Doctoral Dissertation

Study on Physiological Function of Acetic Acid in Skeletal Muscle

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CONTENTS

Abbreviations

OLETF	Otsuka Long-Evans Tokushima Fatty
AceCS	acetyl-CoA synthetase
AMPK	AMP-activated protein kinase
AID	auto-inhibitory domain
CTD	carboxy-terminal domain
CBS	cystathionine β-synthase
LKB1	liver kinase B1
CaMKKβ	calcium-calmodulin-dependent protein kinase kinase β
CBM	carbohydrate-binding module
KD	kinase domain
GLUT4	glucose transporter type 4
LETO	Long-Evans Tokushima Otsuka
ACC	acetyl-CoA carboxylase
FAS	fatty acid synthase
LCACD	long-chain acyl-CoA dehydrogenase
3KACT	3-ketoacyl-CoA thiolase
MYO	myoglobin
KLF15	kruppel-like factor 15
PPARδ	Peroxisome proliferator-activated receptor delta
PPARγ	Peroxisome proliferator-activated receptor gamma
ChREBP	carbohydrate-responsive element binding protein
MEF2	myocyte enhancer factor2
DMEM	Dulbecco's modified eagle medium
FBS	fetal bovine serum
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
AICAR	5-Aminoimidazole-4-carboxamide 1-beta-D-ribofuranoside
araA	adenine 9-β-D-arabinofuranoside
MADS	MCM1-agamous-deficiens-serum response factor
MAPK	mitogen-activated protein kinase
TGF-β	transforming growth factor β

Chapter I Introduction

Acetic acid is a short-chain fatty acid with 2 carbons of carboxyl group and it has a distinctive sour taste and pungent smell. Acetic acid is familiar food-composition that is the main component of vinegar. Vinegar contains about 4 to 8 % acetic acid [1,2]. The existence of vinegar was recorded in Babylonia at 5,000 BC [3] and it has been used for centuries as food preservative, taste enhancing and folk medicine. Vinegar is believed to have several health benefits. In human studies, it has shown an effect on reduced the glycemic index [4], reduced body weight, body fat mass and serum triglyceride levels [5], and prevent of hypertension [6]. The principle bioactive component of vinegar is acetic acid. Previously, it was reported that orally administered acetic acid exhibited to prevent hyperglycemic obesity with hyperinsulinemia and protect against fat accumulation in Otsuka Long-Evans Tokushima Fatty (OLETF) rats [7]. The acetic acid was absorbed by tissues and activated to acetyl-CoA with the concomitant formation of AMP by the catalytic activity of acetyl-CoA synthetase (AceCS) in the cytosol. The increase in AMP concentration led to an increase in the AMP/ATP ratio, following the phosphorylation of AMP-activated protein kinase (AMPK) [7]. AMPK is a heterotrimeric enzyme with α, β and γ subunits. The α-subunit contain a kinase domain at the N-terminal side, including the activation loop that has Thr-172, and an auto-inhibitory domain (AID) at the C-terminal side. On the C-terminal side, there is α-subunit carboxy-terminal domain (α-CTD) which binds to β-subunit via linker. The β-subunit is forming the core of the complex because of having β-subunit carboxy-terminal domain (β-CTD) that binds to the α-CTD of α-subunit and the amino terminus of γ-subunits. The γ-subunit has four tandem repeat of sequences that are named CBS1-CBS4 (CBS; cystathionine β-synthase). These act in pairs to form the binding sites for adenine nucleotides (AMP, ADP and ATP). The binding sites are termed site 1-site 4, correspond to the CBS1-CBS4 domain. Site 2 appears to be empty and site 4 is always occupied by AMP. In the basis of state, sites 1 and 3, that are regulatory sites, are preempted by ATP. Whereas phosphorylation of Thr-

172 is promoted when AMP or ADP replace ATP at site 3. Furthermore, allosteric activation occurs by replacement ATP by AMP at site 1 (Fig 1) [8–13]. Activation of AMPK requires phosphorylation of Thr-172 in the α-subunit [14]. AMP binding to the γ-subunit facilitates Thr-172 phosphorylation by upstream kinase, liver kinase B1 (LKB1) or calcium-calmodulin-dependent protein kinase kinase β (CaMKKβ), and induce significant structural change and protect dephosphorylating by protein phosphatases (Fig 2) [8,11,15]. AMPK is a crucial cellular energy sensor and, can also influence metabolism and energy balance at the whole-body level. Therefore, activation of AMPK has important role in the prevention of obesity and diabetic, since activation of AMPK is involved in many physiological responses involving with anti-obesity and anti-diabetic action.

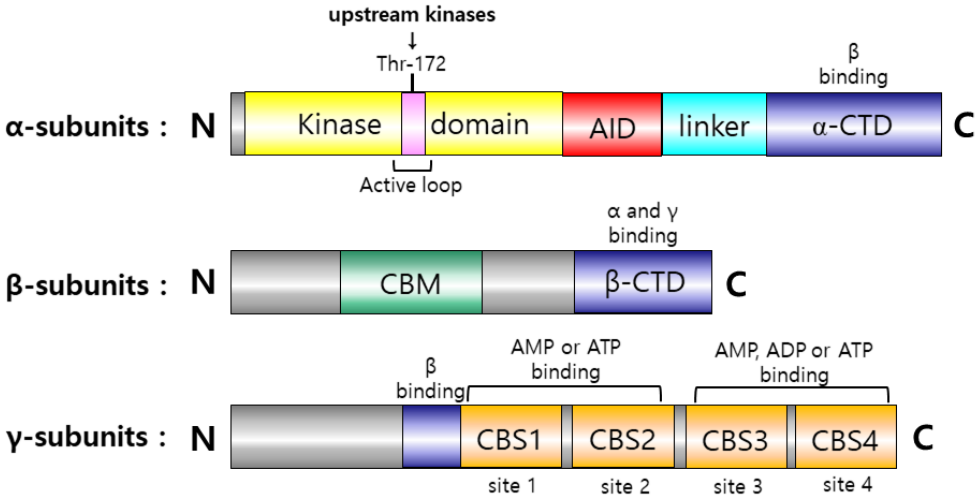

Fig 1. Schematic domain of the components of heterotrimer of AMPKs

The catalytic α-subunits conventional serine/threonine kinase domains containing the threonine residue phosphorylated by upstream kinases. The β-subunit has carbohydrate-binding module (CBM) to which binds starch or glycogen.

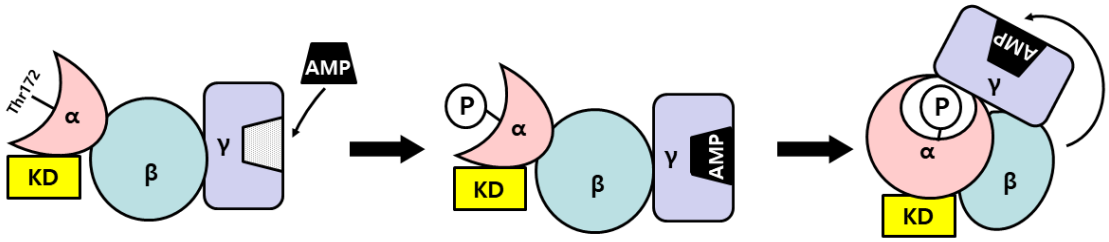

Fig 2. Illustration of the AMPK activation by AMP.

The AMPK complex consists of 3 different subunits: the α catalytic subunit that has a kinase domain (KD) and β and γ regulatory subunits. AMP binding to the γ-subunit causes a conformational change of the AMPK complex, which increases phosphorylation of the α-subunit at Thr-172, primarily by preventing dephosphorylation by protein phosphatases.

Obesity and type 2 diabetes are increasing throughout the world due to change in lifestyles, dietary habits, or lack of exercise. The International Diabetes Federation Diabetes Atlas reported that there were 415 million people living with diabetes in the world at 2015. In addition, the number is expected to rise to 642 million by 2040. Also it has been reported to reach 7.2 million at 2015 in Japan [16]. One of the reason for continuous increase in diabetes is rapid aging in Japan, which is a serious social problem. There are type 1 and type 2 diabetes. Most of the diabetic patients worldwide are type 2 diabetes. Type 2 diabetes is thought to be caused by obesity caused by lifestyle such as overeating and lack of exercise. It is reported that glucose metabolisms in liver, adipose tissues and brain of patients with type 2 diabetes are not different from healthy subjects, whereas it is reduced by about 35 to 40% in muscles of those patients [17]. Skeletal muscle mass decline with lack of exercise and aging, and loss of skeletal muscle directly leads to a decrease in energy metabolism and to an increase in the risk of obesity and diabetes. Skeletal muscle is the largest energy consuming organ and one of the important insulin response organ. Therefore, skeletal muscle is an important target in the study of obesity and diabetes.

The present author attempted to investigate the physiological function of acetic acid in skeletal muscle. The detail experimental data about the effect of acetic acid on laboratory animals shows in Chapter II. The mechanism of acetic acid by using cultured cell shows in Chapter III.

