Creatine supplementation increases glucose oxidation and AMPK phosphorylation and reduces lactate production in L6 rat skeletal muscle cells

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Summary

Recent observations have suggested that creatine supplementation might have a beneficial effect on glucoregulation in skeletal muscle. However, conclusive studies on the direct effects of creatine on glucose uptake and metabolism are lacking. The objective of this study was to investigate the effects of creatine supplementation on basal and insulin-stimulated GLUT4 translocation, glucose uptake, glycogen content, glycogen synthesis, lactate production, glucose oxidation, and AMPK phosphorylation in L6 rat skeletal muscle cells. Four treatment groups were studied: control, insulin (100 nM), creatine (0.5 mM), and creatine + insulin. After 48h of creatine supplementation the creatine and phosphocreatine contents of L6 myoblasts increased by ~9.3- and ~5.1-fold, respectively, however, the ATP content of the cells was not affected. Insulin significantly increased 2-DG uptake (~1.9-fold), GLUT4 translocation (~1.8-fold), the incorporation of D-[U-14C]glucose into glycogen (~2.3-fold), lactate production (~1.5-fold), and 14CO2 production (~1.5-fold). Creatine did not alter the glycogen and GLUT4 contents of the cells nor the insulin-stimulated rates of 2-DG uptake, GLUT4 translocation, glycogen synthesis, and glucose oxidation. However, creatine significantly reduced by ~42% the basal rate of lactate production and increased by ~40% the basal rate of 14CO2 production. This is in agreement with the ~35% increase in citrate synthase activity and also with the ~2-fold increase in the phosphorylation of both α-1 and α-2 isoforms of AMPK after creatine supplementation. We conclude that 48h of creatine supplementation does not alter insulin-stimulated glucose uptake and glucose metabolism; however, it activates AMPK, shifts basal glucose metabolism towards oxidation and reduces lactate production in L6 rat skeletal muscle cells.
Introduction

The physiological roles of creatine in the human body have been extensively investigated. Its main biochemical effect in skeletal muscle, usually described as the “energy shuttle”, is to transfer chemical energy from mitochondria, where ATP is produced, to the myofibrils (Wallimann et al. 1992; Ruggeri 2000). In humans, approximately 95% of the total body creatine is found in skeletal muscle. More than 60% is in the form of phosphocreatine (PCr) and the remainder stored in the nonphosphorylated form (Walker 1979; Mesa et al. 2002). After Harris et al. (1992) demonstrated the efficacy of oral creatine intake to increase skeletal muscle creatine content in humans, interest in the effects of oral creatine supplementation on skeletal muscle contractile performance and metabolism rapidly increased. It is now well established that the ingestion of a high dose (20-25 g per day) of oral creatine can rapidly (3-5 days) raise muscle total creatine content (Mesa et al. 2002). This elevation in muscle creatine storage has been associated with increased muscle power output during repeated short high intensity exercise tasks (Greenhaff et al. 1993; Balsom et al. 1995) and enhanced effects of weight training on muscle volume and strength (Vandenberghe et al. 1997; American College of Sports Medicine 2000). Additionally, it has been shown that the combination of creatine and carbohydrate supplements results in a greater post-exercise muscle glycogen accumulation than carbohydrate alone (Robinson et al. 1999), suggesting that creatine could also exert an effect on peripheral glucose metabolism. Additional evidence suggesting that creatine supplementation might be effective in regulating peripheral glucose metabolism comes from a recent study on transgenic Huntington mice, which are hyperglycemic. The addition of creatine...
to the diet of these mice significantly reduced hyperglycemia, while improving the glucose response to intravenous glucose injection (Ferrante et al. 2000).

It has been suggested that the effects of creatine supplementation on glucose homeostasis may be due to an increase in insulin secretion (Gempel et al. 1996). Although some _in vitro_ studies have indicated that creatine may increase insulin secretion modestly in the perfused rat pancreas (Alsever et al. 1970) and isolated mouse islets (Marco et al. 1976) or insulinoma cells (Gempel et al. 1996), evidence from _in vivo_ human studies indicate that either one 5g-dose of creatine (Green et al. 1996a) or 3 days of creatine supplementation (Green et al. 1996b) does not alter insulin secretion. Therefore, we hypothesized that the glucoregulatory effect of creatine supplementation may be caused by a direct alteration in peripheral glucose metabolism independent of changes in insulin secretion.

