

# Glucose induces de novo lipogenesis in rat muscle satellite cells through a sterol-regulatory-element-binding-protein-1c-dependent pathway

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## Summary

We previously reported that sterol-regulatory-element-binding-protein-1c (SREBP-1c) mediates insulin upregulation of genes encoding glycolytic and lipogenic enzymes in rat skeletal muscle. Here, we assessed whether glucose could regulate gene expression in contracting myotubes deriving from cultured muscle satellite cells. Glucose uptake increased twofold after a 30 minute treatment with a high glucose concentration, suggesting an acute glucose-stimulated glucose uptake. Time-course experiments showed that, within 3 hours, glucose stimulated the expression of hexokinase II, fatty acid synthase and acetyl-CoA-carboxylase-2 proteins, leading to an increased lipogenic flux and intracellular lipid accumulation in contracting myotubes. Furthermore, kinetic experiments indicated that glucose upregulated SREBP-1c precursor and nuclear proteins within 30

minutes, SREBP-1c nuclear translocation being confirmed using immunocytochemistry. In addition, the knockdown of *SREBP-1* mRNA using a RNA-interference technique totally abrogated the glucose-induced upregulation of lipogenic enzymes, indicating that SREBP-1c mediates the action of glucose on these genes in rat skeletal muscle. Finally, we found that glucose rapidly stimulated SREBP-1c maturation through a Jak/STAT dependent pathway. We propose that increased intramuscular lipid accumulation associated with muscle insulin resistance in obesity or type-2 diabetes could arise partly from de novo fatty acid synthesis in skeletal muscle.

Key words: Muscle satellite cells, Glucose, Srebp-1 knockdown, Gene expression, Lipogenesis

## Introduction

Sterol-regulatory-element-binding proteins (SREBPs) regulate the transcription of genes involved in cholesterol and fatty acid metabolism (Brown and Goldstein, 1997). Three members of the SREBP family have been described in mammalian species. SREBP-1a and SREBP-1c are encoded by a single gene and produced through alternative splicing and the use of alternative promoters (Hua et al., 1995), whereas SREBP-2 derives from a different gene. SREBPs are synthesized as precursor forms anchored in endoplasmic reticulum and nuclear membranes. After proteolytic cleavage, a transcriptionally active mature form enters the nucleus, where it can bind both sterol regulatory elements and E-boxes (Kim et al., 1995). SREBP-2 cleavage occurs in response to low sterol levels, whereas factors triggering the cleavage of SREBP-1c remain unknown. In the liver, insulin upregulates SREBP-1c expression, and SREBP-1c has been proposed to mediate the transcriptional effects of insulin on lipogenic and glycolytic enzymes, such as fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), Spot 14 or pyruvate kinase (L-PK) (Foufelle and Ferre, 2002). The effects of insulin on SREBP-1c have been corroborated by in vivo studies showing that SREBP-1c expression and nuclear abundance were low in the liver of streptozotocin-induced diabetic rats, and markedly increased after insulin treatment (Shimomura et al., 1999).

A regulation of SREBP-1c expression by insulin has also been reported in other insulin-responsive tissues. In mouse adipose tissue, the expression of SREBP-1c and its nuclear abundance are positively controlled by insulin, and SREBP-1c mediates the effect of insulin on the *FAS* gene (Kim et al., 1998). In skeletal muscle, SREBP-1c transcript predominates over SREBP-1a in human and rodent species (Ducluzeau et al., 2001; Guillet-Deniau et al., 2002). Its expression in skeletal muscle was reported to be regulated by the nutritional status (Bizeau et al., 2003) and dramatically decreased in streptozotocin-induced diabetic animals (Guillet-Deniau et al., 2002). Using primary cultures of muscle satellite cells, which form spontaneously contracting myotubes within 10 days, we have shown that insulin upregulated the expression of hexokinase II (HKII), FAS and ATP-citrate lyase through SREBP-1c, these effects being mimicked by adenovirus-mediated expression of a transcriptionally active form of SREBP-1c (Guillet-Deniau et al., 2002). However, several glycolytic and lipogenic genes in the liver require both insulin and glucose to be expressed, insulin by itself failing to induce expression in the absence of glucose. In fact, in vivo and in vitro experiments have demonstrated that glucose can upregulate ACC, FAS and stearoyl-CoA desaturase expression in adipose tissue (Foufelle et al., 1992; Jones et al., 1998), and

L-PK, FAS and ACC expression in both liver (Decaux et al., 1989; Prip-Buus et al., 1995) and pancreatic  $\beta$ -cell lines (Andreolas et al., 2002; Brun et al., 1993; Marie et al., 1993; Roche et al., 1998). Although glycolytic and lipogenic genes are expressed in skeletal muscle, their regulation by glucose is poorly understood.

In the present study, we demonstrate, using contracting myotubes deriving from rat muscle satellite cells, that glucose alone stimulates more rapidly than insulin the expression and maturation of SREBP-1c as well as lipogenic gene expression, leading to an increased lipogenic flux and intracellular lipid accumulation. Moreover, successful knockdown of SREBP-1 by small interfering RNAs (siRNAs) totally abolished the glucose-induced upregulation of lipogenic enzymes, suggesting that glucose-induced SREBP-1c expression and maturation could be implicated in muscle lipotoxicity.

