Creatine supplementation increases glycogen storage but not GLUT-4 expression in human skeletal muscle

Luc J. C. van Loon*, Robyn Murphy†, Audrey M. Oosterlaar*, David Cameron-Smith†, Mark Hargreaves†, Anton J. M. Wagenmakers* and Rodney Snow†

*Nutrition Research Institute Maastricht (NUTRIM), Maastricht University, Maastricht, The Netherlands, and †Exercise, Muscle and Metabolism Unit (EMMU), School of Health Sciences, Deakin University, Burwood, Australia

ABSTRACT

It has been speculated that creatine supplementation affects muscle glucose metabolism in humans by increasing muscle glycogen storage and up-regulating GLUT-4 protein expression. In the present study, we assessed the effects of creatine loading and prolonged supplementation on muscle glycogen storage and GLUT-4 mRNA and protein content in humans. A total of 20 subjects participated in a 6-week supplementation period during which creatine or a placebo was ingested. Muscle biopsies were taken before and after 5 days of creatine loading (20 g·day⁻¹) and after 6 weeks of continued supplementation (2 g·day⁻¹). Fasting plasma insulin concentrations, muscle creatine, glycogen and GLUT-4 protein content as well as GLUT-4, glycogen synthase-1 (GS-1) and glycogenin-1 (Gln-1) mRNA expression were determined. Creatine loading significantly increased total creatine, free creatine and creatine phosphate content with a concomitant 18 ± 5% increase in muscle glycogen content (P < 0.05). The subsequent use of a 2 g·day⁻¹ maintenance dose for 37 days did not maintain total creatine, creatine phosphate and glycogen content at the elevated levels. The initial increase in muscle glycogen accumulation could not be explained by an increase in fasting plasma insulin concentration, muscle GLUT-4 mRNA and/or protein content. In addition, neither muscle GS-1 nor Gln-1 mRNA expression was affected. We conclude that creatine ingestion itself stimulates muscle glycogen storage, but does not affect muscle GLUT-4 expression.

INTRODUCTION

It has been established that oral creatine supplementation increases total creatine and creatine phosphate (CrP) content in human skeletal muscle [1–4]. An increased muscle CrP concentration increases its availability for ATP synthesis and, via this mechanism, may lead to the often described improvement in performance during repeated high-intensity exercise tasks [2,5–7]. Subsequently, creatine supplementation has become a common practice in both recreational and professional athletes [7]. As most research on creatine has focused on the ergogenic capacity of creatine loading, far less attention has been attributed to the potential of creatine to affect muscle glucose metabolism.

Creatine feeding has been reported to augment muscle glycogen storage in both rats [8] and fish [9]. However, Rooney et al. [10] failed to reproduce these findings in rats. In agreement, equivocal findings on the effects of creatine supplementation on muscle

Key words: creatine phosphate, glucose metabolism, glucose uptake, glycogen, insulin.

Abbreviations: AMPK, AMP-activated protein kinase; C group, creatine-supplemented group; CrP, creatine phosphate; Cₜ, threshold cycle; FFM, fat-free mass; GS-1, glycogen synthase-1; Gln-1, glycogenin-1; MAPK, mitogen-activated protein kinase; P group, placebo group; V_{O2} max, maximal oxygen uptake capacity; W_{max}, maximal aerobic workload capacity.

Correspondence: Dr L. J. C. van Loon (e-mail L.vanLoon@HB.Unimaas.nl).
glycogen storage have been reported in humans [11–14]. In a recent study by some of the present authors [13], neither creatine loading nor continued creatine use increased muscle glycogen storage. In accordance, Op ‘t Einde et al. [11] showed that creatine ingestion did not affect muscle glycogen content during 2 weeks of immobilization; however, continued creatine use during a consecutive 3-week (rehabilitation) strength-training programme significantly increased muscle glycogen storage [11]. In addition, the combined ingestion of creatine and carbohydrate following exercise has been reported to stimulate post-exercise muscle glycogen storage [12]. More recently, Nelson et al. [14] observed that muscle glycogen super-compensation can be augmented by prior creatine loading. The proposed stimulating effect of creatine use on muscle glycogen synthesis is likely to be mediated by an increase in cell volume secondary to an increase in muscle creatine content [12,14,15]. However, the physiological mechanisms responsible have not yet been established. As an increase in muscle glycogen synthesis should probably be accompanied by an increase in muscle glucose uptake, it has been speculated that creatine supplementation might up-regulate GLUT-4 expression in muscle tissue [11]. Consistent with this, it could be speculated that an increase in the capacity to store glycogen is realized by a concomitant up-regulation of glycogenin-1 (Gln-1) [16] and/or glycogen synthase-1 (GS-1) [17].

