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Metabolomic analysis of C2C12 myoblasts induced by the transcription factor FOXO1

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The transcription factor FOXO1 is considered to play roles in the regulation of energy metabolism in various tissues. To determine the metabolic changes occurring due to FOXO1 activation, we analyzed the metabolic profile of C2C12 myoblasts expressing a FOXO1-estrogen receptor fusion protein using capillary electrophoresis with electrospray ionization time-of-flight mass spectrometry (CE-TOFMS). In FOXO1-activated cells, the metabolite levels during glycolysis are higher and the gene expression of pyruvate dehydrogenase kinase, an enzyme that inhibits glucose utilization, is increased. In addition, the metabolite levels of numerous amino acids are decreased, with increased gene expression of branched chain amino acid metabolism enzymes. Our results suggest that FOXO1 suppresses glucose utilization and promotes the use of proteins/amino acids as energy sources in muscle cells, potentially during starvation.

Keywords: amino acid; C2C12; CE-TOFMS; FOXO1; metabolomic analysis; transcriptional factor

During starvation and excessive exercise, the body adapts to energy shortages in various tissues. In the liver, gluconeogenesis occurs to maintain blood glucose levels. In other peripheral tissues, glucose utilization is suppressed, and lipids and/or proteins are preferentially used to conserve glucose. In skeletal muscles, lipids as well as proteins are degraded and utilized as a source of energy [1].

Forkhead box protein O1 (FOXO1) is a transcription factor, which is expressed in metabolic tissues such as the liver, muscles, and adipose tissue [2,3]. During fasting, FOXO1 activates the expression of gluconeogenesis enzyme genes in the liver [4]. In addition, FOXO1 activates the gene expression of pyruvate dehydrogenase kinase 4, which suppresses the conversion of pyruvate into acetyl CoA, and suppresses glucose utilization during glycolysis [5]. FOXO1 also activates the gene expression of lipoprotein lipase, which hydrolyzes triglyceride to facilitate the utilization of fatty acids in muscles and adipose tissues [6]. In addition, using C2C12 myoblasts-expressing FOXO1-estrogen receptor (ER) fusion protein, which is activated by tamoxifen, Bastie *et al.* showed increased fatty acid incorporation and utilization [7]. Therefore, FOXO1 shifts energy sources from glucose to lipids. Furthermore, FOXO1 activates proteolysis-related genes, such as ubiquitin-proteasome and autophagy–lysosome pathway genes [8–10], which promote the production of amino acids from protein. Therefore, FOXO1 could play some roles in metabolism during periods of energy deprivation.

To the best of our knowledge, there are no reports of metabolomics analyses in the condition of modifying FOXO1 transcriptional activity. Thus, our study aimed to examine global metabolite changes caused by FOXO1 in the muscle in an unbiased manner.

Abbreviations
ACO, acyl CoA oxidase; BCAA, branched chain amino acids; ER, estrogen receptor; FOXO1, forkhead box protein O1; HCA, hierarchical clustering analysis; PCA, principal component analysis; PDK4, pyruvate dehydrogenase kinase 4.

However, metabolite changes occurring *in vivo* are often difficult to interpret due to interorgan effects, such as interactions between the skeletal muscle and liver. Thus, to directly determine metabolic changes (not secondary effects caused by other organs) occurring due to the activation of FOXO1, we used C2C12 cells expressing the FOXO1-ER fusion protein [10]. We then analyzed metabolic profiles using capillary electrophoresis with electrospray ionization time-of-flight mass spectrometry (CE-TOFMS) and conducted related-gene expression analyses.

Methods

Cells

C2C12 mouse myoblasts (Riken Cell Bank, Tsukuba, Japan) stably expressing the FOXO1-ER fusion protein were prepared as previously described [7,10]. In brief, C2C12 cells were stably transfected with either the empty pBABE retroviral vector or pBABE vectors expressing fusion proteins containing a constitutively active form of human FOXO1, in which the AKT phosphorylation sites Thr-24, Ser-256, and Ser-319 are replaced with alanine (FOXO1(3A)) in-frame with a modified tamoxifen-specific version of the ligand-binding domain murine ER [7,10]. FOXO1-ER plasmid was provided by Dr. Terry G. Unterman (Department of Medicine, University of Illinois at Chicago, IL, USA). Cells were selected with puromycin, and the colonies were pooled for analysis. Fusion proteins were restricted to the cytoplasmic compartment until activation with tamoxifen, which caused FOXO1-ER to relocate to the nucleus, where the FOXO1 moiety then functioned as a transcription factor [7,10]. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The medium were replaced every 2 days until cells reached confluence. Two days after confluence, the cells (undifferentiated myoblasts) were treated with tamoxifen for 24 h and used for the metabolomic analysis.

