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Creatine enhances differentiation of myogenic C₂C₁₂ cells by activating both p38 and Akt/PKB pathways

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Deldicque L, Theisen D, Bertrand L, Hespel P, Hue L, Francaux M. Creatine enhances differentiation of myogenic C₂C₁₂ cells by activating both p38 and Akt/PKB pathways. *Am J Physiol Cell Physiol* 293: C1263–C1271, 2007. First published July 25, 2007; doi:10.1152/ajpcell.00162.2007.—In myogenic C₂C₁₂ cells, 5 mM creatine increased the incorporation of labeled [³⁵S]methionine into sarcoplasmic (+20%, *P* < 0.05) and myofibrillar proteins (+50%, *P* < 0.01). Creatine also promoted the fusion of myoblasts assessed by an increased number of nuclei incorporated within myotubes (+40%, *P* < 0.001). Expression of myosin heavy chain type II (+1,300%, *P* < 0.001), troponin T (+65%, *P* < 0.01), and titin (+40%, *P* < 0.05) was enhanced by creatine. Mannitol, taurine, and β-alanine did not mimic the effect of creatine, ruling out an osmolarity-dependent mechanism. The addition of rapamycin, the inhibitor of mammalian target of rapamycin/70-kDa ribosomal S6 protein kinase (mTOR/p70^{s6k}) pathway, and SB 202190, the inhibitor of p38, completely blocked differentiation in control cells, and creatine did not reverse this inhibition, suggesting that the mTOR/p70^{s6k} and p38 pathways could be potentially involved in the effect induced by creatine on differentiation. Creatine upregulated phosphorylation of protein kinase B (Akt/PKB; +60%, *P* < 0.001), glycogen synthase kinase-3 (+70%, *P* < 0.001), and p70^{s6k} (+50%, *P* < 0.001). Creatine also affected the phosphorylation state of p38 (−50% at 24 h and +70% at 96 h, *P* < 0.05) as well as the nuclear content of its downstream targets myocyte enhancer factor-2 (−55% at 48 h and +170% at 96 h, *P* < 0.05) and MyoD (+60%, *P* < 0.01). In conclusion, this study points out the involvement of the p38 and the Akt/PKB-p70^{s6k} pathways in the enhanced differentiation induced by creatine in C₂C₁₂ cells.

protein synthesis; insulin-like growth factor; mitogen-activated protein kinase; extracellular signal-regulated kinase 1/2; 70-kDa ribosomal S6 protein kinase

OVER THE LAST DECADE, the interest in oral creatine supplementation has grown. Creatine has been used in different contexts, not only among athletes as an ergogenic aid for improving muscle power output during high-intensity exercise (5, 17) but also as a potential therapeutic agent for patients suffering from muscle wasting and myopathies (28, 37, 43, 44). Creatine supplementation leads to an increase in lean body mass (19) and in muscle cross-sectional area when the supplementation is associated with resistance exercise training (38, 42) or in the case of rehabilitation after muscle atrophy (18). Evidence accumulated over the past 10 years suggests that creatine exerts an anabolic effect by enhancing the regeneration process sub-

sequent to a degeneration phase induced by exercise, disease, or immobilization. Recently, creatine supplementation has been shown to amplify the increase in satellite cell number and myonuclei concentration in human skeletal muscle fibers during 4–16 wk of resistance training. These creatine-induced modifications were associated with an enhanced muscle fiber growth in response to strength training (35). Nevertheless, the precise mechanism by which creatine affects growth and differentiation of myogenic cells still remains unknown.

Creatine improves growth and differentiation of various other cell types in culture. It stimulates metabolic activity, differentiation, and mineralization of osteoblast-like cells (15). In cultured striatal tissue, creatine increases the density of GABAergic neurons without affecting total cell number (3). In 1972, Ingwall et al. (21) had already reported that creatine increases the expression of myosin heavy chain (MHC) and stimulates muscle-specific protein synthesis in both skeletal and cardiac chicken myotubes in culture. Previous work by our group on C₂C₁₂ myogenic cells has shown that creatine stimulates growth and protein accretion, associated with an increase in insulin-like growth factor I (IGF-I) mRNA and a coordinated upregulation of myogenic regulatory factors mRNA (29).

