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High glucose induces differentiation and adipogenesis in porcine muscle satellite cells via mTOR

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The present study investigated whether the mammalian target of rapamycin (mTOR) signal pathway is involved in the regulation of high glucose-induced intramuscular adipogenesis in porcine muscle satellite cells. High glucose (25 mM) dramatically increased intracellular lipid accumulation in cells during the 10-day adipogenic differentiation period. The expressions of CCAAT/enhancer binding protein-α (C/EBP-α) and fatty acid synthase (FAS) protein were gradually enhanced during the 10-day duration while mTOR phosphorylation and sterol-regulatory-element-binding protein (SREBP)-1c protein were induced on day 4. Moreover, inhibition of mTOR activity by rapamycin resulted in a reduction of SREBP-1c protein expression and adipogenesis in cells. Collectively, our findings suggest that the adipogenic differentiation of porcine muscle satellite cells and a succeeding extensive adipogenesis, which is triggered by high glucose, is initiated by the mTOR signal pathway through the activation of SREBP-1c protein. This process is previously uncharacterized and suggests a cellular mechanism may be involved in ectopic lipid deposition in skeletal muscle during type 2 diabetes. [BMB reports 2010; 43(2): 140-145]

INTRODUCTION

Muscle satellite cells provide a good *in vitro* model for the study of intramuscular adipogenesis because they have myogenic, osteogenic and adipogenic differentiation ability (1). Studies have demonstrated that muscle satellite cells can differentiate into adipocytes following treatments with adipogenic inducers (2, 3).

High intramuscular fat stores and the replacement of myofibers by adipose tissue are strongly correlated with type 2 diabetes in humans (4). High concentration of plasma glucose is another feature of type 2 diabetes (5). High glucose level has been shown to induce adipogenic differentiation in contracting myotubes deriving from rat muscle satellite cells (6, 7) and SREBP-1c plays an important role in this process (6). However, it is unclear how the process of adipogenic differentiation of satellite cells is regulated.

SREBPs including SREBP-1a, SREBP-1c and SREBP-2 regulate the expression of genes involved in lipid and cholesterol metabolism (8-11). In particular, SREBP-1c regulates the transcriptional effects of insulin on lipogenic enzymes such as fatty acid synthase (12). A recent study indicated that SREBP-1c activation and lipogenesis in retinol pigmented epithelial cells required the activity of mammalian target of rapamycin (mTOR) in the presence of glucose (13), and lipid biosynthesis in HEK293T cells also needs to activate mTOR pathway (14). mTOR is a rapamycin-sensitive kinase and its downstream target genes promote protein synthesis through increased translation and elongation (15, 16). Therefore, the mTOR activation, functioning through the activation of SREBP-1c protein, may be required for the regulation of differentiation and lipogenesis in muscle satellite cells induced by high glucose. Pigs are physiologically very similar to human and have been proposed to be one the best models for studies human cardiovascular and metabolic diseases (17). The objective is to test the role of mTOR signal pathway in intramuscular adipogenesis of porcine muscle satellite cells induced by high glucose levels.

RESULTS

The time-course of the effect of high glucose on mRNA expression for adipocyte-specific genes in porcine skeletal muscle satellite cells is shown in Fig. 1. SREBP-1c mRNA expression was low on day 0 but expression increased significantly by day 2. For the remainder of the 10-day differentiation period, SREBP-1c mRNA levels remained fairly constant and no further increases were observed. Similarly, the expression of C/EBP-α mRNA was also induced by day 2 and remained consistent afterwards. Expression of the adipocyte-specific gene, fatty acid synthetase, was at its maximum by day 4 and no change was observed after day 4.

In agreement with the mRNA results, the abundance of both C/EBP-α and fatty acid synthetase were increased during differ-

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entiation (Fig. 2). C/EBP-α protein expression enhanced from day 2 to 4, and remained consistent afterwards. The abundance of fatty acid synthetase protein increased after 2 days of glucose induce, reached maximum at day 6 and then returned datum line gradually by day 8 and day 10. SREBP-1c protein was not detectable on day 0 and day 2, but was highly abundant on day 4 and then decreased below detection limits by day 8. The mTOR was only marginally phosphorylated on day 0 and day 2, but highly phosphorylated on day 4 and was undetectable afterwards, following a similar pattern with SREBP-1c protein.

