

Glucose, glutamine, lactic acid and α -ketoglutarate restore metabolic disturbances and atrophic changes in energy-deprived muscle cells

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Abstract. Skeletal muscle atrophy is often triggered by catabolic conditions such as fasting, malnutrition and chronic diseases; however, the efficacy of nutritional supplementation in maintaining muscle mass and preventing muscle atrophy remains controversial. The present study aimed to compare the inhibitory effects of various nutritional substrates on starvation-induced catabolic changes and muscle cell atrophy. C2C12 muscle cells were starved for up to 24 h in medium lacking serum and main nutrients (glucose, glutamine and pyruvate). To assess the effects of exogenous substrates, the cells were incubated in starvation medium and individually supplemented with each of the following nutrients: Glucose, amino acids, fatty acids, lactate or ketone bodies. The expression of each gene and protein was analyzed by reverse transcription-quantitative PCR and western blotting, respectively. Mitochondrial activity was determined by MTT assay and cell morphology was observed by immunofluorescence staining. The results revealed that starvation for >3 h suppressed mitochondrial activity, and after 5 h of starvation, the expression levels of several metabolic genes were increased; however, the levels of most, with the exception of *Scot* and *Cpt-1b*, were suppressed after 24 h. Protein degradation and a decrease in protein synthesis were observed after 5 h of starvation, followed by autophagy with morphological

atrophy at 24 h. Supplementation with specific substrates, with the exception of leucine, such as glucose, glutamine, lactic acid or α -ketoglutarate, attenuated the suppression of mitochondrial activity, and altered gene expression, protein degradation and myotube atrophy in starved myotubes. Furthermore, the decrease in intracellular ATP production after 24 h of starvation was reversed by restoring glycolysis in glucose-treated cells, and via an increase in mitochondrial respiration in cells treated with glutamine, lactic acid or α -ketoglutarate. In conclusion, increasing the availability of glucose, glutamine, lactic acid or α -ketoglutarate may be beneficial for countering muscle atrophy associated with inadequate nutrient intake.

Introduction

Skeletal muscles require large amounts of energy to generate the force and power needed to sustain physical activity. To maintain a high-energy state, muscle tissues store important fuel substrates, such as proteins (source of amino acids) and glycogen (source of carbohydrates) (1). Thus, an adequate supply of metabolic substrates is crucial for maintaining muscle mass and function. However, due to various physiological and environmental conditions, the supply of such substrates may be insufficient. In particular, undernutrition and starvation are among the primary causes of substrate shortage for energy production in skeletal muscles, leading to muscle weakness and atrophy, which can lead to muscular disorders, such as sarcopenia (2,3).

Various nutritional supplementation methods have been proposed as effective strategies for maintaining muscle mass and preventing muscle dysfunction and atrophy (4,5). Muscle mass largely depends on the balance between protein synthesis and degradation, regulated by cellular energy status through specific anabolic and catabolic signaling pathways (6,7). Among proteins and amino acids, leucine, a branched-chain amino acid, has piqued interest as a nutritional supplement for muscle maintenance owing to its potent ability to stimulate anabolic signals both *in vitro* and *in vivo* (7-9). A recent comprehensive clinical review reported that, although the evidence supporting the recommendation of leucine for increasing muscle mass and strength is low to moderate, it is the best nutrient available among several other nutrients that have been investigated (10).

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Abbreviations: AMPK, AMP-activated protein kinase; ATG1, atrogin-1; Glc, glucose; Gln, glutamine; Glu, glutamic acid; α KG, α -ketoglutarate; LA, lactate; LC3, microtubule-associated protein light-chain 3; Leu, leucine; Murf1, muscle ring finger 1; OA, oleic acid; β OHB, β -hydroxy butyric acid; PA, palmitic acid; p70S6K, 70-kDa ribosomal protein S6 kinase; Val, valine

Key words: muscle cells, starvation, metabolism, nutrient substrates, atrophy

In contrast, no clear recommendations are available for specific nutrients that inhibit catabolic reactions, such as muscle autophagy. When cells and tissues, particularly those with high energy demands, such as the heart and muscles, encounter an energy deficiency, they mobilize fuel substrates in response to the cellular environment, shift the substrate preference to that available for energy production, and attempt to maintain their energy status at a safe level using these fuels (11-14). However, an insufficient supply of extracellular nutrients for such an adaptive reaction activates the autophagy pathway for mobilizing glucose, amino acids, and fatty acids from different organelles for use as a source of energy (15). Therefore, ensuring a sufficient supply of certain nutrients that can be efficiently utilized by muscle tissue may be a viable strategy to prevent muscle catabolism.

Myotubes derived from mouse C2C12 myoblast cell lines are commonly used as a model system for studying skeletal muscle biology. Although the metabolic profile of C2C12-derived myotubes is not identical to that of skeletal muscle, C2C12 myotubes exhibit characteristics similar to those of skeletal muscle in terms of nutrient metabolism (16). Therefore, this cell line has been used in studies investigating muscle metabolism and its alterations (17-19). In addition, when C2C12 myotubes are in a fasting state, they not only display atrophic morphology but also exhibit specific metabolic profiles observed in atrophic muscles, characterized by accelerated protein breakdown through the activation of two major protein degradation systems: the ubiquitin-proteasome and autophagy-lysosome systems (20,21). Several nutrient substrates have been examined for their potential to inhibit myotube atrophy induced by starvation. However, the starvation conditions varied across studies, leading to inconsistent results, even when the same substrates were used.

Therefore, in this study, we aimed to compare the metabolic features of different substrates and their efficiency as energy fuels in C2C12 muscle cells under fasting conditions. The cells were starved by incubating them in a medium devoid of the main nutrients, and their metabolic profile, including anabolic and catabolic signaling, was analyzed in response to treatment with each nutrient substrate.

Materials and methods

Materials. D-glucose (Glc), L-glutamine (Gln), L-glutamic acid (Glu), L-leucine (Leu), L-valine (Val), β -hydroxy butyric acid (β OHB), and FA-free bovine serum albumin (BSA) were purchased from FUJIFILM Wako Pure Chemicals (Osaka, Japan). Palmitic acid (PA), oleic acid (OA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) were purchased from Nacalai Tesque (Kyoto, Japan). Sodium lactate (LA) and dimethyl α -ketoglutarate (α KG; an esterified form of α -ketoglutarate) were purchased from Otsuka Pharmaceutical (Tokyo, Japan) and Combi-Blocks (San Diego, CA, USA), respectively. All chemicals, except PA and OA, were dissolved in deionized distilled water.