Chapter II

Effects of Acetic acid on Lipid Metabolism in Muscles and Adipose Tissues of Type 2 Diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) Rats

Obesity causes excess fat accumulation in adipose tissue, skeletal muscles and the liver. Especially, muscle lipid accumulation causes a predisposition to decrease insulin sensitivity [18–21]. We have reported that acetic acid had a profound anti-obese and anti-diabetic function in animal models of obesity and type 2 diabetes [7]. Acetic acid is an endogenous metabolite of fatty acid β-oxidation in the liver mitochondria under starving conditions [22]. Acetic acid formed in the liver is excreted into the blood stream and is utilized as a biological fuel in the extrahepatic tissues under such conditions [22]. However, under fed conditions, orally administered acetic acid was immediately taken up from the intestine and excreted into the blood stream [7]. The acetic acid induced he phosphorylation of AMPK by increased in the AMP/ATP ratio [7]. AMPK acts as the key metabolic master switch, and regulates a number of enzymes involved in lipid homeostasis [23–28]. The ingested acetic acid activated AMPK by increasing the AMP/ATP ratio in the liver, and decreased the transcripts of the lipogenic genes [7]. Acetic acid administration protected the OLETF rats against obesity. It contributed to lowering the accumulation of abdominal fat and protected from the accumulation of lipid in the liver. An accumulation of excess lipid in the liver or skeletal muscle disturbs insulin signaling [29]. The marked reduction of lipid content in the adipose tissue and liver due to treatment with acetic acid contributed to improved glucose tolerance and insulin resistance as secondary effects in OLETF rats.

The skeletal muscle is one of the most important insulin-responsive organs in the body [29,30], and it is possible that accumulation of locally derived fat metabolites in it is an important factor contributing to insulin resistance [29]. In the present study, we investigated the effects of acetic acid on the skeletal muscle and adipose tissues.

MATERIALS AND METHODS

Acetic acid administration experiment

Four-week-old male OLETF rats and Long-Evans Tokushima Otsuka (LETO) rats as a non-diabetic control were obtained from Otsuka Pharmaceutical (Tokushima, Japan) and fed a normal laboratory diet (CE2, Clea, Tokyo) for 1 week to stabilize the metabolic conditions. The rats were housed individually in an air-conditioned room at approximately 25°C with alternating 12 hr periods of light and dark (light, 8:00-20:00). All the animals were allowed free access to water and the appropriate diet. The OLETF rats were randomly assigned to two groups: water-injected and acetic acid-injected. The water-injected group was given distilled water at 5ml/kg of body weight, and the acetic acid-injected group was given 52.5 mg/kg of body weight (1% v/v) acetic acid of 5ml/kg of body weight) between 9:30 and 10:30 daily 5 day a week for 6 months. The initial body weights of the LETO, water-administered OLETF, and acetic acid-administered OLETF rats were 84 ± 26, 126 ± 10, and 125 ± 4 g respectively. Food consumption and body weight were recorded every day. At 32 weeks of age, the rats were anesthetized by intraperitoneal injection of Nembutal, (100 μl/100g of body weight), and muscles, white and brown adipose tissues were immediately isolated, weighed, frozen in liquid nitrogen, and stored at -80˚C for subsequent isolation of RNA. Part of the white adipose and brown adipose tissues of each rat was subjected to histochemical analysis. Tissue samples were taken 24 hr after injection of water or acetic acid. The care and use of the animals in this study followed the guidelines of Okayama Prefectural University and the laws and notifications of the Japanese government.

O_2 consumption rates of OLETF rats administrated water or acetic acid and LETO rats.

Oxygen consumption was measured with an O_2/CO_2 metabolism measuring system (model MK-5000, Muromachi Kikai, Tokyo). Each rat was in a sealed chamber with an air flow of 3.5 L/min for 24 hr at 25˚C with free access to water and the diet. The consumed oxygen concentration (VO_2) was calculated.

Preparation of cRNA probe for Northern blotting.

The cDNA fragments for rat acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), long-chain acyl-CoA dehydrogenase (LCACD), 3-ketoacyl-CoA thiolase (3KACT), myoglobin (MYO), GLUT4, krupple-like factor (KLF15), peroxisome proliferator-activated receptor delta (PPARδ) , and peroxisome proliferator-activated receptor gamma (PPARγ) were obtained by polymerase chain reaction from a cDNA library for the rat heart (Takara Shuzo, Shiga, Japan) and rat brown adipose tissue mRNA that had been isolated with an mRNA isolation kit (Roche, Mannheim, Germany) from total RNA of rat brown adipose tissue. First-strand cDNA was prepared using Superscript II transcriptase primed with oligo-dT. The PCR primers used were as follows: ACC: 5' primer, 5'-GTTTGGCCTTTCACATGAGGTC-3', and 3' primer, 5' -GTGGGGATACCTGCAGTTTGAG-3'. FAS: 5' primer, 5' -GACCCCTGTGGTGTTTGAGAAC-3', and 3' primer, 5' -CTTGAGTGTCTCCCTCCAGCAT-3'. LCACD: 5' primer, 5' -CAAAAGGTCTGGGAGTGATTGG-3', and 3' primer, 5' -GTCCAGACGTTTGGTTTCATGC-3'. 3KACT: 5' primer, 5' -AGAAGACTGCGACAGATACGCC-3', and 3' primer, 5' -TATTTTCCACCTCGACGCCTTA-3'. PPARδ: 5' primer, 5'-ACAACGCTATCCGCTTTGGAAG-3', and 3' primer, 5'-

AAGAACTCATGGGTGACGAAGC-3'. PPARγ: 5' primer, 5'-GGTTGACACAGAGATGCCATTC-3', and 3' primer, 5'-ACTTCTGAAACCGACAGTACTG-3'. MYO: 5' primer, 5'-GGAGTGGCAGATGGTGCTAAAC-3', and 3' primer, 5' -GGAAGCCCAGCTCCTTGTACTT-3'. GLUT4: 5' primer, 5'-GCCCCACAGAAAGTGATTGAAC-3', and 3' primer, 5' -CAGGTTCCGGATGATGTAGAGG-3'. KLF15: 5' primer, 5'-AAGCCTTCTGTTCCTGCTACAG-3', and 3' primer, 5' -TGCTTCCTGCTTCACAGCAACA-3'. The polymerase chain reaction was performed with Taq DNA polymerase (Takara Shuzo). Forty-one cycles of amplification were made following this program: 94°C, 1min; 66°C, 1 min; and 72°C, 2 min. The amplified products were subcloned into pGEM-T Easy vector (Promega, Madison, WI). These plasmid DNAs were used in DIG-RNA labeling by DIG labeling system (Roche, Schwiiz), and the cRNA probes were used as probes in Northern blotting.

Northern blotting.

Total RNA was extracted from each tissue using Isogen (Nippon Gene, Toyama, Japan). The RNA was dissolved in diethylpyrocarbonate-treated water and quantified by its absorbance at 260 nm. Total extracted RNA was denatured with formaldehyde and electrophoresed in 1% agarose gel containing formaldehyde; the amounts of loaded RNAs were checked by staining the 28 and 18 S rRNA bands with ethidium bromide. After capillary transfer to a nylon membrane (Roche, Schwiiz) and UV crosslinking, the membrane was hybridized overnight at 68˚C with DIG-labeled cRNA probes recognizing ACC, FAS, LCACD, 3KACT, PPARδ, PPARγ, MYO, GLUT4, and KLF15 respectively. The filter was washed twice with 2 × SSC and 0.1% SDS at room temperature, further washed twice with 0.1 × SSC and 0.1% SDS at 68˚C for 30 min, incubated with a blocking

solution and anti-DIG antibody-conjugated alkaline phosphatase for 30 min at room temperature, and then detected using CDP-Star (Roche, Schwiiz) by exposure to an X-ray film. The amount of each transcript was quantified with an image analyzer (Gel Print 2000i, Genomic Solutions, Tokyo) and was expressed as relative intensity. Each membrane was reprobed with β-actin to ensure that the changes observed did not reflect any unequal loading of the samples.

Histological analysis of white adipose and brown adipose tissues.

Small samples of tissues were fixed with a 20% formalin neutral buffered solution (Wako, Osaka, Japan) and embedded in paraffin. Sections (4.5 μm) were cut and stained with hematoxylin and eosin. Images were captured with a CCD camera (Olympus Optical, Tokyo) at a magnification of × 100 or 200.

AMP, ADP, and ATP assays.

The removed muscles were frozen quickly in liquid nitrogen, lyophilized, homogenized with ice-cold 0.5 N perchloric acid, neutralized, and centrifuged. The concentrations of AMP, ADP, and ATP in the various muscle extracts were determined by reverse-phase HPLC analysis.

Western blotting

The rat muscle samples were cut with scissors into small pieces, suspended in 20 mM potassium phosphate (pH 7.4), and homogenized with potter-Elvehjem equipment. The homogenate was centrifuged (20,000 × g, 10 min) to remove the tissue debris. An aliquot (30 μg of protein) of each muscle extract from the OLETF and LETO rats was applied to 12% SDS acrylamide gel electrophoresis and then transferred to an Immobilon-P membrane (Millipore, Osaka, Japan). Immunoblot analysis was performed using the ECL Western blotting detection system (GE Healthcare, UK Ltd.). Membrane sheets were

first incubated with the antibody against phosphopeptides based on the amino-acid sequence surrounding Thr-172 of the α-subunit of human AMPK (Cell Signaling, Beverly, MA) for 1 hr at room temperature, then washed several times and incubated with biotinylated goat anti-rabbit IgG, and subsequently with streptavidin-conjugated horseradish peroxidase, according to the protocol supplied by the manufacturer. We also determined the protein level of AMPKα using a specific antibody for the α subunit of AMPK.

Statistical analysis

The data values are presented as mean ± SE. Results were treated with one-way ANOVA followed by the Tukey-Kramer's post hoc test for multiple comparisons (Figs. 1, 2, 5), or analyzed by unpaired Student's t test (Table 1). P values < 0.05 were considered to represent statistical significance.

