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ORIGINAL ARTICLE

Effects of creatine supplementation on muscle wasting and glucose homeostasis in rats treated with dexamethasone

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Abstract We aimed to investigate the possible role of creatine (CR) supplementation in counteracting dexamethasone-induced muscle wasting and insulin resistance in rats. Also, we examined whether CR intake would modulate molecular pathways involved in muscle remodeling and insulin signaling. Animals were randomly divided into four groups: (1) dexamethasone (DEX); (2) control pair-fed (CON-PF); (3) dexamethasone plus CR (DEX-CR); and (4) CR pair-fed (CR-PF). Dexamethasone (5 mg/kg/day) and CR (5 g/kg/day) were given via drinking water for 7 days. Plantaris and extensor digitorum longus (EDL) muscles were removed for analysis. Plantaris and EDL muscle mass were significantly reduced in the DEX-CR and DEX groups when compared with the CON-PF and CR-PF groups ($P < 0.05$). Dexamethasone significantly decreased phospho-Ser^{473}-Akt protein levels compared to the CON-PF group ($P < 0.05$) and CR supplementation aggravated this response ($P < 0.001$). Serum glucose was significantly increased in the DEX group when compared with the CON-PF group (DEX 7.8 ± 0.6 vs. CON-PF 5.2 ± 0.5 mmol/l; $P < 0.05$). CR supplementation significantly exacerbated hyperglycemia in the dexamethasone-treated animals (DEX-CR 15.1 ± 2.4 mmol/l; $P < 0.05$ vs. others). Dexamethasone reduced GLUT-4 translocation when compared with the CON-PF and CR-PF ($P < 0.05$) groups and this response was aggravated by CR supplementation ($P < 0.05$ vs. others). In conclusion, supplementation with CR resulted in increased insulin resistance and did not attenuate muscle wasting in rats treated with dexamethasone. Given the contrast with the results of human studies that have shown benefits of CR supplementation on muscle atrophy and insulin sensitivity, we suggest caution when extrapolating this animal data to human subjects.

Keywords Glucocorticoid · Atrophy · Glucose homeostasis · GLUT-4

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Introduction

Over the last decade, the therapeutic role of creatine (CR) supplementation has been largely investigated (for a review please see Gualano et al. 2010a). In this regard, there is a growing body of evidence showing that CR may counteract muscle wasting in animal models (Menezes et al. 2007) as well as in dystrophic patients (Kley et al. 2008, 2007; Tarnopolsky and Martin 1999). Furthermore, the group has recently observed that CR can also improve glycemic control in diabetic subjects most likely by enhancing GLUT-4 translocation to the sarcolemma (Gualano et al. 2010b). In view of these findings, CR supplementation has emerged as a potential adjuvant nutritional strategy for treating conditions characterized by progressive muscle loss and insulin resistance.

Glucocorticoids (GC) are widely prescribed drugs that present well-known anti-inflammatory and immunosuppressive effects. Paradoxally, the long-term use of GC may provoke several adverse effects in rodents and humans, namely insulin resistance (Bernal-Mizrachi et al. 2003; Giorgino et al. 1993) and muscle wasting (Clarke et al. 2007; Gilson et al. 2007; Hasselgren 1999; Waddell et al. 2008). The potential role of CR supplementation in alleviating such effects remains to be elucidated.

A few studies have suggested that CR supplementation may attenuate muscle wasting in rats treated with GC (Campos et al. 2006; Menezes et al. 2007; Roy et al. 2002) although the mechanisms underlying this response are not clear. In addition, the effects of CR supplementation on glucose homeostasis have not been evaluated. Importantly, these studies have used a relatively weak GC (e.g., prednisone and methylprednisone) and an intraperitoneal route of administration. The ability of CR supplementation to attenuate severe muscle wasting, caused by an aggressive oral dexamethasone administration protocol, has not yet been investigated, nor has the effects of supplementation on glucose homeostasis under these conditions.

Therefore, the aim of this study was to investigate the therapeutic role of CR supplementation on muscle wasting and insulin sensitivity in rats treated with dexamethasone. We also examined whether CR intake would modulate molecular pathways involved in muscle remodeling and insulin signaling.