Although some targets have been identified, it is not clear whether IGF or other signaling pathways are involved in creatine action. Therefore, the purpose of the present study was to identify the signaling of creatine by using a myogenic cellular model. Since the p38 and the ERK1/2 mitogen-activated protein kinase (MAPK) as well as the phosphatidylinositol 3-kinase (PI3K) pathways are known to be key signaling cascades in the differentiation process of myoblasts (16, 25, 26, 49), we tested their possible involvement in creatine action.

MATERIALS AND METHODS

Cell Culture

C₂C₁₂ murine skeletal muscle myoblasts (ATCC, Manassas, VA) were seeded in 100-mm-diameter culture dishes and grown in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (5,000 U/5,000 μg/ml), and 100 μM nonessential amino acids. For immunohistochemistry, coverslips were placed on the bottom of the plates at the beginning of the proliferation phase. When cells were 70% confluent, the proliferation medium was replaced by a differentiation medium containing 1% horse serum, 1% penicillin/streptomycin

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(5,000 U/5,000 $\mu\text{g/ml}$), and 100 μM nonessential amino acids. At the beginning of the differentiation phase, creatine (Flamma) was added to reach a final concentration of 5 mM to half of the plates, with the other plates serving as controls. This concentration of creatine was chosen because it had the largest effect on the growth of C₂C₁₂ cells (29). To test a potential activation of cell signaling by disturbance of extracellular or intracellular osmolarity, we used 5 mM mannitol, taurine, or β -alanine (Sigma). Rapamycin [100 nM, inhibitor of mammalian target of rapamycin (mTOR); Alexis Biochemicals], SB 202190 (10 μM , inhibitor of p38; Sigma), or picropodophyllin (1–10 nM, inhibitor of IGF-I receptor; Calbiochem) was added to the culture medium at the beginning of the differentiation phase in control and creatine conditions.

Protein Extraction

Sarcoplasmic proteins. Cells were rinsed with phosphate-buffered saline (PBS) and harvested in a lysis buffer containing 20 mM Tris, pH 7.0, 270 mM sucrose, 5 mM EGTA, 1 mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium β -glycerophosphate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM 1,4-dithiothreitol, and a protease inhibitor cocktail (Roche Applied Science). The lysate was immediately centrifuged at 10,000 g for 10 min at 4°C. The supernatants were stored at –80°C, and protein concentration was determined using the DC protein assay kit (Bio-Rad Laboratories) with bovine serum albumin (BSA) as a standard.

Nuclear proteins. Plates were rinsed with PBS and washed with cold hypotonic buffer (20 mM HEPES, 5 mM sodium fluoride, 1 mM sodium molybdate, and 0.1 mM EDTA). Cells were then scraped in a lysis buffer (20 mM HEPES, 0.5% Nonidet P-40, 5 mM sodium fluoride, 1 mM sodium molybdate, and 0.1 mM EDTA) and immediately centrifuged for 30 s at 10,000 g. The pellet was resuspended in 100 μl of a buffer containing 20 mM HEPES, 5 mM sodium fluoride, 1 mM sodium molybdate, 0.1 mM EDTA, and 20% glycerol, and 150 μl of a saline buffer (20 mM HEPES, 5 mM sodium fluoride, 1 mM sodium molybdate, 0.1 mM EDTA, 20% glycerol, and 0.8 M NaCl) were added. The lysate was mixed for 30 min at 4°C and centrifuged for 10 min at 10,000 g. The supernatant was stored at –80°C, and protein concentration was determined as described above.

Myofibrillar proteins. Myofibrillar proteins were extracted according to the method described by Artaza (4). Briefly, plates were rinsed with PBS and scraped in a homogenization buffer (250 mM sucrose, 100 mM potassium chloride, 5 mM EDTA, and 20 mM Tris, pH 6.8). Cells were then centrifuged at 10,000 g for 10 min at 4°C. The pellet was washed (175 mM potassium chloride, 2 mM EDTA, 0.5% Triton, and 20 mM Tris, pH 6.8), centrifuged, and resuspended in a buffer containing 150 mM potassium chloride and 20 mM Tris (pH 7.0). Protein content was measured as described above.