Preparation of fatty acid complex. The cells were treated with fatty acids (FAs) using a previously described method, wherein the fatty acids were individually complexed with BSA in phosphate-buffered saline (PBS) (22). Briefly, FAs (PA or

OA) were added to 100 mM NaOH solution and dissolved in a heat-block at 75°C for 30 min to obtain the FA solution. The prepared FA solution was added to 10% (w/v) FA-free BSA in PBS at a ratio of 1:9 (v/v) to obtain a 10 mM FA solution. This solution was added to the medium at a final concentration of 0.1 or 0.2 mM FA. A mixture of 100 mM NaOH and 10% BSA/PBS (1:9) was used as the vehicle control. Each prepared solution was heated up to 55°C before being added to the cells.

Cell culture and treatment. Mouse C2C12 myoblast cell line (RCB0987) was purchased from the RIKEN BioResource Research Center (Tsukuba, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 5.5 mM glucose (041-29775; FUJIFILM Wako Pure Chemicals) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO₂. To induce differentiation of myoblasts into myotubes, C2C12 cells at 80% confluency were maintained in the differentiation medium (DMEM containing 2% horse serum; Thermo Fisher Scientific, Tokyo, Japan) for 6 days. For starvation, the differentiation medium was replaced with 'starvation medium'-DMEM lacking glucose, glutamine, and pyruvate (A14430; Thermo Fisher Scientific)- and the cells were incubated for the specified durations before harvesting. The control cells were incubated in regular DMEM without serum for the same durations. For some experiments, a specific nutrient was added to the starvation medium at concentrations within the medium to high physiological range, which are typically used in cell experiments. The concentrations of each nutrient used in this study are shown in Table I.

MTT assay. Cell viability was assessed using the MTT assay. The tetrazolium salt MTT is metabolized to insoluble purple formazan crystals in the mitochondria, which can be quantified upon solubilization using a spectrophotometer. The incubation medium was replaced with a medium containing MTT (0.5 mg/ml) 3 h before analysis. After the 3-h incubation period, the medium was replaced with dimethyl sulfoxide (250 μ l/well in a 24-well plate) to dissolve the formazan crystals. The absorbance was measured at 570 nm using a microplate reader (Multiskan FC; Thermo Fisher Scientific JP, Tokyo, Japan).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from cells using the RNAiso Plus reagent (TAKARA BIO, Shiga, Japan), and first-strand cDNA was synthesized using the ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan), according to the manufacturer's instructions. qPCR was performed using TB Green Premix Ex Taq II (TAKARA BIO) in 10- μ l reactions on a StepOne Plus Real-Time PCR System (Thermo Fisher Scientific). The qPCR cycling conditions were as follows: One cycle of 30 sec at 95°C, followed by 40 cycles of 5 sec at 95°C, and finally, one cycle of 30 sec at 60°C. Gene expression was normalized to that of a standard housekeeping gene (β -actin) and analyzed with the 2^{- $\Delta\Delta$ C_q} method (23). Experiments were performed in duplicate. The primer sequences are listed in Table SI.

Western blotting. Cells were washed and lysed with 50 μ l of lysis buffer [1% Triton X-100, 0.45% sodium pyrophosphate, 100 mM NaF, 2 mM Na₃VO₄, 50 mM HEPES (pH 8.0),

Table I. Type and concentration of substrates added to the cells.

Type	Substrate (abbreviation)	Concentration in medium
Monosaccharide	Glucose (Glc)	5.5 mM
Amino acid	Glutamine (Gln)	2.0 mM
	Glutamic acid (Glu)	0.2 mM
	Leucine (Leu)	0.2 mM
	Valine (Val)	0.2 mM
	Palmitic acid (PA)	0.1 and 0.2 mM
Fatty acid	Oleic acid (OA)	0.1 and 0.2 mM
	Lactate (LA)	10 mM
Lactic acid	Lactate (LA)	10 mM
Ketone	β -hydroxy butyric acid (β OHB)	0.5 mM
TCA cycle intermediate	α -ketoglutarate (α KG)	2.0 mM

147 mM NaCl, 1 mM EDTA, and a protease inhibitor mixture (cOmplete; Sigma-Aldrich, Tokyo, Japan)]. Then, the lysed cells were centrifuged at 13,000 $\times$ g, 4°C, for 15 min to obtain the supernatant, which was used as the cell lysate. Equal amounts of cell lysate proteins were loaded (10 μ g per lane) onto a 15% acrylamide gel for microtubule-associated protein light-chain 3 (LC3) and onto a 10% acrylamide gel for other proteins, separated by SDS-PAGE electrophoresis, and transferred onto PVDF membranes (Hybond-P; GE Healthcare Life Science, Tokyo, Japan). The membranes were blocked with Blocking One-P (Nacalai Tesque) for 60 min at room temperature (22–24°C) and then incubated overnight (16–18 h) at 4°C with primary antibodies against each of the following proteins: LC3 (#2775), 70-kDa ribosomal protein S6 kinase (p70S6K; #9202), phosphorylated-p70S6K (#9205), AMP-activated protein kinase (AMPK; #2532), and phosphorylated-AMPK (#2535), all purchased from Cell Signaling (Danvers, MA, USA), or β -actin (#sc47778; Santa Cruz Biotechnology, Dallas, TX, USA). After washing with PBS containing 0.1% Tween 20 (TBS-T), the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature (22–24°C). Specific proteins were detected by chemiluminescence using the ECL Select Western Blotting Detection Reagent (GE Healthcare). Images were captured and quantified using the iBright Imaging System (Thermo Fisher Scientific JP).

Fluorescence imaging of myotubes. Differentiated C2C12 cells were fixed and permeabilized for 10 min at room temperature (22–24°C) using 1% Triton-X containing 4% paraformaldehyde in PBS. Then, the cells were incubated with MF-20 anti-MHC antibody (1:100; Hybridoma Bank, Iowa, IA, USA) for 90 min at room temperature, washed with PBS-T, and incubated for 1 h with fluorescein isothiocyanate-conjugated anti-mouse IgG (1:500; #115-095-062; Jackson ImmunoResearch, West Grove, PA, USA). For each condition, representative images of the cells were captured using a 10x objective lens on a fluorescence microscope (BZ-X700; Keyence, Osaka, Japan).

Analysis of ATP production and glycolytic capacity. Cellular ATP production and glycolytic capacity were evaluated using the Glycolysis/OXPHOS Assay Kit (G270; Dojindo,

Kumamoto, Japan), following the manufacturer's instructions. Differentiated myotubes were incubated without or with 2.5 μ M oligomycin (an inhibitor of the mitochondrial respiratory chain) in a 96-well culture plate for 3 h. Then, ATP working solution was added to each well to assess ATP production. The plate was incubated for 10 min at 25°C, and relative ATP content was measured as luminescence intensity, using a multimode plate reader (EnSpire, Perkin Elmer Japan, Yokohama, Japan).