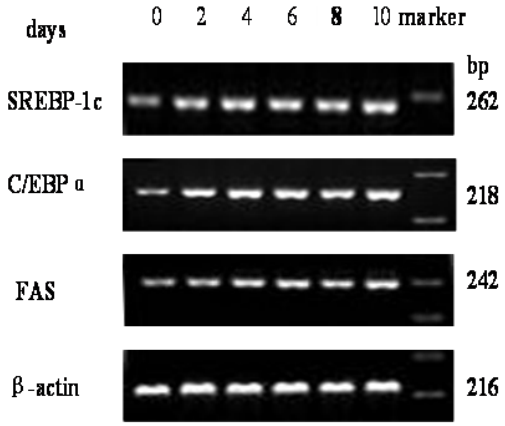

Fig. 1. Effect of 25 mM glucose on the relative expression of genes during the process of adipogenic differentiation of porcine skeletal muscle satellite cells. Total RNA was isolated and analyzed for sterol-regulatory-element-binding protein (SREBP)-1c, CCAAT/enhancer binding protein-α (C/EBP-α) and fatty acid synthetase (FAS) mRNA levels by RT-PCR. β-actin was used as the internal control. Marker: 2,000 bp DNA ladder (Takara, Shiga, Japan).

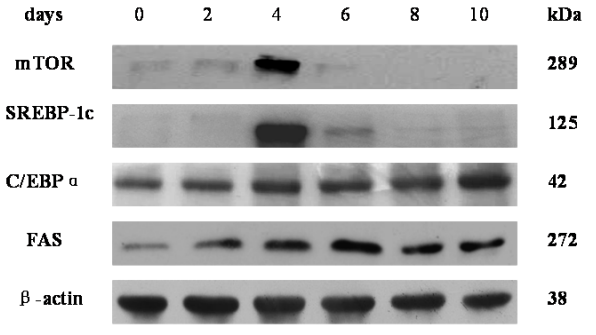

Fig. 2. Effect of 25 mM glucose on the levels of sterol-regulatory-element-binding protein (SREBP)-1c, CCAAT/enhancer binding protein-α (C/EBP-α) and fatty acid synthetase (FAS) during the process of adipogenic differentiation of pig muscle satellite cells. Total protein content was extracted, separated by SDS-PAGE and analyzed by Western blot. The phosphorylation state of mTOR protein was also analyzed by Western blot. Western blots represent four independent experiments.

As shown in Fig. 3B, mTOR protein was highly phosphorylated in control group on day 4 during differentiation. Whereas in the group treated with rapamycin, no mTOR phosphorylation was detected, indicating the mTOR signal pathway was completely inhibited. In addition, the SREBP-1c protein was expressed in the control cells, but was undetectable in the cells treated with rapamycin. However, the mRNA expression of SREBP-1c did not differ between the control and treated cells (Fig. 3A).

Fig. 4 shows the extent of lipid accumulation in cells obtained from the mTOR negative and mTOR positive groups after 10 days of adipogenic differentiation using Oil Red-O staining. Large amounts of lipid droplets were observed in the mTOR positive group cells while little lipid accumulation was observed in the mTOR negative cells.

DISCUSSION

Diabetes is characterized by high blood glucose levels. The capacity of a high glucose concentration to partly activate an adipogenic differentiation program has been previously observed in pancreatic β cells (18) and in rat muscle-derived cells (6). The present study demonstrated high concentration of glucose could induce an obvious adipogenesis in porcine muscle satellite cells within 10 days. In contrast, in rat cells, the process required almost 18-25 days to show significant lipid storage (7). These differences are likely due to specific differences among species. Because the metabolism of pigs is very similar to that of humans, our results obtained in porcine muscle satellite cells are better mimic human situation.

Members of the CCAAT/enhancer binding protein (C/EBP) family play an important role in adipogenesis. For example, ectopic expression of C/EBP-β promotes the differentiation of fibroblastic cells (19). Over expression of C/EBP-α in the presence of peroxisome proliferator-activated receptor-γ, can convert mouse fibroblastic cells into adipocytes (20). Differentia-

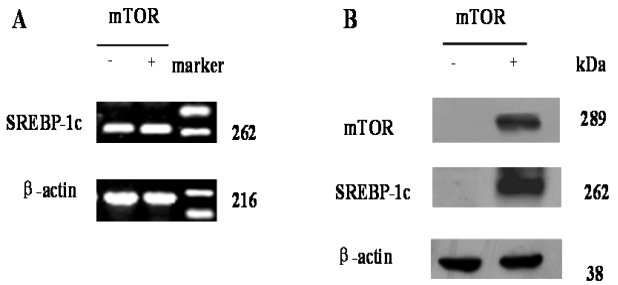

Fig. 3. Phosphorylated mTOR regulates the expression of sterol-regulatory -element-binding protein (SREBP-1c) Cells were treated with 20 nM rapamycin in medium containing 25mM glucose on day 2. Total RNA and protein was collected on day 4 to analyze for mRNA expression of SREBP-1c (A) and Phosphorylated mTOR, SREBP-1c protein levels (B). Note - denotes with the inhibitor while + denotes without.