Recently, it was reported that oral creatine supplementation increased by ~40% the GLUT4 content in vastus lateralis muscle after rehabilitation training in subjects which previously had one of their legs immobilized (Op ‘t Eijnde et al. 2001a). It was also demonstrated that muscle glycogen and total creatine contents were higher in creatine-supplemented subjects, but no data were presented regarding glucose uptake and other aspects of glucose metabolism in this study (Op ‘t Eijnde et al. 2001a). In rats, it has also recently been reported that creatine supplementation reduces the PCr:total(TCr) ratio suggesting an alteration in the energy state in muscle cells but with no effect on glucose uptake in isolated plantaris muscles (Young & Young 2002).

Energy and metabolic sensing in muscle cells have been attributed to AMP-activated protein kinase (AMPK) [Hardie et al. 1998; Winder 2001], which has been
shown to regulate glucose uptake and metabolism in skeletal muscle. An alteration of the energy state of the cell is likely to alter the activity of AMPK and the demand for glucose. Investigation of GLUT4 translocation and glucose uptake as well as the fate of glucose via the pathways of glycogen synthesis, oxidation, and lactate production are crucial to characterize the implications of altering the energy state of muscle cells by creatine supplementation. However, the possible effects of creatine supplementation on AMPK activation and its implications for glucose uptake and metabolism have not been fully investigated. This study was designed to enhance our understanding of the metabolic response of skeletal muscle with respect to glucose uptake and metabolism in response to creatine supplementation. Accordingly, we investigated the in vitro effects of short-term (48 hour) creatine supplementation on glucose uptake, GLUT4 translocation, lactate production, glycogen synthesis, glucose oxidation, citrate synthase activity, and AMPK phosphorylation in L6 rat skeletal muscle cells.

Methods

Chemicals

5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) was purchased form Toronto Research Chemicals, Inc. Amyloglucosidase, Creatine hydrate, Creatine Kinase (CK), Cytochalasin B, DTNB (5,5′Dithiobiis(2-nitrobenzoic acid)), glycogen, Hexokinase (HK)/glucose-6-P dehydrogenase (G6P-DH) conjugated enzymes, 3-phospho-glyceric acid, 3-phospho-glycerate kinase (PGK), glyceraldehydes-3-phosphate dehydradegase (GPDH), lactate dehydrogenase (LDH)/pyruvate kinase (PK) conjugated enzymes, O-phenylenediamine di-hydrochloride (OPD), oxaloacetic acid, phenylethylamine, phosphoenolpyruvate, and triethanolamine were purchased from
SIGMA (St Louis, MO, USA), Acetyl-CoA, ADP, ATP, NADH, and NADP from Bioshop Canada Inc., Human insulin (Humulin®R) from Eli Lilly (Canada, Inc.), and D-[U-\textsuperscript{14}C]glucose and \textsuperscript{3}H-2-deoxy-glucose from Amersham (Quebec, Canada). \(\alpha\)-MEM and all other cell culture components from Wisent (Quebec, Canada). Anti-myc antibody 9E10 was purchased from Santa Cruz (Santa Cruz, CA). All other chemicals were of the highest grade available.

*Cell culture conditions for L6 Glut4-myc myoblasts*

For all experiments L6 rat skeletal muscle cells were grown in minimum essential medium (\(\alpha\)-MEM) supplemented with 10% (v/v) fetal bovine serum in a humidified atmosphere of 95% air and 5% \(\text{CO}_2\) at 37\(\degree\)C. Cells were transfected to stably overexpress GLUT4 harbouring a myc epitope on the first exofacial loop of the transporter. This facilitated accurate quantitative analysis of GLUT4 translocation in intact cells. Myoblasts were grown to confluence and the following experimental conditions were assigned: control, cells cultivated with medium not supplemented with creatine and not subsequently stimulated with insulin; insulin, cells cultivated with medium not supplemented with creatine but subsequently stimulated with insulin; Creatine, cells cultivated with medium supplemented with creatine but not subsequently stimulated with insulin; Creatine + insulin, cells cultivated with medium supplemented with creatine and subsequently stimulated with insulin. The final creatine concentration in all media was 0.5 mM. For all experiments, creatine-supplemented medium was changed every 24h and the cultivation, either with or without creatine, was maintained for 48h. Cells were serum-deprived for 4h (in the continued presence of creatine) prior
to all experimental manipulations. The final concentration of insulin in all experiments was 100 nM.