Methods

Animals

The experiments were conducted in accordance with the National Research Council's Guidelines for the Care and Use of Laboratory Animals. This study was approved by the Ethical Committee of the University of Sao Paulo. Wistar male rats (400–450 g) were housed under controlled environmental conditions (temperature, 22°C; 12-h dark period starting at 18:00 h). They were given free access to commercial laboratory chow and water before the experiments were performed. Rats were randomly divided into the following groups: (1) dexamethasone (DEX), (2) control pair-fed (CON-PF), (3) dexamethasone plus creatine (DEX-CR), and (4) creatine pair-fed (CR-PF). Dexamethasone (5 mg/kg/day) and CR (5 g/kg/day) were given daily (at 0900 h) via drinking water for 7 days. All groups were pair-fed to the DEX-treated group according to individual body weight. Animals were killed by decapitation after an overnight fast of 12 h, except for GLUT-4 experiments in which the animals had free access to food and water. Plantaris and extensor digitorum longus (EDL) muscles from both limbs were isolated, weighed, and frozen at −80°C for further analysis.

Muscle dry:wet weight ratio

Muscle tissues were desiccated for 5 days in a drying oven set at 50°C prior to determination of dry weight. Dry:wet weight ratio was determined as previously described (Kauvar et al. 2007).

Serum insulin and glucose levels

Blood was collected and serum samples were separated after allowing blood to clot on ice. Serum was stored at −80°C for further analysis. Serum insulin was measured using RIA commercial kits (DPC®, Brazil). Serum glucose levels were measured using an automatized method (Accu-Chek Active System, Roche Diagnostics, Mannheim, Germany).

HOMA-IR

The homeostasis model for assessment of insulin resistance index (HOMA-IR) was calculated as follows: HOMA-IR index (mmol mU/l^2) = fasting insulin (mU/l) × serum glucose (mmol/l)/22.5 (Adami et al. 2004).

Cellular fractionation for GLUT-4 protein expression

Muscle samples were minced and homogenized in ice-cold lysis buffer (2 mM EDTA, 10 mM EGTA, 0.25 M Sucrose, 1:300 Sigma protease inhibitor cocktail, and 20 mM Tris–HCl at pH 7.5). The homogenate was centrifuged at 100,000×g for 30 min (4°C) to obtain the membrane fraction.

Western blot

Bradford assays were used to determine sarcoplasmic protein concentrations after which, samples were standardized to 1 mg/ml by dilution with 3× Laemmli loading buffer. In brief, samples were subjected to SDS-PAGE in 8% polyacrylamide gel. After electrophoresis, proteins were electrotransferred to the nitrocellulose membrane through Transblot Semi Dry Transfer Cell (BioRad Biosciences, NJ, USA). Equal loading of samples and transfer efficiency were monitored with the use of 0.5% Ponceau S staining of the blot membrane. The blotted membrane was then blocked (with 5% low-fat milk (total) or 5% BSA (for phospho-antibodies) in TBS-T for 1 h, membranes were rotated overnight with the following primary antibodies: GLUT-4 (1:1,000; Millipore, MA, USA), phospho-Ser^{473}-Akt, total-FoxO3a, phospho-Ser^{253}-FoxO3a, and MuRF-1

(1:1,000; Cell Signaling). Membranes were then washed with TBS-T and incubated for 1 h at room temperature with a HRP-conjugated anti-rabbit secondary antibody (1:10,000; Cell Signaling), before further washing with TBS-T and incubation for 1 min with ECL. Quantification analysis of blots was performed with the use of Image J. Protein expressions were normalized against GAPDH.

Statistical analysis

The results are expressed as means ± SEM. Data were graphically examined for normality. The dependent variables presented normal distribution and were tested by either two-way (food consumption and body weight) or one-way (all other variables) ANOVA. Whenever a significant F value was obtained, a post hoc test with a Tukey adjustment was performed for multiple comparison purposes. The significance level was set at $P < 0.05$. Statistical analyses were performed using SAS 8.2 (SAS Institute Inc., Cary, NC, USA).

Results

Effects of dexamethasone and CR supplementation on body weight, muscle wasting, and food intake

Baseline body weight was not significantly different between groups ($P > 0.05$). CON-PF and CR-PF presented significant weight loss (4.3 and 5.4%, respectively; $P < 0.05$). DEX and DEX-CR groups also presented progressive weight loss throughout the study (~25%) when compared with the CON-PF and CR-PF groups ($P < 0.05$; Fig. 1a). As shown in Fig. 1b, food intake was comparable between groups ($P > 0.05$). As expected, dexamethasone caused a significant reduction in food intake by ~45% ($P < 0.05$).

Dexamethasone significantly reduced plantaris and EDL muscle mass in DEX-CR and DEX when compared with the CON-PF and CR-PF groups ($P < 0.05$; Table 1). There were no significant differences in the muscle dry:wet weight ratio between groups.