RESULTS

Effects of acetic acid on O2 uptake and energy metabolism

At the age of 30 weeks, the average body weight was lower in the LETO and acetic acid-injected OLETF rats than in the water-injected OLETF rats (LETO, 535 ± 39 g; OLETF-acetic acid, 530 ± 35 g; OLETF-water, 679 ± 39 g). These results are similar to previous data. In order to determine whether acetic acid administration affects the total body metabolic rate, oxygen consumption in whole animals was measured. The acetic acid-administered OLETF rats showed higher rates of oxygen consumption than the water-administered OLETF rats in the active state (Fig. 1).

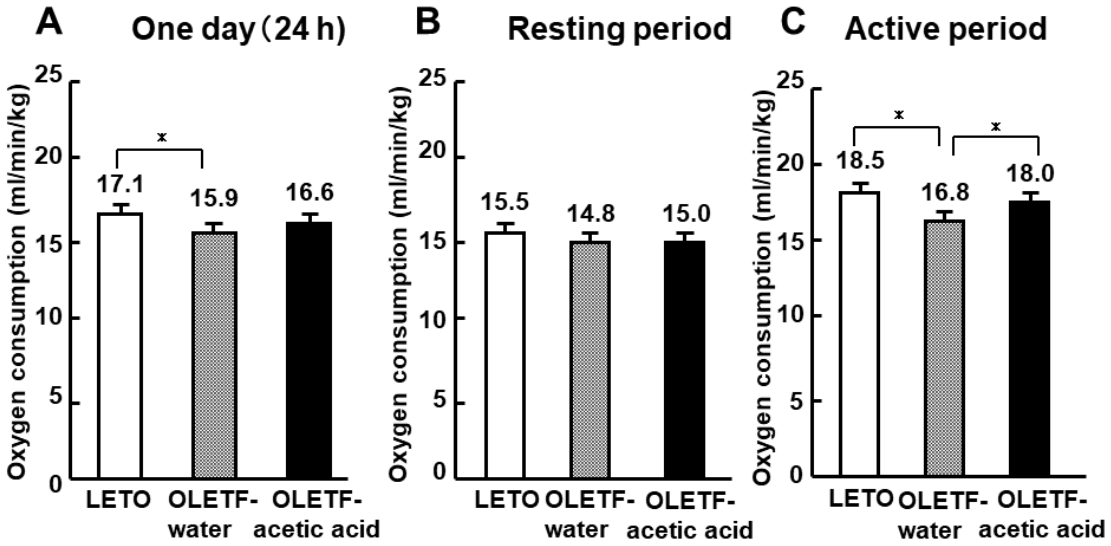

Fig 1. Effects of Acetate Administration on the Oxygen Consumption of OLETF Rats.

Acetic acid (52.5 mg/kg of BW) was orally administered to OLETF rats for 6 months. Oxygen consumption was measured at 20 weeks of age. Resting and active periods were from 7:30 to 19:30 and from 19:30 to 7:30 respectively. Each data value is expressed as the mean ± SE for 3-4 rats. $^{*}p<0.05$ as compared with the water-injected OLETF rats or LETO rats. Reprinted and revised by *BBB* 73(3): 570-576, 2009.

Effects of acetic acid on mRNA expression involved in lipid metabolism in the skeletal muscle

To determine the effects of acetic acid administration on energy metabolism in the skeletal muscles of abdominal and forelegs, mRNA levels associated with energy metabolism were measured by Northern blotting (Fig 2). The genes associated with fatty acid β-oxidation did not change between the water-administered and the acetic acid-administered OLETF rats (Fig 2B), but, as compared to the water-administered OLETF rats, the acetic acid-administered rats were about 2 times higher in transcripts of the myoglobin and GLUT4 genes in the abdominal muscle (Fig 2A). The transcript of the KLF15 gene tended to increase in the abdominal muscle of the acetic acid-administered rats. As for the forelegs, in a similar way, the transcripts of the myoglobin, GLUT4, and KLF15 genes were stimulated by the injection of acetic acid (Fig 3). As for the lipolytic genes, the LCACD and 3KACT mRNA levels were not significantly different between the water- and acetic acid-administered OLETF rats (Fig 3).

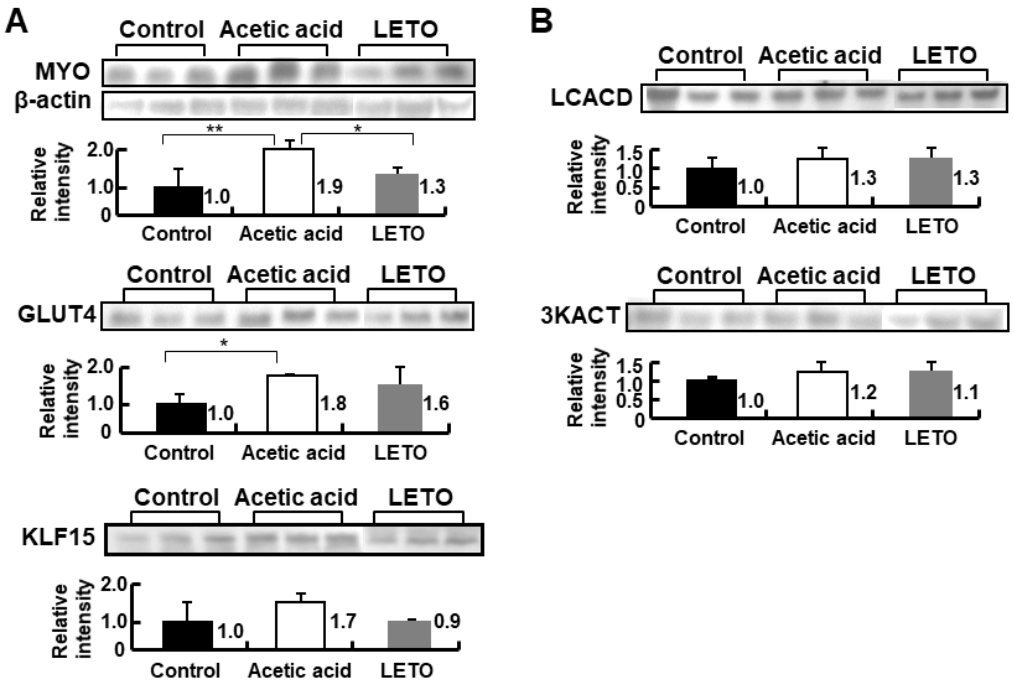

Fig 2. Effects of Acetate Administration on mRNA Levels of the Genes Involved with Energy Metabolism in Abdominal Muscles of OLETF Rats.

Experimental conditions were the same as in Fig. 2. Total RNA from abdominal muscle was isolated from OLETF rats administered water or acetate (52.5 mg/kg of BW) and from LETO rats at 32 weeks of age. Twenty-μg aliquots of total RNA were subjected to electrophoresis and transferred to nylon membranes. The membrane filters were hybridized with the indicated DIG-labeled cRNA probes. Each lane represents a sample from an individual rat. Data values are expressed as mean ± SE for 3 rats. *$p<0.05$; **$p<0.01$ as compared with water-injected OLETF rats or LETO rats. Reprinted and revised by *BBB* 73(3): 570-576, 2009.

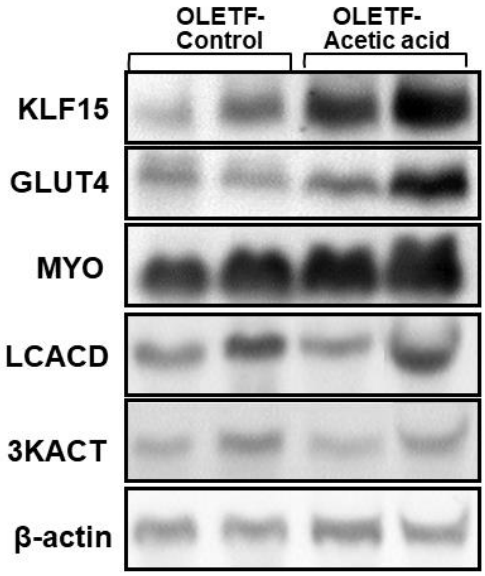

Fig 3. Effects of Acetate Administration on mRNA Levels of the Genes Involved in Energy Metabolism in the Forelegs of OLETF Rats.

Experimental conditions were the same as in Fig 2. Reprinted and revised by *BBB* 73(3): 570-576, 2009.

Effects of acetic acid on the change in the AMP/ATP ratio and the phosphorylation of AMPK in abdominal muscle

We determined the adenine nucleotide concentration in perchloric acid extracts of the abdominal muscle after intragastric injection of 105 mg/kg of BW of acetic acid. The AMP content of the muscle increased about 3-fold 2 min after injection of the acetic acid (Table 1). An increase in the AMP/ATP ratio should induce phosphorylation of AMPK, following AMPK activation. Phosphorylated AMPK in the muscles of the water- and acetic acid-injected OLETF rats was determined by western blotting. In the acetic acid-injected OLETF rats, the protein level of phosphorylated AMPK was higher than in the water-injected rats (Fig 4A). In a similar way, in the acetic acid-injected SD rats, phosphorylation of AMPK was seen 3 min after injection of acetic acid (Fig 4B).

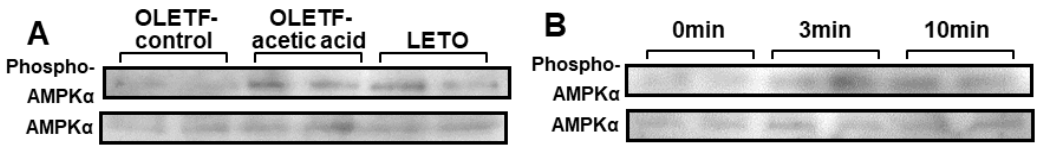

Fig 4. Effects of Acetate Administration on the Phosphorylation of AMPK in the Abdominal Muscles of Rats.