**Determination of glycogen, creatine, phosphocreatine, and ATP contents**

L6Glut4myc myoblasts were seeded in 6-well plates and grown to confluence. Subsequently, the cells were incubated either in the absence or presence of creatine (0.5 mM final concentration). After 0, 24, and 48h of creatine treatment, the cells were washed 5x with cold PBS and lysed in ice-cold perchloric acid. Subsequently, the cell lysates were neutralized with triethanolamine/K$_2$CO$_3$ solution, filtered, kept on ice and immediately used for assays (Wahlefeld *et al.* 1985; Heinz *et al.* 1985). Glycogen content was determined with amylo-glucosidase as previously described (Passoneau *et al.* 1974; Keppler *et al.* 1974). Total free creatine (TCr) and phosphocreatine (PCr) were determined by enzymatic assays using lactate dehydrogenase (LDH)/pyruvate kinase (PK)/creatine kinase (CK) and glucose-6-phosphate dehydrogenase (G6P-DH)/hexokinase (HK)/creatine kinase (CK), respectively, as previously described (Harris *et al.* 1974; Wahlefeld *et al.* 1985; Heinz *et al.* 1985). The PCr:TCr ratio was calculated by dividing the values obtained for phosphocreatine and free creatine, respectively. Creatine content in aliquots of the PBS used after the fifth wash was also determined to ensure that creatine was completely removed from the incubation medium before cell lysis. Adenosine-5'-triphosphate (ATP) was determined in the neutralized cell extract by an enzymatic assay with 3-phosphoglycerate kinase (Jaworek *et al.* 1974). Aliquots of the cell lysates were used to determine the protein content of the samples.
**Glucose uptake**

L6 myoblasts were cultivated in 12-well plates with or without creatine for 48h and serum starved for 4h before being incubated for 20min with or without insulin. Subsequently, myoblasts were washed twice, and glucose transport was assayed in HEPES-buffered saline solution (140 mM NaCl, 20 mM HEPES-Na, 2.5 mM MgSO₄, 1 mM CaCl₂, 5 mM KCl, pH 7.4) containing 10µM 2-deoxy-D-glucose (0.5 µCi/ml 2-deoxy-D-[³H]glucose) as previously described (Somwar *et al.* 2002). The incubation medium was aspirated, the cells were washed with ice-cold saline, and 1ml of NaOH (0.05 M) was added to each well. Cell lysates were transferred to scintillation vials for radioactivity counting. Non-specific uptake was determined in the presence of Cytochlasin B (10 µM) and was subtracted from all values.

**Determination of Cell Surface GLUT4myc**

The L6 GLUT4-myc cells are stably transfected to express GLUT4 tagged on its first exofacial loop with a myc epitope (received as a kind gift from Dr. Amira Klip, The Hospital for Sick Children, Toronto). The exofacial location of the myc epitope on GLUT4 in these cells allows the analysis of GLUT4 localization in intact cells. GLUT4myc levels at the cell surface were measured by an antibody-coupled colorimetric assay as described previously (Somwar *et al.* 2002). Briefly, after 4h of serum-starvation, cells were incubated for 20 min in the presence or absence of insulin. Subsequently, cells were quickly washed in ice-cold phosphate-buffered saline buffer (PBS) and incubated with anti-c-myc antibody (9E10, 1:200 dilution) for 60 min at 4°C. Cells were washed and fixed in 3% paraformaldehyde for 3 min on ice. The fixative was neutralized by incubation in 10 mM glycine in ice-cold PBS for 10 min. Cells were
blocked in 10% goat serum for 10 min and then incubated with horseradish peroxidase-conjugated donkey anti-mouse IgG (1:1000 dilution, 4°C) for 60 min. Cells were washed 5x with ice-cold PBS and incubated for 30 min at room temperature with 1 ml of OPD reagent (0.4 mg/ml O-phenylenediamine di-hydrochloride and 0.4 mg/ml urea hydrogen peroxide in 0.05 M phosphate citrate buffer) per well. The reaction was stopped by adding 0.25 ml of 3 M HCl. The supernatant was collected and the absorbance was measured at 492 nm.

*Lactate production*

After the 48h creatine-supplementation-period, cells were serum-starved for 4h then incubated with or without insulin for 2h and the media were collected for lactate determination. Total lactate released in the medium was measured by the lactate oxidase assay using a lactate kit (Sigma Diagnostics, St. Louis, Mo).