Statistical analysis. Data are expressed as the mean $\pm$ standard error of the mean. All statistical analyses were performed using the IBM SPSS Statistics for Windows (version 29; IBM Japan, Tokyo, Japan). The unpaired Student's t-test was used to identify significant differences between two groups, and one-way analysis of variance followed by Tukey's post-hoc test was performed to determine differences among three or more groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Nutrient starvation suppresses mitochondrial activity in muscle cells. Mitochondrial dehydrogenases reduce MTT to formazan; thus, formazan production depends on the redox activity of the cell and reflects mitochondrial function (24). Consequently, the amount of formazan produced can be interpreted as an indicator of mitochondrial activity and can also be used to assess cell viability. In this study, the mitochondrial activity of C2C12 myotubes was suppressed by incubation in the starvation medium in a time-dependent manner (Fig. 1A). After 3, 8, and 24 h of starvation, the mitochondrial activity in myotubes decreased to approximately 60, 44, and 7%, respectively, when compared with that in untreated cells. The decrease in cell viability after 5 h of starvation was restored to levels comparable to those of control cells by replacing the medium with regular DMEM for 3 h (Fig. 1B). This indicates that short-term starvation of up to 5 h causes temporary suppression of mitochondrial activity rather than irreversible cell damage. In contrast, the decrease in cell viability after 8 h of starvation was not fully recovered by replacing the medium with regular DMEM (Fig. 1B). This irreversible decline in

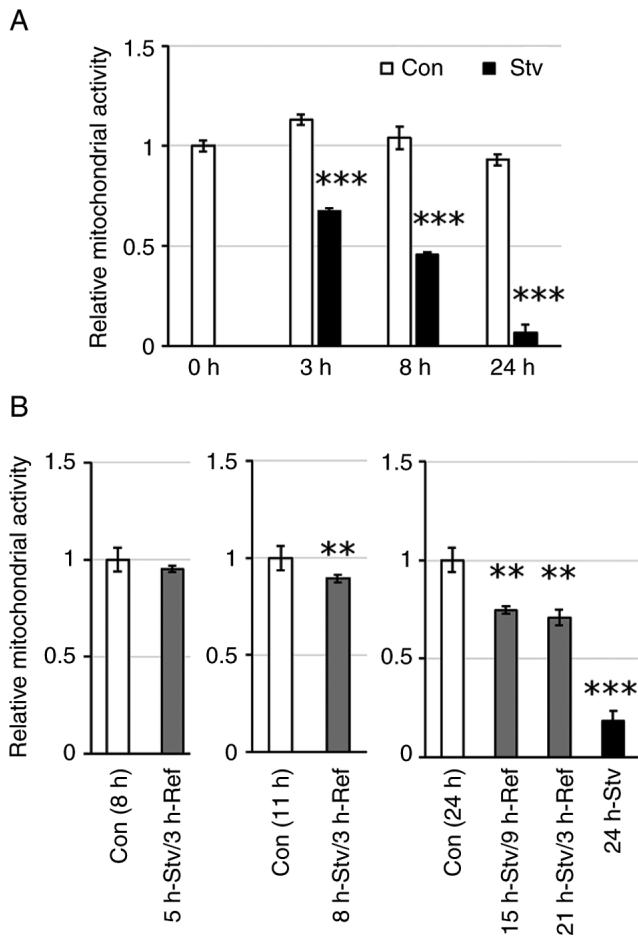


Figure 1. Effect of starvation duration on metabolic activity. (A) Cells were incubated in starvation medium for 3, 8, or 24 h. (B) Cells were incubated in starvation medium for 5, 8, 15, 21, or 24 h, followed by the replacement of the medium with regular DMEM and incubated for an additional 3 or 9 h. Control cells were incubated in regular DMEM for the same duration. Metabolic activity was assessed by MTT assay, and the values are expressed as fold-change compared with the values for control cells. Values represent the mean $\pm$ SEM ($n=4-6$). ** $P<0.01$, *** $P<0.001$ vs. control cells at the same time point. Con, control; Stv, starvation; Ref, refeeding.

mitochondrial activity became more pronounced with longer starvation times (Fig. 1B), suggesting a transition from reversible suppression of mitochondrial respiration to irreversible cell damage due to prolonged starvation.

Nutrient starvation directly affects the expression of genes related to substrate metabolism in muscle cells. To evaluate the cellular metabolic response to nutrient starvation, we examined the expression levels of key metabolic genes using RT-qPCR. The names and roles of these genes are listed in Table SI. After 1 h of starvation, none of the genes tested showed a significant change in their expression levels (Fig. 2A). After 5 h of starvation, the gene expression of hexokinase 2 (*Hk*) was suppressed, whereas that of several genes involved in the uptake and degradation of fuel substrates, such as succinate-CoA ligase, subunit β (*Sucla2*), cluster of differentiation 36 (*Cd36*), medium-chain acyl-CoA dehydrogenase (*Mcad*), branched chain α -keto acid dehydrogenase E1 α (*Bckdha*), and succinyl CoA 3-oxoacid CoA transferase (*Scot*), increased (Fig. 2B). In contrast, the expression of most metabolic genes

was suppressed after 24 h of starvation, with the exception of *Scot* and carnitine palmitoyl transferase 1-b (*Cpt1b*), the latter showing a notable increase in expression (Fig. 2C).

Nutrient starvation regulates signaling pathways involved in protein metabolism in muscle cells. The balance between protein degradation and synthesis is crucial for maintaining muscle mass and is regulated by the energy status of muscle tissue. Therefore, we investigated alterations in protein metabolism. After 1 h of starvation, the expression levels of atrogin-1 (*Atg1*) and muscle ring finger 1 (*Murf1*), which contribute to muscle proteolysis, remained unchanged. However, after 5 h of starvation, the expression levels of these genes increased significantly, accompanied by a trend toward AMPK activation ($P=0.07$) (Fig. 3A and B), whereas the LC3II/LC3I ratio, a representative autophagy marker, showed no change (Fig. 3B).

After 24 h of starvation, *Atg1* and *Murf1* expression remained elevated when compared with that in controls; however, the difference was less pronounced than that after 5 h of starvation (Fig. 3A). In contrast, the LC3II/LC3I ratio increased significantly, accompanied by a marked increase in AMPK activity (Fig. 3C). Regarding protein synthesis, the activity of p70S6K, which enhances the protein synthesis pathway, was significantly suppressed after both 5 and 24 h of starvation (Fig. 3B and C). In addition, the protein levels of AMPK, LC3, and p70S6K decreased after 24 h of starvation. When cultured cells are deprived of serum, a selective proteolytic pathway is activated for specific proteins, which applies to approximately 30% of cytosolic proteins (25,26). Therefore, these proteins may have been partially degraded by this pathway.

Glc, Gln, LA, and α KG alleviated mitochondrial inactivity and alteration of metabolic gene expression in starved muscle cells. Next, we evaluated whether any of the nutrient substrates could improve the metabolic disturbances induced by nutrient starvation. MTT assay was utilized to assess the toxic effects of each nutrient at the concentrations used on mitochondrial function in myotubes maintained in a differentiation medium. PA treatment significantly impacted mitochondrial activity after 24 h of incubation (Fig. 4A); therefore, we excluded PA from further studies.