At present, it is not clear whether creatine supplementation itself (in the absence of exercise and/or dietary intervention) can up-regulate muscle GLUT-4 expression and/or increase muscle glycogen content in humans, despite the fact that such an effect could be clinically relevant. We assessed the effects of creatine loading and continued supplementation on muscle creatine, glycogen and GLUT-4 protein content as well as GLUT-4, GS-1 and Gln-1 mRNA expression in vivo in humans. This present study is part of a project that investigated the effects of creatine supplementation on body composition, sprint and endurance performance [18]. A total of 20 subjects participated in a 6-week supplementation period during which either creatine or a placebo was ingested. Muscle biopsies were collected before and after creatine loading (20 g·day$^{-1}$) as well as after 6 weeks of continued creatine use.

METHODS

Subjects

Twenty fit, young and non-vegetarian male subjects, with no history of participation in any regular exercise training regimen, were recruited for this study. Subjects were assigned in a group-matched fashion for maximal aerobic workload capacity [Wmax; expressed per kg of fat-free mass (FFM)] and maximal oxygen uptake capacity ($\dot{V}$O$_{2\text{max}}$; expressed per kg of FFM) to either a creatine-supplemented (C) or placebo (P) group. Characteristics of subjects are provided in Table 1. All subjects were informed about the nature and risks of the experimental procedures before their informed consent was obtained. All research has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association, and has been approved by the local Medical Ethical Committee.

$\dot{V}$O$_{2\text{max}}$ and body composition

Wmax and $\dot{V}$O$_{2\text{max}}$ were determined 1 week before the start of the intervention on an electronically braked cycle ergometer (Lode Excalibur) during an incremental exhaustive exercise test [19]. Body composition was assessed using the hydrostatic weighing method in the morning after an overnight fast. Body fat percentage was calculated using the Siri’s equation [20]. FFM was calculated by subtracting fat mass from total body mass.

Creatine supplementation

Subjects were supplemented over a 6 week period, either with creatine-containing supplements (C group) or a placebo (P group). Supplements were provided in a double-blind fashion. The C group received 20 g (4 × 5 g) of creatine monohydrate per day (100 % pure creatine monohydrate; Pfannstiel Laboratories Inc., Wachegom, IL, U.S.A.) during an initial 5 day loading phase, followed by 2 g·day$^{-1}$ during the subsequent 37 day maintenance phase (supplementation dosage according to the guidelines of the American College of Sports Medicine Roundtable [7]). The provided creatine was mixed with 15 g of glucose (as glucose monohydrate; AVEBE, Veendam, The Netherlands) and 10 g of maltodextrin (AVEBE). The P group received the same packaged supplements without the addition of creatine monohydrate. Supplements could not be distinguished from each other, as a poorly soluble maltodextrine was chosen.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Subjects’ characteristics</th>
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<tbody>
<tr>
<td>Parameter</td>
<td>C group (n = 9)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>20.7 ± 0.3</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>66.5 ± 1.7</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.82 ± 0.02</td>
</tr>
<tr>
<td>BMI (kg·m$^{-2}$)</td>
<td>20.1 ± 0.6</td>
</tr>
<tr>
<td>Body fat content (%)</td>
<td>13.6 ± 2.1</td>
</tr>
<tr>
<td>Wmax (W)</td>
<td>274 ± 7</td>
</tr>
<tr>
<td>Wmax (W·kg of FFM$^{-1}$)</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td>(V\dot{O}_{2\text{max}}) (litre·min$^{-1}$)</td>
<td>3.6 ± 0.1</td>
</tr>
<tr>
<td>(V\dot{O}_{2\text{max}}) (ml·min$^{-1}$·kg of FFM$^{-1}$)</td>
<td>68.4 ± 1.6</td>
</tr>
<tr>
<td>Maximal heart rate (beats·min$^{-1}$)</td>
<td>194 ± 2</td>
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to mask the low solubility of creatine monohydrate. All subjects were instructed to dissolve the supplements in (lukewarm) water before ingestion. During the loading phase, subjects were instructed to ingest one supplement package with breakfast, lunch and dinner and the fourth package at 22:00 hours (approx. 4 h intervals). In the maintenance phase, subjects ingested the supplement during dinner (i.e. evening meal). Subjects in both the C group and P group were instructed to maintain normal dietary habits and physical activity throughout the entire 6 week period. In addition, food intake diaries were collected for 2 days before the first biopsy and blood sample collection, and the identical dietary intake was repeated before collection of the other biopsies after the loading phase and following 6 weeks of continued supplementation (see below).