*Glycogen synthesis*

Glycogen synthesis was assessed by the incorporation of D-[U-14C]glucose into glycogen as previously described (Cuendet *et al.* 1976). Briefly, cells were cultivated in 6-well plates in the absence or presence of creatine. After 4h of serum starvation, cells were incubated for 2h with medium containing 0.15 μCi/ml of D-[U-14C]glucose with or without insulin. Cells were then quickly washed with ice-cold PBS and lysed in 0.5 ml of KOH (1M). Cell lysates were used for overnight glycogen precipitation with ethanol. Precipitated glycogen was dissolved in water and transferred to scintillation vials for radioactivity counting.
Glucose oxidation

Glucose oxidation was measured by the production of $^{14}$CO$_2$ from D-[U-$^{14}$C]glucose as previously described (Ceddia et al. 1999) with a few modifications. Briefly, cells were incubated for 2h in 60 x 15 mm petri dishes with medium containing 0.15 µCi/ml of D-[U-$^{14}$C]glucose with or without insulin. Each petri dish was sealed with parafilm having a piece of Whatman paper taped facing the inside of the petri dish. The whatman paper was wet with 100 µl of Phenylethylamine/Methanol (1:1) to trap the CO$_2$ produced during the incubation period. After 2h of incubation, 200 µl of H$_2$SO$_4$ (4M) was added to the cells and incubated for 1 h at 37°C. Finally, the pieces of whatman paper were removed and transferred to scintillation vials for radioactivity counting.

Citrate synthase (E.C.4.1.3.7.) maximum activity

L6Glut4myc myoblasts were seeded in 12-well plates and grown to confluence. Subsequently, the cells were incubated either in the absence or presence of creatine (0.5 mM final concentration). After 0, 24, and 48h, the cells were washed 3x with ice cold PBS (1ml/well) and subsequently lysed in 200 µl of ice-cold extraction buffer containing Tris/HCl (50mM), MgCl$_2$ (1mM), KCl (100 mM), sucrose (250 mM), and 2-mercaptoethanol (30 mM). The suspension was kept on ice and centrifuged (9000g, 15 min, 4°C). The supernatant was collected and the pellet of cells debris was discarded. For the measurement of citrate synthase activity, the assay buffer (950 µl final volume) contained tris-hydroxymethyl-aminomethane (100 mM), DTNB (0.2 mM), acetyl CoA (0.1 mM), and triton X-100 (0.05% v/v). The reaction was initiated by the addition of 20µl of the enzyme extract and 30µl of oxaloacetate (10 mM final concentration) as previously described (Srere et al. 1963; Alp et al. 1976; Ceddia et al. 2000). Absorbance
at 412 nm (25°C) was then measured during 10 min. An aliquot of the enzyme extract was used to determine the protein content of each sample. The maximum activity of citrate synthase was expressed as nmoles of substrate converted to product per minute per mg of protein (nmol/min/mg of protein).

**Western blot determination of GLUT4, P-AMPKα, and P-ACC**

Cells were grown in 6-well plates and after 48h in the presence or absence of creatine, incubated for 30 min with or without insulin as indicated, and then washed twice with ice-cold PBS. AICAR (1mM, 30 min) was used as a positive control for ACC phosphorylation. Subsequently, cells were lysed in 1x SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue, and protease and phosphatase inhibitors (1µM Na<sub>3</sub>VO<sub>4</sub>, 1µM leupeptin, 1µM pepstatin, 1µM Okadaic acid, and 1 µM PMSF), passed through a syringe several times and heated (65 °C, 5min). Aliquots (15µl) of cell lysates were then subjected to SDS-PAGE (8% resolving gels), and then transferred to PVDF membranes (Bio-Rad Laboratories). The phosphorylation of AMPK and ACC (acetyl-CoA carboxylase) were determined by using phospho-AMPK(Thr172)-specific (1:1000 dilution, Cell Signaling Technology) and phospho-ACC(S79)-specific (1:500 dilution, Upstate) antibodies, respectively. Equal loading of samples was confirmed by coomassie blue staining of the gels.

**Determination of phosphorylation of the α-1 and α-2 subunits of AMPK**

The phosphorylation of the α-1 and α-2 subunits of AMPK was determined after immunoprecipitation with specific antibodies (Santa Cruz Biotechnology, Inc) against the a-1 and a-2 catalytic subunits as previously described (Stapleton *et al.* 1996) and
protein A sepharose (Amersham Biosciences AB) beads. Immunoprecipitates were washed 5 times with lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na$_3$VO$_4$, 1 µg/ml leupeptin, 1 mM PMSF) and the pellet was resuspended in 40 µl of SDS sample buffer and centrifuged for 30 seconds. Twenty µl of the supernatant was used for western blot using phospho-AMPK(Thr172)-specific antibody (1:200 dilution, Cell Signaling Technology).

**Statistical analysis**

Data are presented as means ± s.e.m. Statistical analyses were performed by $t$ test or one- or two-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparison test or Bonferroni post-tests as indicated in the figure legends. The level of significance was set at $P<0.05$.

