Induction of Autophagy and Changes in Cellular Metabolism in Glucose Starved C2C12 Myotubes

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Summary Mouse myoblast C2C12 cells are commonly used as a model system for investigating the metabolic regulation of skeletal muscle. As it is therefore important to understand the metabolic features of C2C12 cells, we examined the effect of glucose starvation on autophagy in C2C12 myotubes. After culture of C2C12 myotubes with high (HG, 25.0 mM) or low (LG, 5.6 mM) glucose concentrations, the concentration of glucose in the LG group had decreased to 0 mM after 24 h of culture and was around 17 mM after 48 h of culture in the HG group. The concentration of lactate increased from 0 to approximately 9 mM at 24 h and then dropped slightly in the LG group, while it increased linearly to 21 mM in the HG group at 48 h. The phosphorylation of p70 S6 kinase, marker for the protein translation initiation was significantly lower and the ratio of LC3-II/LC3-I, marker for the induction of autophagy was significantly higher in the LG group. GLUT1 and hexokinase II expression were significantly higher in the LG group. Together, these changes in glucose and lactate concentrations in the culture media suggest that C2C12 myotubes depend on anaerobic glycolysis. Our findings also suggest that glucose depletion stimulates the expression of key molecules involved in glycolysis and that cellular autophagy is also activated in C2C12 myotubes.

Key Words glycolysis, hexokinase, lactate, protein synthesis, skeletal muscle

The regulation of extracellular glucose levels is important for proper cellular functioning, and it is well known that skeletal muscle is the major tissue responsible for whole-body glucose homeostasis. In the postprandial period, increased blood glucose is transported into skeletal muscle cells, which account for approximately 75% of insulin-stimulated glucose uptake (1). Conversely, skeletal muscle provides glycogenic amino acids via the breakdown of its own cellular proteins during prolonged starvation (2).

Mouse myoblast C2C12 cells are commonly used as a model system for investigating the metabolic regulation of skeletal muscle. When C2C12 myoblasts are cultured in media with low serum concentrations, they differentiate into multinucleated myotubes, express sarcomeric proteins, and develop contractile properties. Although differentiated C2C12 myotubes exhibit similar metabolic features to skeletal muscle, previous reports have shown that they possess quite distinct glucose utilization profiles (3). Thus, it is important to understand the metabolic features of C2C12 cells for the continued use of this cell line as a model for skeletal muscle.

Autophagy is a lysosome-dependent intracellular catabolism process responsible for the bulk degradation of cytoplasmic proteins and organelles and the recycling of their components (4). In mammalian cells, the main regulators of autophagy are hormonal and nutritional factors. While the exact nutrient sensor has yet to be identified, the deprivation of amino acids, glucose, and other nutrients can serve as metabolic stimuli to activate autophagy. Although the regulation of autophagy by amino acids has been extensively studied, knowledge regarding the effects of glucose is more limited.

Therefore, in the present study, we aimed to investigate the effect of glucose starvation on the cellular responses of C2C12 myotubes, including autophagy, by measuring levels of key molecules involved in various metabolic pathways.

MATERIALS AND METHODS

Materials. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), and antibiotic solution (penicillin and streptomycin sulfate) were purchased from FUJIFILM Wako Pure Chemical Corporarion (Osaka, Japan). Antibodies against glucose transporter 1 (GLUT1/SLC2A1), hexokinase II (HK-II), phospho-p70 S6 kinase (p70S6K, T389), phospho-AMP-activated protein kinase (AMPKα, T172), total AMPKα, and β-actin were obtained from Cell Signaling Technol-
ology (Beverly, MA). Antibodies against total p70S6K and L-type amino acid transporter 1 (LAT1/SLC7A5) were obtained from Santa Cruz Biotechnology (Dallas, TX). Another antibody against LAT1 was provided by Dr. Yoshikatsu Kanai (Osaka University, Osaka, Japan) (5).

C2C12 cell culture. Mouse myoblast C2C12 cells were plated in 6-well culture plates in DMEM (25 mm glucose), supplemented with 10% FBS, 100 units/mL penicillin, and 100 mg/mL streptomycin sulfate in a 5% CO₂-humidified chamber at 37°C. Cells were grown to approximately 90% confluence, and then the culture medium was replaced with differentiation medium (DMEM supplemented with 2% horse serum, 100 units/mL penicillin, and 100 mg/mL streptomycin sulfate). Cells were cultured in differentiation medium for 5 d to form multi-nucleated myotubes, and the medium was changed every 24 h. Then, cells were further cultured in 3 mL of differentiation medium (phenol red free), containing 25 mM glucose (high glucose, HG) or 5.6 mM glucose (low glucose, LG) for 48 h without changing the medium.