A; The phosphorylation of AMPK and the total protein level of AMPK are shown in abdominal muscles of the water- and the acetate-injected OLETF rats and the LETO rats at 32 weeks of age. Abdominal muscle was excised 24 hr after injection of water or acetate. B; The phosphorylation of AMPK and the total protein level of AMPK are shown in abdominal muscles of 10-week-old male SD rats (body weight, 350–450 g) administered acetic acid at concentrations of 52.5 mg/kg of BW. Abdominal muscle was excised at each time point indicated after the injection of 52.5 mg/kg of BW of acetate into individual rats. Reprinted and revised by *BBB* 73(3): 570-576, 2009.

Table 1. Stimulation of the AMP/ATP Ratio in Abdominal Muscle after Oral Administration of Acetate

Time (min)	ATP	ADP	AMP	Total	AMP/ATP
	µmol/ g				
0	24.1 ± 1.9	3.3 ± 0.1	0.14 ± 0.02	27.6 ± 2.0	0.006 ± 0.001
0.7	26.2 ± 0.4	3.4 ± 1.1	0.18 ± 0.04	29.7 ± 0.9	0.007 ± 0.002
1	25.3 ± 2.1	3.4 ± 0.3	0.16 ± 0.03	28.8 ± 2.3	0.006 ± 0.001
2	24.6 ± 3.0	3.9 ± 0.1**	0.41 ± 0.18*	28.9 ± 2.9	0.017 ± 0.008*
3	25.7 ± 2.4	3.6 ± 0.3	0.15 ± 0.03	29.5 ± 2.5	0.006 ± 0.001
10	24.4 ± 2.5	4.8 ± 1.2	0.17 ± 0.07	29.3 ± 3.1	0.007 ± 0.002

The effects are shown of an acetate injection on the adenine nucleotide content and the AMP/ATP ratio in abdominal muscle. Acetate at concentration of 10.5 mg/kg of BW was orally injected into 10-week-old male SD rats (body weight, 350–450 g; n=5-7 for each point). The abdominal muscle was excised at each time point indicated after the injection of 10.5 mg/kg of BW of acetic acid into an individual rat, then frozen and lyophilized, and the nucleotides were extracted. Each data value is expressed as the mean ± SE for 5-7 rats. *$p<0.05$, **$p<0.01$, compared with the 0 min point for the control rats.

Effects of acetic acid on the mRNA expression involved in lipid metabolism in white adipose and brown adipose tissues

To determine the effects of acetic acid administration on fatty acid metabolism in adipose tissues, the mRNA levels associated with lipid metabolism were measured by Northern blotting (Fig 5). The genes associated with lipogenic enzymes did not change significantly between the water-administered and the acetic acid-administered OLETF rats (Fig 5A-2), but compared to the water-administered OLETF rats, the acetic acid-administered ones tended to be enhanced in transcripts of the lipolytic genes, LCACD, 3KACT, and PPARδ, higher by about 1.7, 1.6, and 1.7 times respectively (Fig 5A-1). Also, in brown adipose tissue, the transcripts of the LCACD, 3KACT, and PPARδ genes increased significantly (Fig 5B).

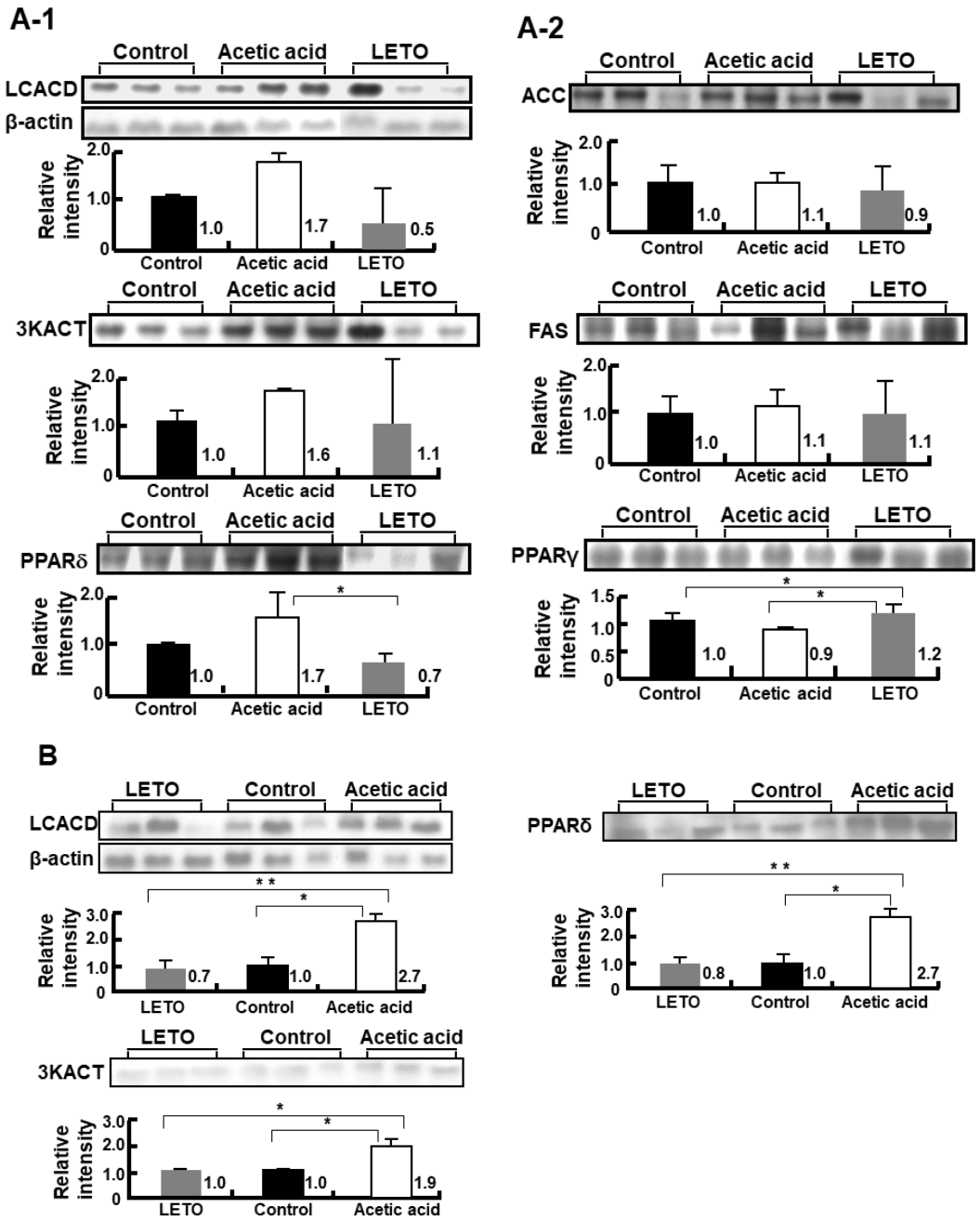

Fig 5. Effects of Acetate Administration on mRNA Levels of Lipolytic and Lipogenic Genes in the White Adipose (A) and Brown Adipose (B) Tissues of OLETF Rats.

The experimental conditions were the same as in Fig 2, except that 10 μg aliquots of total RNA were subjected to electrophoresis. Reprinted and revised by *BBB* 73(3): 570-576, 2009.

Effects of acetic acid on lipid accumulation in white adipose and brown adipose tissues.

Histological analysis indicated that the water-administered OLETF rats accumulated large-size droplets of lipid, although the acetic acid-administered OLETF rats showed smaller lipid droplets than the water-administered OLETF rats (Fig 6A). In a similar way, lipid droplets of a smaller size were observed in brown adipose tissue in acetic acid-administered OLETF rats than the water-administered OLETF rats (Fig 6B).

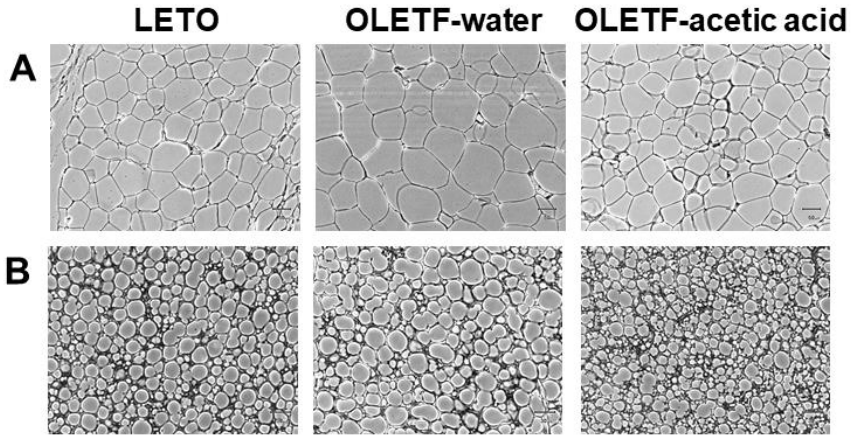

Fig 6. Histological Sections of White Adipose (A) and Brown Adipose (B) Tissues from LETO, Water-Administered OLETF, and 52.5 mg/kg of BW of Acetic Acid-Administered OLETF Rats.

A; White adipose tissue (×100 magnification). B; Brown adipose tissue (×200 magnification). Sections were cut and stained with hematoxylin and eosin. Scale bars: A, 50 μm; B, 30 μm. Reprinted and revised by *BBB* 73(3): 570-576, 2009.