Metabolomic analysis

The cells treated with vehicle (control, $N = 3$) or tamoxifen (FOXO1-activated, $N = 3$) were used for the metabolomic analysis (Human Metabolome Technologies Inc., Tsuruoka, Japan) [11–13]. Cells (3.9×10^6) were transferred into 500 μL of methanol containing 50 mM external standard. After homogenization by BMSM10N21 (BMS, Tokyo) at 2038 ***g*** for 120 s performed five times, 500 μL of chloroform and 200 μL of ultrapure water were added to the homogenate. The solution was mixed well and centrifuged at 2300 ***g*** for 5 min at 4 °C. The resultant water phase was ultrafiltered using a Millipore Ultrafree-MC PLHCC HMT Centrifugal Filter Device, 5 kDa (Millipore, Billerica, MA, USA). The filtrate was dried and then dissolved in 50 μL of ultrapure water. The samples obtained were then subjected to CE-TOFMS analysis using the Agilent CE-TOFMS system (Agilent Technologies, Santa Clara, CA, USA) at 4 °C. The detected peaks were aligned according to their m/z values and normalized migration times. The peaks were mean-centered and scaled using their standard deviations on a per peak basis as a pretreatment. After autoscaling, principal component analysis (PCA) and hierarchical clustering analysis (HCA) were conducted using SAMPLESTAT ver. 3.14 and PeakStat ver. 3.18 (Human Metabolome Technologies Inc., Tsuruoka, Japan), respectively. In the PCA, a score plot of the first and second principal components was generated. In the HCA, the resulting datasets from each group were clustered by Euclidean distance using Ward method [11,14]. Heat maps were generated by coloring the data across their value ranges. The relative area of each peak was calculated and compared between the control and FOXO1-activated cells. The metabolomics data was submitted to Metabolomics Workbench (www.metabolomicsworkbench.org).

Quantitative real-time RT-PCR analysis

Total RNA was isolated from tissue homogenates using the TRIzol reagent (Thermo Fisher Scientific Inc., Tokyo, Japan). cDNA was synthesized using 500 ng of each RNA sample with a ReverTra Ace qPCR RT Master Mix Transcription Kit (Toyobo, Tokyo, Japan). Gene expression levels were measured as described previously [11]. The mouse-specific primer pairs used are as shown in Table S1.

Results

Metabolomic analysis

C2C12 cells stably overexpressing FOXO1-ER fusion protein were treated with vehicle (control) or tamoxifen (FOXO1-activated). To evaluate whether FOXO1 is activated in the cells, FOXO1-target cathepsin L gene expression was examined. As expected, we observed a marked increase in cathepsin L mRNA following treatment with tamoxifen (Fig. S1). In contrast, in control C2C12 cells, tamoxifen treatment did not change cathepsin L mRNA level (Fig. S1). The data indicate tamoxifen did not nonspecifically increase cathepsin L levels, confirming the validity of the cell system. In the metabolomic analysis, 199 peaks (91 cations and 108 anions) were detected by the cation and anion modes of CE-TOFMS. The PCA results for the detected peaks are presented in Fig. 1. The first principal component effectively and distinctly separated the two groups (x-axis), suggesting that FOXO1

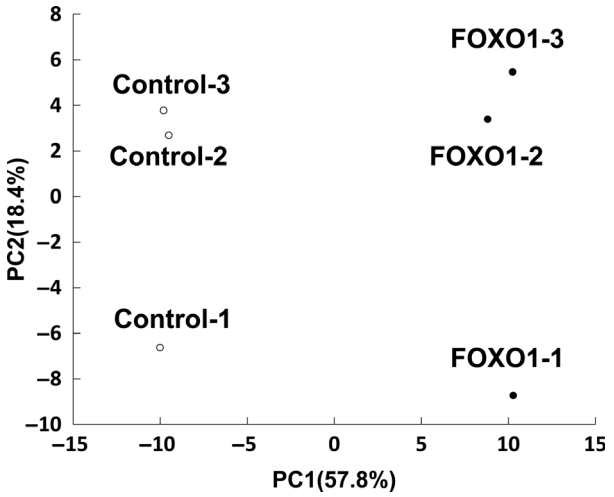

Fig. 1. Principal component analysis (PCA) of metabolomic datasets of samples from vehicle-treated (control) and tamoxifen-treated (FOXO1-activated) cells. Three cell dishes were used in each group (Control-1 to Control-3 for vehicle and FOXO1-1 to FOXO1-3 for tamoxifen-treated cells). PCA was conducted with the determined data peaks using SAMPLESTAT ver. 3.14. Plots of the control (open circles) and the FOXO1-activated (filled circles) cells are clearly distinguished on the PC1 axis (*x*-axis).