Fusion Index

The fusion index corresponds to the proportion of nuclei present within myotubes that contain three or more nuclei (23). To determine clearly the boundaries of each myotube, the cells were incubated with a specific antibody directed against the membrane protein desmin (see *Immunohistochemistry*), and the nuclei were stained with 300 nM 2-(4-amidinophenyl)-6-indolecarbamidine (Sigma) for 5 min (see Fig. 1, A and B). The fusion index was determined after 4 days of differentiation by counting at least 1,000 nuclei per culture in three independent cultures.

Incorporation of Labeled [³⁵S]Methionine

Cells were cultured in control and creatine conditions for 4 days of differentiation and then incubated for 24 h in the presence of 1 mM [³⁵S]methionine (100 $\mu\text{Ci/ml}$). Sarcoplasmic and myofibrillar proteins were extracted, and the incorporation of labeled methionine was measured separately. Proteins were precipitated with 10% trichloro-

acetic acid (TCA), subsequently dissolved in 0.1 N NaOH, and precipitated again. Proteins were washed twice with 5% TCA. The final pellet was resuspended in 1 ml of formic acid. Five milliliters of scintillating liquid (Ultimagold; Perkin Elmer) were then added, and [³⁵S]incorporation was counted in a scintillation counter (Beckman).

SDS-PAGE and Immunoblotting

Cell lysates were combined with Laemmli sample buffer and separated by SDS-PAGE (8–15%). After electrophoretic separation, the proteins were transferred to a polyvinylidene difluoride membrane for Western blot analysis, except for titin, the expression of which was assessed using 3% SDS-PAGE strengthened with agarose (39). Membranes were incubated in a 5% Blotto solution. After the blocking step, they were incubated with the following antibodies overnight at 4°C (Supplementary Fig. 1): anti-phospho-Akt/PKB Ser473 (1:2,000; Upstate Biotechnology), total Akt/PKB (1:1,000; Upstate Biotechnology), anti-phospho-p70^{S6k} Thr389 (1:1,000; Santa Cruz Biotechnology), total p70^{S6k} (1:1,000; Santa Cruz Biotechnology), 4E-BP1/PHAS-I recognizing all phosphorylated forms between 19 and 25 kDa (1:1,000; Calbiochem), anti-phospho-p38 Thr180/Tyr182 (1:1,000; Cell Signaling), total p38 (1:1,000; Cell Signaling), anti-phospho-ERK1/2 Thr202/Tyr204 (1:1,000; Cell Signaling), total ERK1/2 (1:1,000; Cell Signaling), anti-phospho-GSK-3 Ser21 (1:1,000; Upstate Biotechnology), MyoD (1:500; Santa Cruz Biotechnology), MEF-2 (1:1,000; Santa Cruz Biotechnology), MHC II (1:100; Alexis Biochemicals), and troponin T fast isoform (1:500; Santa Cruz Biotechnology). (Supplemental data for this article is available online at the *American Journal of Physiology-Cell Physiology* website.)

Membranes were washed in TBST (Tris-buffered saline and 0.1% Tween 20) and incubated for 1 h at room temperature with a secondary antibody conjugated to horseradish peroxidase (1:10,000; Calbiochem). After an additional three washes, chemiluminescent detection was carried out using an ECL Western blotting kit (ECL Plus; Amersham Biosciences). Steady expression of the analyzed proteins through the differentiation was verified with a total antibody. The films were scanned with an image scanner using LabScan software and quantified with the ImageMaster 1D image analysis software (Amersham Biosciences). The results represent the phosphorylated form of the protein.