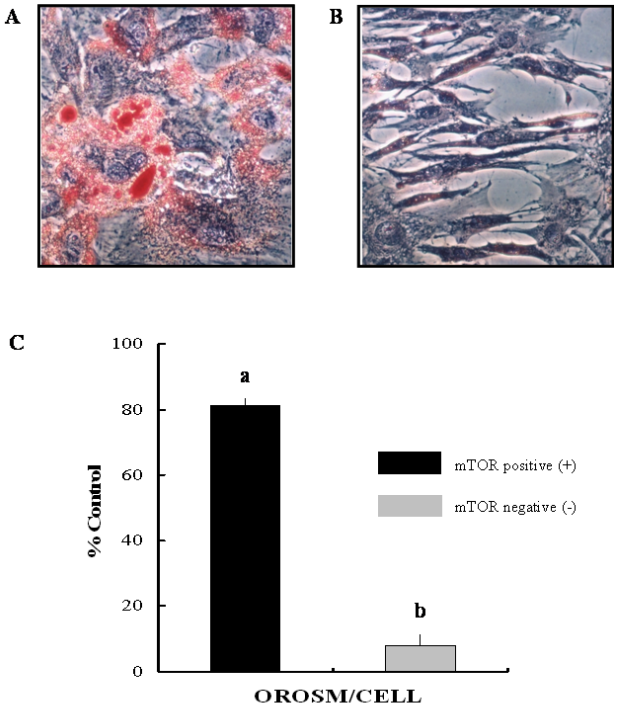

Fig. 4. Lipid accumulation in cells obtained from the mTOR positive (+) and mTOR negative (−) groups after 10 d of adipogenic differentiation using Oil Red-O staining. Cells were cultured and treated with glucose samely and then divided into two groups including an mTOR positive group and a negative group. On day 2, rapamycin (20 nM) was added into the culture medium of the mTOR negative group, while no additions were made to the mTOR positive group. (A) Cells were cultured in medium containing 25 mM glucose for 10 days (B) Cells were treated with 20 nM rapamycin in medium containing 25 mM glucose on day 2, after two days cells were cultured in medium containing 25 mM glucose for 6 days. Adipogenic differentiation was confirmed by Oil Red O staining. (C) Lipid was quantified by determining the integrated optical density of the Oil Red O-stained material (OROSM) in each 2003 microscopic field. The data are expressed as the amount of OROSM per cell in each group as a percentage of that in the control group (Cells cultured in normal culture medium for 10 days). Statistical differences reported are within tissue. Values are means ± SEM; n = 4. Values with different letters are significantly different ($P < 0.05$). Lipid droplets are in red, and nuclei are in blue (Magnification: × 20).

tion of myoblasts into adipocytes requires the coordinated expression of C/EBP-α and peroxisome proliferators-activated receptor-γ as well as the addition of fatty acids and adipogenic hormones to the culture medium (21).

In experiment 1, the C/EBP-α mRNA and protein expression were gradually increased which corresponded with lipid accumulation and the maturity of the porcine skeletal muscle satellite cells, indicating that cells were differentiated into adipocytes by the high level of glucose. Results also support that C/EBP-α plays an important role in the adipogenic differentiation process of porcine skeletal muscle satellite cells induced by high glucose.

FAS is the key enzyme of de novo lipogenesis and high glucose levels have previously been shown to increase FAS expression in myotubes from rats (6). We observed the first time that the expression of FAS was induced by high glucose during the adipogenic differentiation of porcine skeletal muscle satellite cells prior to myotube formation. This suggests an ectopic regulation of lipogenesis by high glucose in mammalian muscle.

Interestingly, C/EBP-α and FAS mRNA and protein expressed low in porcine muscle satellite cells, while they were dramatically enhanced by high glucose induction. Only little lipid accumulation was observed both in differentiated myogenic cells receiving no glucose induction and mTOR negative cells. It suggests that a low level of adipogenesis existing during muscle satellite cell differentiation is independent of the mTOR pathway. Otherwise, it implies that few adipose precursor cells were immingled in muscle satellite cells. However, it has been clear demonstrated that high glucose-induced mTOR phosphorylation initiated an extensive lipid accumulation in cells.