**Results**

*Creatine, phosphocreatine, and ATP contents*

The creatine and PCr contents of the cells increased from 6.75 ± 0.73 and 4.5 ± 0.36 µg/mg of protein before supplementation to 46.39 ± 1.23 and 22.10 ± 1.48 µg/mg of protein after 24h and 62.36 ± 2.04 and 23.0 ± 0.78 µg/mg of protein after 48h of creatine supplementation (Figure 1A), respectively. The expansion of the creatine and PCr contents, however, was not accompanied by an increase in the total ATP concentration in L6 muscle cells after creatine supplementation (5.40 ± 0.67 vs. 5.07 ± 0.28 nmols/mg of protein in the control and creatine supplemented cells, respectively) (Figure 1B).
Glucose uptake, GLUT4 translocation, glycogen synthesis, and GLUT4 and glycogen contents

In the present study, glucose uptake (Figure 2A), GLUT4 translocation (Figure 2B), and the incorporation of D-[U-14C]glucose into glycogen (Figure 2C) increased by ~1.9-fold, ~1.8-fold, and 2.3-fold, respectively, after insulin stimulation. AICAR (1mM, 30 min) was used as positive control and it significantly increased glucose uptake by ~1.7-fold (1.5 ± 0.19 and 2.53 ± 0.17 nmol/µg of protein for the control and AICAR-treated cells, respectively) in L6 muscle cells. However, creatine supplementation neither altered the basal nor the insulin-stimulated rates of glucose uptake, GLUT4 translocation, and glycogen synthesis. Furthermore, the GLUT4 protein content (Figure 2D) was not altered by creatine supplementation in L6 rat skeletal muscle cells. The glycogen content of the cells increased from 3.38 ± 0.32 µmoles/mg of protein before supplementation to 4.36 ± 0.22 and 4.63 ± 0.55 µmoles/mg of protein after 24 and 48h of creatine supplementation, respectively. However, there was no significant difference between the control and creatine-supplemented cells (Figure 3).

Lactate production, glucose oxidation, and citrate synthase maximal activity

In the presence of insulin, the L6 myoblasts elicited a significant ~1.5-fold increase in lactate production as compared to control cells. Interestingly, creatine supplementation reduced the basal production of lactate by ~42%, but did not alter the insulin stimulated lactate production by the cells (Figure 4A). Concomitantly, creatine supplementation significantly increased (1.4-fold) the basal production of $^{14}$CO$_2$ from D-[U-14C]glucose but did not alter the effect of insulin on this variable (Figure 4B). These results are supported by the significant increase (~30%) in citrate synthase activity after creatine
supplementation (Figure 5). In fact, the maximal activity of citrate synthase increased from 21.22 ± 0.84 nmol/min/mg of protein before supplementation to 23.40 ± 2.48 and 27.54 ± 1.4 nmol/min/mg of protein after 24 and 48h of creatine supplementation, respectively. Although there was a tendency towards an increase after 24h, it only reached statistically significant values after 48h of creatine supplementation (Figure 5).

**AMPK phosphorylation**

Insulin did not alter AMPK phosphorylation in L6 skeletal muscle cells (Figure 6A), however, AMPK phosphorylation was significantly increased (~1.8-fold) under basal or insulin stimulated conditions after creatine supplementation (Figure 6A). The increase in AMPK activity in our experiments was confirmed by the ~2.2-fold increase in phosphorylation of a well characterized substrate of AMPK, namely ACC (Figure 6B), after creatine supplementation. Additionally, we used AICAR as a positive control for AMPK and ACC phosphorylation. The magnitude of ACC phosphorylation caused by creatine supplementation was similar to that obtained after the incubation of L6 muscle cells with AICAR (Figure 6B). We also investigated if creatine regulated the phosphorylation of both α-1 and α-2 isoforms of AMPK. To do this, we immunoprecipitated α-1 and α-2 using isoform specific antibody and then western blotted immunoprecipitates with phospho-AMPK(Thr172)-specific antibodies. Interestingly, phosphorylation of both isoforms was significantly increased by creatine supplementation. We detected a significant ~2-fold increase in the phosphorylation state of both AMPK α-1 and α-2 isoforms, with no alteration in total AMPK α-1 and α-2 protein contents (Figure 6C and D).
Discussion