## Materials and Methods

### Animals

Eight-week-old Sprague Dawley male rats fed ad libitum with a standard laboratory chow diet were housed at 22°C with light from 7:00 am to 7:00 pm. All procedures were performed in accordance with the guidelines established by the European Convention for the Protection of Laboratory Animals.

### Primary culture of muscle satellite cells

Satellite cells from rat hindlimb muscle fibres were prepared and cultured as previously described (Guillet-Deniau et al., 1994). Cells were plated onto growth-factor-reduced Matrigel™-coated flasks (BD-Biosciences) in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) horse serum, 5 mM glucose, antibiotics and 2 mM glutamine. Cells were fed fresh medium the day after plating and every other day. At day 6, the medium was replaced by a differentiation medium (2% horse serum) and myoblasts differentiated into spontaneously contracting myotubes within 3 days, then serum was totally removed.

### Glucose uptake

The uptake assay was performed as previously described (Guillet-Deniau et al., 1994). Cells were grown on 13 mm Matrigel-coated Thermanox coverslips (Nunc) placed in 24-well plates. Coverslips were washed three times in warm PBS and then incubated at 37°C for 30 minutes in 1 ml Krebs-Ringer phosphate buffer (KRP) (130 mM NaCl, 5 mM KCl, 1.3 mM CaCl<sub>2</sub>, 1.3 mM MgSO<sub>4</sub>, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4) supplemented with either glucose (5 mM or 25 mM) or mannitol (25 mM). Myotubes were rinsed and 10 mM 2-deoxy-D-glucose (2-DG) containing 3  $\mu$ Ci ml<sup>-1</sup> 2-deoxy-D-[1-<sup>3</sup>H]glucose (13.9 Ci mmole<sup>-1</sup>; Amersham Pharmacia Biotech) was added. Uptake was measured for 15 minutes at 37°C, then coverslips were rinsed three times in cold PBS, immediately immersed in scintillant liquid and counted. Six coverslips were used for each experimental condition. Proteins were measured using the Bradford method (Bradford et al., 1976).

### Preparation of total cell lysates, cytoplasmic and nuclear protein extracts

Cells were rinsed three times with cold PBS, immediately frozen in liquid nitrogen, scraped on ice in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM Na<sub>3</sub>P<sub>2</sub>O<sub>7</sub>, 1% Triton X-100, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM PMSF, 1  $\mu$ g ml<sup>-1</sup> pepstatin A, 2  $\mu$ g ml<sup>-1</sup> leupeptin, 5  $\mu$ g ml<sup>-1</sup> aprotinin) and lysed for 2 hours at 4°C

under rotational agitation. Total cell lysates were recovered after centrifugation at 16,000 *g* for 30 minutes at 4°C. Cytoplasmic and nuclear protein extracts were prepared using the NE-PER™ Nuclear and Cytoplasmic Extraction Reagents kit (Pierce) according to the supplier's instructions. Protein concentration was determined using the Bradford method.

### Western blot analysis

Proteins (30  $\mu$ g) were separated by sodium-dodecyl-sulphate polyacrylamide-gel electrophoresis (SDS-PAGE) in a 7% gel and electrotransferred onto nitrocellulose membrane. Membranes were blotted with the relevant antibody and then revealed using the enhanced chemiluminescence system (Supersignal; Pierce). Signals were scanned and quantified using a ChemiGenius Apparatus (Syngene).

### Antibodies

The precursor and mature forms of SREBP-1c were detected using a monoclonal antibody raised against the N-terminus (amino acids 301-407) of human SREBP-1 (NeoMarkers). The anti-human-HKII antibody was purchased from Santa Cruz Biotechnology. Anti-rat-FAS and -CPT2 polyclonal antibodies were gifts from I. Dugail (Unité INSERM 465, Paris, France) and J. D. McGarry (University of Southwestern Medical Center, Dallas, USA), respectively. ACC2 and Ser<sup>79</sup> phospho-ACC polyclonal antibodies were from Alpha Diagnostic International. Active mitogen-activated-protein kinase (pTEpY) was detected using a polyclonal antibody (Promega). Polyclonal antibodies against phospho-STAT3 (Tyr<sup>705</sup> or Ser<sup>727</sup>) and phospho-STAT1 (Tyr<sup>701</sup>) were purchased from Upstate Biotechnology.

### Lipogenesis and intracellular lipid accumulation

Contracting myotubes were cultured for 24 hours in a serum-free medium containing 5 mM glucose in the absence or presence of 100 nM insulin. Lipogenesis rate from [2-<sup>14</sup>C]acetate (5 mM, 10  $\mu$ Ci per flask; Amersham Pharmacia Biotech) was determined in duplicate in the absence or presence of 25 mM glucose and 5  $\mu$ M 5-tetradecyloxy-2-furoic acid (TOFA) during the last 3 hours of culture. Cells were rinsed twice with ice-cold PBS, immediately frozen in liquid nitrogen and then scrapped off in 30% KOH. Labelled fatty acids were extracted as described by Stansbie et al. (Stansbie et al., 1976). Lipid accumulation was detected in myotubes using Oil Red-O staining following fixation in 3% paraformaldehyde according to Koopman et al. (Koopman et al., 2001). After counterstaining of nuclei with DAPI (Molecular Probes), fluorescence of Oil Red-O was observed as described below.