The importance of the transcription factor SREBP-1c in the regulation of adipogenic differentiation has been well established *in vitro* (22, 23). SREBP-1c is a key transcription factor linking the nutritional status and hormones to the expression of genes that regulated systemic energy metabolism (24). In similar with a previous study (6), we confirmed that SREBP-1c mRNA expression was induced by high glucose in porcine skeletal muscle satellite cells. In pancreatic β cells, the expression of SREBP-1c protein can be stimulated by glucose (25 mM) (18, 25), with a relatively short exposure to glucose (6-48 h). However, we found that SREBP-1c protein was induced by 25 mM glucose in porcine skeletal muscle satellite cells only after 2-4 days. Moreover, the expression of SREBP-1c in β cells was unresponsive to acute glucose stimulation (18), similar to what we have observed in the present experiment with porcine skeletal muscle satellite cells. There was not a consecutive regulation in SREBP-1c protein expression induced by high glucose. This is possibly because mTOR-mediated SREBP-1c protein expression was mainly on the translation level even though SREBP-1c mRNA level was relatively constant within the 10-day period. Collectively, these findings also indicate a significant role for SREBP-1c in the process of high glucose-induced lipogenesis in porcine skeletal muscle satellite cells.

Recently, it has been reported that SREBP-1c activation and lipogenesis in retinal pigmented epithelial cells requires the activity of mTOR (13). Interestingly, in experiment 1, we observed a similar expression trend between SREBP-1c and mTOR phosphorylation during the 10-day adipogenic differentiation period. This finding suggests that phosphorylated mTOR is involved in the adipogenesis of muscle satellite cells and is closely connected with SREBP-1c.

Additionally, phosphoprylated mTOR and SREBP-1c protein expressions dramatically increased on day 4 during glucose induction and then rapidly reduced to undetected during the adipogenic differentiation. What made lipid accumulation continuing remains unknown, which warrants further identify.

To further test our hypothesis, we inhibited the activity of mTOR on day 4. In contrast to the control group, almost no lipid accumulation was observed in the mTOR negative group, clearly indicating that mTOR is necessary for the adipogenic differentiation of muscle satellite cells induced by high glucose. The presence of SREBP-1c mRNA but disappearance of SREBP-1c protein in the mTOR negative group suggests that SREBP-1c translation may be inhibited with mTOR activity inhibited. Since both the mTOR and SREBP-1c protein were expressed at their highest level on day 4 during the differentiation process, we believe that this mTOR-mediated adipogenesis, induced by 25 mM glucose, was initiated after 2 to 4 days of exposure to glucose. mTOR mediated regulation of a extensive adipogenesis requires the SREBP-1c protein expression. However, exactly how mTOR regulates SREBP-1c translation remains to be elucidated, which warrants further study.

High concentrations of plasma glucose and intramuscular fat content are highly related with type 2 diabetes (4). In the present experiment, we demonstrated that high glucose-induced intramuscular adipogenesis is regulated by the phosphorylated mTOR signal pathway. This finding implies that mTOR might be associated with type 2 diabetes and may provide new strategies or ideas for type 2 diabetes therapy.

In summary, we have demonstrated the effects of high glucose on adipogenic differentiation in porcine skeletal muscle satellite cells and clarified that mTOR participates in this process, possibly through the activation of SREBP-1c protein expression. As such, the work provides new insight into the cellular mechanisms involved in ectopic lipid deposition in skeletal muscle during obesity and type 2 diabetes.

MATERIALS AND METHODS

Primary culture of porcine muscle satellite cells

A total of four, 3-day old piglets (Dalland, Sino-Dutch Animal Husbandry Training and Demonstration Center, Beijing, China) were killed by lethal injection of sodium pentobarbital. Primary cultures of porcine femoral muscle satellite cells were prepared as described by Balan et al (25). Briefly, isolated muscle tissues were washed with DMEM/F12 medium (HyClone, Logan UT) and treated for 40 minutes with 0.2% collagenase (Sigma, St Louis, MO) at 37°C. This was followed by centrifugation at 1,500 × g for 4 minutes. The pellet was washed and centrifuged three more times at 400 × g for 10 minutes in order to separate the mononuclear cells from the muscle fibers and myofibril fragments. The resulting supernatants were pooled and centrifuged at 1,500 × g for 6 minutes. At this point, the supernatant fluid was discarded. The porcine muscle cells were pre-plated repeatedly for removal of fibroblasts as described previously (27).