Akt/PKB Activity

Akt/PKB activity was measured as the phosphorylation of a synthetic peptide after immunoprecipitation (100 μg of protein extract) with a total Akt/PKB antibody recognizing the PH domain (Upstate Cell Signaling) as previously described (6). In short, the assay was performed in a final volume of 50 μl in the presence of 10 mM MOPS (pH 7.0), 0.5 mM EDTA, 10 mM magnesium acetate, 0.1% β -mercaptoethanol, 0.1 mM Mg-[³²P]ATP (specific radioactivity 1,000 cpm/pmol; Amersham Biosciences) and 0.25 mM substrate peptide RPRATF (1). After 20 min at 30°C, the reaction mixture was quickly centrifuged, and 20- μl samples were spotted on P81 phosphocellulose paper, followed by washes in cold 75 mM phosphoric acid. ³²P incorporation was counted in a scintillation counter (Beckman).

Immunohistochemistry

Cells were fixed on the coverslips with 4% paraformaldehyde for 20 min, washed twice with PBS, and permeabilized for 15 min with 0.2% Triton. After two washes, the blocking step was performed with goat serum for 30 min, followed by a 1-h incubation with an antibody against MHC II (1:10; Alexis Biochemicals) or an antibody against desmin (1:100; DAKO) diluted in PBS containing 0.5% BSA. Coverslips were washed three times, and a fluorescein isothiocyanate secondary anti-mouse antibody (1:100; Sigma) was applied for 1 h. After several washes, the coverslips were mounted with Vectashield (Vector Laboratories) on glass slides. The slides were examined with an Axiovert 40 fluorescent microscope (Zeiss).

Fig. 1. Effect of creatine on differentiation, incorporation of labeled methionine, and expression of troponin T and titin in C₂C₁₂ cells. *A* and *B*: control (CTRL; *A*) and creatine (CR)-treated myotubes (*B*) after 4 days of differentiation. To determine the boundaries of each myotube, the cells were incubated with a specific antibody directed against the membrane protein desmin, and the nuclei were stained with DAPI. *C*: creatine induced an increase in the fusion index. The fusion index was defined as the percentage of nuclei located within myotubes (at least 3 nuclei) with respect to the total number of nuclei. *D*: effects of creatine on [³⁵S]methionine ([³⁵S]Met) incorporation into C₂C₁₂ myotubes for 24 h. Results are expressed as a percentage of the control condition after 5 days of differentiation ($n = 3$). *E* and *F*: creatine increased the expression of troponin T (*E*) and titin (*F*). Results are means $\pm$ SE ($n = 4$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, creatine vs. control at same time.

ATP, Phosphocreatine, Free Creatine, and Total Creatine Contents

Cells were rinsed with cold PBS and scraped with 3 N perchloric acid on ice. Cell lysates were centrifuged at 10,000 g for 3 min at 4°C, and the supernatants were neutralized with 2 N KHCO₃. ATP, phosphocreatine (PCr), and free creatine were analyzed enzymatically with standard fluorometric assays (30). Muscle total creatine content was calculated as the sum of free creatine and PCr.

Statistical Analysis

The difference between control and creatine conditions was tested for significance using either one- or two-way analyses of variance (ANOVA). When appropriate, ANOVA for repeated measures were applied. When the significance threshold, set to $P < 0.05$, was reached, Bonferroni post hoc tests were used. Results are means $\pm$ SE.

RESULTS

Myogenic Effects of Creatine

To quantify the effect of creatine on the differentiation of myogenic C₂C₁₂ cells, we assessed the index of fusion by measuring the number of nuclei within myotubes. After 4 days of differentiation, creatine increased the number of nuclei within myotubes by $\sim 40\%$ ($P < 0.001$; Fig. 1, *A–C*). The

increase in fusion of myoblasts was accompanied by an increase in the incorporation of [³⁵S]methionine into both sarcoplasmic ($P < 0.05$) and myofibrillar proteins ($P < 0.01$) after 5 days of differentiation (Fig. 1*D*). After 4 days of differentiation, troponin T and titin expressions were increased in the plates treated with creatine ($P < 0.01$ and $P < 0.05$, respectively; Fig. 1, *E* and *F*). At the same time, the expression of MHC II in the myotubes was markedly enhanced ($P < 0.001$; Fig. 2, *A–C*). Since this increase has already been observed by Ingwall et al. (21), and since MHC is the most abundant and one of the most important myofibrillar proteins in the muscle, we used MHC II as a marker of the creatine effect in the experiments described below. On the other hand, creatine did not influence the activity of citrate synthase and lactate dehydrogenase (data not shown).