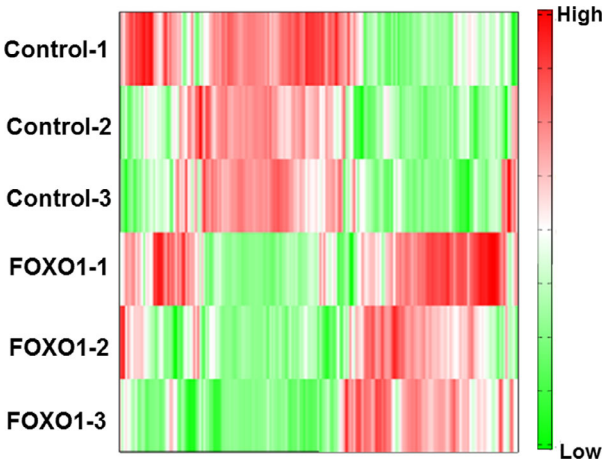

Fig. 2. A heat map comparing metabolite changes between control and FOXO1-activated cells. The vertical axis shows sample names corresponding to the samples used in Fig. 1 (Control-1 to Control-3 for vehicle and FOXO1-1 to FOXO1-3 for tamoxifen-treated cells). The heat map patterns between the control (upper three lanes) and FOXO1-activated (lower three lanes) are clearly distinguishable. Red indicates that the relative contents of metabolites are high, whereas green indicates that the relative contents of metabolites are low.

activation caused a significant change in the overall metabolite profile of myoblasts. As demonstrated by a heat map analysis (Fig. 2), cell specimens from the control and the tamoxifen treatment segregated into two groups, indicating that FOXO1 activation had profound effects on the metabolite profiles of the cells. Considered together, the PCA and heat map results indicate that FOXO1 activation significantly influenced the metabolite profile of myoblasts and clearly distinguished the two groups. The relative area values of the detected metabolic products in the control and the FOXO1-activated cells are listed in Table S2. Of these, the metabolites that exhibited significant changes are listed in Table 1. The numbers of metabolites that exhibited significant decreases and increases in FOXO1-activated cells relative to the control cells were 36 and 41, respectively. The metabolites are involved in various pathways, particularly pathways associated with glucose and amino acids.

Glycolysis

Glycolysis-related metabolite levels were changed. In FOXO1-activated cells, increased levels of glucose-1-phophate, glucose-6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, dihydroxyacetone phosphate, glyceraldehyde 3-phophate, 3-phosphoglyceric acid, 2-phosphoglyceric acid, phosphoenolpyruvic acid, lactic acid, and acetyl CoA were observed (Fig. 3A). The gene expression in rate-limiting enzymes of the glycolytic pathway, such as hexokinase 2, 6-phosphofructokinase, and pyruvate kinase [15], did not change (Fig. 3B). With regard to the glycolysis pathway, the gene expression in phosphoglucomutase and lactate dehydrogenase did not change (Fig. 3B). In addition, the gene expression in pyruvate dehydrogenase kinase 4 (PDK4), which suppresses pyruvate dehydrogenase activity (catalyzes conversion of pyruvate to acetyl CoA) [5], markedly increased in FOXO1-activated cells (Fig. 3B).

Amino acid metabolism and TCA cycle

Branched chain amino acids (BCAA), including valine (Val), leucine (Leu), and isoleucine (Ile), are mainly catabolized in skeletal muscles, and are metabolized into acetyl CoA and succinyl CoA and used as sources of energy [16]. In FOXO1-activated cells, BCAA levels tended to decrease (Val; $P = 0.056$, Leu; $P = 0.073$, Ile; $P = 0.052$) (Fig. 4A). Consistently, the levels of gene expression in metabolic enzymes of BCAA including branched chain amino acid aminotransferase (BCAT2) and branched chain alpha-keto acid dehydrogenase (BCKDHa) significantly increased (Fig. 4B).

Concentrations of numerous other amino acids decreased in FOXO1-activated cells (Fig. 5). The metabolites of the TCA cycle (acetyl CoA, fumaric

Table 1. List of significant changes in metabolites in FOXO1-activated cells 'Ratio' is the comparative value of the relative areas (FOXO1-activated vs. control). The *P*-value was calculated using the Welche's *t*-test (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$).