DISCUSSION

We have reported that acetic acid has an inhibitory effect on fatty acid synthesis in the liver and that it protects against lipid accumulation in adipose tissue and ameliorates obesity and diabetes in OLETF rats [7]. Orally administered acetic acid was immediately taken up from the intestine, absorbed by the liver, and this increased the AMP/ATP ratio. An increase in the AMP/ATP ratio stimulates the phosphorylation of AMPK, which regulates a number of enzymes involved in lipid homeostasis [23–28]. It is possible that acetic acid administration inactivated carbohydrate-responsive element binding protein (ChREBP), which is involved with the transcription of lipogenic genes via phosphorylation by AMPK in the liver [31–35]. Accumulation of excess lipid in the liver or skeletal muscle disturbs insulin signaling [29]. In this study, we focused on the effects of acetic acid in the skeletal muscle and adipose tissues. Acetic acid-administered OLETF rats showed increased oxygen consumption (Fig 1). The transcripts of myoglobin and GLUT4 increased significantly in the abdominal muscles of the OLETF rats administered acetic acid as compared with the OLETF rats administrated water. The ingested acetic acid activated AMPK by increasing the AMP/ATP ratio in abdominal muscle, as in the liver [7]. Acetic acid is converted to acetyl-CoA by a catalytic activity of acetyl-CoA synthetase in the cytosol. In our previous study, cytosolic type acetyl-CoA synthetase (AceCS1) was widely distributed in tissues containing skeletal muscle [36]. After acetic acid administration, the AMP content increased within 5 min of injection (Table 1), then AMPK was phosphorylated in the muscle by stimulation of a higher AMP/ATP ratio. Myoglobin is a cytosolic hemoprotein expressed selectively in cardiac and skeletal myocytes, where it functions to facilitate the diffusion of oxygen and to maintain mitochondrial respiration during muscle contraction [37]. In adult mammals, myoglobin expression is modulated by environmental stimuli, including chronic hypoxia and

endurance exercise training [38–41].

Myocyte enhancer factor2 (MEF2) is a transcription factor involved in skeletal muscle differentiation [42–44] and the regulation of myoglobin transcription [45–47]. Activation of MEF2 in skeletal muscle is regulated via parallel intracellular signaling pathways, including activation of AMPK [48]. It has been reported that MEF2 plays an important role in GLUT4 expression in skeletal muscle and that GLUT4 gene regulation is associated with AMPK activation [49,50]. Increases in the transcripts of the myoglobin and GLUT4 genes by acetic acid-treatment might be mediated by activation of MEF2 via AMPK activation. Transcription of the KLF15 gene tended to increase in the muscle with the administration of acetic acid. KLF15 has been reported to regulate transcription of the GLUT4 gene [51]. The physiological relationship between KLF15 and AMPK is now under investigation.

The important role of AMPK is also shown by its association with adiponectin [52]. Adiponectin enhances insulin sensitivity and increases fatty-acid oxidation [53]. That function was shown to involve the activation of AMPK. The anti-diabetic drugs metformin and rosiglitazone can also activate AMPK, suggesting that AMPK plays an important role in the regulation of glucose and lipid metabolism [54,55].

On the other hand, acetic acid treatment had an effect in protecting against hypertrophy of the adipocytes. Lipid accumulation in brown adipose tissue was also inhibited by acetic acid treatment. In white adipose tissue, mRNA expression of fatty acid oxidizing enzymes was stimulated in the OLETF rats administered acetic acid. Activation of AMPK in rodent adipocytes was reported to lead to decreased lipogenic flux, decreased triglycerides synthesis, and an increase in fatty acid oxidation [56]. Activation of AMPK also had an inhibitory action on free fatty acid release [57] and decreased the availability of fatty acid in the plasma, and consequently it is beneficial in insulin-resistant states such as type 2 diabetes. Activation of AMPK in adipocytes has been found the lower

expression and secretion of pro-inflammatory cytokines TNF-α and interleukin-6 [58,59]. Whether acetic acid treatment activates AMPK in white and brown adipose tissues is now under investigation.

The data obtained here prompt the suggestion that acetic acid has anti-obese and anti-diabetic functions in the skeletal muscles and adipose tissues of an animal model of obesity and type 2 diabetes.

Chapter III

Activation of AMP-activated Protein Kinase and Stimulation of Energy Metabolism by Acetic Acid in L6 Myotube Cells

AMPK, a heterotrimeric protein kinase, has been found to play a key role in regulation of whole-body energy balance by phosphorylating key metabolic enzymes in both biosynthetic and oxidative pathways [23–25,28,60–62]. AMPK is activated by a high AMP/ATP ratio in the cytosol, which occurs under heat shock, hypoxia, starvation, or physical exercise. AMPK activation results both from phosphorylation of Thr-172 on AMPK's α-subunits via upstream AMPK kinase and by way of allosteric activation of phosphorylated AMPK by 5'-AMP [23–25,28,60–63]. Administered acetic acid increased AMP concentration, resulting in an increase of the AMP: ATP ratio, and led to the activation of AMPK in skeletal muscle [63]. This phenomenon is very similar to that induced by endurance exercise training. Furthermore, treatment with acetic acid increased the gene expression of myoglobin and GLUT4 in skeletal muscle of rats. GLUT4 is a well-known gene that is induced by activation of AMPK in skeletal muscle [25]. Expression of the myoglobin gene is modulated by environmental stimuli including chronic hypoxia or endurance exercise training [38–41]. Mutational analysis of the myoglobin promoter confirmed that A/T-rich MEF2-binding motifs were important in its gene regulation [64]. The human GLUT4 promoter is also regulated by the cooperative function of MEF2A [65], which is a transcription factor that plays a key role in skeletal muscle differentiation [42–44]. In differentiated myotubes, MEF2 is localized to the nucleus, indicating the importance of this transcription factor in specific skeletal muscle gene expression [46].

To investigate the function of acetic acid on AMPK activation and expression of genes such as myoglobin and GLUT4 that are involved with energy metabolism of skeletal muscle, we used L6 myotube cells and examined the effect of acetic acid. When added to the culture medium, acetic acid was rapidly taken up by L6 cells, and the phosphorylation of AMPK was stimulated. Transcripts and protein levels of myoglobin and GLUT4 were increased upon treatment with acetic acid. Furthermore, MEF2A levels

in the nuclear fraction were increased. The uptake of glucose and fatty acid by cells were increased, while triglyceride accumulation was decreased upon treatment with acetic acid. These results indicate that treatment with acetic acid increases the expression of myoglobin and GLUT4 via the activation of AMPK and MEF2A, thus enhancing fatty acid metabolism and glucose uptake.

MATERIALS AND METHODS

Materials

Rat L6 myoblasts (JCRB9081) were purchased from JCRB cell bank (Osaka, Japan). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS) and 0.02% EDTA were from MP Biomedical (CA, USA); penicillin, streptomycin, and 0.25% trypsin, from Invitrogen (CA, USA). Antibodies against AMPKα, phosphorylated AMPKα, ACC, phosphorylated ACC, and GLUT4 were purchased from Cell Signaling (MA, USA), antibodies against myoglobin, MEF2A, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and Sp1 were from Santa Cruz Biotechnology (CA, USA), and α-tubulin antibody was from Wako Pure Chemical Industries Ltd. (Osaka, Japan). AMPK agonist 5-amino-4-imidazolecarboxamide-1-beta-D-ribofuranoside (AICAR) and AMPK inhibitor adenine 9-β-D-arabinofuranoside (araA) were purchased from Sigma-ALDRICH (MO, USA). AMPK inhibitor Compound C was purchased from Merck (DA, Germany)

L6 cell culture

L6 myoblasts were grown in DMEM containing 10% (v/v) FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 at 37 °C. For myotube differentiation, the medium was changed to DMEM containing 2% (v/v) horse serum when myoblasts were 80% confluent. Myotubes were harvested 8-11 days after differentiation, and experimental procedures were initiated.

Amount of acetic acid incorporated in cells

Differentiated L6 myotube cells were treated with 0.5 mM (1 μmol/2ml) acetic acid for 0-30 min, and then the each conditioned medium that treated with acetic acid for each time period was collected and measured the concentration of acetic acid. The

concentration of acetic acid remaining in the media was measured using the acetic acid UV-method kit (R-Biopharm AG, Darmstadt, Germany) according to the manufacturer's instructions. The rates of acetic acid uptake were calculated by using the amount of acetic acid remaining in the medium, which averaged 473.1 μM (ca. 0.95 μmol/2ml), 394.0 μM (ca. 0.79 μmol/2ml), 390.0 μM (ca. 0.78 μmol/2ml) in 10sec, 2min, 30min of the treatment of acetic acid, respectively.

Amount of glucose uptake into cells

Differentiated L6 myotube cells were treated with 0.5 mM acetic acid and 100 nM insulin for 24 and 48 hr in the medium that the glucose concentration was 25 mM (50 μmol/2ml). Each conditioned medium that treated with acetic acid or insulin for 24 and 48 hr was collected and measured the concentration of glucose. The concentration of glucose remaining in the media was measured using a commercial assay kit (Glucose CII-Test Wako; Wako Pure Chemical Industries Ltd., Osaka, Japan). The amount of glucose uptake was calculated by using the amount of glucose remaining in the medium, which averaged 39 μmol and 32 μmol in 24 hr and 48 hr of the treatment of acetic acid, respectively, and 34 μmol and 20 μmol in 24 hr and 48 hr of the treatment of insulin, respectively. The amount of glucose remaining in the control medium averaged 44 μmol and 40 μmol in 24 hr and 48 hr, respectively.