### Localization of SREBP-1c by immunocytochemistry

Muscle satellite cells were cultured on Matrigel-coated Permanox four-chamber slides (Lab-Tek, Nunc) for 11 days (contracting myotubes). Cells were incubated for 0 minutes, 15 minutes, 30 minutes or 60 minutes in the presence of 25 mM glucose, immediately washed three times with cold PBS and fixed with 3% paraformaldehyde for 20 minutes. After an extensive PBS wash and quenching for 10 minutes with 50 mM NH<sub>4</sub>Cl, cells were permeabilized with 0.1% Triton X-100 in PBS for 4 minutes. Myotubes were incubated overnight at 4°C with the anti-SREBP-1 antibody, washed three times with PBS and then treated for 1 hour with Alexa-488-conjugated goat-anti-mouse immunoglobulin G (Molecular Probes). Nuclei were counterstained with DAPI (Molecular Probes). Slides were mounted in Cityfluor mounting medium (BioRad) and pictures were taken using a Nikon TS100 fluorescence microscope equipped with a CF1 APO PLAN 60 $\times$ /1.40 oil objective and a Coolpix digital camera (Nikon, Tokyo, Japan).

### siRNA preparation and transfection of myotubes

Target-specific siRNA duplex was designed by selecting sequences of the type AAN<sub>19</sub>UU from the open reading frame of *Rattus* sp. *ADD1* mRNA (GenBank accession number L16995) (Tontonoz et al., 1993) in order to obtain a 21-nucleotide sense and a 21-nucleotide antisense strand with symmetric 2-nucleotide 3' overhangs of identical sequence. The siRNA targeting SREBP-1 was from position +455 to +476 relative to the start codon. The selected siRNA sequence was submitted to a BLAST search against the rat genome sequence to ensure that only SREBP-1 was targeted. Double-stranded 21-nucleotide siRNA was purchased from Dharmacon (Lafayette, CO) in deprotected and desalted form. Contracting myotubes were cultured in six-well plates in DMEM without serum, and the medium was changed to antibiotic-free medium the day before transfection. Transfection of myotubes with pEGFP-C3 plasmid (Clontech, Palo Alto, CA) encoding green fluorescent protein (GFP) indicated that over 70% of the cells were transfected. siRNAs (50 pmol per well) were transfected using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Experiments were performed 48 hours after transfection of the cells.

### Statistical analysis

Results are expressed as means $\pm$ s.e.m. Statistical significance was evaluated using analysis of variance.

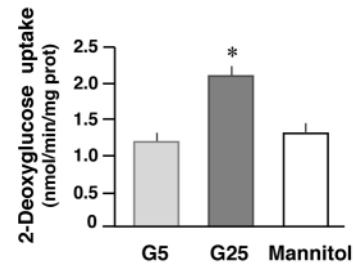
## Results

### Glucose increases glucose uptake in contracting myotubes

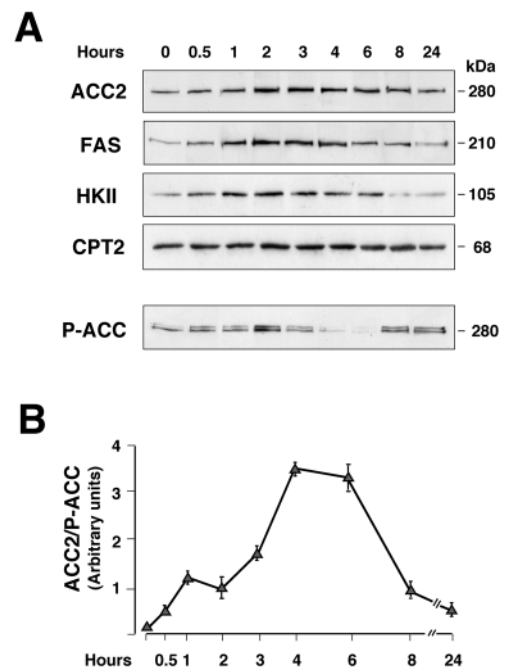
We first assessed the effect of glucose concentration on glucose uptake by incubating myotubes for 30 minutes with 25 mM glucose in absence of insulin. This resulted in a twofold increase in 2-DG uptake, whereas mannitol was inefficient, ruling out an osmotic effect of glucose (Fig. 1). Thus, high glucose concentration induced in contracting myotubes a rapid increase in glucose uptake.