Determination of glucose, lactate, and branched chain amino acid (BCAA) concentrations in culture medium. A small sample of culture medium was harvested from the HG and LG groups at 0, 5, 24, and 48 h of culture. The glucose concentration in the culture medium was determined using a glucose C-II test (FUJIFILM Wako Pure Chemical Corporarion). The lactate concentration was determined using N-assay L LAC (Nittobo Medical Co. Ltd., Tokyo, Japan). The BCAA concentration was determined using a BCAA colorimetric assay kit (BioVision Incorporated, Milpitas, CA).

Protein separation and immunoblotting. After 48 h of cultured under high (HG) and low glucose (LG) conditions, cells were rinsed with ice-cold phosphate-buffered saline (PBS) and lysed in RIPA lysis buffer (Santa Cruz Biotechnology) with a cell scraper. The cell lysates were centrifuged for 10 min at 15,000 × g at 4°C. The protein concentration in the supernatant was determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL). Equal amounts of total cellular protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Merck KGaA, Darmstadt, Germany) for immunoblotting. Nonspecific binding to the membrane was blocked with Tris-buffered saline (pH 7.4) containing 0.05% (v/v) Tween-20 and 5% bovine serum albumin (BSA) or 5% skim milk. Following incubation overnight at 4°C with the indicated primary antibody, the membranes were washed and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG for 1 h at room temperature. Immunoreactive protein bands were visualized by ECL-prime (GE Healthcare, Buckinghamshire, UK) with an ImageQuant LAS500 image analyzer (GE Healthcare). The band intensity was quantified ImageJ (National Institutes of Health, Bethesda, MD).

Lactate dehydrogenase (LDH) isoenzyme analysis. LDH isoenzymes were analyzed as previously described by Gan et al. (6). Briefly, After 48 h of cultured under HG and LG conditions, cells were lysed in a solution of 0.9% NaCl and 5 mM Tris-HCl (pH 7.4) with a cell scraper and homogenized. The homogenate were centrifuged for 30 min at 15,000 ×g at 4°C. Equal amounts of protein in the supernatant were loaded onto a 6% non-denaturing polyacrylamide gel. After electrophoresis, the gel was soaked in staining solution containing 0.1 M sodium lactate, 1.5 mM NAD, 0.1 M Tris-HCl (pH 8.6), 10 mM NaCl, 5 mM MgCl₂, 0.03 mg/mL phenazine methosulfate, and 0.25 mg/mL nitro blue tetrazolium. Protein lysed from the mouse heart was loaded as a control.

Statistical analysis. Data are presented as mean ± SD (Figs. 2–6). Statistical analysis was performed using unpaired Student’s t-tests. Differences were considered statistically significant at p<0.05. Each experiment included 3–6 biological replicates.

RESULTS

Glucose and lactate concentrations in culture medium

In order to determine the effects of glucose starvation on C2C12 cellular responses, C2C12 cells were cultured under HG and LG conditions. Glucose and

![Fig. 1. Changes in glucose and lactate concentrations in culture media during 48 h of culture. C2C12 myotubes were cultured in differentiation medium containing 25 mM glucose (high glucose, HG) or 5.6 mM glucose (low glucose, LG) for 48 h without changing the medium. Concentrations of glucose (a) and lactate (b) were measured at 0, 5, 24, and 48 h. Data are presented as mean (n=3). Error bars are not presented since standard deviation (SD) values are very small. See SD values in result section.](image-url)
lactate concentrations in the culture media of the HG and LG groups were determined at 0, 5, 24, and 48 h of culture (Fig. 1). In the HG group, the concentration of glucose gradually decreased from 27.3±0.5 mM initially to 27.0±0.4 mM at 5 h, 24.4±0.9 mM at 24 h, and 16.9±0.4 mM at 48 h of culture (Fig. 1a). In the LG group, the concentration of glucose also decreased, from 5.3±0.1 mM initially to 4.5±0.1 mM at 5 h, 0 mM at 24 h, and 0 mM at 48 h of culture. Thus, cells in the LG group were starved for glucose for at least 24 h. The concentration of lactate in the HG group increased linearly over the culture period, from 0.3±0.3 mM initially to 2.1±0.1 mM at 5 h and 9.0±0.2 mM at 24 h, and then dropped slightly to 8.5±0.1 mM at 48 h of culture.