Amounts of fatty acid uptake and triglyceride accumulation

Differentiated L6 myotube cells were incubated with the medium containing 0.6 μmol palmitic acid (300 μmol/L) for 24 and 48 hrs in the presence or absence of 0.5 mM acetic acid or 0.5 mM AICAR. After the incubation, mediums and cells were collected separately and the concentration of NEFA in the mediums and the concentration of TG in the cells were determined by using commercial assay kits (NEFA C-Test Wako and

Triglyceride E-Test Wako, respectively; Wako Pure Chemical Industries Ltd., Osaka, Japan). The amount of fatty acid uptake was calculated by using the concentration of fatty acid remaining in the medium, which averaged 0.263 μmol and 0.075 μmol in 24 hr and 48 hr of the treatment of acetic acid, respectively, and 0.183 μmol and 0.149 μmol in 24 hr and 48 hr of the treatment of AICAR, respectively. The amount of fatty acid remaining in the control medium averaged 0.316 μmol and 0.159 μmol in 24 hr and 48 hr, respectively.

Nucleotide assay

Differentiated L6 myotube cells were treated with 0.5 mM acetic acid for 0-30 min and added to ice-cold 0.5 M perchloric acid, neutralized, and centrifuged. Concentrations of AMP, ADP, and ATP in the extracts of myotube cells were determined by reverse-phase HPLC analysis (SPD-10A, respectively; Shimazu, Kyoto, Japan) with an ODS column (HPLC PACKED COLUMN C18 CAPCELLPAK, respectively; Shimadzu Corporation, Kyoto, Japan). The mobile phase consisted of 100 mM phosphate buffer, pH 6.3 and 0.89% methanol. Quantification was performed at $\lambda = 259$ nm. All chromatographic assays were carried out at room temperature with a flow of 1.0 ml/min. Adenosine nucleotides (ATP, ADP, and AMP) were identified and quantified based on the corresponding standard compounds.

Nuclear extraction

Myotubes were grown in 10-cm dishes, treated with acetic acid or AICAR as described above, and then washed immediately with ice-cold PBS. Hypotonic buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1% NP-40, 1 mM DTT, 1 mM PMSF, and protease inhibitors (Nacalai Tesque, Kyoto, Japan)) were added and the cells were collected. After homogenization, the extract was centrifuged for 5 min at 3,000 rpm

at 4 °C, and the supernatant (cytoplasmic fraction) was collected. The nuclear pellet was resuspended in hypertonic buffer (20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 400 mM NaCl, 0.1 mM EDTA, 0.1% NP-40, 10% glycerol, 1 mM DTT, 1 mM PMSF, and protease inhibitors) by pipetting. The suspension was incubated and shaken for 1 hr on ice. Then the supernatant (nuclear fraction) was collected by centrifugation at 15,000 rpm for 5 min. Protein concentration of the nuclear extract was determined by Bradford assay.

AMPK activity

To measure AMPK activity, AMPK was immunoprecipitated from cell extracts with specific antibodies against the α2-subunits bound to protein G agarose beads. The kinase activity of the immunoprecipitates was measured using SAMS peptide [HMRSAMSGLHLVKRR] and the Kinase-Glo Luminescent Kinase Assay kit (Promega, Madison, USA) according to the manufacturer's instructions.

Western blotting

L6 myotube cells were washed with ice-cold PBS and lysed with RIPA buffer (1× TBS pH 7.4, 0.5% deoxycholic acid, 0.1% SDS, 1% NP-40, 1 mM PMSF, 1 mM Na_3VO_4, 10 mM NaF, and protease inhibitors). Following centrifugation, supernatants were used for western blotting. Protein content of supernatants was determined by Bradford assay and an aliquot (15-30 μg of protein) of each extract from L6 cells was used for western blot to determine the contents of total AMPKα, phosphorylated Thr-172 AMPKα, total ACC, phosphorylated ACC, myoglobin, GLUT4, MEF2A, PGC-1α and α-tubulin. Samples were applied to 10-15% SDS-PAGE, and then proteins on the gel were transferred onto a polyvinylidene difluoride membrane (Merck, DA, Germany). The membranes were first incubated with primary antibodies overnight at 4°C, washed three times with TBST, and they were incubated with HRP-conjugated secondary antibodies

for 60 min. For highly sensitive system, after the membranes were incubated with primary antibodies, they were incubated with biotin-conjugated secondary antibodies for 15-30min, and they were incubated with HRP-conjugated streptavidin for 15min. After washing three times with TBST, the chemiluminescent reaction was performed for 5 min with ImmunoStar LD (Wako Pure Chemical Industries Ltd., Osaka, Japan), according to the protocol supplied by the manufacturer. Chemiluminescent signals were visualized and quantified with ImageQuant LAS-4000 and Multi Gauge V3.2 analyzing software (Fujifilm, Tokyo, Japan).

Quantitative RT-PCR analysis

Differentiated myotubes were incubated with 0.5 mM acetic acid and/or other reagents for the indicated time, and then the cells were washed three times with RNase free PBS and harvested for RNA extraction. Total RNA was isolated from L6 cells by using Sepasol RNA I super G (Nacalai Tesque, Kyoto, Japan), and RNase inhibitor (TOYOBO, Osaka, Japan) was added according to the manufacturer's instructions. Total RNA concentration was measured and cDNA was prepared with PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Real-time quantitative PCR analyses were performed using the StepOnePlus detection system (Applied Biosystems, CA, USA) with KAPA SYBR FAST qPCR Kits (Kapa Biosystems, Wilmington, MA) for quantification of specific mRNA content. Data were normalized to β-actin mRNA and expressed relative to untreated control cells. The oligonucleotide primers were as follows: rat β-actin (*actb*) forward: 5'-GGAGATTACTGCCCTGGCTCCTA-3', reverse: 5'-GACTCATGTACTCCTGCTTGCTG-3', rat GLUT4 (*Slc2a4*), forward: 5'-GGGCGATTTCTCCCACATAC-3', reverse: 5'-CTCATGGGCCTAGCCAATG-3', rat MEF2A (*mef2a*), forward: 5'-ATGAGAGGAACCGACAGGTG-3', reverse: 5'-TATCCGAGTTCGTCCTGCTT-3',

rat myoglobin (*Mb*), forward: 5'- CTAACAGCCGGCCTACACTC-3', reverse: 5'-CGTGCTTCTTCAGGTCCTCT-3', PGC-1α (*ppargc1a*) forward: 5'-GACCCCA GAGTCACCAAATGA-3', reverse: 5'-GGCCTGCAGTTCCAGAGAGT-3'.

Immunofluorescence

L6 cells were fixed with 4% formaldehyde solution, incubated with 0.1% Triton X-100/PBS for 3 min, and blocked with 3% BSA/PBS for 10 min. Samples were incubated at room temperature for 1 hr with primary anti-MEF2A antibody (Santa Cruz Biotechnology) and anti-skeletal myosin antibody (SIGMA-ALDRICH, MO, USA). Then samples were washed three times with PBS and incubated with secondary antibody conjugated to Alexa Fluor at room temperature for 1 hr. Samples were counterstained with Hoechst 33258 (Polysciences, Inc., PA, USA) for 5 min and imaged using a confocal microscope (OLYMPUS FLUOVIEW FV1000, respectively; OLYMPUS, Tokyo, Japan).

Statistical analysis

For analysis of AMPK activity, specific activities of AMPK were compared using unpaired Student's t-test. For the remaining analysis, one-way ANOVA followed by the Tukey-Kramer post hoc test for multiple comparisons was performed. P values < 0.05 were considered to represent statistical significance (*$p< 0.05$, **$p< 0.01$ compared to control). Groups without the same letter represent significantly different ($p< 0.05$).

RESULTS

Absorption of acetic acid by differentiated L6 myotube cells

To assess the ability of L6 myotubes to absorb acetic acid, 0.5 mM acetic acid was added to cultured cells and the amount of acetic acid taken up by the cells was measured. Acetic acid was immediately taken up within 2 min of incubation (Fig 1).

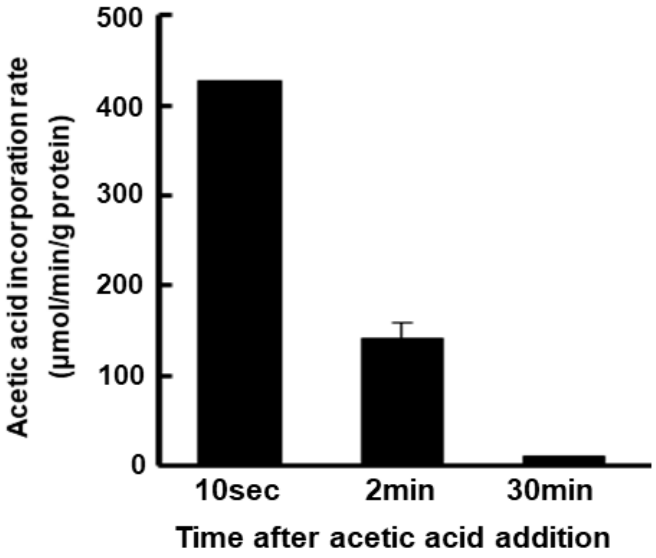

Fig 1. Uptake of acetic acid by L6 myotube cells.
After treatment with acetic acid (0.5 mM), acetic acid content in the medium was determined at each indicated time point and the amount of uptake by the cells was calculated. Each value is shown as the mean ± SE (n=3-6). Reprinted and revised by *PLOS ONE* 11(16): e0158055, 2016.