### Time-course effects of glucose on the expression of lipogenic and glycolytic enzymes

ACC catalyses the carboxylation of acetyl-CoA to malonyl-CoA, a major precursor for de novo fatty acid synthesis (Hardie, 1989). Two ACC isoforms with molecular masses of 265 kDa (ACC1) and 280 kDa (ACC2) were described, the latter being dominant in heart and skeletal muscle. To determine the effects of glucose on gene expression, contracting myotubes were treated with 25 mM glucose for times ranging from 30 minutes to 24 hours. Within 3 hours, glucose increased three- to fourfold ACC2, FAS and HKII protein levels, whereas the mitochondrial carnitine palmitoyltransferase 2 (CPT2) remained constant (Fig. 2A). Phosphorylation of ACC by cAMP-dependent protein kinase or AMP kinase resulted in the inactivation of the enzyme (Munday et al., 1988). The phosphorylation state of ACC was assessed using a specific antibody raised against the Ser<sup>79</sup> phospho-ACC. After a transient increase within the first 2 hours of glucose challenge, ACC phosphorylation decreased to an undetectable level between 4 hours and 6 hours (Fig. 2A). From these time-course experiments, we evaluated the activity of ACC2 through the ACC2/P-ACC ratio. This ratio showed a drastic increase in response to glucose, corresponding to an equivalent rise in the active form of ACC2 (Fig. 2B). These



**Fig. 1.** Glucose increases glucose uptake in contracting myotubes. After 48 hours in serum-free medium containing 5 mM glucose, 2-deoxyglucose (2-DG) uptake was measured after a 30 minute incubation of the cells with 5 mM glucose (G5), 25 mM glucose (G25) or 25 mM mannitol. Results are the means $\pm$ s.e.m. of 10-13 independent experiments. \* $P$ <0.05 versus G5.

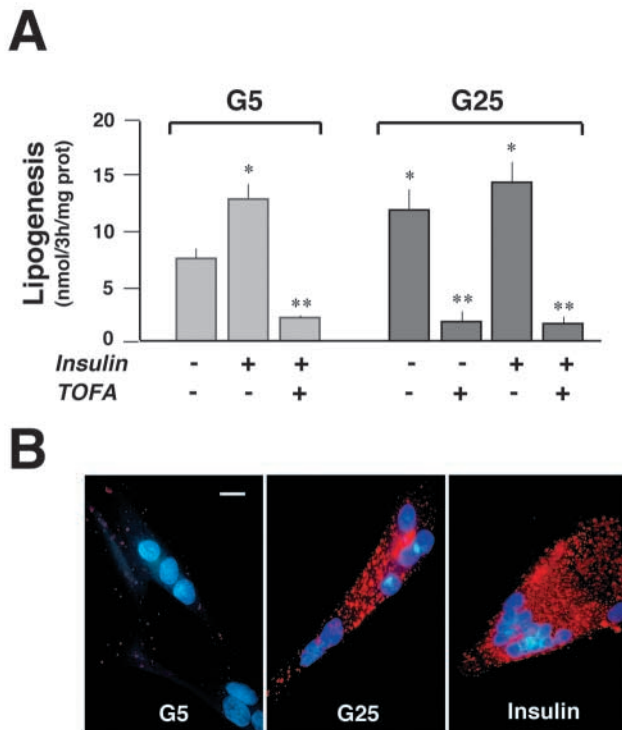


**Fig. 2.** Time course of the effect of 25 mM glucose on lipogenic enzymes in myotubes. After 48 hours in serum-free medium containing 5 mM glucose, contracting myotubes were incubated for the indicated times with 25 mM glucose. Total protein content was then extracted, separated by SDS-PAGE and analysed for ACC2, FAS, HKII and CPT2 protein levels, and for the phosphorylation state of ACC protein (A). (B) ACC activity, deduced from the ratio between ACC2 and phosphorylated ACC protein level. Western blots represent three independent experiments.

results revealed that glucose rapidly upregulated ACC, FAS and HKII enzymes in rat skeletal muscle cells.

### Glucose stimulates lipogenesis in contracting myotubes

Consistent with the stimulation of ACC and FAS expression, 25 mM glucose increased de novo fatty acid synthesis within 3 hours at a rate similar to that observed after a 24-hour treatment with 100 nM insulin (Fig. 3A). The glucose-stimulated lipogenesis was not significantly potentiated by insulin. Both basal and stimulated lipogenesis were inhibited



**Fig. 3.** Glucose induces de novo lipogenesis and lipid accumulation in contracting myotubes. (A) After 48 hours in serum-free medium containing 5 mM glucose (G5), cells were treated or not with 100 nM insulin for the last 24 hours. The lipogenesis rate from [2-<sup>14</sup>C]acetate (5 mM) was measured in the presence or absence of 25 mM glucose (G25) and/or 5 μM TOFA during the last 3 hours of culture. Results are the means±s.e.m. of 3-13 independent experiments. \**P*<0.05 compared with G5; \*\**P*<0.01 compared with the respective controls. (B) After a 24-hour treatment with 100 nM insulin or 25 mM glucose (G25), myotubes were stained with Oil Red-O to detect the presence of intracellular lipid droplets. Nuclei were counterstained with DAPI. Scale bar, 15 μm.

by 90% by TOFA, a specific inhibitor of ACC (Panek et al., 1977). Moreover, intracellular lipid accumulation was detected following a 24-hour treatment with either 25 mM glucose or insulin, whereas very few lipid droplets could be detected in myotubes cultured under basal conditions (Fig. 3B). Taken together, these results suggest that accumulation of intracellular lipids resulted from increased de novo lipogenesis in contracting myotubes.