Glc replacement notably recovered the decrease in mitochondrial activity induced by 5 h of starvation, as expected. Starvation-induced metabolic inactivity in mitochondria was also attenuated by adding Gln, LA, or α KG to the starvation medium. However, other substrates did not achieve similar improvements at the tested concentrations (Fig. 4B). Even after 24 h of starvation, which caused suppression of redox activity to less than 5% of that of control cells, the addition of each of these four substrates (Glc, Gln, LA, or α KG) alleviated the decline in mitochondrial activity to varying extents (Fig. 4C).

We also examined the expression levels of genes whose expression changed after the cells were starved for 24 h. All four substrates tested (Glc, Gln, LA, or α KG) attenuated the decrease in the expression of the following genes in starved cells: Glucose transporter 4 (*Glut4*), oxoglutarate dehydrogenase (*Ogdh*), glutamate dehydrogenase 1 (*Glud1*), branched-chain amino transaminase 2 (*Bcat2*), *Bckdha*, lactate dehydrogenase (*Ldh*), and monocarboxylic acid transporter 1 (*Mct1*) (Fig. 5). Additionally, LA or α KG attenuated fasting-induced decrease in

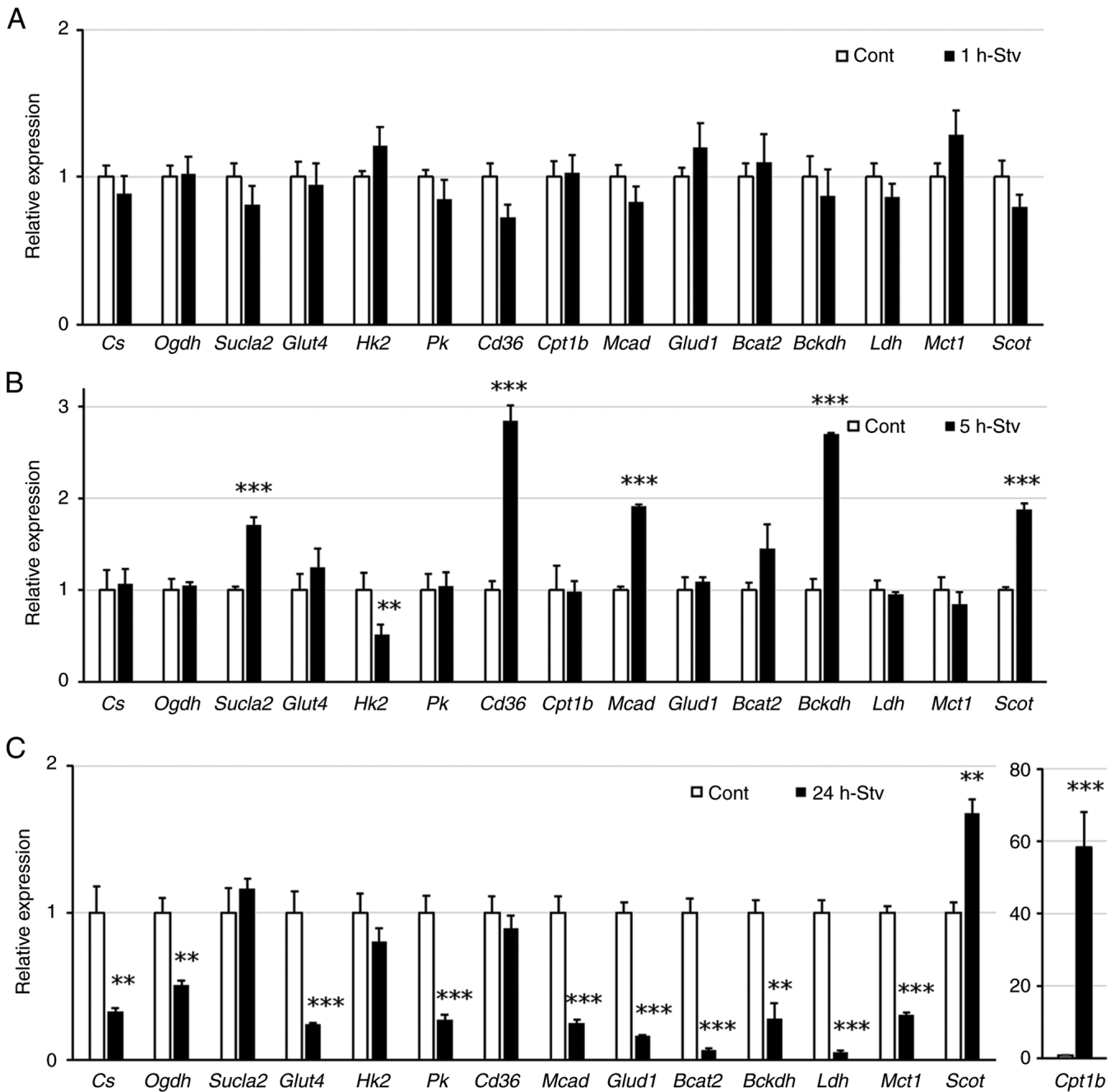


Figure 2. Effect of starvation on metabolic gene expression. Cells were incubated in regular DMEM or starvation medium for (A) 1, (B) 5, or (C) 24 h. The expression levels of representative genes related to metabolic fuel utilization were quantified. Values are expressed as fold-change compared with the values of control cells incubated in regular DMEM. The gene names for each abbreviation are presented in Table SI. Values represent the mean $\pm$ SEM (n=4). **P<0.01; ***P<0.001 vs. control cells at the same time point. Cont, control; Stv, starvation.

the expression of pyruvate kinase (*Pk*) and citrate synthase (*Cs*). Glc, Gln, or α KG attenuated that of *Mcad*, while they suppressed the fasting-induced increase in *Cpt1b* expression (Fig. 5).

Glc, Gln, LA, and α KG inhibit protein degradation and promote protein synthesis in starved muscle cells, thereby alleviating myotube atrophy. We examined the effects of selected nutrients, including β OHB, which generates acetoacetic acid as a substrate for SCOT, and Leu, as a major activator of anabolic signaling, to compare anabolic and catabolic signals. The expression of genes related to the ubiquitin-proteasome system, which peaked in fasted cells after 5 h of starvation, was

suppressed upon treatment with Glc, Gln, or α KG (for *Atg1*), and with Gln, LA, or α KG (for *Murf-1*) (Fig. 6A). The increase in AMPK activity after 24 h of starvation was significantly inhibited by the administration of Glc, Gln, LA, or α KG, but not by that of β OHB or Leu (Fig. 6B). After 24 h of starvation, the enhancement of autophagy, as assessed by the LC3II/LC3I ratio, was inhibited by Glc, LA, or α KG treatment, while the inhibition of protein synthesis, as assessed by phosphorylation of p70S6K, was restored by treatment with all the tested substrates, except β OHB (Fig. 6B).