Muscle biopsies

Percutaneous muscle biopsies [21] were taken from the vastus lateralis muscle (left leg) immediately before supplementation (at day 0), after the 5-day loading phase (on day 6) and after the entire 6 weeks of supplementation (on day 43). All subjects were instructed to fill in a food intake diary for 2 days prior to the first biopsy and to keep their dietary intake as identical as possible prior to the day of the other two biopsies. Subjects were instructed to refrain from demanding physical labour and any sort of physical exercise for at least 48 h before biopsy sampling. A standardized meal (2.7 kJ; relative contribution of total energy content of the meal: protein, 15%; carbohydrate, 75%; fat, 10%; [Honig, Koog a/d Zaan, The Netherlands]) was given to each subject and eaten at 20:00 hours on the evening before each biopsy collection. Thereafter, subjects were allowed to drink only water. After the overnight fast, subjects arrived at the laboratory at 08:00 hours by car or public transport, where, after 1 h of supine rest, a blood sample and muscle biopsy were collected. Plasma and muscle samples were immediately frozen in liquid nitrogen and stored at $-80 \, ^\circ C$ until analysis.

Analysis

Creatine content, glycogen and plasma insulin

One fraction of muscle sample [approx. 50 mg (w/w)] was freeze-dried, after which collagen, blood and other nonmuscle material were removed from the muscle fibres under a light microscope. Thereafter, each sample was pulverized and powdered extracts were used for spectrophotometric determination of creatine, CrP, total creatine and ATP content [22]. For glycogen content analysis, 3-4 mg of isolated muscle fibre mass was weighed and 500 $\mu l$ of 1 M HCl was added. After heating for 3 h at 100 $\, ^\circ C$ to hydrolyse the glycogen to glycosyl units and cooling down to room temperature, the solution was neutralized by adding 267 $\mu l$ of Tris/KOH. Thereafter, a portion (150 $\mu l$) was analysed for glucose (Uni Kit III, 07367204; Roche, Basel, Switzerland) with a COBAS FARA semi-automatic analyser (Roche). Blood samples (7 ml) were collected in EDTA-containing tubes and centrifuged at 1000 $\times g$ for 10 min. Aliquots of plasma were frozen immediately in liquid nitrogen and stored at $-80 \, ^\circ C$ until analysis for insulin by RIA (ultrasensitive human insulin RIA kit; Linco Research, St. Charles, MO, U.S.A.).

PCR methodology

Total RNA was extracted from 10–15 mg of wet muscle as described previously [23]. cDNA was obtained using AMV Reverse Transcriptase and Oligo(dT)$_{15}$ Primers (Promega, Madison, WI, U.S.A.) and stored at $-80 \, ^\circ C$ until subsequent analysis. Samples were analysed in triplicate to detect GS-1, Gln-1, GLUT-4 and cyclophilin mRNA transcripts using real-time reverse transcription-PCR (GeneAmp 5700 sequence detection system; Applied Biosystems, Foster City, CA, U.S.A.). GenBank accession numbers are shown in parentheses. Table 2 Primer sequences