	FOXO1 vs Control	
Compound name	Ratio	*P*-value
Dihydroxyacetone phosphate	8.1	0.025*
Glyceraldehyde 3-phosphate	6.3	0.002**
XA0055	6.3	0.014*
Fructose 6-phosphate	4.2	2.4E-04***
Glucose 6-phosphate	3.1	0.001**
Acetyl CoA_divalent	3.0	0.002**
Sedoheptulose 7-phosphate	2.9	0.006**
Uric acid	2.7	1.8E-03**
Ribose 5-phosphate	2.6	0.041*
Ribulose 1,5-diphosphate	2.4	0.029*
Ribulose 5-phosphate	2.4	0.028*
Gluconic acid	2.0	2.4E-02*
XA0002	1.9	0.004**
3-Phosphoglyceric acid	1.8	0.001**
2,3-Diphosphoglyceric acid	1.8	0.019*
PRPP	1.8	2.1E-02*
Glucose 1-phosphate	1.8	3.5.E-04***
Fumaric acid	1.8	0.004**
GDP-glucose	1.7	0.006**
GDP-mannose		
GDP-galactose		
Phosphoenolpyruvic acid	1.7	4.5E-02*
Malic acid	1.7	0.010**
O-Succinylhomoserine	1.6	0.025*
Glycerol 3-phosphate	1.6	5.6.E-04***
N-Acetylserine	1.4	1.8E-02*
6-Phosphogluconic acid	1.4	0.007**
Choline	1.4	0.003**
Sorbitol 6-phosphate	1.4	3.7.E-04***
CoA_divalent	1.3	3.4E-02*
myo-Inositol 2-phosphate	1.3	0.030*
Ethanolamine	1.3	0.025*
Thiamine diphosphate	1.2	0.039*
Carnitine	1.2	9.5E-03**
CMP-*N*-acetylneuraminate	1.2	0.009**
GMP	1.2	0.037*
Rhein	1.2	0.038*
Pro	1.0	2.2E-02*
Gly	0.9	0.009**
β-Ala	0.8	8.2.E-04***
Phosphocreatine	0.8	0.008**
3-Guanidinopropionic acid	0.8	1.5E-02*
XA0033	0.8	0.009**
Phosphorylcholine	0.8	2.1.E-04***
2-Aminoisobutyric acid	0.8	0.022*
2-Aminobutyric acid		
Glutathione (GSH)	0.8	4.7E-02*
N-Acetylglucosamine 1-phosphate	0.8	0.003**
NAD^+	0.8	0.011*
Glutathione (GSSG)_divalent	0.7	0.049*
Thr	0.7	7.4E-05***
Ala	0.7	5.1.E-04***
Glycerophosphocholine	0.7	0.001**
GABA	0.7	0.004**
1-Methylhistidine	0.7	3.8E-02*
3-Methylhistidine		
UDP-*N*-acetylgalactosamine	0.7	0.013*
UDP-*N*-acetylglucosamine		
Methionine sulfoxide	0.6	0.003**
Hydroxyproline	0.6	0.002**
His	0.6	0.017*
Asn	0.6	0.015*
dTDP-glucose	0.6	0.013*
Met	0.6	0.052
Ophthalmic acid	0.6	7.3E-05***
1-Methylnicotinamide	0.6	3.5.E-04***
Asp	0.6	0.004**
XC0016	0.6	4.2.E-05***
Hypotaurine	0.6	1.7E-04***
Argininosuccinic acid	0.6	0.038*
Cystathionine	0.6	5.6E-04***
Gln	0.5	5.6.E-06***
4-Guanidinobutyric acid	0.5	0.004**
Phe	0.5	0.026*
*N*6-Methyllysine	0.5	2.7E-03**
Lys	0.5	1.4.E-05***
Ornithine	0.5	0.003**
Arg	0.5	6.2.E-05***
Trp	0.5	2.1E-03**
Tyr	0.5	0.005**
Guanidoacetic acid	0.4	0.001**
O-Phosphoserine	0.4	0.008**
Pantothenic acid	0.3	0.007**

acid, and malic acid) are significantly increased in the FOXO1-activated cells (Fig. 6A). The gene expression in TCA cycle enzyme succinate dehydrogenase marginally increased, but malate dehydrogenase, citrate synthase, aconitase, and isocitrate dehydrogenase was not altered (Fig. 6B).

Discussion

The CE-TOFMS is a highly sensitive method to separate many different molecules in a complex mixture, such as cellular extract, as described here. A limitation of this method is the exclusion of hydrophobic molecules from the analysis. Despite this limitation, this study used the best possible method to determine water-soluble metabolites in cell extracts. As described in the Results section, we observed that various metabolites changed in FOXO1-activated cells.