Additionally, we evaluated the effect of nutrients, which restored the starvation-induced inhibition of p70S6K

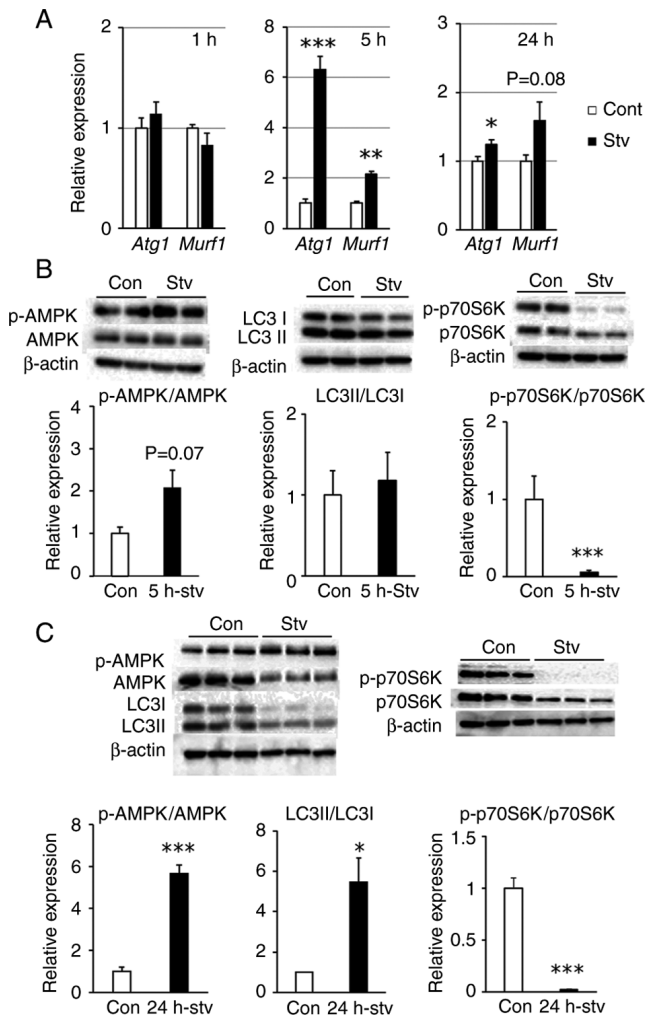


Figure 3. Effect of starvation on protein synthesis and degradation. Cells were incubated in starvation medium for 1, 5, or 24 h. (A) The relative mRNA expression of Atg1 and Murf1 was quantified by reverse transcription-quantitative PCR. (B and C) Protein phosphorylation levels of AMPK and p70S6K, and the protein expression ratio of LC3II/LC3I after (B) 5, or (C) 24 h of starvation were analyzed by western blotting. Representative blot images are shown in the upper part. Values are expressed as fold-change compared with the values of control cells incubated in regular DMEM. Values are presented as the mean $\pm$ SEM (n=3). * P <0.05, ** P <0.01, *** P <0.001 vs. control. Atg1, atrogenin-1; Con, control; Murf1, muscle ring finger 1; p-, phosphorylated; Stv, starvation.

phosphorylation on myotube morphology (Fig. 6C). Generally, atrophy occurs when protein breakdown exceeds protein synthesis in muscle cells. Consistent with the results for anabolic and catabolic signaling in the previous section, the incubation of cells in the starvation medium for 24 h led to severe myotube atrophy. Notably, treatment with Glc not only prevented fasting-induced atrophy but also promoted hypertrophic changes in myotubes. Treatment with Gln, LA, or α KG also prevented myotube atrophy but to a lesser extent than that with Glc. Unexpectedly, Leu showed no protective effects against myotube atrophy caused by nutrient starvation.

Gln, LA, and α KG enhance ATP production in starved muscle cells via oxidative phosphorylation. Finally, we investigated the metabolic pathways through which each nutrient contributes to cellular energy production. In the regular differentiation medium, ATP production in myotubes primarily

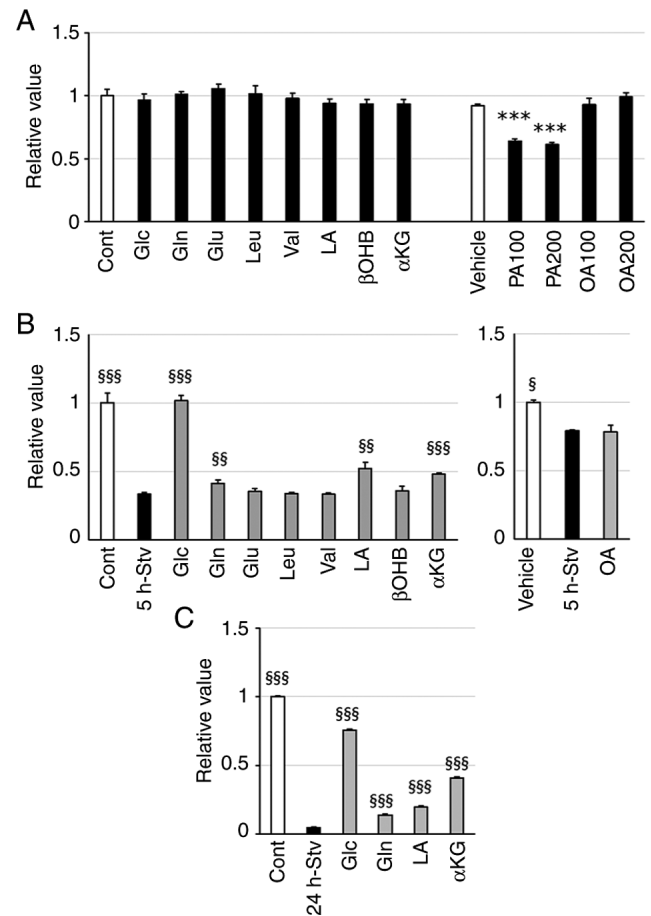


Figure 4. Effect of single nutrient supplementation on metabolic activity. (A) Cells were incubated in regular DMEM supplemented with the indicated nutrient for 24 h. (B and C) C2C12 myotubes were cultured in starvation medium only or with the indicated nutrient for (B) 5 or (C) 24 h. Albumin and NaOH were added as vehicle controls in both regular and starvation media in the experiment for the fatty acid (PA and OA). Metabolic activity was assessed using the MTT assay. Values are expressed as fold-change compared with the values of the control cells incubated in regular DMEM. Values are presented as the mean $\pm$ SEM (n=3-4). *** P <0.001 vs. vehicle control; § P <0.05, §§ P <0.01, §§§ P <0.001 vs. starved cells in the same group. Cont, control; Vehicle, vehicle control; Stv, starvation; Glc, glucose; Gln, glutamine; Glu, glutamic acid; Leu, leucine; Val, valine; LA, lactate; β OHB, β -hydroxy butyric acid; α KG, α -ketoglutarate; PA, palmitic acid; OA, oleic acid.