Effects of dexamethasone and CR supplementation on the expression of key proteins related to muscle remodeling and insulin signaling

As fast-twitch fibers are the most affected by dexamethasone, molecular analyses were restricted to plantaris muscle. Dexamethasone significantly decreased phospho-Ser^{473}-Akt protein levels compared to the CON-PF group ($P < 0.05$; Fig. 2a) and CR supplementation aggravated this response ($P < 0.001$). Total-FoxO3a protein expression did not change among groups but phospho-Ser^{253}-FoxO3a was significantly reduced by the same extent in the DEX and DEX-CR groups in relation to the CON-PF and CR-PF groups ($P < 0.01$; Fig. 2b). MuRF-1 protein expression was equally enhanced in the DEX and DEX-CR when compared with the CON-PF and CR-PF groups ($P < 0.001$; Fig. 2c).

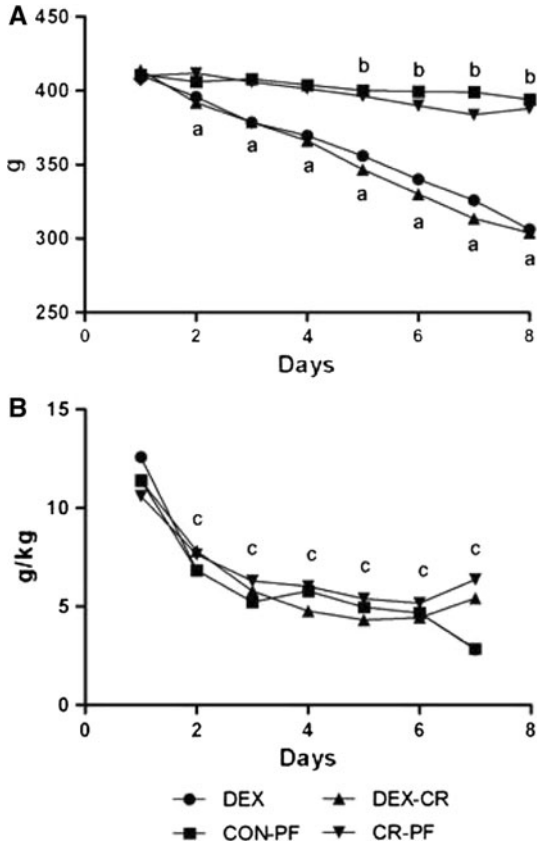

Fig. 1 **a** Body weight (g) and **b** food intake data (g/kg) among groups. In **a**, *a* indicates a significant difference from baseline in both DEX and DEX-CR groups ($P < 0.05$); *b* indicates a significant difference from baseline in both CON-PF and CR-PF groups ($P < 0.05$). In **b**, *c* indicates a significant difference from baseline in all groups ($P < 0.05$)

Effects of dexamethasone and CR supplementation on glucose homeostasis

Serum glucose was significantly increased in the DEX group when compared with the CON-PF group (DEX 7.8 ± 0.6 vs. CON-PF 5.2 ± 0.5 mmol/l; $P < 0.05$; Fig. 3a). CR supplementation significantly exacerbated hyperglycemia in the dexamethasone-treated animals (DEX-CR 15.1 ± 2.4 mmol/l; $P < 0.05$ vs. others). CR supplementation per se increased serum glucose when compared with the CON-PF group (CR-PF 8.2 ± 0.9 mmol/l; $P < 0.05$). Serum glucose did not differ between DEX and CR-PF groups ($P > 0.05$).

Table 1 Animal's characteristics

Variable	Group			
	DEX ($n = 6$)	CON-PF ($n = 6$)	DEX-CR ($n = 6$)	CR-PF ($n = 6$)
Basal body weight (g)	409 ± 7	412 ± 9	414 ± 12	410 ± 7
Final body weight (g)	306 ± 1[a,b,c]	394 ± 5[a]	304 ± 10[a,b,c]	388 ± 10[a]
Plantaris weight (mg)	274.2 ± 27[b,c]	406 ± 27	264 ± 24[b,c]	396 ± 28
Plantaris wet:dry ratio (mg)	0.25 ± 0.001	0.24 ± 0.005	0.25 ± 0.02	0.20 ± 0.10
EDL weight (mg)	160 ± 17[b,c]	206 ± 15	147 ± 11[b,c]	216 ± 14
EDL wet:dry ratio (mg)	0.24 ± 0.01	0.24 ± 0.01	0.25 ± 0.03	0.26 ± 0.09