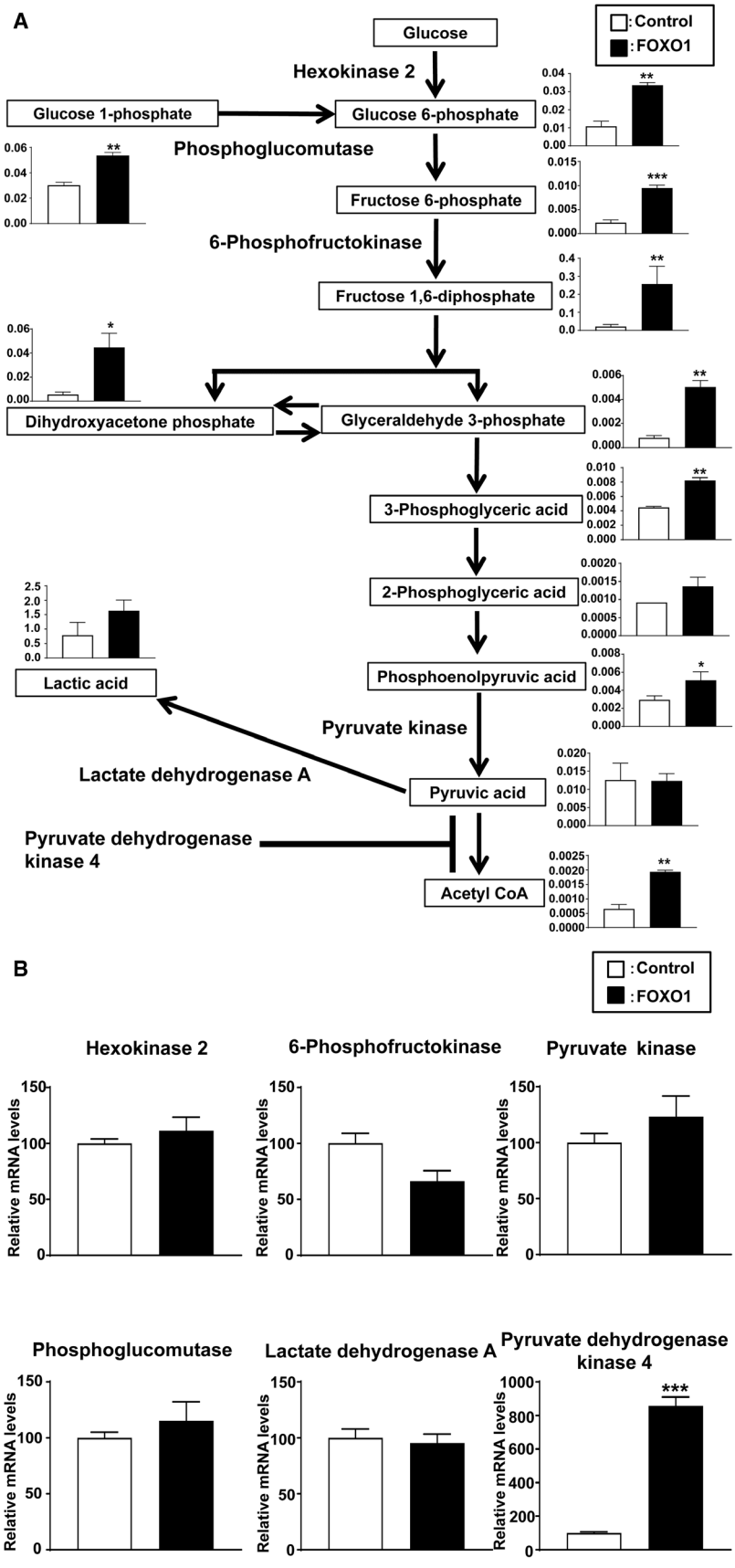

Fig. 3. Metabolic changes involved in glucose metabolism. A) Metabolite changes in the control and FOXO1-activated cells are shown. Relative metabolite changes shown in the graphs were obtained by CE-TOFMS (Table S2). Open bars, control; filled bars, FOXO1-activated C2C12 cells. Data are expressed as mean ± SD ($N = 3$); $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$. B) Gene expression of glucose metabolism in the control and FOXO1-activated cells. Open bars, control; filled bars, FOXO1-activated C2C12 cells. Data are expressed as mean ± SE ($N = 3$); $^{***}P < 0.001$.