Oral creatine supplementation in humans (Mesa et al. 2002) and rats (Young & Young 2002) has been proven to increase the contents of creatine and phosphocreatine in skeletal muscle. In humans, creatine is usually administered as a dosage regimen consisting of a loading phase of 20 g/day (four times 5g) for 5-7 days and a maintenance dosage of 3-5 g/day thereafter (Mesa et al. 2002). It has been reported that 1h after a single oral dose (5g) of creatine the serum concentration of this substance rises from 0.05 – 0.1 mmol/L (fasting serum values) to 0.6 – 0.8 mmol/L (Persky et al. 2003). In response to a 20g-oral dose, plasma creatine concentration reaches peak values of 2.17 mmol/L (50-fold increase) after 2.5h of ingestion (Mesa et al. 2002). The uptake of creatine by muscle cells presents Michaelis-Menten kinetics with a maximum rate of creatine uptake (Vmax) obtained at concentrations higher than 0.3 – 0.4 mmol/L (Sora et al. 1994). Based on these data, we decided to supplement the incubation medium with 0.5 mM/L of creatine, which corresponds to the concentration achieved in humans after a single 5g-oral-dose of creatine. This value is also within the range that seems to elicit the maximal rate of muscle creatine uptake (Sora et al. 1994). We incubated the L6 muscle cells in the presence of 0.5 mM creatine for 48h, since it has been established that the majority of muscle creatine accumulation is maximal in the two days of oral supplementation (Kamber et al. 1999). The effectiveness of our supplementation regimen was confirmed by the ~9.6- and ~5.4-fold increases in creatine and PCR contents of the cells after 48h of creatine supplementation, respectively. In fact, these increases were significantly higher than what has been reported in humans (15 – 20% increase in muscle total creatine content).
after supplementation (Febraio et al. 1995; Hultman et al. 1996; Casey et al. 1996; Robinson et al. 1999). Therefore, although we have attempted to recreate the physiological milieu as closely as possible, care must be extended when extrapolating our data regarding the effects of increased intracellular creatine and PCr on glucose uptake and metabolism to intact muscle tissue.

We did not find any significant alteration in GLUT4 content, basal or insulin-stimulated GLUT4 translocation or glucose uptake, and glycogen content in these cells upon creatine supplementation. Additionally, contrary to what has been observed in humans (Robinson et al. 1999; Op `t Eijnde et al. 2001a) and rats (Op `t Eijnde et al. 2001b), we did not observe any alteration on either basal or insulin-stimulated glycogen synthesis after creatine supplementation. Interestingly, creatine supplementation has been reported to cause an increase in glycogen accumulation in humans only in muscles that are submitted to exercise (Robinson et al. 1999; Op `t Eijnde et al. 2001a). This suggests that some muscle contraction is necessary in order to observe any additive effect of creatine on glycogen synthesis in muscle. This could be one reason why creatine supplementation did not alter glycogen content and the basal or insulin-stimulated rates of glycogen synthesis in our in vitro model. In fact, our experiments were performed in non-contracting cells and the results obtained are in agreement with what has been reported in non-exercising rats in which creatine supplementation did not alter both basal or insulin-stimulated glucose uptake (Young & Young 2002), glycogen synthase activity (Op `t Eijnde et al. 2001b; Rooney et al. 2002) and the incorporation of $[^{14}\text{C}]$-glucose into glycogen (Op `t Eijnde et al. 2001b) in skeletal muscle.
The GLUT4 and glycogen contents of the cells were not altered by creatine supplementation, and although we did not observe any effect of creatine on basal and insulin-stimulated GLUT4 translocation and glucose uptake, L6 muscle cells supplemented with creatine significantly increased (~40%) their basal production of $^{14}\text{CO}_2$ from D-[U-$^{14}$C]glucose. This suggests that creatine supplementation shifted basal glucose metabolism towards oxidation, which is compatible with the significant reduction (~42%) in basal lactate production observed in the creatine-supplemented cells. These observations are also in agreement with data obtained from mitochondria isolated from muscles of creatine supplemented human subjects (Walsh et al. 2001) and from mice cardiac skinned muscle fibers incubated with creatine (Saks et al. 2000). In both models, oxygraphic measurements point towards an increase in mitochondrial respiration in the presence of creatine (Saks et al. 2000; Walsh et al. 2001). It has been demonstrated that in the presence of creatine the mitochondrial isoform of creatine kinase (mi-CK) uses ATP to produce ADP, which is channeled directly to adenine nucleotide translocase for regulation of respiration (Saks et al. 2000). This means that creatine, by activating mi-CK, changes the energy state of the cell and directly controls the mitochondrial energy production (Saks et al. 2000). The increase in CO$_2$ production that we observed in our creatine-supplemented L6 muscle cells could have resulted from the elevation of creatine concentration in the cell leading to higher mi-CK activity and increased oxidative phosphorylation. It has been repeatedly reported that creatine supplementation increases total creatine concentration in muscle in both humans (Greenhaff et al. 1994; Green et al. 1996a,b; Hultman et al. 1996; Steenge et al. 1998; Robinson et al. 1999) and rats (Brannon et al. 1997; McMillen 2001; Young & Young...
Interestingly, the expansion of the total creatine (TCr) pool in muscle is due to an increase in both the free and phosphorylated (PCr) forms of creatine. However, PCr does not seem to increase in the same proportion as free creatine in the muscle cell, hence the PCr:TCr ratio actually falls after supplementation (Greenhaff et al. 1994; Green et al. 1996a,b; Hultman et al. 1996; Steenge et al. 1998). This was also the case in our experiments, since the PCr:TCr ratio was 0.67 and 0.37 for the control and the 48h-creatine-supplemented cells, respectively. Considering that the PCr:TCr ratio reflects cellular energetics (Connet 1988; Op ‘t Eijnde et al. 2001a,b), a decrease in PCr:TCr might have been interpreted as an altered energy state in our resting muscle cells, which in turn increased their basal glucose oxidation rate. This is compatible with our data showing increases (~30%) in both glucose oxidation and maximal activity of citrate synthase after 48h of creatine supplementation.