relied on glycolysis. After 24 h of starvation, glycolytic ATP production significantly decreased, leading to a reduction in total ATP production; however, ATP production by oxidative phosphorylation (OxPhos) increased (Fig. 7). This indicates that nutritional inadequacy triggers a metabolic shift in muscle cells. The starvation-induced decrease in intracellular ATP production was fully restored by the addition of Glc, Gln, LA, or α KG to the starvation medium, resulting in higher intracellular ATP production when compared with that in control cells. This restoration of ATP production was primarily driven by the recovery of glycolysis in Glc-treated cells, whereas it was facilitated by an increase in OxPhos-derived ATP generation in cells treated with Gln, LA, or α KG (Fig. 7).

Discussion

In muscle tissues under starvation conditions, the rate of protein degradation exceeds the rate of protein synthesis,

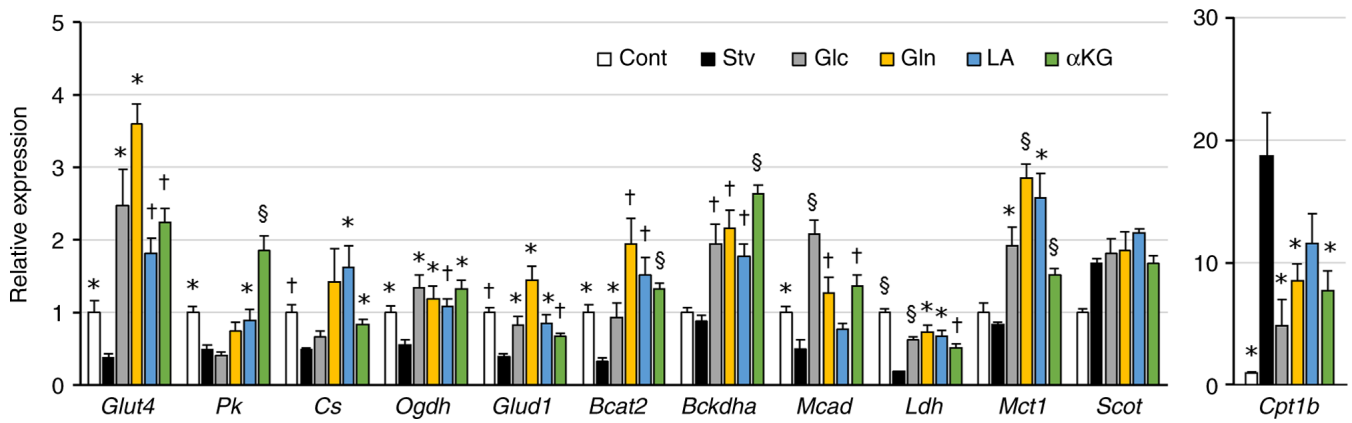


Figure 5. Effect of single nutrient supplementation on metabolic gene expression. Cells were incubated in starvation medium with (colored bars) or without (black bars) the indicated nutrients for 24 h. The expression levels of representative genes related to metabolic fuel utilization were quantified. Values are expressed as fold-change compared with the values of the control cells incubated in regular DMEM (empty bars). Values are presented as the mean $\pm$ SEM (n=3-4). * P <0.05, † P <0.01, § P <0.001 vs. starved cells. Cont, control; Stv, starvation; Glc, glucose; Gln, glutamine; LA, lactate; α KG, α -ketoglutarate.