Data are expressed as mean ± SEM

[a] $P < 0.05$ vs. basal body weight

[b] $P < 0.05$ vs. CON-PF

[c] $P < 0.05$ vs. CR-PF

Dexamethasone treatment increased serum insulin when compared with the CON-PF group (DEX 47.7 ± 3.3 vs. CR-PF 26.6 ± 4.5 μU/ml; CON-PF 15.2 ± 2.0 μU/ml; $P < 0.001$; Fig. 3b), with the addition of CR supplementation resulting in a further increase in insulin (DEX-CR 53.6 ± 3.9 μU/ml; $P < 0.05$ vs. CON-PF; $P < 0.001$ vs. others). CR supplementation per se also increased serum insulin concentration versus the CON-PF group ($P < 0.001$).

HOMA-IR indicated that the DEX group presented impaired insulin sensitivity when compared to the CON-PF group (DEX 6.20 ± 0.14 vs. CON-PF 1.94 ± 0.13; $P < 0.01$; Fig. 3c). Similar to insulinemia and glycemia, CR supplementation significantly increased HOMA-IR above dexamethasone alone (DEX-CR 9.60 ± 1.3; $P < 0.05$ vs. DEX; $P < 0.001$ vs. others). CR-PF and CON-PF groups presented similar values of HOMA-IR (CR-PF 3.72 ± 0.28; $P > 0.05$).

Effects of dexamethasone and CR supplementation on GLUT-4 translocation to the sarcolemma

As shown in Fig. 3b, neither dexamethasone nor CR supplementation changed the total GLUT-4 protein content ($P > 0.05$). In contrast, dexamethasone significantly reduced GLUT-4 translocation when compared with the CON-PF and CR-PF ($P < 0.05$) groups, with CR supplementation resulting in a further significant decrease in GLUT-4 translocation (Fig. 2d; $P < 0.05$ vs. others). GLUT-4 translocation was comparable between the CR-PF and CON-PF groups ($P > 0.05$).

Discussion

By using a 7-day orally administered dexamethasone protocol, we were able to induce severe muscle wasting and insulin resistance in rats. Further supporting this data, we also noted a drastic modulation in proteins involved in both muscle proteolysis and insulin signaling (i.e., reductions in the phospho-Ser^{473}-Akt, phospho-Ser^{253}-FoxO3a/total FoxO3a ratio, membrane GLUT-4 expression, and an increase in the MuRF-1 expression). The key finding was that CR supplementation did not counteract dexamethasone-induced muscle wasting. Moreover, CR supplementation aggravates dexamethasone-induced insulin resistance. These findings seem to be contradictory to the recent body of literature pointing out the therapeutic role of CR in muscle disorders and homeostasis regulation (Gualano et al. 2008; Tarnopolsky and Martin 1999).

In contrast to previous isotopic investigations showing that CR supplementation does not affect protein turnover (Louis et al. 2003; Parise et al. 2001), recent evidence has suggested that CR intake may attenuate GC-induced muscle wasting (Menezes et al. 2007). In this regard, Roy et al. (2002) demonstrated that the CR supplementation can attenuate prednisone-induced EDL atrophy in rats. Accordingly, Campos et al. (2006) and Menezes et al. (2007) also verified that CR supplementation can counteract dexamethasone-induced muscle wasting in rodents. Conversely, we failed to find any effect of CR supplementation in GC-induced muscle wasting. The following factors may explain the discrepancy between these results and ours. First, Campos et al. (2006) used a biologically weak GC (e.g., predinisone). In contrast, we used an aggressive treatment (i.e., high-dose dexamethasone via drinking water), which led to severe muscle wasting (e.g., 32% in the study in plantaris muscle vs. ~5.4% in the study of Campos et al. (2006) in medial gastrocnemius muscle). Therefore, these results suggest that CR may not prevent muscle wasting in severe conditions of muscle catabolism. Second, the length of the supplementation protocol (i.e., 7 days) was shorter than those used in aforementioned studies (e.g., 18 days in the Menezes's