The AMP-activated protein kinase (AMPK) has been established as a protein that monitors the metabolic and energetic states of the muscle cells (Hardie et al. 1998; Winder 2001). Additionally, it has been shown that AMPK activity is modulated by the PCr:TCr ratio (Ponticos et al. 1998). A fall in the PCr:TCr ratio within the cell would be expected, therefore, to cause activation of AMPK as a result of the release of inhibition exerted by PCr (Ponticos et al. 1998). Interestingly, we found an ~1.8-fold increase in AMPK phosphorylation. Previous studies have reported specific induction of AMPK α-2 isoform activity in skeletal muscle by exercise (Wojtaszewski et al. 2000, Fuji et al. 2000, Stephens et al. 2002), suggesting that this isoform may be involved in the metabolic responses observed in contracting skeletal muscles. A more detailed analysis of changes in activity of the catalytic AMPK isoforms in our study revealed an ~2-fold
increase in phosphorylation of both $\alpha$-1 and $\alpha$-2 isoforms in creatine supplemented muscle cells. Additionally, we demonstrated that similar amounts of the $\alpha$-1 and $\alpha$-2 AMPK isoforms were present in L6 muscle cells. Therefore, the metabolic effects we observed in the present study with non-contracting L6 muscle cells are likely to be due to the activation of both $\alpha$-1 and $\alpha$-2 AMPK isoforms. This is again different from exercise conditions that lead to the degradation of intracellular glycogen content (Fuji et al. 2000) and other energy substrates and may cause isoform-specific activation of the catalytic subunits of AMPK. In fact, the exercise-induced increase in $\alpha$-2-specific activity of AMPK has been reported to be intensity dependent (Wojtaszewski et al. 2000, Fuji et al. 2000) and inversely correlated with glycogen depletion in skeletal muscle (Fuji et al. 2000, Stephens et al. 2002), suggesting that the glycogen content of muscle cells may indeed play an important role in triggering the multiple cellular effects of AMPK.

AMPK activation has been suggested to play a key role in increasing glucose uptake in contracting skeletal muscle (Zierath 2002). Yet we do not observe an increase in glucose uptake by creatine supplementation, despite activation of AMPK. In order to demonstrate that L6 muscle cells have the ability to react to increased AMPK activity by elevating glucose transport, we treated cells with AICAR and observed a significant increase (~1.7-fold) in glucose uptake. However, it has been reported that AMPK activity and glucose uptake may be completely dissociated in contracting perfused slow-twitch rat muscle (Derave et al. 2000). It seems that the major factor that dissociates AMPK activation from an increase in glucose uptake in skeletal muscle is the glycogen content of the cells (Derave et al. 2000). In our experiments the glycogen content was similar in the control and creatine-supplemented conditions and this may be the reason
why the increased phosphorylation of AMPK in our creatine-supplemented cells was not followed by an increment in glucose uptake. Furthermore, there are many instances where activation of a given mediator of glucose uptake does not lead to glucose uptake, e.g. PI3-kinase (Jiang et al. 1998). It may also be that creatine activates AMPK in a compartment specific manner such that only a subset of AMPK not involved in stimulating glucose uptake is activated.

One intriguing point in our results is the fact that creatine increased basal glucose oxidation rate and this may have resulted from AMPK activation; however, when the glycolytic flux was acutely increased by insulin in the creatine-supplemented cells the rate of glucose oxidation was not affected at all by creatine, despite a similar increase in AMPK phosphorylation. At the present time we don not have a precise explanation for this finding. However, it is possible that the increase in glucose oxidation observed under basal conditions, which was compensated for by a proportional reduction in lactate production, was already sufficient to meet the new energetic demands of the cells to adjust for the reduction in the PCr:TCr ratio caused by creatine supplementation. Therefore, no additional increases in glucose oxidation were necessary, even though the glycolytic flux in the cells was acutely increased by insulin stimulation.