Effect of glucose starvation on total cellular protein

The total cellular protein in lysate was 707.5±30.6 μg/well in HG group and 632.5±41.8 μg/well in LG group. There was significant difference between HG and
LG groups (p<0.05).

Effect of glucose starvation on LC3

LC3 is a mammalian ortholog of yeast ATG8. The conversion of LC3-I to LC3-II serves as a specific marker for autophagy in mammalian cells (7, 8). Both the LC3A-II/LC3A-I ratio (Fig. 2a) and the LC3B-II/LC3B-I ratio (Fig. 2b) were markedly higher in the LG group than in the HG group after 48 h of culture.

Effect of glucose starvation on GLUT1 and HK-II

GLUT1 is responsible for the basal-level uptake of glucose through facilitated diffusion (9). HK catalyzes the first step of glycolysis, which is the conversion of glucose to glucose-6-phosphate. Four distinct mammalian HK isoforms (I, II, III, and IV) have been identified. HK-II is the predominant isoform in insulin-sensitive tissue such as cardiac, skeletal muscle, and adipose tissue (10). GLUT1 (Fig. 3a) and HK-II (Fig. 3b) protein expression levels were significantly higher in the LG group than in the HG group.

Effect of glucose starvation on phosphorylation of p70S6K

The activation of the mTOR/p70S6K pathway has been identified as a key step in the stimulation of protein synthesis. The phosphorylation of p70S6K at threonine 389 (T389) most closely correlates with kinase activity (11) and is associated with the increased activation of protein translation initiation (12). The phosphorylation level of p70S6K at T389 was significantly lower in the LG group than in the HG group (Fig. 4). The amount of total p70S6K was not statistically different between the LG and HG groups.

Effect of glucose starvation on phosphorylation of AMPKα

AMPKα plays a key role in the regulation of energy homeostasis (13). AMPKα is activated by an increase in the AMP/ATP ratio, which is required for the phosphorylation of threonine 172 (T172). The phosphorylation of AMPKα at T172 was significantly lower in the LG group than in the HG group (Fig. 5a), whereas the amount of total AMPKα was not statistically different between the LG and HG groups (Fig. 5b).

Fig. 4. Glucose starvation suppressed the phosphorylation of p70 S6 kinase. C2C12 myotubes were cultured in HG or LG medium for 48 h. A representative western blot image for phospho (P)-p70S6K, total (T)-p70S6K and β-actin are shown in the upper panel. β-Actin was used as a loading control. Quantitative data for P-p70S6K are shown in the lower panel. Data are expressed as percentages relative to the HG group (100%). Data are presented as mean±SD (n=3–6). *p<0.05 vs. HG group.

Fig. 5. Glucose starvation suppressed the phosphorylation of AMPKα. C2C12 myotubes were cultured in HG or LG medium for 48 h. Representative western blot images for phospho (P)-AMPKα, total (T)-AMPKα and β-actin are shown in panel (a). β-Actin was used as a loading control. Quantitative data for P-AMPKα and T-AMPKα are shown in panel (b) and panel (c), respectively. Data are expressed as percentages relative to the HG group (100%). Data are presented as mean±SD (n=3–6). *p<0.05 vs. HG group.
Effect of glucose starvation on LAT1

L-type amino acid transporter 1 (LAT1), also known as solute carrier family 7 member 5 (SLC7A5), is a multipass-membrane protein responsible for sodium-independent, high-affinity neural transport of large, neural amino acids. LAT1 protein expression was markedly higher in the LG group than in the HG group (Fig. 6).

LDH isoenzyme in C2C12 myotubes

LDH catalyzes the final step in anaerobic glycolysis through the conversion of pyruvate to lactate. LDH functions as a tetrameric complex composed of two distinct isoforms, LDH A and LDH B (14). LDH isoenzyme complexes are classified into LDH1–LDH5 based on different combinations of the LDH A and LDH B isoforms (see Fig. 7), with the LDH A isoenzyme favoring the reaction that converts pyruvate to lactate (Fig. 7b) (15). The LDH5 isoenzyme, which is composed of four LDH A subunits, was dominantly expressed in C2C12 myotubes (Fig. 7a).