#### SREBP-1c expression and processing are regulated by glucose

We have previously shown that insulin increased both the precursor and mature forms of SREBP-1c within 3 hours in contracting myotubes, leading to the upregulation of FAS, ATP-citrate lyase and HKII proteins (Guillet-Deniau et al., 2002). We then tested whether the glucose-induced upregulation of lipogenic enzymes could also be mediated by SREBP-1c. Indeed, the precursor form of SREBP-1c was enhanced two- to threefold after a 30-minute exposure to 25 mM glucose, concomitant with a threefold increase in the nuclear mature form (Fig. 4A), whereas mannitol (25 mM) and

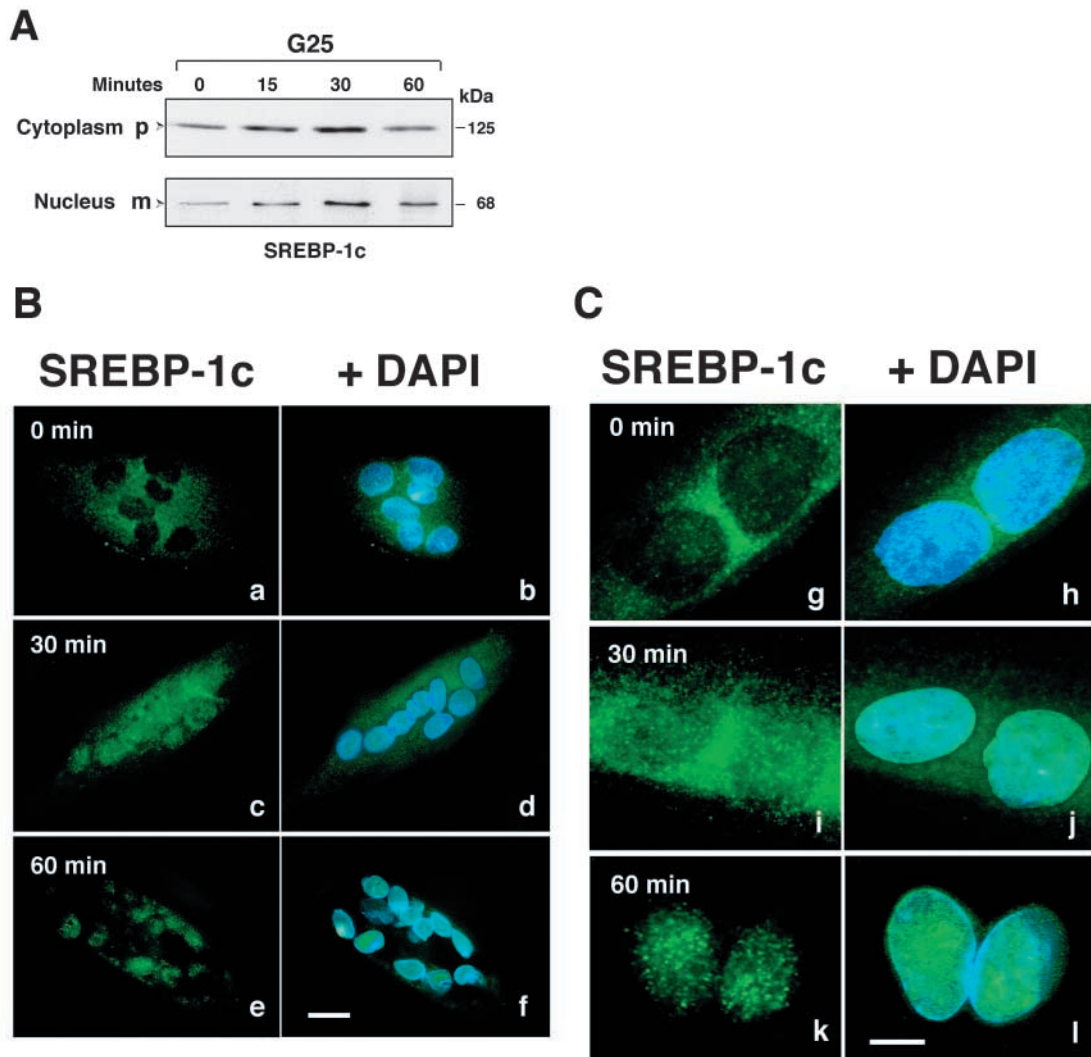
2-DG (10 mM) had no effect (not shown). However, the glucose-induced increase in the precursor and mature forms of SREBP-1c was not consecutive to an upregulation of *SREBP-1* gene expression because no variation in *SREBP-1* mRNA level was observed within 2 hours of the application of a high glucose concentration (not shown). The nuclear translocation of SREBP-1c detected by immunofluorescence staining is shown in Fig. 4B,C. In absence of 25 mM glucose, SREBP-1c was mainly observed in the cytoplasm. 30 minutes after the addition of glucose, SREBP-1c was detected into the nucleus, and it remained nuclear for at least 60 minutes (Fig. 4C). Thus, glucose rapidly increased the precursor form of SREBP-1c and the nuclear translocation of the mature form.