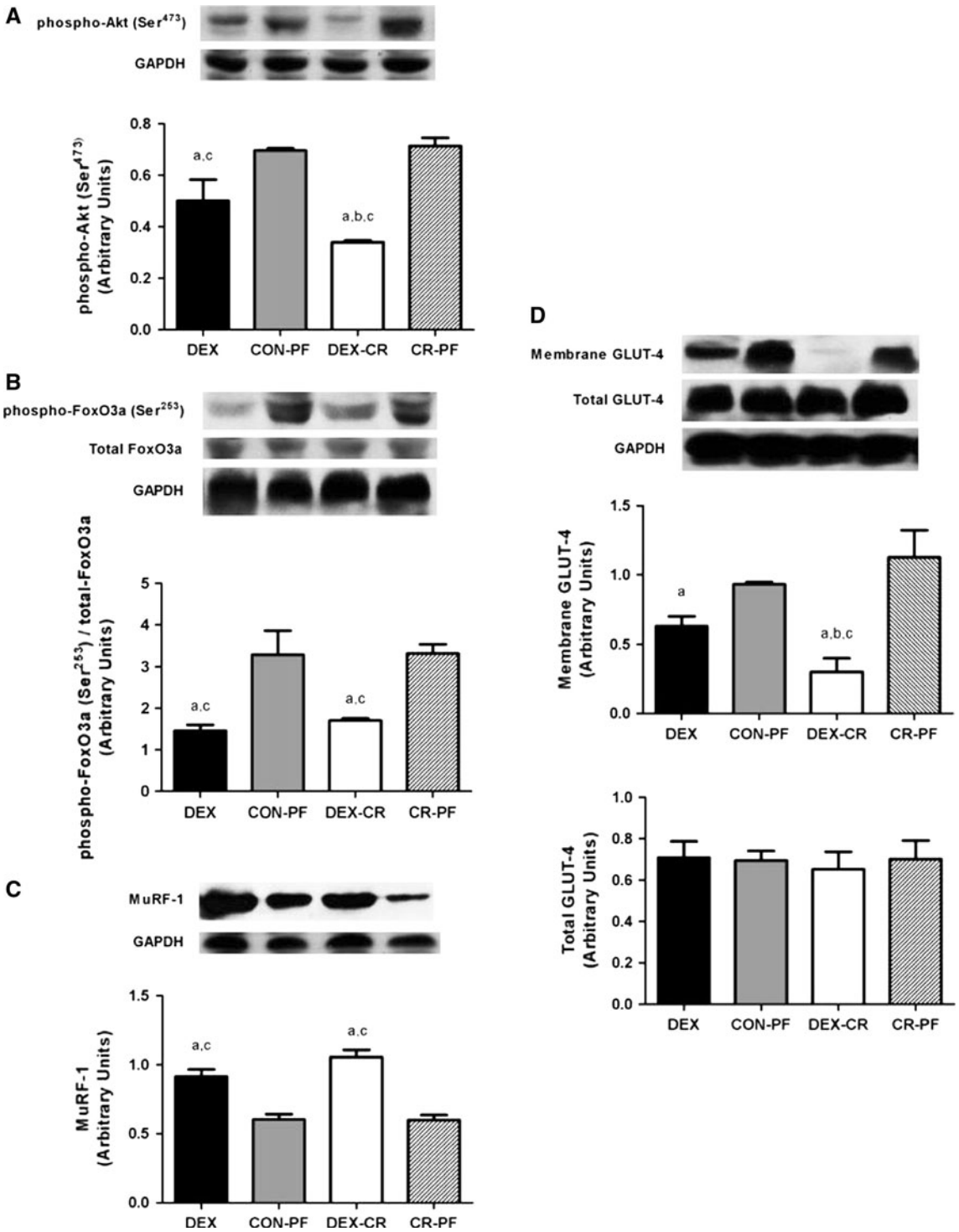

Fig. 2 **a** phospho-Ser^{473}-Akt protein levels; **b** ratio of phospho-Ser^{253}-FoxO3a protein/total-FoxO3a; **c** MuRF-1 protein levels; **d** membrane and total GLUT-4 protein expression in plantaris muscle of rats after 7 days of dexamethasone and CR treatment. A typical blot is shown in the *inset*. Data are expressed as mean ± SEM; $^{a}P < 0.05$ vs. CON-PF; $^{b}P < 0.05$ vs. DEX; $^{c}P < 0.01$ vs. CR-PF. Data were normalized to GAPDH protein expression

study. It is possible that a more extensive CR protocol might result in greater increase in muscle CR content and associated benefits that it confers. However, given the absence of a sufficient time-response studies in rodent species, this is difficult to confirm. Third, it is imperative to emphasize that responses to CR supplementation are highly