Primers complementary to selected regions of the genes of interest were designed by using the Primer Express Software (PerkinElmer Biosystems, Foster City, CA, U.S.A.). GenBank accession numbers are shown in parentheses.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’ to 3’)</th>
</tr>
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<tbody>
<tr>
<td>Cyclophilin</td>
<td>CAC ACC GTC TGC TGC GAC AT</td>
</tr>
<tr>
<td></td>
<td>CCA GTG CTC AGA GCA CCA AA</td>
</tr>
<tr>
<td>GLUT-4</td>
<td>GCC GGA CGT TTG ACC AGA T</td>
</tr>
<tr>
<td></td>
<td>GGT TTG TCA CCT CCT GCT CTA</td>
</tr>
<tr>
<td>Gln-1</td>
<td>CTA TGG CAC AGC GGT TTG TAT C</td>
</tr>
<tr>
<td></td>
<td>CCT CTG GAT GTT GTC AAA GGA ATC T</td>
</tr>
<tr>
<td>GS-1</td>
<td>TGA GTG CAC GGT TAT GGG AAT</td>
</tr>
<tr>
<td></td>
<td>TGC GAG CAG GAA TCA TCC A</td>
</tr>
</tbody>
</table>

Immunoblotting

Crude membrane protein was extracted from another fraction of muscle sample [20–30 mg (w/w)]. Samples

Table 2 Primer sequences

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’ to 3’)</th>
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<tbody>
<tr>
<td>Cyclophilin</td>
<td>CCC ACC GTC TGC TGC GAC AT</td>
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<tr>
<td></td>
<td>CCA GTG CTC AGA GCA CCA AA</td>
</tr>
<tr>
<td>GLUT-4</td>
<td>GCC GGA CGT TTG ACC AGA T</td>
</tr>
<tr>
<td></td>
<td>GGT TTG TCA CCT CCT GCT CTA</td>
</tr>
<tr>
<td>Gln-1</td>
<td>CTA TGG CAC AGC GGT TTG TAT C</td>
</tr>
<tr>
<td></td>
<td>CCT CTG GAT GTT GTC AAA GGA ATC T</td>
</tr>
<tr>
<td>GS-1</td>
<td>TGA GTG CAC GGT TAT GGG AAT</td>
</tr>
<tr>
<td></td>
<td>TGC GAG CAG GAA TCA TCC A</td>
</tr>
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</table>
Table 3 Muscle creatine content

<table>
<thead>
<tr>
<th></th>
<th>C group (n = 9)</th>
<th>P group (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 6</td>
</tr>
<tr>
<td>Free creatine</td>
<td>42.6 ± 1.5</td>
<td>63.3 ± 2.5*</td>
</tr>
<tr>
<td>CrP</td>
<td>78.3 ± 4.6</td>
<td>94.7 ± 4.0†</td>
</tr>
<tr>
<td>Total creatine</td>
<td>120.9 ± 5.3</td>
<td>150.0 ± 4.4‡</td>
</tr>
<tr>
<td>ATP</td>
<td>21.2 ± 1.8</td>
<td>23.1 ± 1.1</td>
</tr>
<tr>
<td>Glycogen</td>
<td>598 ± 42</td>
<td>679 ± 52†</td>
</tr>
</tbody>
</table>

RESULTS

Creatine and ATP content

Muscle creatine and ATP content before supplementation (day 0), after the initial loading phase (day 6) and after 6 weeks of continued supplementation (day 43) have been published previously [18] and are summarized in Table 3. One subject in the C group (n = 10) did not respond to creatine supplementation. This subject was regarded a non-responder [1] and, as such, was excluded from further statistical analyses (n = 9). Exclusion of the non-responder did not significantly change the average creatine content data.

Muscle glycogen

Muscle glycogen concentrations at days 0, 6 and 43 are shown in Table 3. A significant treatment effect on muscle glycogen content between groups was observed (P < 0.02), with post-hoc analysis showing a significant difference between groups at day 6 (Table 3). In the C group, eight out of nine subjects showed an increase in muscle glycogen content after 5 days of creatine loading, whereas a variable response was observed in the P group. Within groups, a significant time effect was observed in the C group only (P = 0.02), with post-hoc analysis showing a significant effect between days 6 and 43 (P < 0.05). Due to relatively large inter-individual differences in muscle glycogen content, data were expressed relative to the individual baseline values, consistent with the mRNA and protein data (Figure 1).