The porcine muscle satellite cells were plated in a medium containing 90% DMEM/F12, 10% fetal bovine serum (Gibco, Grand Island, NY), 15 mmol/ml HEPES, 100 U/ml penicillin-streptomycin and 2 mM glutamine (all reagents from Invitrogen, Carlsbad, CA). Cells were then incubated at 37°C and 5% CO_2 in a standard cell culture incubator (Thermo Scientific, Waltham, MA).

Experiment 1. High glucose induction assay

Primary cultured cells were grown to confluence in 24-well plates (Corning Life Sciences, St Louis, MO). Four replicates were run and the cells were isolated from a different piglet for each replicate. Cells were cultured in a high glucose medium containing 90% DMEM/F12, 10% fetal bovine serum and 25 mM glucose. The medium was changed every two days. Cells were harvested to measure total RNA and protein on day 0, 2, 4, 6, 8 and 10. On day 10, cells were stained with Oil Red O (Amresco Inc., Solon OH) and lightly counterstained with hematoxylin to monitor lipid accumulation in the cells. The cellular expression levels of phosphorylated mTOR protein, SREBP-1c protein, FAS and C/EBP-α were determined by semi-quantitative RT-PCR and western blot analysis.

Experiment 2. mTOR phosphorylation inhibition assay

During the high glucose-induced adipogenic differentiation which occurred in experiment 1, we observed a high cellular concentration of both phosphorylated mTOR and SREBP-1c protein on day 4. For experiment 2, we blocked the mTOR activity by adding the mTOR inhibitor rapamycin to the culture medium before day 4 to confirm whether or not mTOR regulates lipogenesis in muscle satellite cells and to determine when this regulation takes effect.

Cells were cultured and treated with glucose as described in experiment 1, and then divided into two groups including a control group and an mTOR positive group. On day 2, rapamycin (20 nM) was added into the culture medium of the mTOR negative group, while no additions were made to the mTOR positive group. Importantly, we did not observe significant changes in cell growth under the conditions used here (data not shown). Cells from the two groups were cultured for 2 more days and total RNA and protein were measured on day 4. The expression of phosphorylation of mTOR and SREBP-1c in the cells on day 4 was determined by semi-quantitative RT-PCR and Western blot analysis.

On day 10, the cells were stained with Oil Red O as described above to monitor lipid accumulation. Oil Red O-stained cells were completed using 2003 magnified fields. We used computer-assisted image analysis (Image-Pro Plus; Media Cybernetics, Inc., Silver Spring, MD) to determine the integrated optical density of the Oil Red O-stained material (OROSM). Lipid content was expressed as the integrated optical density of OROSM per cell in each 2003 magnified field. A minimum of five microscopic fields for each dish were quantified (28).

Total RNA isolation, cDNA preparation and semi-quantitative RT-PCR

Total RNA was isolated from the cultured cells using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Reverse transcription and RT-PCR were carried out according to Supplementary material 1.

Western blot assay

Cells were homogenized using a lysis buffer containing complete protease inhibitor (Hoffmann-La Roche, Nutley, NJ). Homogenates were centrifuged at 14,000 × g for 15 minutes at 4°C, and the protein concentration in the supernatants was determined using a BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). Protein samples were boiled for 3 minutes and then subjected to SDS-PAGE in gels (Bio-Rad Laboratories, Hercules, CA). Proteins were electro-blotted onto nitrocellulose. Membranes were blocked for 1 h in 5% non-fat dry milk and TBS buffer (20 mmol/l Tris and 500 mmol/l NaCl, pH 7.6). The membranes were incubated overnight at 4°C with a rabbit polyclonal antibody against phosphorylated mTOR (S2448, Abzoom, Dallas, TX) diluted 1 : 500, mouse monoclonal antibody against SREBP-1C (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1 : 200, fatty acid synthetase (H-300; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1 : 200, rabbit polyclonal antibody against C/EBP-α (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1 : 50, or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1 : 5,000. All dilutions were made in TBST buffer (TBS buffer with 0.05% Tween-20). Membranes were washed three times for 10 minutes with TBST buffer followed by incubation for 1 h with horseradish peroxidase-labeled anti-mouse and anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were visualized with the ECL-Plus Western blotting Reagent (Amersham Pharmacia Biotech, Piscataway, NJ) and exposure to Kodak X-Omat film. The density of bands was analyzed using Image Analysis Software (Alpha-Imager 2,200, Alpha Innotech, San Leandro, CA).

Statistical analysis

All analyses were performed using SAS (version 8.1; SAS Institute, Cary, NC). Significance of differences was tested by Student's t-test analysis. A significant difference was defined as $P < 0.05$.

Acknowledgements

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