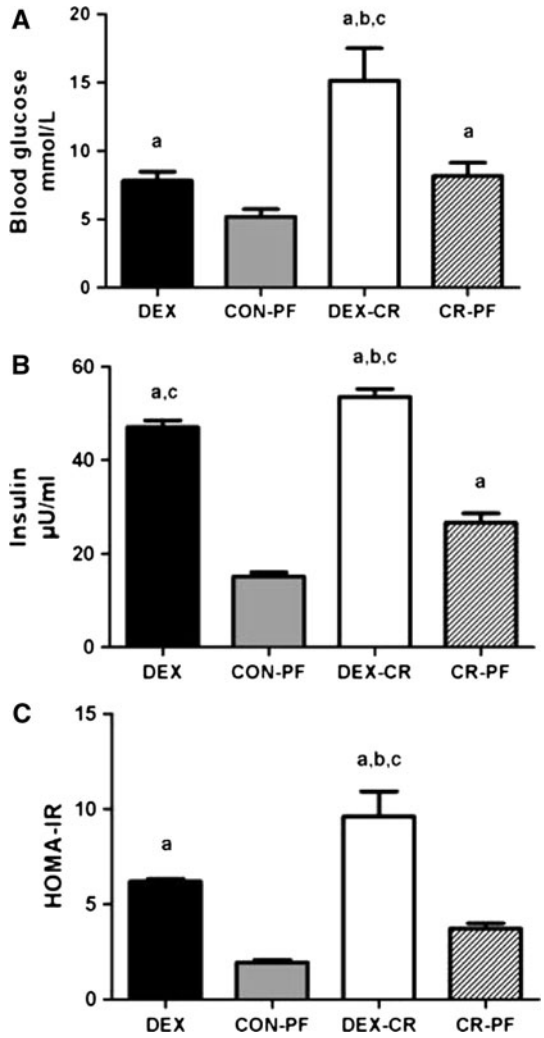

Fig. 3 **a** Serum glucose (mmol/l), **b** serum insulin (µU/ml) and **c** HOMA-IR index among groups. Data are expressed as mean ± SEM; $^{a}P < 0.05$ vs. CON-PF; $^{b}P < 0.05$ vs. DEX; $^{c}P < 0.001$ vs. CR-PF

different among varied species. In this respect, Tarnopolsky et al. (2003) demonstrated that CR administration may induce hepatitis in mice, but not in rats, suggesting substantial differences in CR metabolism even for closely related species. Caution is therefore recommended when interpreting the relevance of these findings since animal observations may not reflect the similar alterations in humans. In contrast to the current findings in rats, for example, Tarnopolsky and Martin (1999) reported strength and weight gain in patients with muscle dystrophies, cytopathies, inflammatory myopathies, and peripheral neuropathy disorders.

The most intriguing results refer to the effects of CR supplementation on insulin sensitivity. Namely, CR intake increased dexamethasone-induced both hyperglycemia and hyperinsulinemia. Interestingly, CR and dexamethasone per se also provoked hyperglycemia to the same extent. These observations diverge from previous reports in which CR has been showed to improve glucose homeostasis (Gualano et al. 2010b, 2008; Op't Eijnde et al. 2006, 2001a, b). Similar to the above discussion regarding muscle mass data, the possible explanation for these contradictory observations lies in the fact that response to CR supplementation varies according to the experimental model. For instance, Rooney et al. (2002) reported hyperinsulinemia and abnormal glucose homeostasis in CR-supplemented Wistar rats. In contrast, Op't Eijnde et al. (2006) showed that CR intake may improve insulin sensitivity in Goto-Kakizaki rats (an animal model of inherited type 2 diabetes). Accordingly, we (Gualano et al. 2010b) and others (Op 't Eijnde et al. 2001b) have provided promising evidence that CR supplementation can improve glucose uptake and glycemic control in healthy and type 2 diabetic subjects. Further corroborating this discrepancy, human studies have demonstrated that CR may increases Akt expression and GLUT-4 translocation to the sarcolemma, whereas the opposite was found in the current study. Thus, there is a clear dissonance among experimental models applied in studies involving CR supplementation, precluding from generalizing animal findings to humans.

In conclusion, we demonstrated that CR supplementation aggravated insulin resistance and did not attenuate muscle wasting in rats treated with dexamethasone. In light of the previous human findings showing benefits of CR in both muscle atrophy and insulin sensitivity, we also reinforce earlier evidence (Tarnopolsky et al. 2003) suggesting caution in extrapolating animal data to humans in CR investigations.

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