#### Silencing of SREBP-1 in contracting myotubes

In order to confirm the role of SREBP-1c, myotubes were transfected with a SREBP-1 siRNA duplex, and the effects of glucose on the expression of lipogenic enzymes were assessed 48 hours later (Fig. 5). *SREBP-1* mRNA (not shown) and protein (Fig. 5) were hardly detectable in siRNA-transfected myotubes compared with control cells, indicating an efficient knockdown of *SREBP-1* expression. Under these conditions, the basal protein levels of FAS and ACC2 were also decreased, whereas CPT2 expression was not affected (Fig. 5). Furthermore, a high glucose concentration failed to upregulate SREBP-1c, FAS and ACC2 proteins upon silencing of SREBP-1. These results demonstrate that glucose-induced upregulation of lipogenic enzymes was mediated by SREBP-1c in contracting myotubes.

#### SREBP-1c processing by glucose is dependent on phosphorylation of STAT3

STAT proteins are latent cytoplasmic-signal-dependent transcription factors that are phosphorylated by Janus kinase (Jak) proteins. Through the Jak/STAT pathway, extracellular molecules bound to cell-surface receptors can change nuclear gene expression patterns within minutes (Levy and Darnell, 2002). We have already shown that glucose uptake could be enhanced through a Jak2/STAT3 pathway in contracting myotubes (Guillet-Deniau et al., 1997). Therefore, we determined whether the Jak/STAT pathway was involved in the glucose-induced upregulation of SREBP-1c expression. After 15 minutes of glucose challenge, cytoplasmic and nuclear STAT3 were phosphorylated on Tyr<sup>705</sup>, nuclear STAT3 phosphorylation increasing up to 3 hours (Fig. 6A). By contrast, no Ser<sup>727</sup> phosphorylation of STAT3 (not shown) or STAT1 phosphorylation (Fig. 6A) occurred. Furthermore, the phosphorylation state of Erk1/Erk2 proteins remained unaffected by glucose (Fig. 6A). When myotubes were incubated with 25 mM glucose in the presence of AG490, a specific inhibitor of Jak2 phosphorylation, no phosphorylation of STAT3 occurred and nuclear SREBP-1c was barely detectable, although SREBP-1c precursor was still increased (Fig. 6B). In contrast to AG490, PD98059 (a specific inhibitor of MEK phosphorylation) had no inhibitory effect on the glucose-induced processing of SREBP-1c (Fig. 6B). Taken together, these results show that glucose induced the processing of SREBP-1c through the Jak2/STAT3 pathway.



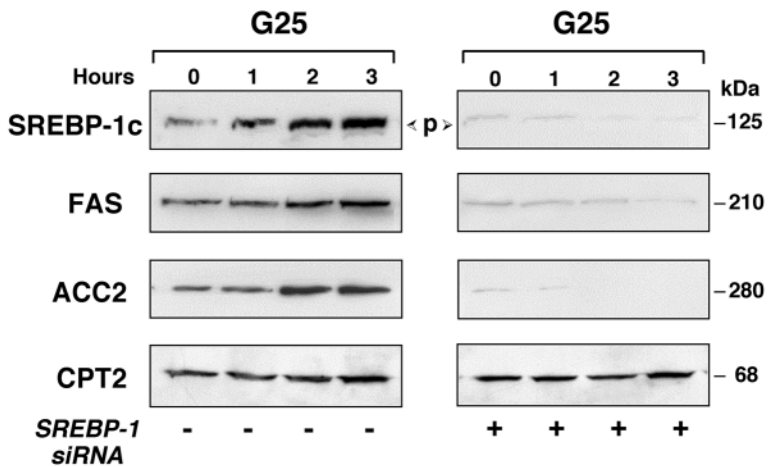
**Fig. 4.** Glucose induces the processing of SREBP-1c precursor (p) and the nuclear translocation of the mature form (m). Contracting myotubes were cultured in 5 mM glucose without serum for 48 hours. (A) Cytoplasmic (top) and nuclear (bottom) extracts were prepared at 0 minutes, 15 minutes, 30 minutes or 60 minutes after the addition of 25 mM glucose. The western blots represent three separate experiments. (B,C) Detection of the precursor and nuclear forms of SREBP-1c by immunofluorescence in contracting myotubes. In absence of 25 mM glucose, SREBP-1c precursor (green) was observed in the cytoplasm (a,b,g,h). Following the addition of 25 mM glucose, nuclear translocation of SREBP-1c was detected in myotubes after 30 minutes (c,d,i,j) and remained up to 60 minutes (e,f,k,l). Nuclei were counterstained with DAPI. Scale bars, 20  $\mu$ m (B), 5  $\mu$ m (C).

## Discussion

The present study provides the first demonstration that glucose, in the absence of insulin, regulates both the expression and processing of SREBP-1c protein in skeletal muscle. This is in contrast to what occurs in liver, where insulin, but not glucose, induced SREBP-1c expression (Foretz et al., 1999). Furthermore, glucose effects occurred rapidly in muscle cells compared with those induced by insulin (30 minutes versus 3 hours) (Guillet-Deniau et al., 2002). In pancreatic  $\beta$ -cell lines, a stimulation of *SREBP-1c* expression by glucose has also been reported (Andreolas et al., 2002; Wang et al., 2003). However, this required a long exposure to glucose (6–48 hours) and SREBP-1c processing in  $\beta$ -cells was unresponsive to acute glucose stimulation (Wang et al., 2003), unlike what we report here in myotubes.