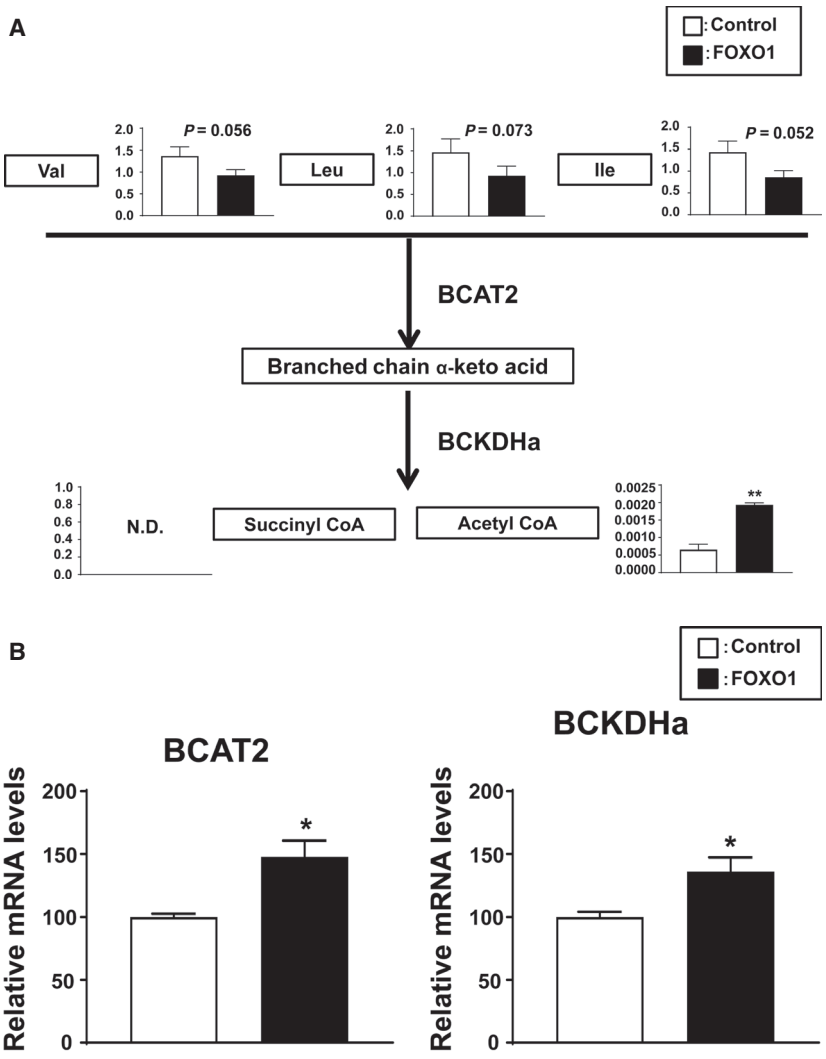

Fig. 4. Metabolic changes involved in the BCAA metabolism. A) Metabolite changes in the control and FOXO1-activated cells are shown. Relative metabolite changes shown in the graphs were obtained by CE-TOFMS (Table S2). Open bars, control; filled bars, FOXO1-activated C2C12 cells. Data are expressed as mean ± SD ($N = 3$); $^{**}P < 0.01$. B) Gene expression of BCAA metabolism in the control and FOXO1-activated cells. Open bars, control; filled bars, FOXO1-activated C2C12 cells. N.D., not detected. Data are expressed as mean ± SE ($N = 3$); $^{*}P < 0.05$.

Combined with representative gene expression analysis, by quantitative reverse-transcription PCR analysis, the metabolic change occurring in FOXO1-activated cells are surmised below:

Concerning glycolysis, because of increased expression of PDK4 (Fig. 3B), the rate of conversion of pyruvate into acetyl CoA was likely decreased, which could have led to an increase in glycolysis-related metabolite levels in FOXO1-activated cells. Namely, the glycolytic pathway is likely suppressed, which does not contradict the role of FOXO1 in muscle cells during fasting (saving glucose for other tissues such as brain). An increase in acetyl CoA (Fig. 3A) could be attributed to an increase in acetyl CoA production from other sources, such as fatty acids and amino acids (described later).

Generally, amino acids (and fatty acids) are metabolized and integrated into the TCA cycle. Ala, Ile, Leu, Lys, Phe, and Trp are metabolized into acetyl CoA; Arg, Gln, and His are metabolized into 2-oxoglutaric acid; Ile, Met, Thr, and Val are metabolized into succinyl CoA; Tyr is metabolized into fumaric acid; and Asn and Asp are metabolized into oxaloacetic acid [17] (Fig. 6A). The decreased levels of amino acids, observed in the FOXO1-activated cells, were probably because they were used in the TCA cycle and the electron-transfer system to produce ATP. To support this, a flux-type study using a stable isotope tracer for the relevant amino acids should be performed to demonstrate accelerated amino acid catabolism and incorporation into downstream metabolites (e.g., TCA cycle intermediates). Nevertheless, it can be surmised that the amino acids could be sources of energy. Since FOXO1 activates proteolysis, for instance, in the ubiquitin–proteasome and autophagy–lysosome pathways [8,9], FOXO1 activation may cause protein