PCr, free creatine, and total creatine contents were increased three- to sixfold both after 3 and 4 days of differentiation ($P < 0.001$; Table 1), whereas ATP content was not modified in the presence of creatine (Table 1). To test whether the enhancement of differentiation by creatine was caused by a disturbed osmolarity, we added mannitol, taurine, or β -alanine to the medium. Increased extracellular osmolarity by 5 mM mannitol,

Fig. 2. Effect of creatine and disturbance of osmolarity on the expression of myosin heavy chain (MHC) II. *A* and *B*: control (*A*) and creatine-treated myotubes (*B*) immunostained with an antibody against MHC II after 96 h of differentiation. *C*: creatine increased the expression of MHC II after 96 h of differentiation. *D*: effect of disturbance in intracellular (5 mM taurine or 5 mM β -alanine) or extracellular osmolarity (5 mM mannitol) on the expression of MHC II after 96 h of differentiation. Tau, taurine; Ala, β -alanine; Man, mannitol. Results are means $\pm$ SE ($n = 5$). * $P < 0.05$; *** $P < 0.001$ vs. control.

which is not taken up by the cell, did not change MHC II expression (Fig. 2D). Taurine or β -alanine (5 mM) increased their intracellular concentration by ~ 16 -fold (data not shown), but in contrast with creatine, MHC II expression tended to be decreased (Fig. 2D). These data demonstrate that changes in osmolarity were not able to mimic the action of creatine.

Signaling of the Myogenic Effects of Creatine

To test the involvement of IGF-I in creatine action, we used picropodophyllin, a specific inhibitor of IGF-I receptor (41). We had previously tested the effectiveness of this inhibitor in our experimental conditions and confirmed that it prevented the increase in p38 phosphorylation induced by IGF-I (data not shown). Incubation of the cells with picropodophyllin (1–10 nM) at the beginning of the differentiation phase did not prevent the enhancement of MHC II induced by creatine (Fig. 3). This suggests that IGF-I is not initiating the signaling leading to the enhancement of MHC II expression.

The time course of p38 phosphorylation was biphasic under control conditions (Fig. 4A): it decreased during the first 48 h of differentiation and then increased again after 72 h. In the presence of creatine, the time course was accelerated, with an

earlier decrease and a larger re-increase ($P < 0.05$). The potential implication of p38 in the differentiation process of myogenic cells was also suggested by the use of SB 202190, an inhibitor of p38, which abolished the expression of MHC II in control conditions (Supplementary Fig. 2B). Creatine did not reverse this effect, suggesting that the p38 pathway is likely involved in the enhanced differentiation induced by creatine. In contrast to p38, which was affected throughout the differentiation by creatine, ERK1/2 phosphorylation only increased after 4 days of differentiation in creatine conditions ($P < 0.01$; Fig. 4B).

The nuclear expression of myocyte enhancer factor-2 (MEF-2), a downstream target of p38, followed the pattern of p38 phosphorylation with a 24-h delay and was affected by creatine ($P <$

Table 1. ATP, PCr, free creatine, and total creatine content

	72 h		96 h	
	Control	Creatine	Control	Creatine
ATP	45 $\pm$ 3.2	37 $\pm$ 3.8	52 $\pm$ 6.8	47 $\pm$ 0.4
PCr	73 $\pm$ 8.9	287 $\pm$ 3.5*	80 $\pm$ 5.3	418 $\pm$ 1.8*
Cr	106 $\pm$ 12.0	424 $\pm$ 3.5*	63 $\pm$ 7.0	427 $\pm$ 22.6*
Total Cr	179 $\pm$ 20.5	712 $\pm$ 0.9*	143 $\pm$ 11.4	845 $\pm$ 21.2*

ATP, phosphorylcreatine (PCr), free creatine (Cr), and total creatine (total Cr) contents were determined in control and creatine-treated cells after 72 and 96 h of differentiation. Results are means $\pm$ SE in nmol/mg protein ($n = 3$). * $P < 0.001$, creatine vs. control.