The early response made by contracting myotubes to a high

glucose concentration was an acute increase in glucose uptake, suggesting that increased glucose uptake is a prerequisite for glucose action on SREBP-1c. In liver, glucose must be metabolized in order to generate a signal metabolite that mediates its hormone-independent transcriptional effects (Decaux et al., 1989; Prip-Buus et al., 1995). Recently, the carbohydrate-responsive-element-binding protein (ChREBP) cloned in rat liver (Yamashita et al., 2001) and xylulose-5-phosphate (Kabashima et al., 2003) were proposed to be a glucose-responsive transcription factor and a signal metabolite responsible for L-PK gene regulation in liver, respectively. The suggested scenario is that glucose activates ChREBP through dephosphorylation catalysed by xylulose-5-phosphate-activated protein phosphatases (Kabashima et al., 2003). Such a glucose signal metabolite is likely to exist in skeletal muscle but remains to be identified. By contrast, ChREBP is unlikely



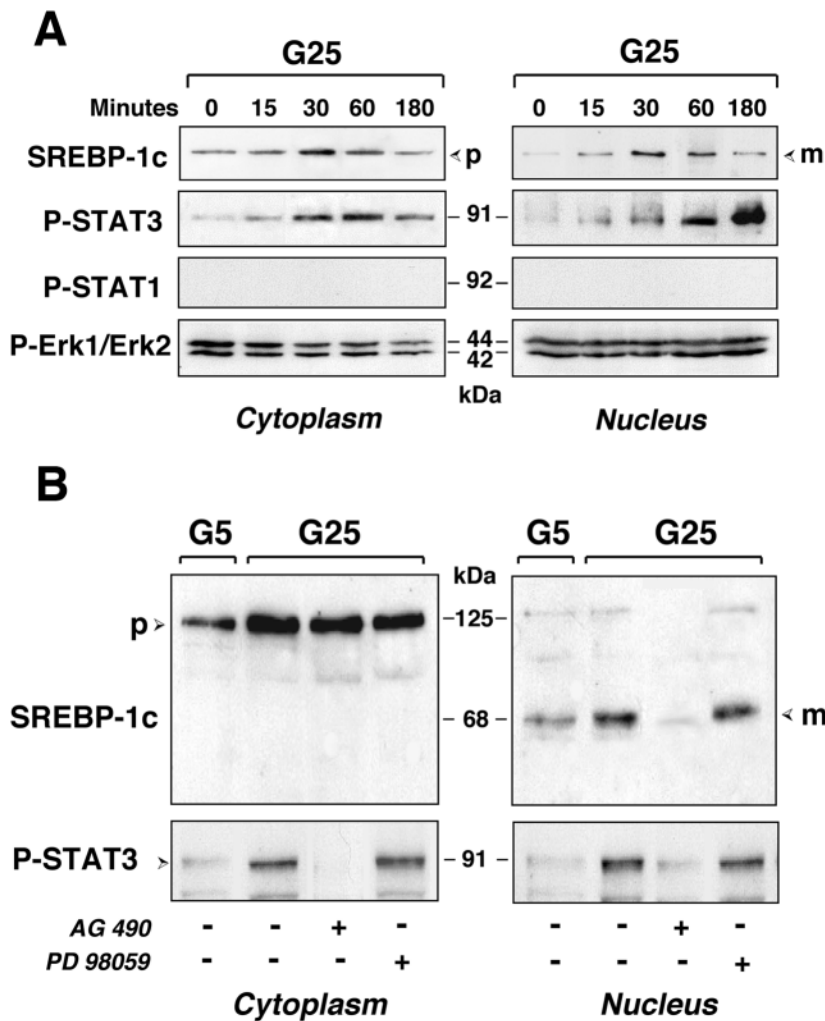
**Fig. 5.** Silencing of SREBP-1 protein in contracting myotubes. After 48 hours in serum-free medium containing 5 mM glucose, myotubes were transfected with a *SREBP-1* siRNA duplex. After 48 hours, total protein extracts were prepared at 0 hours, 1 hour, 2 hours or 3 hours after the addition of 25 mM glucose (G25). Western blotting was performed to detect SREBP-1c precursor (p), FAS, ACC2 and CPT2 proteins. Representative western blots of three independent experiments are shown.

to be the mediator of glucose effects in contracting myotubes because its expression was not detected in skeletal muscle (Yamashita et al., 2001). Nevertheless, the existence of an unidentified ChREBP isoform in skeletal muscle cannot be ruled out.

The present study allows new insights into the signalling pathway involved in the regulation of SREBP-1c expression

and processing in skeletal muscle. In liver, the effects of insulin on SREBP-1c expression and synthesis involved mainly the phosphatidylinositol-3-kinase pathway (Azzout-Marniche et al., 2000; Matsumoto et al., 2002) but the identity of the downstream effector is controversial. In our study, glucose-induced maturation of SREBP-1c was prevented by neither wortmannin, an inhibitor of phosphatidylinositol-3-kinase (I. Guillet-Deniau, unpublished), nor by PD98059, an inhibitor of the mitogen-activated-protein-kinase (MAPK) pathway, confirming that these pathways are not involved. Conversely, processing of SREBP-1c by glucose in myotubes specifically required Tyr<sup>705</sup> phosphorylation of STAT3. This result strengthens previous evidence that glucose can stimulate the Jak/STAT pathway.