Acetic acid raises AMP concentration in differentiated L6 myotube cells

Following the absorption of acetic acid by cells, acetic acid is converted to acetyl-CoA concomitantly with the formation of AMP, a well-known activator of AMPK, via the catalytic activity of acetyl-CoA synthetase in the cytosol. AMP concentration in cells incubated with 0.5 mM acetic acid was consistently higher than that in non-treated steady state control cells (0 min), and significantly increased in 2 min compared to the steady state concentration (Table 1). The AMP/ATP ratio during incubation with acetic acid was significantly increased in 2 min of the addition of acetic acid.

Table 1. Stimulation of the AMP/ATP ratio in L6 myotube cells by treatment with acetic acid.

Time	ATP	ADP	AMP	Total	AMP/ATP
	(μmol / g of protein)				
0 min	20.95 ± 3.65	5.53 ± 0.71	0.86 ± 0.07	27.35 ± 4.45	0.041 ± 0.007
0.5 min	18.52 ± 2.83	6.48 ± 0.16	2.06 ± 0.51	27.06 ± 2.61	0.111 ± 0.051
2 min	19.73 ± 1.91	6.81 ± 0.49	4.43 ± 0.47*	30.96 ± 1.17	0.224 ± 0.061*
30 min	18.95 ± 1.01	8.66 ± 0.31*	3.38 ± 1.43	30.99 ± 0.57	0.179 ± 0.018

Adenine nucleotides (μmol/g of protein) in L6 cells treated with 0.5 mM acetic acid for the indicated times. Each value is shown as the mean ± SE (n=3-4). Results were analyzed with one-way ANOVA followed by the Tukey-Kramer post hoc test for multiple comparisons. Statistical differences are shown as *$p < 0.05$ compared to the 0 min time point.

Acetic acid induces phosphorylation of AMPK and ACC in differentiated L6 cells

To determine whether the treatment with acetic acid can induce phosphorylation of AMPK at Thr172 in L6 cells, the change of phosphorylated AMPK levels was analyzed. Phosphorylation of AMPK was significantly increased in acetic acid-treated cells compared to in non-treated control cells. Ten minutes after the addition of the indicated amount of acetic acid to the culture medium, phosphorylated AMPK increased in a dose-dependent manner (Fig 2A).

A time-course study revealed that incubation with 0.5 mM acetic acid increased the phosphorylation of AMPK after the addition of acetic acid (Fig 2B). Treatment with AICAR, an AMPK activator, also increased the phosphorylation of AMPK by about 2.2-fold at 12 hr after the addition. Pre-treatment of cells with compound C and araA, potent AMPK inhibitors, suppressed the acetic acid induced phosphorylation of AMPK (Figs 2C and D). Phosphorylation of AMPK was also analyzed after treatment with other acid compounds such as 0.5 mM lactic acid and 0.5 mM citric acid. Both compounds did not enhance the phosphorylation of AMPK (Fig 2E).

One downstream target of AMPK is phosphorylation of ACCβ at Ser79. Phosphorylated ACC was significantly increased at 10 min after treatment with 0.5 mM acetic acid (Fig 2F), and the addition of compound C and araA together with acetic acid led to suppression of this phosphorylation (Figs 2F and G). This increase in phosphorylation was also seen in the treatment with AICAR, and inhibited by addition of compound C.

Moreover, we also conducted an evaluation of AMPK activity in L6 myotube cells using the SAMS peptide as a substrate of AMPK. AMPK activity was significantly increased in cells treated with acetic acid compared to non-treated control cells (Fig 2H).

Acetic acid treatment increases mRNA and protein expression of myoglobin and GLUT4 in L6 myotube cells

Transcripts of myoglobin and GLUT4 were increased in cells treated with acetic acid. Time-course studies revealed that mRNA levels of myoglobin gene, *Mb* and GLUT4 gene, *Slc2a4* were significantly higher than those of non-treated steady state control cells (Figs 3A and B). Furthermore, pre-treatment with compound C or araA and acetic acid completely suppressed the increase in expression of *Mb* and *Slc2a4* genes (Figs 3C-F). Expression of these proteins were also significantly increased and this stimulation was completely suppressed in the presence of compound C and araA (Figs 3G-J)

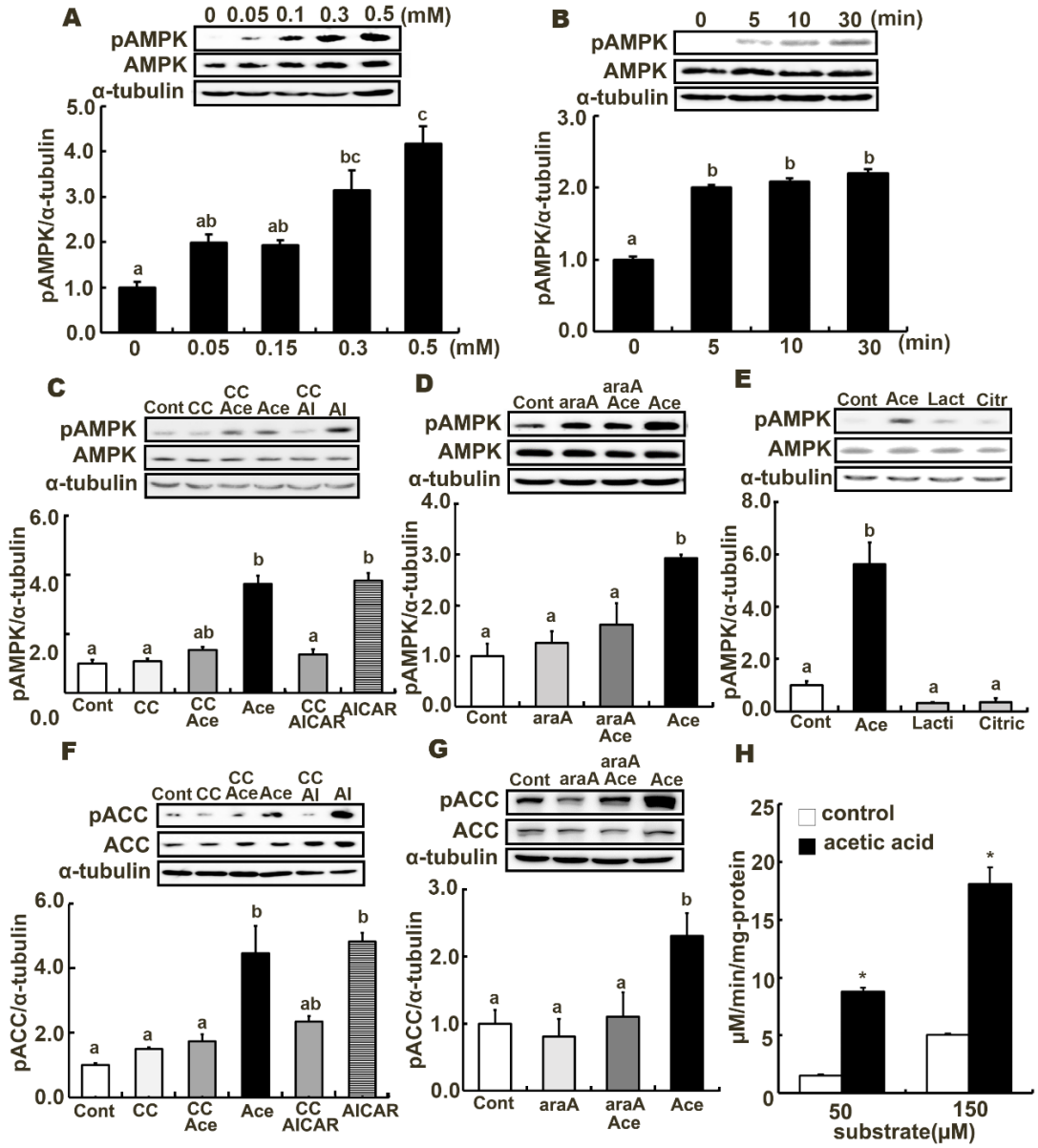

Fig 2. Acetic acid induces phosphorylation of AMPK and ACC in L6 myotube cells.

L6 myotube cells were treated with 0, 0.05, 0.15, 0.3, and 0.5 mM acetic acid for 10 min (A). Cells were incubated with 0.5 mM acetic acid for the indicated time (B), 0.5 mM AICAR for 12 hr, and 10 μM compound C for 30 min (C), 2 mM araA for 20 min (D), and 0.5 mM lactic acid or 0.5 mM citric acid for 10 min (E), and analyzed for the phosphorylation of AMPK. Cells were treated with 0.5 mM acetic acid for 10 min and 0.5 mM AICAR for 12 hr, and 10 μM compound C for 30 min (F) and 2 mM araA for 20min (G), and analyzed for phosphorylation of ACC and ACCβ. After L6 myotube cells were treated with 0.5 mM acetic acid for 2 min, cell lysates were prepared. Then, AMPK was immunoprecipitated from cell extracts and AMPK activity was measured using the synthetic SAMS peptide as a substrate (50 μM and 150 μM) (H). Each bar represents the mean ± SE (n=3-4). Results were analyzed with one-way ANOVA followed by the Tukey-Kramer post hoc test for multiple comparisons (A-G), or analyzed by an unpaired Student's t-test (H). Statistical differences are shown as *p< 0.05, **p< 0.01 compared to non-treated (A). Groups without the same letter are significantly different (p< 0.05) (B-G). Reprinted and revised by *PLOS ONE* 11(16): e0158055, 2016.