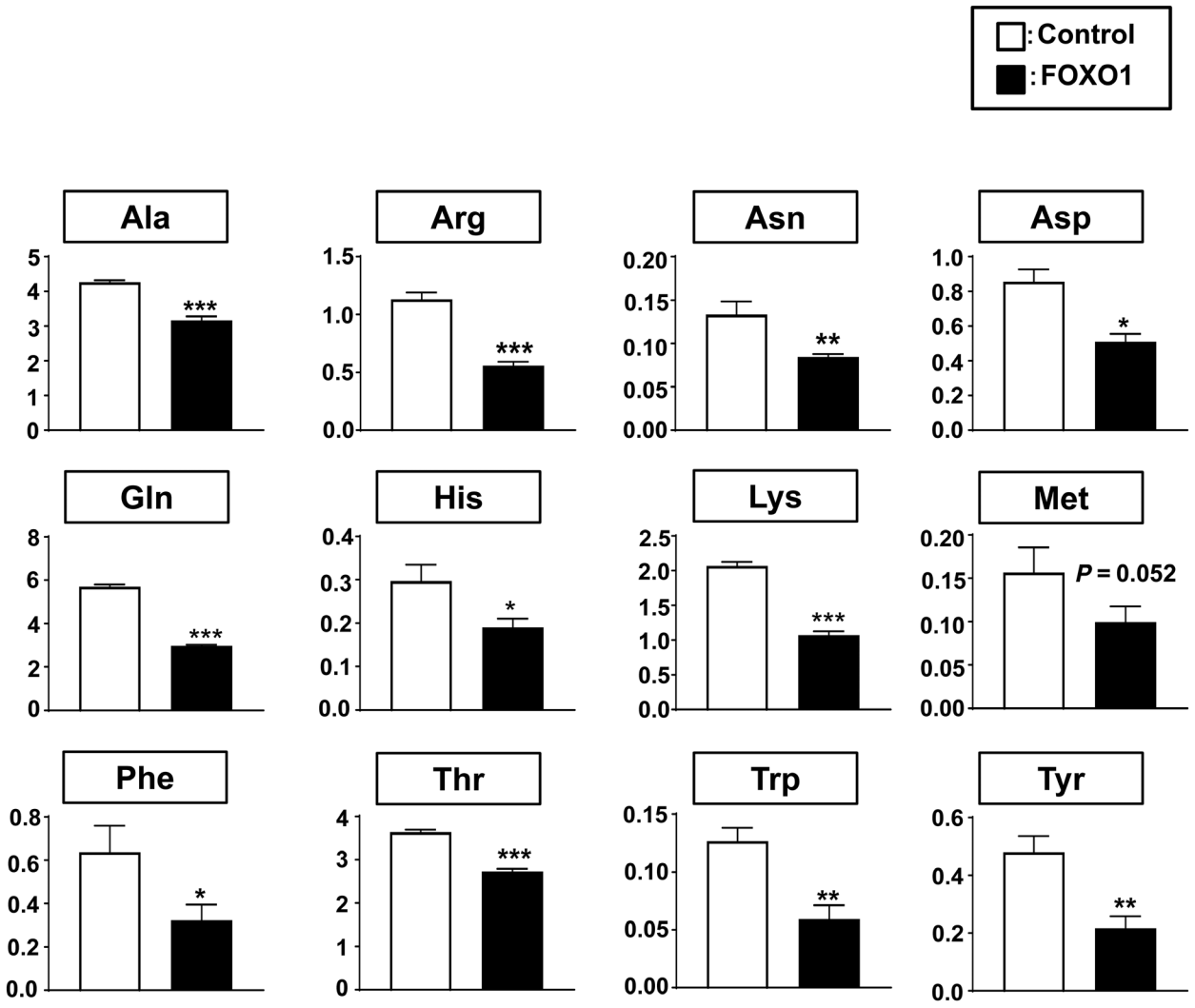

Fig. 5. Changes in amino acid levels. Amino acid levels in the control and FOXO1-activated cells are shown. Relative amino acid changes shown in the graphs were obtained by CE-TOFMS (Table S2). Open bars, control; filled bars, FOXO1-activated C2C12 cells. Data are expressed as mean ± SD ($N = 3$); ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

degradation and provide an energy source from the generated amino acids.

It is well known that fatty acids are used in the skeletal muscle during fasting. Indeed, a previous study clearly showed that the activation of FOXO1 in C2C12 cells increased the capacity for fatty acid oxidation [7]. Namely, Bastie *et al.* used C2C12 cells stably overexpressing FOXO1-ER fusion protein treated with tamoxifen (as used in the present study) and reported increases in fatty acid oxidation activity, triglyceride content, fatty acid uptake, and expression of acyl CoA oxidase (ACO) and CD36 (fatty acid transporter) [7]. Indeed, we observed increased ACO and CD36 mRNA levels in the FOXO1-activated cells (Fig. S2). Although our method described in this paper (CE-TOFMS) is not able to directly analyze hydrophobic metabolites, it is possible to speculate with the change of hydrophilic metabolites vicinity surrounding them. Namely, we observed increases in dihydroxyacetone phosphate (Fig. 3A) and glycerol 3-phophate (Table 1), which may lead to increased triglyceride content. During fatty acid beta-oxidation, acetyl CoA is produced and NAD^+ is metabolized to NADH. Consistent with this, in our data, the acetyl CoA level increased (3.0-fold), NAD^+ decreased (0.8-fold), and NADH increased (1.3-fold) (Table 1 and Table S2). These findings also suggest the importance of FOXO1 in fatty acid metabolism. In our data, BCAA