DISCUSSION

To examine the responses of C2C12 myotubes to glucose starvation, cells were cultured in either high (25.0 mM) or low (5.6 mM) glucose-containing medium for 48 h. The concentration of glucose in the culture medium decreased to 0 mM after 24 h in the LG group and was maintained at 0 mM after 48 h of culture. These results suggest that C2C12 myotubes in the LG group were starved for glucose for more than 24 h. Lactate, the end product of anaerobic glycolysis, is highly produced by C2C12 myotubes. The concentration of lactate increased linearly in the HG group throughout the experimental period. By contrast, the increase in lactate in the LG group was arrested at 24 h of culture, when all of the glucose had been consumed. These results suggest that C2C12 myotubes are highly dependent on glucose, and that most of the glucose is metabolized into lactate, even in the presence of oxygen. This phenomenon is similar to the “Warburg effect” (16, 17), which is observed in tumor cells. Warburg proposed that tumor cells exhibit a permanent impairment of oxidative metabolism, resulting in a compensatory increase in glycolytic flux. Warburg-like glycolysis has been observed in differentiated C2C12 myotubes (18) and skeletal muscle satellite cells (19).

Analysis of the LDH isoenzymes in C2C12 myotubes from the HG and LG groups revealed that the LDH5 isoenzyme was dominantly expressed in these cells. This indicates that C2C12 myotubes favor the reaction that catabolizes pyruvate to lactate. Lactate production is important in working muscle to maintain the glycolytic flux for ATP production (20). The conversion of pyruvate to lactate rapidly regenerates NAD+ from NADH. As skeletal muscle cells have a limited pool of cytosolic NAD+ (21). We assume that the increased production
of lactate in C2C12 myotubes may contribute to the provision of NAD\textsuperscript{+} for recycling in glycolysis.

The conversion of LC3-I to LC3-II serves as a specific marker for autophagy in mammalian cells (7, 8). The LC3A-II/LC3A-I and LC3B-II/LC3B-I ratios were significantly elevated in the LG group, suggesting that glucose starvation induced autophagy. Activation of autophagy may resulted in the lower total cellular protein in LG group than HG group. Autophagy in mammalian cells is accelerated by glucose starvation (22–24). It has been reported that the activation of AMPK by intracellular low-energy status induces autophagy (25, 26). A recent study by Nwadike et al. (27) showed that glucose starvation had inhibitory effects on both the early and late stages of autophagy and that the activation of AMPK and phosphorylation of ULK1 were responsible for this phenomenon. In the present study, the phosphorylation of AMPK was downregulated in the LG group, suggesting that glucose starvation reduces AMPK activity. Thus, AMPK is not responsible for glucose starvation-induced autophagy in C2C12 myotubes.

Furthermore, the phosphorylation of p70S6K was lower in the LG group than in the HG group, suggesting that mTORC1 was inhibited by glucose starvation. It has been established that glucose deprivation activates autophagy through mTORC1 inhibition. Thus, glucose starvation-induced autophagy in C2C12 myotubes may be regulated by mTORC1. Roberts et al. (23) reported that, in response to glucose deprivation, HK-II binds to and inhibits the autophagy suppressor mTORC1. HK-II is an essential enzyme that catalyzes the first step of glycolysis, the conversion of glucose to glucose-6-phosphate. The expression of HK-II is abundant in insulin-sensitive cells, such as mammalian adipocytes and myocytes (10, 28, 29). In the present study, HK-II was markedly upregulated in the LG group compared to levels in the HG group, suggesting that glucose starvation-induced autophagy in C2C12 myotubes was mediated via the induction of HK-II and subsequent suppression of mTOR activity. Interestingly, levels of LAT1 were markedly increased upon glucose starvation. Previous studies have shown that LAT1 mRNA expression is elevated in C2C12 myotubes cultured in glucose-free medium (30). BCAAs are major substrates for LAT1 (31). The catabolism of BCAA in skeletal muscle is promoted by prolonged starvation (32, 33). We had hypothesized that the utilization of BCAA would be accelerated during glucose starvation. However, concentrations of BCAA in the culture media did not differ between the HG and LG groups. Although extracellular amino acid is sufficient, the uptake of BCAA was not increased during glucose starvation-induced autophagy. It has been reported that LAT2 is responsible for amino acid uptake in normal cells, instead of LAT1 in cancer cells (5). Protein expression of LAT2 was shown in mouse skeletal muscle (34). In future study, the effect of glucose starvation on the expression of LAT2 should be determined.

In conclusion, the disappearance of glucose and lactate production in the culture medium suggest that C2C12 myotubes depend on anaerobic glycolysis and that the oxidative mitochondrial utilization of pyruvate is low. Our findings also suggest that glucose depletion stimulates the expression of key molecules involved in glucose and amino acid uptake, despite the fact that cellular autophagy is activated in C2C12 myotubes. Moreover, glucose starvation-induced autophagy in C2C12 myotubes may be mediated via the suppression of mTOR activity induced by the upregulation of HK-II.

Disclosure of state of COI

The authors declare no conflicts of interest.

Acknowledgments

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