Indeed, in vascular smooth muscle cells (VSMCs), a high glucose concentration has been shown to potentiate angiotensin II induction of VSMC proliferation by increasing signal transduction through the Jak/STAT pathway (Amiri et al., 1999). More recently, in glomerular mesangial cells, Wang et al. provided direct evidence for linkages between Jak2/STAT1 and the glucose-induced overproduction of transforming growth factor  $\beta$  as well as fibronectin, two factors involved in the mechanism of diabetic nephropathy (Wang



**Fig. 6.** Processing of SREBP-1c by glucose depends on the phosphorylation of STAT3. Contracting myotubes were cultured for 48 hours in 5 mM glucose without serum. (A) Cytoplasmic and nuclear extracts were prepared at 0 minutes, 15 minutes, 30 minutes, 60 minutes and 180 minutes after the addition of 25 mM glucose (G25). The western blots were probed using antibodies against SREBP-1c precursor (p) and mature (m) forms, Tyr<sup>705</sup>-phosphorylated STAT3, Tyr<sup>701</sup>-phosphorylated STAT1 or phosphorylated Erk1/Erk2 proteins. (B) Contracting myotubes were incubated in 5 mM (G5) or 25 mM (G25) glucose for 30 minutes in the absence or presence of AG490 (a specific inhibitor of Jak2 phosphorylation) or PD98059 (an inhibitor of MAPK phosphorylation). Cytoplasmic and nuclear extracts were prepared, analysed by SDS-PAGE and immunoblotted using antibodies against SREBP-1c and phosphorylated-STAT3. Western blots are representative of three separate experiments.

et al., 2002). Nevertheless, the glucose effects observed in both studies were long-term effects (24-48 hours) compared with the findings reported here (30 minutes). However, insulin and an osmotic shock have both been reported to activate STAT3 on Ser<sup>727</sup> through a MEK-dependent pathway in CHO/IR cells, whereas no tyrosine phosphorylation occurred (Ceresa et al., 1997). In our study, the glucose-induced processing of SREBP-1c was prevented by an inhibitor of Jak2 phosphorylation but not by PD98059, confirming that glucose-induced tyrosine phosphorylation of STAT3 is independent of the MAPK pathway in myotubes. Thus, glucose regulates SREBP-1c expression in skeletal muscle cells through a pathway different from the one described for insulin in liver. The mechanisms by which glucose, or a signal metabolite, stimulates the Jak/STAT pathway remain to be elucidated. There might be an interaction between Jak2 and reactive oxygen species (ROS) that are induced by high glucose (Nishikawa et al., 2000), because ROS stimulate the activity of Jak2 in both fibroblasts and A-432 cells (Simon et al., 1998). Furthermore, recent studies in VSMCs suggested that high glucose, via the polyol pathway, induced a rapid increase in intracellular ROS through the activation of PKC, leading to NADPH oxidase activation and then ROS production (Shaw et al., 2003). These ROS could act as signal transducers via protein-tyrosine phosphatases in the activation of Jak2 (Simon et al., 1998) and could be responsible for the effects we observed in muscle cells under high glucose conditions.

Finally, increased expression of SREBP-1c by glucose was associated in contracting myotubes with upregulation of lipogenic enzymes, enhanced lipogenic flux and intracellular lipid accumulation, as observed in  $\beta$ -cell lines (Brun et al., 1993; Roche et al., 1998; Wang et al., 2003). The direct implication of SREBP-1c was examined by performing SREBP-1 knockdown using RNA interference. Indeed, our primary culture of satellite cells is an excellent model for silencing the expression of specific genes expressed in terminally differentiated muscle cells. We successfully transfected contracting myotubes with a synthetic siRNA directed against *SREBP-1* mRNAs. Owing to a large common sequence between SREBP-1a and SREBP-1c, we could not design a specific *SREBP-1c* siRNA. Nevertheless, SREBP-1 knockdown clearly demonstrated that this transcription factor directly mediated the glucose upregulation of lipogenic enzymes in skeletal muscle. This also emphasizes that gene silencing can be used for functional analysis of muscle gene products within the context of a biologically relevant muscle cell culture model.

Excessive deposition of lipids in non-adipose tissues contributes in humans to the aetiology of lipotoxic disorders including dyslipidaemia, insulin resistance, type-2 diabetes and heart disease, together referred as 'metabolic syndrome X' (Unger and Orci, 2001). In genetically obese diabetic animals, lipotoxicity is accompanied by an increase in the SREBP-1c mature form in liver and pancreas (Kakuma et al., 2000). It remains to be explored whether there are alterations in the regulation of SREBP-1c expression or maturation in skeletal muscle and heart in obese diabetic animals and type-2 diabetic humans. Investigation of SREBP-1c regulation by glucose or insulin should provide new insights into muscle insulin resistance.

In conclusion, the present study suggests that glucose-

induced increase in SREBP-1c expression and maturation might play an important role in muscle lipotoxicity by promoting de novo lipogenesis. This opens new possibilities for unravelling the cellular mechanisms involved in ectopic lipid deposition in skeletal muscle during obesity and type-2 diabetes.

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