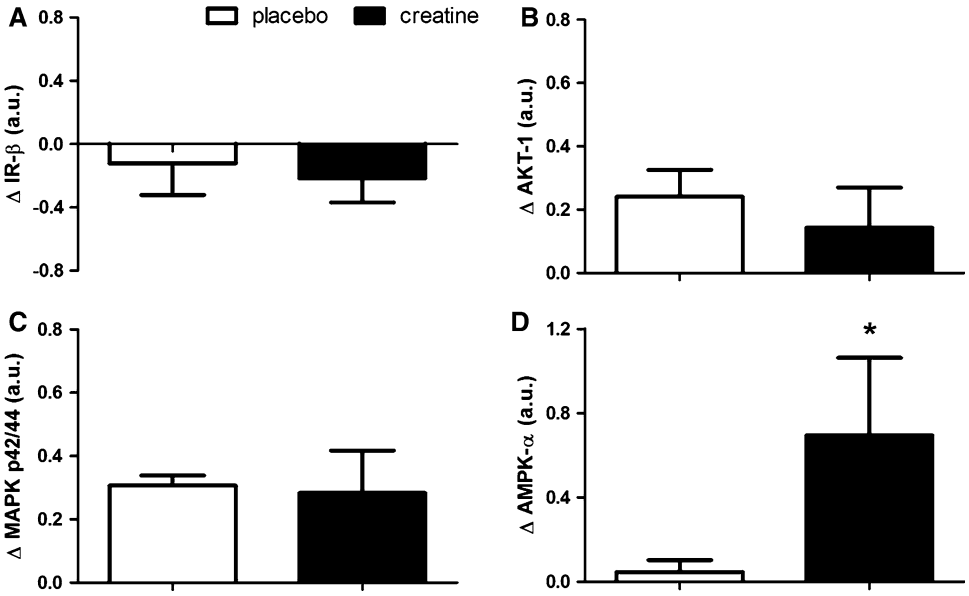

Fig. 1 Effects of Cr supplementation on IR-β (**a**), AKT-1 (**b**), and MAPK p42/44 (**c**) and AMPK-α (**d**) protein expression in type 2 diabetic patients undergoing exercise training. *Asterisk* denotes $p = 0.06$ between groups. Creatine $n = 6$; Placebo $n = 4$

(PI3-kinase), which is an intermediate effector that seemingly mediates glucose transport via signaling to protein kinase C or AKT (Alessi et al. 1997). In fact, AKT inhibition, either by dominant-negative expression (Cong et al. 1997) or microinjection of AKT antibody (Hill et al. 1999), decreases insulin-stimulated GLUT-4 translocation. Importantly, there is evidence suggesting that glucose transport may be also partially mediated by MAPKs, which can be separated into three major subdivisions: MAPK p42/44, MAPK p38, and the c-jun NH2 terminal kinase (JNK). In fact, MAPK signaling cascades have been recognized as one of the most important cellular signaling mechanisms mediating exercise-induced adaptations in skeletal muscle (Henriksen 2002). Furthermore, in vitro experiments have shown that the activation of MAPK p42/44 parallels that of MAPK p38 in response to osmotic shock under supraphysiological conditions (Sabio et al. 2004). Supposedly, Cr, as an "osmotic active" compound, could elicit a similar response on MAPK activation and/or protein content.

Interestingly, Safdar et al. (2008) observed an up-regulation of MAPK p42/44 mRNA and protein content in the skeletal muscle of Cr-supplemented healthy subjects. Moreover, these authors also showed that Cr induced overexpression in AKT-1 mRNA and protein content, leading them to speculate that increased AKT-1 expression could ultimately affect GLUT-4 translocation. However, neither MAPK p42/44 nor AKT-1 protein expression were altered in the current study, suggesting that our previous observations showing increased GLUT-4 translocation and reduced Hb1Ac (Gualano et al. 2011) cannot be explained by modulation in these two candidate proteins. This conclusion holds true for IR, which also remained unchanged after Cr supplementation.

Conversely, we did observe a significant relation between increased AMPK-α expression and (1) decreased Hb1aC levels and (2) increased GLUT-4 translocation. These findings altogether suggest that AMPK-α may play an important role in facilitating Cr-induced glucose uptake in diabetic patients. AMPK signaling is activated following a rise in the AMP:ATP ratio within the cell, and responds by adjusting the rates of ATP-consuming and ATP-generating pathways (Winder 2001; Xiao et al. 2011). Importantly, AMPK signaling has been implicated as an important mediator of muscle contraction-induced GLUT-4 translocation (Hardie et al. 1999) and a target for pharmacological intervention to treat altered glucose homeostasis (Narkar et al. 2008).