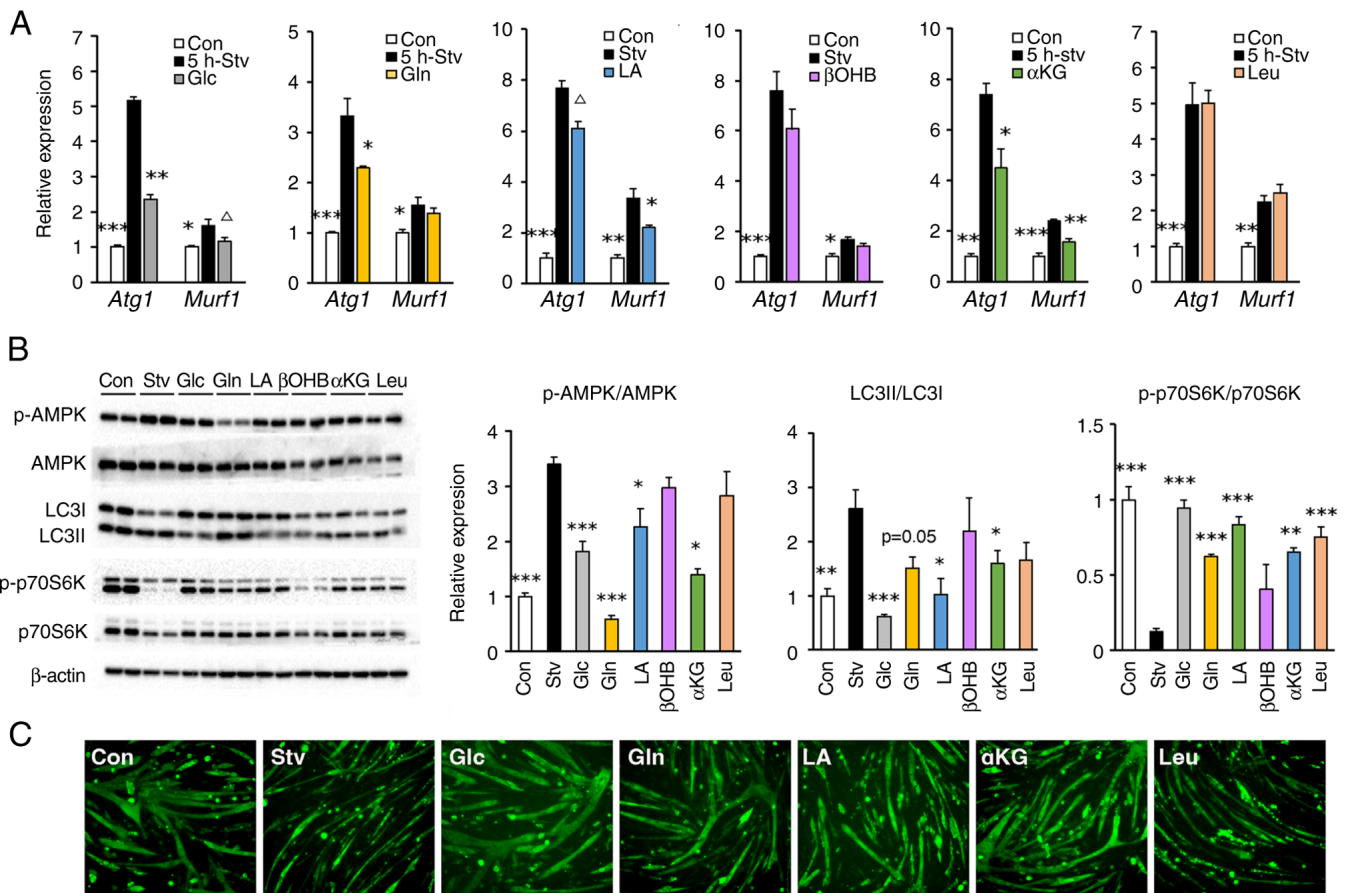


Figure 6. Effect of single nutrient supplementation on protein metabolism and morphological atrophy. Cells were incubated in starvation medium with (colored bars) or without (black bars) the indicated nutrients for (A) 5 or (B and C) 24 h. (A) Relative mRNA expression of Atg1 and Murf1 was quantified by reverse transcription-quantitative PCR. (B) Protein phosphorylation levels of AMPK and p70S6K, and the protein expression ratio of LC3II/LC3I were analyzed by western blotting. Representative blot images are shown on the left side. (C) Representative fluorescence images of MHC antibody-stained myotubes at x200 magnification were captured using a fluorescence microscope (BZ-X700; Keyence). Values are expressed as fold-change compared with the values of the control cells incubated in regular DMEM. Values are presented as mean $\pm$ SEM (n=3-4). ΔP <0.1, * P <0.05, ** P <0.01, *** P <0.001 vs. starved cells. Atg1, atrogen-1; Con, control; Murf1, muscle ring finger 1; p-, phosphorylated; Stv, starvation; Glc, glucose; Gln, glutamine; LA, lactate; β OHB, β -hydroxy butyric acid; α KG, α -ketoglutarate; Leu, leucine.

leading to a decrease in skeletal muscle mass. To assess muscle cell biology in atrophied muscles, myocytes derived from C2C12 cells deprived of nutrient substrates have been

utilized (20,27-30). However, starvation conditions varied across these studies; either saline (PBS or HEPES buffered saline) containing only inorganic salts (28,29) or containing

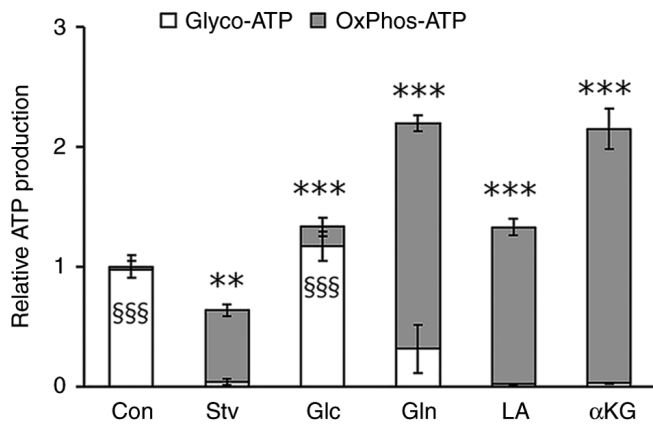


Figure 7. Effect of single nutrient supplementation on ATP production. C2C12 myotubes were incubated in starvation medium with or without the indicated nutrients for 24 h. The ATP production rates from glycolysis (Glyco-ATP) and OxPhos (OxPhos-ATP) were determined. Values are expressed as fold-change compared with the values of the control cells incubated in regular DMEM. Values are presented as mean $\pm$ SEM ($n=3-4$). ** $P<0.01$, *** $P<0.001$ vs. control cells for total ATP production; §§§ $P<0.001$ vs. starved cells for Glyco-ATP production. Con, control; Glyco-ATP, glycolytic ATP; OxPhos, oxidative phosphorylation; Stv, starvation.

2% horse serum (20) was used as the starvation medium, or serum-free DMEM with (27) or without (30) Glc. We confirmed that 6 h of incubation with PBS resulted in the detachment of more than half of the cells from the bottom of the dish. In contrast, incubation with serum-free DMEM for up to 24 h had no effect on cell morphology (data not shown). Therefore, in this study, we utilized DMEM as the starvation medium, wherein the serum and main energetic nutrients are absent, following the method used by Zeidler *et al.* (31). The authors stated that short-term starvation in such a medium was an appropriate approach for evaluating the influence of fuel substrates on cellular metabolism.

Short-term (1 h) starvation using this medium did not impact mitochondrial metabolic activity in C2C12 myotubes, as assessed by the MTT assay. Similarly, 1 h of starvation caused no changes in the expression levels of the metabolic genes that were tested. These findings, consistent with the results of a previous study (31), suggest that a 1-h starvation period is unlikely to induce adaptive changes as the presence of residual substrates within the cells prevents energy depletion. Extending the starvation period to 5 h led to a reversible decrease in mitochondrial activity and an increase in the expression of genes involved in fuel utilization, particularly *Sucla2*, *Cd36*, *Mcad*, *Bckdha*, and *Scot*. CD36 is a glycoprotein that facilitates FA transport into cells, and MCAD is an enzyme that catabolizes FA. Both of these play a role in FA oxidation for mitochondrial ATP production (32). Studies have shown that the expression of genes related to FA oxidation, including these two, is upregulated in skeletal muscles during starvation across several animal species (33-35). During starvation, animal bodies attempt to maintain ATP production by rapidly shifting fuel substrates from glucose to lipids (13). This aligns with our findings of increased *Cd36* and *Mcad* expression and decreased *Hk2* expression, a key enzyme for glycolysis, supporting this adaptive phenomenon. Additionally, BCKDHA and SCOT metabolize branched-chain amino acids

and ketone bodies, respectively, providing substrates for TCA cycle, while SUCLA2 is an enzyme involved the TCA cycle. Thus, the increased expression of these genes may represent an adaptive response to enhance intracellular substrate supply for mitochondrial ATP production.