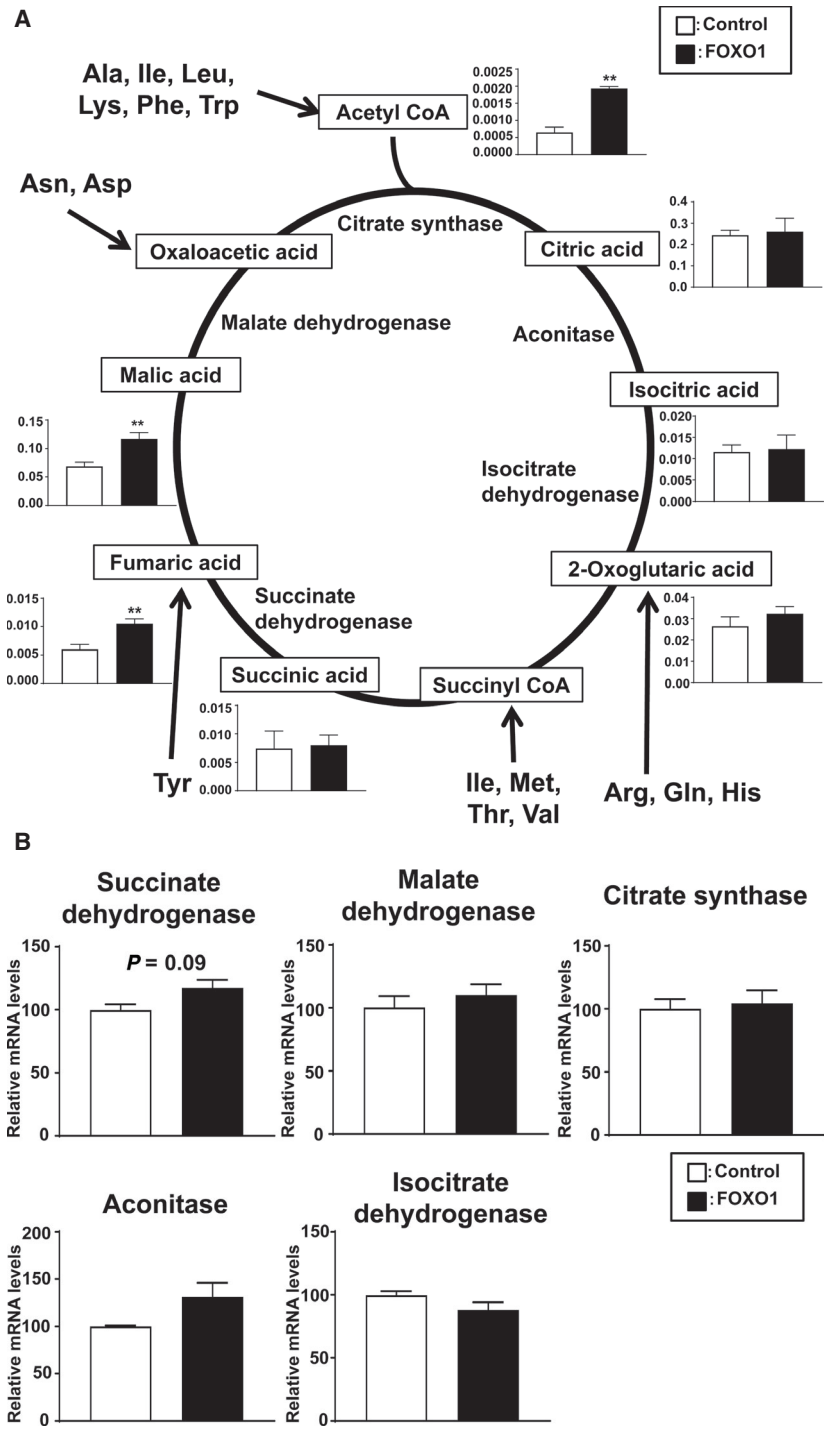

Fig. 6. Metabolic changes involved in the TCA cycle. (A) Metabolite changes in the control and FOXO1-activated cells are shown. Relative metabolite changes shown in the graphs were obtained by CE-TOFMS (Table S2). Open bars, control; filled bars, FOXO1-activated C2C12 cells. Data are expressed as mean ± SD ($N = 3$); $^{**}P < 0.01$. (B) Gene expression of TCA cycle in the control and FOXO1-activated cells. Open bars, control; filled bars, FOXO1-activated C2C12 cells. Data are expressed as mean ± SE ($N = 3$).

catabolism was suggested to be activated. In our method, we do not have information about the contribution (comparison) of BCAA and fatty acids, but FOXO1 may play roles in BCAA metabolism, as BCAA is mainly catabolized in skeletal muscles [16], and used as a source of energy in certain conditions, such as exercise and fasting [16,18].

Conclusion

Our study aimed to examine global metabolite changes caused by FOXO1 in muscle cells in an unbiased manner. In the metabolomics analysis using tamoxifen-inducible FOXO1-activating myoblasts, we observed suppression of glucose utilization and increased protein/

amino acid utilization. The results of this study offer insights into the role of FOXO1 in the muscle cells, particularly during adaptation to low energy conditions.

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Author contributions

RM and RU analyzed the data and undertook the statistical analyses. MO and YH, performed cell experiment and collected the data. YK prepared the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Gene expression of FOXO1-target cathepsin L in the C2C12 cells (left graph) and C2C12 cells-expressing FOXO1-ER fusion protein (right graph).
Fig. S2. Gene expression of CD36 and acyl CoA oxidase in the control and FOXO1-activated cells.
Table S1. Primer pairs used for quantitative RT-PCR.
Table S2. List of metabolites detected in CE-TOFMS.