In the C group, muscle glycogen content significantly increased by 18 ± 5 % following 5 days of creatine loading (P < 0.05). After the entire 6 week supplementation period, glycogen content had decreased again and was not significantly different from baseline values. In the P group, no changes in muscle glycogen content were observed over time. Between groups, muscle glycogen content was significantly higher in the C group following creatine loading. Regression analysis revealed a significant correlation between the relative increase and subsequent decrease in muscle CrP (R = 0.60) and total creatine

Statistics

The results are expressed as means ± S.E.M. The statistical significance of differences between groups was assessed by a two-way repeated ANOVA. Statistical differences over time within each group were analysed by a single-factor repeated ANOVA. Tukey’s post-hoc test was applied to locate differences. Simple regression analysis was performed to assess the relative changes in muscle creatine/CrP content and glycogen concentrations observed during the loading and subsequent maintenance stage in the C group. Statistical significance was set at P < 0.05.
Creatine use and muscle glucose metabolism

Figure 1  Muscle glycogen content before and after creatine loading and after 6 weeks of prolonged supplementation in the C and P groups
Data were normalized to pre-supplementation values. *, significant difference compared with values observed at day 0 within group; ∧, significant difference compared with values observed at day 43 within groups; #, significant difference between groups at day 6 (P < 0.05).

Figure 2  Regression analysis of the changes in muscle CrP (A) and total creatine (B) content and the concomitant change in muscle glycogen concentration following creatine loading and during the subsequent maintenance phase

\[ R = 0.67 \] content, with the corresponding changes in muscle glycogen content between days 0–6 and days 6–43, in the C group (Figure 2; P < 0.05).

Plasma insulin
Fasting plasma insulin concentrations averaged 7.0 ± 0.8, 7.2 ± 0.6 and 7.5 ± 1.0 compared with 7.4 ± 1.0, 7.0 ± 0.6 and 6.7 ± 0.8 µunits/ml at days 0, 6 and 43 in the C and P group respectively. No changes in fasting plasma insulin concentrations were observed within or between groups (P > 0.05).

mRNA content
Skeletal muscle Gln-1, GS-1 and GLUT-4 mRNA content at day 0, 6 and 43 is shown in Figure 3. Muscle mRNA contents were expressed relative to the corresponding baseline values, set to equal 1. Gln-1, GS-1 and GLUT-4 mRNA expression did not change over time in either the C or P group and were not significantly different between groups (P > 0.05).

GLUT-4 protein content
Skeletal muscle (membrane-bound) GLUT-4 protein content at days 0, 6 and 43 is shown in Figure 4. Muscle GLUT-4 protein content was expressed relative to the corresponding baseline values, set to equal 1. No significant changes in GLUT-4 content were observed within or between groups (P > 0.05).

DISCUSSION
Muscle free creatine, CrP and total creatine content were significantly increased following 5 days of creatine loading (20 g·day⁻¹; Table 3). These increases are in
in accordance with earlier reports [1–4]. Concomitantly, we observed a significant 18 ± 5 % increase in muscle glycogen content following the 5 days of creatine loading (Figure 1). This is in accordance with a previous report [8] on high-dose creatine feeding in rats, showing an increase in glycogen concentration in both soleus (40 %) and red gastrocnemius (15 %), but not white gastrocnemius, muscle. Consistent with the subsequent decline in CrP and total creatine content observed during the remaining 37 days of the 6 week supplementation period (Table 3), muscle glycogen content returned to baseline values at the end of this period (Figure 1). Interestingly, the relative changes in muscle total creatine and CrP content were significantly correlated with the changes in muscle glycogen content, as observed during the creatine loading and subsequent maintenance period (Figure 2).

Our present findings appear to be in contrast with reports on creatine supplementation in humans [13,14]. In a recent study by some of the present authors [13], no significant changes in muscle glycogen content were observed following 5 days of creatine loading (20 g · day$^{-1}$) and 28 days of continued creatine use (3 g · day$^{-1}$) in humans (n = 6). However, this apparent contradictory finding can be explained by the efficiency by which creatine loading increased muscle CrP and total creatine content, which was substantially lower in the study by Newman et al. [13]. In the latter, muscle CrP and total creatine content increased by 6 % (P = not significant) and 12 % (P < 0.05) respectively, in contrast with the 24 % (P < 0.05) and 32 % (P < 0.05) increases observed in the present study. Concomitantly, muscle glycogen content was increased by 9 % (P = not significant) in contrast with the 18 % increase (P < 0.05) in the present study. As such, these findings seem to correspond with the suggested link between an increase in muscle creatine content and muscle glycogen storage (Figure 2).