Fig. 3. Effect of picropodophyllin (PPP) on the expression of MHC II. The effect of increasing doses of PPP (1–10 nM), a specific inhibitor of the insulin-like growth factor I (IGF-I) receptor, on the expression of MHC II in control and creatine conditions after 96 h of differentiation. No inh, no inhibitor. Results are means $\pm$ SE ($n = 3$). * $P < 0.05$, creatine vs. control.

Fig. 4. Time course of p38 and ERK1/2 phosphorylation and time course of myocyte enhancer factor-2 (MEF-2) and MyoD expression in the nucleus during the differentiation phase in C₂C₁₂ cells incubated with creatine. A: p38 phosphorylation on Thr180/Tyr182 ($n = 4$). B: ERK1/2 phosphorylation on Thr202/Tyr204 ($n = 4$). C: MEF-2 expression ($n = 3$). D: MyoD expression ($n = 6$). Results are means $\pm$ SE. * $P < 0.05$; ** $P < 0.01$, creatine vs. control at same time.

0.05; Fig. 4C). Another putative target of p38, the myogenic regulatory factor MyoD, was also modified by creatine. In control conditions, the expression of MyoD in the nucleus increased throughout the differentiation (Fig. 4D). At the end of the differentiation, this increase was larger in the myotubes treated with creatine ($P < 0.01$).

Incubation with creatine led to an increased activity (2.5-fold) of Akt/PKB after 72 h of treatment ($P < 0.01$; Fig. 5A) and an increased Ser473 phosphorylation at 72 h ($P < 0.01$) and 96 h of differentiation ($P < 0.001$; Fig. 5B). Creatine also increased the phosphorylation state of glycogen synthase kinase-3 (GSK-3), a downstream effector of Akt/PKB, at 72 h ($P < 0.01$) and 96 h ($P < 0.001$; Fig. 5C). Downstream of the cascade are 70-kDa ribosomal S6 kinase (p70^{s6k}) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). The percentage of 4E-BP1 in γ -isoform, which represents the most phosphorylated form of the protein, was not affected by creatine (Fig. 5D), but creatine increased the phosphorylation state of p70^{s6k} on Thr389 from the second day of differentiation until the end of the period studied ($P < 0.05$ at 48 and 72 h and $P < 0.001$ at 96 h; Fig. 5E). The phosphorylation state of p70^{s6k} was only barely detectable after 24 h of differentiation in two of four cultures. Therefore, the basal state has been referred to as 48 h rather than to 24 h (Fig. 5E). As observed for the p38 pathway, the Akt/PKB-mTOR-p70^{s6k} cascade is required for the occurrence of the differentiation process. Indeed, the addition of rapamycin, an inhibitor of mTOR/p70^{s6k} pathway, completely repressed differentiation and the expression of MHC II in control conditions. Creatine did not reverse this effect, indicating that the Akt/PKB-mTOR-p70^{s6k}

pathway is also needed for the enhanced differentiation induced by creatine (Supplementary Fig. 2B).

An interaction between p38 and Akt/PKB in myogenic cells has previously been described in the literature (11). To confirm that p38 and the Akt/PKB-mTOR-p70^{s6k} pathways could cross talk in our model, we studied the effects of SB 202190 (inhibitor of p38) on the phosphorylation state of Akt/PKB and p70^{s6k} (Fig. 6). SB 202190 reduced Akt/PKB and p70^{s6k} phosphorylation in both control and creatine conditions.