In summary, our study demonstrates that 2-day treatment of L6 rat skeletal muscle cells with creatine increased the proportion of glucose being oxidized and caused a corresponding reduction in lactate production (Figure 8) in L6 rat skeletal muscle cells. The ability of creatine to phosphorylate, and activate AMPK may be responsible for this effect. Although creatine supplementation caused an increase in
AMPK activity, GLUT4 and glycogen contents, the ability of insulin to stimulate GLUT4 translocation, glucose uptake, and glycogen synthesis were not affected in these cells.
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References


Figure 1. (A) Creatine (Cr), phosphocreatine (PCr), and (B) ATP contents of L6-Glut4myc myoblasts after incubation for 0, 24, and 48h either in the absence (control) or presence of 0.5 mM creatine (creatine). Data are representative of four independent experiments with quadruplicates in each experiment. Data are expressed as means ± sem. *P<0.05 vs. PCr control at 0, 24, and 48h; **P<0.05 vs. Cr control at 0, 24, and 48h; #P<0.05 vs. Cr control at 0, 24, and 48h and Cr at 24h (two-way ANOVA).
Figure 2. Effects of creatine (Cre), insulin (Ins), and insulin plus creatine (Ins + Cre) on 
(A) 2-Deoxyglucose uptake, (B) GLTU4 translocation, and (C) glycogen synthesis. (D) 
Representative blot of GLUT4 (55 kDa) in L6 rat skeletal muscle cells. Prior to insulin 
stimulation (20 min, 100 nM where indicated), cells were cultivated for 48h either in the 
presence or absence of creatine. Data representative of 4 independent experiments 
with quadruplicates in each experiment (A, B, and C). For GLUT4 content we performed 
4 independent experiments with duplicates in each experiment (D). *P<0.05 vs. control 
(Con) and creatine (one-way ANOVA).
Figure 3. Glycogen content of L6 rat skeletal muscle cells incubated for 0, 24, and 48h either in the absence (control) or presence of creatine (0.5 mM). Data representative of 4 independent experiments with quadruplicates in each experiment. *$P<0.05$ vs. control and creatine time 0h (two-way ANOVA).
Figure 4. Effects of creatine (Cre), insulin (Ins), and insulin plus creatine (Ins + Cre) on the production of (A) lactate and (B) $^{14}$CO$_2$ from D-[U-$^{14}$C]glucose in L6 rat skeletal muscle cells. Prior to insulin stimulation (2h, 100 nM), cells were cultivated for 48h either in the absence or presence of creatine (0.5 mM). Data representative of 4 independent experiments with quadruplicates in each experiment. *$P<0.05$ vs. control (Con). $^\#P<0.05$ vs. control, insulin and insulin + creatine (one-way ANOVA).
Figure 5. Maximum activity of citrate synthase in L6 rat skeletal muscle cells incubated for 0, 24, and 48h either in the absence (control) or presence of creatine (0.5 mM). Data representative of 4 independent experiments with quadruplicates in each experiment. *P<0.05 vs. control 0, 24, and 48h (two-way ANOVA).
Figure 6. Representative blots and their respective densitometric quantification of the effects of insulin (Ins) and creatine (Cre) on (A) P-AMPKα (62 kDa), (B) P-ACC (257 kDa), (C) P-AMPK α-1 (62 kDa) and AMPK α-1 (62 kDa) protein content, (D) P-AMPK α-2 and AMPK α-2 protein content. P-AMPK α-1 (panel C) and P-AMPK α-2 (panel D) are expressed as relative to AMPK α-1 and AMPK α-2 contents, respectively. Data representative of 4 independent experiments with duplicates in each experiment. AICAR (1mM, 30 min) was used as a positive control for ACC phosphorylation. *P<0.05 vs. control and insulin (A and B, ANOVA) and vs. control (C and D, t-test).
Figure 7. Schematic representation of the effects of creatine on glucose uptake and metabolism in L6 rat skeletal muscle cells. Creatine supplementation did not alter either basal or insulin-stimulated Glut4 translocation (1), glucose uptake (2), or glycogen synthesis (3). The Glut4 and glycogen contents of the cells were not altered by creatine supplementation either. However, the basal production of lactate was reduced while the maximum activity of citrate synthase and the production of CO$_2$ were increased after creatine supplementation. $\uparrow$ = increase; $\downarrow$ = reduction; $\leftrightarrow$ = no effect; PM = plasma membrane; CT = creatine transporter.