The mechanism(s) behind the observed link between creatine supplementation and glycogen storage probably involves the observed increase in CrP and/or total creatine content (Figure 2). It has been suggested that a decrease in the CrP/free creatine ratio following creatine supplementation stimulates AMP-activated protein kinase (AMPK) activity [24]. The latter has been shown to increase GLUT-4 protein content, hexokinase activity, muscle glucose transport and glycogen content in rats [25–27]. However, in the present study, CrP/free creatine ratios were not significantly reduced after creatine loading. Hence the most likely explanation would be an indirect stimulation of muscle glucose uptake and/or glycogen synthesis by increased cellular osmolarity, and a subsequent increase in cellular hydration status, secondary to the increase in total creatine content following creatine loading [28]. In vitro studies have described well the stimulating effect of increased cell volume on glycogen synthesis [15]. Cell swelling has been identified as an anabolic proliferative signal, stimulating the mitogen-activated protein kinase (MAPK) signalling cascade [29–31], which could also be linked to the up-regulation of muscle glycogen synthesis. In addition, muscle glucose transport rate in isolated rat muscle has been shown [27] to be acutely increased by contraction and hypoxia as well as hyperosmolarity. As such, AMPK may well play a role in the underlying mechanism, as all these stimuli activate both the α1 and α2 isoforms of AMPK [27].

Activation of the AMPK and/or MAPK signalling cascade controls the expression of many genes involved in glucose transport and glycogen synthesis. As an increase in muscle glycogen synthesis is likely to be accompanied by an increase in muscle glucose uptake, it has been speculated that creatine supplementation up-regulates GLUT-4 expression in muscle tissue [11]. However, in the present study, we observed no changes in GLUT-4 mRNA and total GLUT-4 protein content following either short-term creatine loading or prolonged creatine use (Figures 3 and 4). The latter is in accordance with observations in rats in which creatine supplementation failed to increase muscle GLUT-4 content and the maximal capacity for insulin-stimulated glucose transport [8]. However, in humans, the same group [11] reported that creatine supplementation during 2 weeks of immobilization prevented the decrease in muscle GLUT-4 content due to muscle disuse atrophy. In addition, during a subsequent 10 week rehabilitation strength-training programme, creatine supplementation resulted in a progressive increase in muscle GLUT-4 content [11]. Although the present study shows that creatine supplementation itself does not increase muscle GLUT-4 expression, more research is warranted to investigate the effects of creatine supplementation in combination with exercise training on muscle GLUT-4 expression and glucose homoeostasis in humans.
In addition to GLUT-4, we also investigated the potential effect of creatine supplementation on muscle GS-1 and Gln-1 mRNA expression. However, no significant changes were observed following creatine or placebo use (Figure 3). Due to limited muscle sample size, GS-1 and Gln-1 protein content and/or activity were not determined.

In addition to the proposed mechanisms, which could explain the observed effect of creatine use on muscle glycogen content, in vitro studies in rodents have shown that creatine has a modest insulinotropic capacity [32]. In addition, Rooney et al. [10] have reported that prolonged creatine supplementation in rats increases fasting plasma insulin levels. As such an effect would stimulate muscle glycogen storage, we also determined fasting plasma insulin levels. However, in accordance with others [13], neither creatine loading nor prolonged creatine use altered fasting plasma insulin levels in humans.

In conclusion, the present study shows that short-term (5 day) creatine loading significantly increased muscle total creatine, free creatine and CrP content with a concomitant increase in muscle glycogen storage. The subsequent use of a 2 g·day⁻¹ maintenance dose for 37 days did not maintain CrP, total creatine or glycogen content at elevated levels. The increase in muscle glycogen accumulation was not explained by a concomitant increase in muscle GLUT-4 mRNA and/or protein content. In addition, neither muscle GS-1 nor Gln-1 mRNA expression was increased following creatine supplementation. Although creatine ingestion itself stimulates muscle glycogen storage, it does not affect GLUT-4 expression in humans.

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