An elegant study demonstrated that Cr supplementation increases GLUT-4 protein content by ~40% following a rehabilitation training program in healthy subjects who had one leg immobilized (Op 't Eijnde et al. 2001b). In a subsequent report, the same group showed that the augment in GLUT-4 protein content was not paralleled by changes in AMPK protein expression and phosphorylation (Eijnde et al. 2005). In contrast, an in vitro study did show an increase in AMPK phosphorylation, but not GLUT-4 protein content or glucose uptake in L6 rat skeletal muscle cells incubated with Cr (0.5 mM) for 48 h (Ceddia and Sweeney 2004). Considering the large discrepancy among the experimental models, it is hard to reconcile these results. Hypothetically, Cr supplementation might affect AMPK signaling by inducing a fall in phosphorylcreatine (PCr):total Cr ratio, which would represent an alteration in energy state in muscle cells (Eijnde et al. 2005; Ceddia and Sweeney 2004; Ponticos et al. 1998). However, the impact of Cr supplementation on PCr:total Cr ratio has been

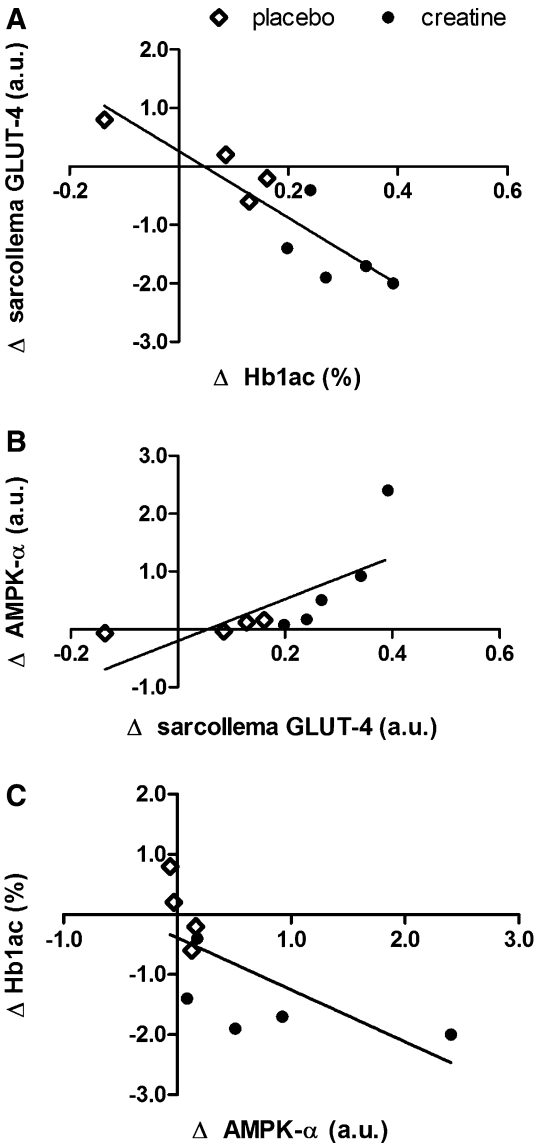

Fig. 2 **a** Correlation between changes in AMPK-α protein content and changes in sarcollema GLUT-4 protein content (or GLUT-4 translocation) ($r = 0.71$; $p < 0.001$); **b** correlation between changes in AMPK-α protein content and changes in Hb1Ac levels ($r = -0.68$; $p < 0.001$); and **c** correlation between changes in Hb1Ac levels and sarcolemma GLUT-4 protein content ($r = -0.89$; $p < 0.001$). Creatine $n = 5$; Placebo $n = 4$

controversial in vivo (Green et al. 1996; Greenhaff et al. 1994; Brannon et al. 1997; Op 't Eijnde et al. 2001a). Unfortunately, we were unable to measure total muscle Cr, thus we cannot confirm as to whether a fall in PCr:total Cr ratio occurred in the current study. Indeed, the mechanisms by which Cr supplementation (per se or combined with exercise training) modulates AMPK signaling cascade, GLUT-4 translocation and glycemic control in type 2 diabetes require additional investigations.

In conclusion, Cr supplementation seems to modulate AMPK-α protein content in type 2 diabetic patients undergoing exercise training. The changes in AMPK-α protein content significantly related to the changes in GLUT-4 translocation and Hb1Ac levels, suggesting that AMPK signaling may be implicated in the previously reported beneficial effects of Cr supplementation on glucose uptake in diabetic patients.

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Conflict of interest The authors declare no conflict of interests.

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