In the present study, starvation for more than 8 h led to partial irreversible cellular damage. Prolonged starvation (24 h) suppressed the expression of almost all genes tested, which contrasts the results of short-term (5 h) starvation. Consistently, studies focusing on rodent muscles have reported that the expression of genes involved in the catabolic pathway, such as glycolytic flux and mitochondrial respiration, was suppressed by prolonged starvation, and this suppression was enhanced with an increase in starvation duration (36,37). In contrast, *Cpt-1* expression significantly increased after 24 h starvation. These results align with those obtained in studies with *C. elegans* (38,39), which reported that the expression of genes associated with catabolic pathways, including the proteasome, OxPhos, and the tricarboxylic acid (TCA) cycle, was suppressed in *C. elegans* fasted for 16 h, while that of *Cpt-1* was markedly elevated. In these studies, survival rates during fasting reduced in the *Cpt-1* knockout individuals, suggesting that the elevation of *Cpt-1* expression may be a crucial biological response to endure prolonged fasting. In the present study, the expression of *Scot*, a key enzyme in ketone metabolism, remained elevated even after 24 h of starvation. Nevertheless, in this study, a ketone body, β OHB, could not ameliorate starvation-induced myotube atrophy. Few studies have examined the changes in *Scot* expression in starved muscles, but one study reported its upregulation in chicken sartorius muscle after 24 h of fasting (40). Recently ketone bodies have been reported to induce a quiescent state of muscle cells during starvation to enhance their resilience (41). An increase in *Scot* expression may play a role in ketone signaling to prevent muscle cells from starvation-induced damage.

Regarding the effects of individual nutrients on starvation, among those tested in this study, the addition of Glc, Gln, LA, or α KG mitigated the decrease in metabolic activity observed in myotubes subjected to 5 h of starvation. Supplementation with each of these four nutrients attenuated metabolic alterations, such as the decrease in mitochondrial activity and changes in metabolic gene expression to varying degrees, even after 24 h of starvation. In contrast, OA administration did not improve cellular metabolism under starvation conditions, despite a significant increase in the expression of *Cpt-1*, a key molecule for transporting long-chain FAs into the mitochondria, after prolonged nutritional deprivation. In the OA supplementation experiments, starvation-induced metabolic changes were not observed (Fig. 4B), probably due to the addition of albumin as a vehicle control in the starvation medium, which may have masked any potential effects of starvation or OA treatment.

The mechanism by which certain nutrients, such as Glc, Gln, LA, and α KG, improve metabolic disturbance in muscle cells may involve AMPK, a crucial sensor of energy status in skeletal muscles. In the present study, ATP content decreased in 24-h-starved cells, leading to significant activation of AMPK, while administration of Glc, Gln, LA, or α KG, but not β OHB and Leu, suppressed this starvation-induced activation of AMPK. Activated AMPK inhibits mTOR activity

via phosphorylation of the adaptor protein, Raptor (42), resulting in decreased protein synthesis via inhibition of the mTOR/p70S6K pathway (43) and increased proteolysis via autophagy (44,45), ultimately leading to muscle fiber atrophy. In the present study, Glc, Gln, LA, or α KG inhibited AMPK activation, reversing the reduction in protein synthesis and preventing autophagy, thereby ameliorating the histological atrophy of myotubes caused by starvation. These results suggest that Glc, Gln, LA, and α KG contribute to ATP production, which inhibits AMPK activation, thereby preventing muscle atrophy. Additionally, Glu and its metabolite α KG have been reported to directly activate mTORC1, promoting protein synthesis (46), which may contribute to their beneficial effects on muscle cell atrophy. On the other hand, Leu is also a potent stimulator of mTORC1 (47,48) and is widely recognized as a crucial nutritional factor for preventing muscle atrophy (49,50). Leu administration could restore experimentally induced atrophy in C2C12 myotubes (51,52). However, in the present study, although Leu enhanced mTOR-S6K signaling, it was unable to ameliorate the metabolic abnormalities and histological atrophy induced by starvation. Thus, an adequate supply of ATP to muscle cells may be essential for leveraging the potential of Leu as a protein synthesis stimulator to prevent muscle atrophy.

In the present study, treatment with Glc not only prevented starvation-induced atrophy but also promoted hypertrophic changes in myotubes. Consistent with this, Nakai *et al* (30) reported that addition of Glc markedly increased the phosphorylation of p70S6K only in starved C2C12 myotubes and that starvation-induced autophagy accounts for the activation of p70S6K by adding Glc. Gln, LA, and α KG, also improve atrophic changes in nutrient-deficient muscle cells. These findings are consistent with those of several studies demonstrating that these nutrients prevent muscle atrophy or promote muscle hypertrophy both *in vitro* and *in vivo* (53-57). These studies reported as the underlying mechanisms that α KG as well as Gln directly activated the mTOR pathway (53), or that α KG inhibited proline hydroxylase 3 to stimulate β 2 adrenergic receptor (54). Regarding LA, Ohno *et al* (57) proposed that the activation of GPR81 (with LA acting as a ligand)-ERK signaling in muscle cells is involved in the mechanistic pathway. Thus, Gln, LA, or α KG may function not only as energy fuels but also as signaling molecules that help to prevent muscle atrophy.

To maintain energy supply, energy-consuming organs such as heart and muscles can switch between and adapt to energy substrates in response to external or internal environmental factors. Glucose is the most preferred energy substrate for skeletal muscles and when a sufficient amount of glucose is available after feeding, skeletal muscles increase glucose utilization and storage while reducing fatty acid oxidation. In contrast, when nutrient supply to the muscles is interrupted, such as during starvation or sleep, the rate of fatty acid oxidation rapidly increases (13,58). In addition, several nutrients other than fatty acids, such as lactate and glutamine are also gaining attention as alternative energy sources for muscles during fasting. One study using intravenous infusions of ^{13}C -labelled nutrients showed that lactate contributes significantly to the TCA cycle carbon in muscle tissues of fasted mice, suggesting it as a primary source for maintaining muscle

ATP levels (59). Additionally, Li *et al* (60) reported that in the muscles of fasted mice, glutaminolysis was upregulated before an increase in FA oxidation, indicating the crucial role of a substrate shift from glucose to glutamine in sustaining muscle energy supply under starvation condition. Metabolic shifting is generally believed to be regulated by neurogenic and serum factors, such as hormones. However, in the present study, we observed a similar phenomenon in cultured muscle cells-ATP production shifted from glycolysis to OxPhos when nutrient supply was interrupted. Moreover, LA, Gln, and its metabolite α KG effectively supported cellular ATP production through OxPhos. This suggests that even in the absence of neural and serum regulators, muscle cells can switch their metabolism based on the available substrates.

In conclusion, we examined the metabolic characteristics of various nutritional substrates and their efficiency in C2C12 muscle cells under starvation conditions. Our findings revealed that certain nutrients, such as Gln, LA, and α KG, help to improve metabolic imbalances and counteract atrophic changes caused by energy deprivation in muscle cells. To the best of our knowledge, this is the first study to compare the effects of multiple nutrients on metabolic changes in muscle cells experiencing energy substrate deficiency. Our findings could potentially be translated into an effective nutritional strategy to prevent muscle atrophy.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

MI, MM and KI designed and conceived the study, analyzed the results and drafted the manuscript. MI, MM and MT conducted the experiments and collected the data. MK contributed to the study design and supervised the experiments. KI supervised the entire project and obtained the research grants. MI and KI confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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