DISCUSSION

The present study shows that in the myogenic C₂C₁₂ cell line, as in skeletal and cardiac chicken myotubes in culture (20–22, 29), creatine promotes differentiation. It also shows that creatine enhances incorporation of labeled methionine (Fig. 1D) and fusion of myoblasts (Fig. 1C). The effect of creatine was rather specific, since the expression of troponin T, titin, and MHC II were clearly increased after 4 days of differentiation (Fig. 1, E and F, and Fig. 2C), whereas the activities of citrate synthase and lactate dehydrogenase were unchanged by creatine (data not shown). The enhancement of differentiation by creatine was independent of a change in intracellular ATP content and could be mediated by an increase in free creatine as well as PCr levels (Table 1). The present data confirm previous data on endothelial (34), myogenic L6 (12), and C₂C₁₂ cells (2) showing that ATP levels are not affected by creatine, whereas free creatine and PCr are increased. It seems that intracellular free creatine concentration rapidly (<24 h) increases and reaches a maximal level after addition of creatine into the cell culture medium. This maximal

Fig. 5. Time course of Akt/PKB, glycogen synthase kinase-3 (GSK-3), eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), and 70-kDa ribosomal S6 protein kinase (p70^{S6k}) phosphorylation in C₂C₁₂ cells incubated with creatine. **A**: Akt/PKB activity after 72 h of differentiation ($n = 3$). **B**: Akt/PKB phosphorylation on Ser473. **C**: GSK-3 phosphorylation on Ser21. **D**: 4E-BP1 in gamma form. **E**: p70^{S6k} phosphorylation on Thr389 ($n = 4$). Results are means $\pm$ SE. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, creatine vs. control at same time.

content is, to a certain extent, independent of the dose of creatine added and the duration of incubation period. Indeed, the addition of 0.5 mM creatine for 24–48 h (12), 5 mM in the present study for 72–96 h, or 20 mM for 16 h (2) leads to an intracellular free creatine concentration of ~ 400 nmol/mg protein. This is not the case for PCr, which increases through the differentiation phase from 175 (2) to 400 nmol/mg protein (Table 1), implying an increase in the PCr/Cr ratio throughout the differentiation phase. This could be partially explained by muscle creatine kinase, which only reaches maximal expression and activity after 4–5 days of differentiation in C₂C₁₂ myotubes (36, 40), allowing the cell to accumulate more PCr throughout the differentiation phase.

Since the experiments with mannitol, taurine, and β -alanine ruled out an osmolarity-dependent action of creatine, we further focused our work on the signaling enhancing growth and differentiation. The p38 pathway is potentially one major signaling cascade activated by creatine, since p38 is known to regulate the expression of muscle-specific genes and fusion of

myoblasts into myotubes (14). In the present study, creatine essentially affected the fusion of myoblasts (Fig. 1C) and the expression of muscle-specific proteins (Fig. 1, E and F, and Fig. 2C).

The phosphorylation state of p38 during the differentiation phase of C₂C₁₂ cells is known to follow a pattern similar to that presented in Fig. 3A in control conditions. The phosphorylation of p38 decreases at the beginning of the differentiation before increasing at the end (26). The addition of creatine to the culture medium anticipated the changes in the phosphorylation state of p38. Creatine also affected the nuclear expression of MEF-2 and MyoD (Fig. 4, C and D), two muscle-specific transcription factors whose expression and activation are known to be potentially controlled by p38 (16, 48). MyoD is a member of the myogenic basic helix-loop-helix (bHLH) proteins that forms heterodimers with a ubiquitous class of bHLH transcription factors called E proteins to bind a consensus DNA sequence referred to as an E-box in the control regions of muscle-specific genes (9). All myogenic regulatory factors

Fig. 6. Cross talk between p38 and Akt/PKB-p70^{S6K}. A: phosphorylation state of Akt/PKB on Ser473 and of p70^{S6K} on Thr389 after addition of 10 μ M SB 202190 (SB; inhibitor of p38) in control and creatine conditions at the beginning of the differentiation phase for 48 h. B: the total (tot) form of p38 was not affected by SB. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. control.

(MyoD, Myf5, MRF4, and myogenin) bind to an E-box, and our group (29) has previously shown that creatine potentiates the transcription of all the members of this family. MEF-2 is an essential coactivator of MyoD to initiate and control skeletal myogenesis. By binding to MyoD, MEF-2 increases the myogenic activity of the latter (8). Nearly all skeletal muscle genes possess a MEF-2 binding site in their control regions (9). These genes encode a wide variety of proteins, including GLUT-4 (27), troponin T (45), and MyoD itself (47), as well as other myogenic regulatory factors (7).

The ERK pathway has been shown to be involved in the hypertrophy of terminally differentiated myotubes (48), and creatine increased the phosphorylation state of ERK1/2 after 4 days of differentiation (Fig. 4B). Our results suggest that creatine accelerates the differentiation program via p38 and that ERK1/2 could potentially act at the very end of the differentiation to induce myotube hypertrophy.

Rapamycin completely inhibited cell differentiation, and creatine did not reverse this inhibition, indicating that mTOR activity is required for the effect of creatine. Therefore, involvement of the Akt/PKB-mTOR-p70^{S6K} pathway in creatine signaling was tested. Akt/PKB is an upstream kinase of mTOR-p70^{S6K} that plays a central role in the signal transduction pathways stimulated by growth factors and insulin. It mediates a wide range of cellular functions, including nutrient metabolism (13), cell growth, and apoptosis (10, 24, 32). The phosphorylation state of Akt/PKB increases throughout the differentiation and is potentiated by creatine (Fig. 5B). The first identified substrate for Akt/PKB is GSK-3. Creatine increases the phosphorylation state of GSK-3 at the end of the differentiation (Fig. 5C).

Akt/PKB is known to activate p70^{S6K} and inhibit 4E-BP1 through mTOR (33). In the present study, creatine potentiated

the increase in Akt/PKB activity at the end of the differentiation phase (Fig. 5, A and B), and a similar activation pattern was observed on p70^{S6K} (Fig. 5E). A possible cross talk with the p38 pathway could contribute to the higher phosphorylation state of Akt/PKB and p70^{S6K} at the end of the differentiation. Indeed, SB 202190, the inhibitor of p38, diminished the phosphorylation state of Akt/PKB and p70^{S6K} (Fig. 6), as already reported (14). Contrary to Akt/PKB and p70^{S6K}, the phosphorylation state of 4E-BP1 remained unchanged by creatine (Fig. 5D). Recent work has revealed that 4E-BP1 and p70^{S6K} are regulated by distinct signaling events downstream of mTOR (46) that may be regulated differently by creatine.

The involvement of IGF-I in creatine signaling, reported in a previous study (29), is probably minimal, because inhibition of IGF-I receptor by picropodophyllin had no effect (Fig. 3A), indicating that creatine recruits additional mediators to enhance differentiation and activate the p38 and Akt/PKB cascades. Since IGF-I transcription is regulated by MyoD (31), the expression of which is enhanced by creatine (Fig. 4D), the increase in IGF-I expression is probably a consequence of a nuclear accumulation in MyoD. By its autocrine action, IGF-I would then amplify creatine signaling through the MAPK and Akt/PKB pathway.

In summary, we have identified two major signaling cascades by which creatine promotes differentiation of C2C12 cells (Fig. 7). The first is the p38 MAPK pathway, which would in turn activate MEF-2, MyoD, and probably other unidentified transcription factors. Through this cascade, creatine would promote the fusion of the myoblasts and the expression of muscle-specific genes (MHC II, troponin T, and titin). At the end of the differentiation, creatine also increases the phosphorylation state of ERK1/2 MAPK, which is known to promote myotube growth (48). Through activation of the Akt/PKB pathway, creatine would increase protein synthesis. More specifically, the activation of p70^{S6K} could prime the machinery required to accelerate the translation of specific mRNA increased by creatine.

Creatine has often been studied for its beneficial effect on human muscle performance or as a treatment for patients, but only a few studies have tried to understand the mechanisms by which creatine leads to muscle hypertrophy or stimulates muscle regeneration. Putting in perspective the results of Olsen et al. (35) and the present findings, future research should be directed toward the highlighting of the mechanisms by which creatine stimulates satellite cells in vivo and the possible involvement of the p38 and Akt/PKB pathways.

Fig. 7. Hypothetical model for creatine signaling. PI3K, phosphatidylinositol 3-kinase. Solid lines represent the effect of creatine; dashed lines indicate no effect of creatine or an untested effect. See DISCUSSION for details.

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