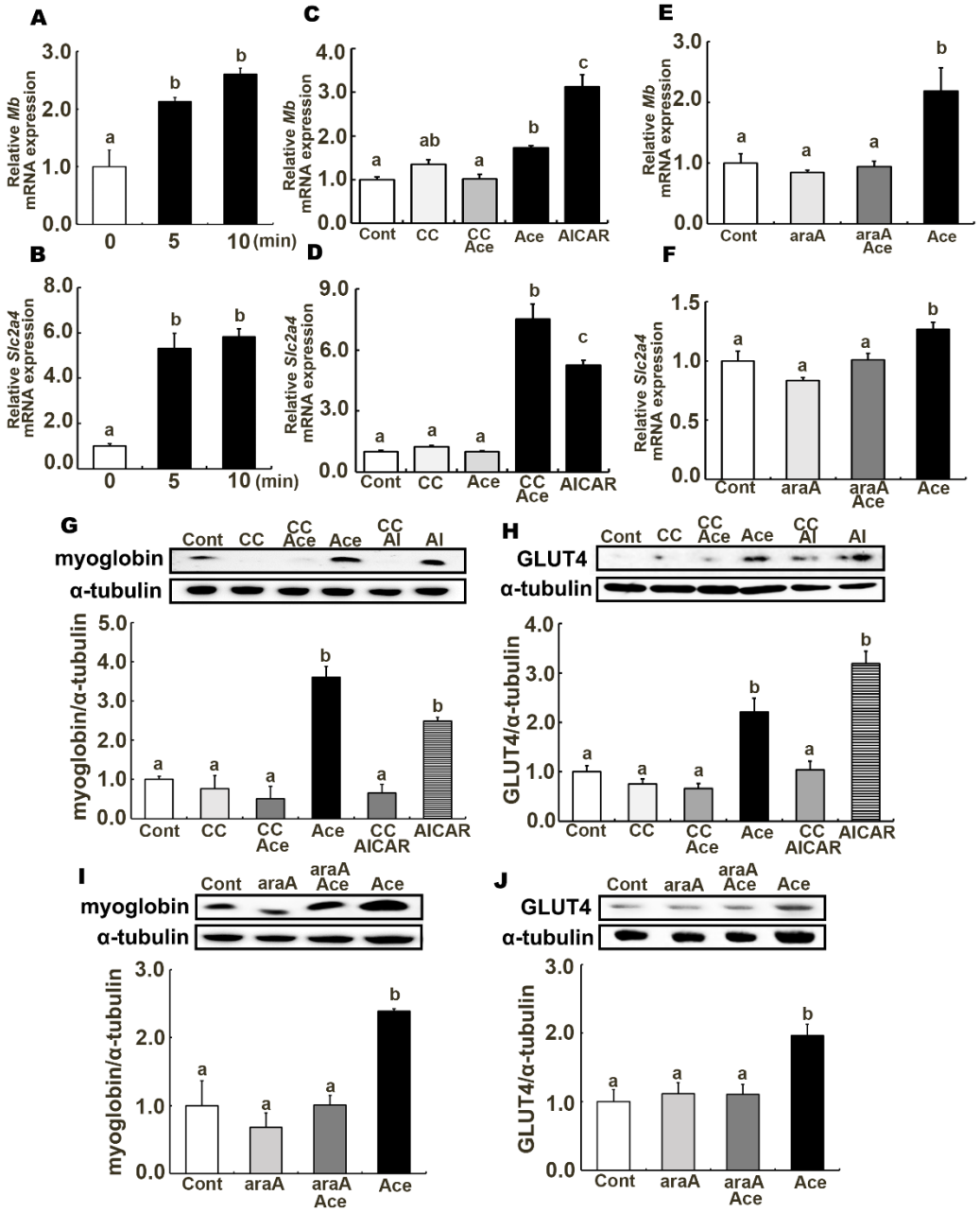

Fig 3. Effects of acetic acid on the expression of myoglobin and GLUT4 in L6 myotube cells. Total RNA was extracted from untreated L6 myotube cells or those treated with 0.5 mM acetic acid for the indicated time period (A; *Mb*, B; *Slc2a4*) or for 5 min after the addition of acetic acid (C; *Mb*, D; *Slc2a4*) or 0.5 mM AICAR for 12 hr, and10 μM compound C for 30 min or 2mM araA for 20min. Real-time PCR analysis was carried out for determination of *Mb* (A, C, E) and *Slc2a4* (B, D, F) mRNA levels in L6 myotube cells. Myoglobin or GLUT4 proteins were analyzed by western blotting on the treatment of 0.5 mM acetic acid for 10 min, 0.5 mM AICAR for 12 hours, and 10 μM compound C for 30 min (G, H) or 2 mM araA for 20 min (I, J). Each bar represents the mean ± SE (n=3-6). Results were analyzed with one-way ANOVA followed by the Tukey-Kramer post hoc test for multiple comparisons. Groups without the same letter are significantly different ($p < 0.05$). Reprinted and revised by *PLOS ONE* 11(16): e0158055, 2016.

Acetic acid increases glucose and fatty acid uptake, and suppresses triglyceride accumulation in L6 myotube cells

To investigate whether the activation of AMPK by acetic acid stimulates uptake of glucose, glucose clearance was examined in cells treated with acetic acid. After insulin treatment, glucose uptake was approximately 3 times higher than that of non-treated controls. Similarly, acetic acid as well as AICAR treatment significantly increased glucose uptake (Fig 4A). Treatment with acetic acid tended to increase the utilization of fatty acid, but reduced their accumulation as triglyceride in the cells compared to non-treated control or AICAR treatment (Figs 4B and C).

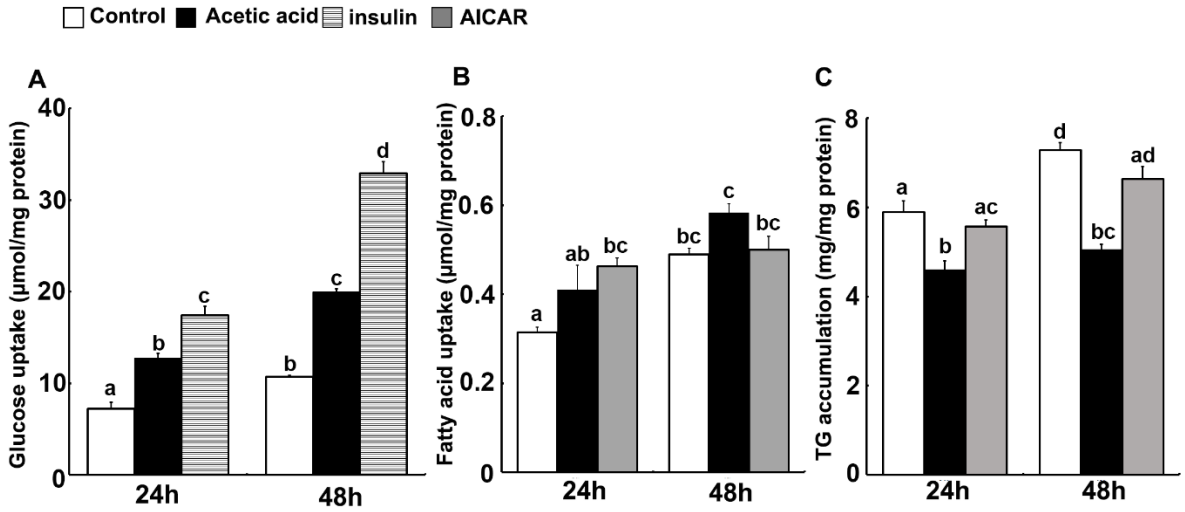

Fig 4. Effects of acetic acid treatment on glucose, fatty acid uptake and triglyceride accumulation in L6 myotube cells.

(A) Glucose uptake by L6 cells. Differentiated L6 myotube cells were treated with 0.5 mM acetic acid and 100 nM insulin for 24 and 48 hrs in the medium (50 μmol/2 ml). Each conditioned medium was collected and measured the concentration of glucose. Amount of glucose uptake was calculated by using the amount of glucose remaining in the medium. (B) Fatty acid uptake by L6 cells. (C) TG accumulation in L6 cells. Differentiated L6 myotube cells were incubated with the medium containing 0.6 μmol palmitic acid (300 μmol/L) for 24 and 48 hrs in the presence or absence of 0.5 mM acetic acid or 0.5 mM AICAR. After the incubation, mediums and cells were collected separately and the concentration of NEFA in the mediums and the concentration of TG in the cells were determined. Each bar represents the mean ± SE (n=3-4). Results were analyzed with one-way ANOVA followed by the Tukey-Kramer post hoc test for multiple comparisons. Groups without the same letter are significantly different ($p < 0.05$). Reprinted and revised by *PLOS ONE* 11(16): e0158055, 2016.

Acetic acid treatment increases mRNA and protein expression of MEF2A in L6 myotube cells

MEF2A plays an important role in the regulation of gene expression of myoglobin and GLUT4 in skeletal muscle [26]. MEF2 proteins are transcription factors involved in the differentiation of skeletal muscle, and possess diverse cellular functions in skeletal muscles or neurons. We focused on MEF2A and hypothesized that the increase in *mb* and *Slc2a4* gene expression by acetic acid treatment might be associated with the function of MEF2A, as well as with the activation of AMPK.

Transcript levels of *mef2a* were significantly higher in cells treated with acetic acid than those of non-treated control (Fig 5A). Pre-treatment with compound C or araA completely suppressed the induction of *mef2a* mRNA by acetic acid (Figs 5B and C). Protein expression of MEF2A was also increased upon acetic acid treatment (Figs 5D and E), which was abolished by compound C and araA.

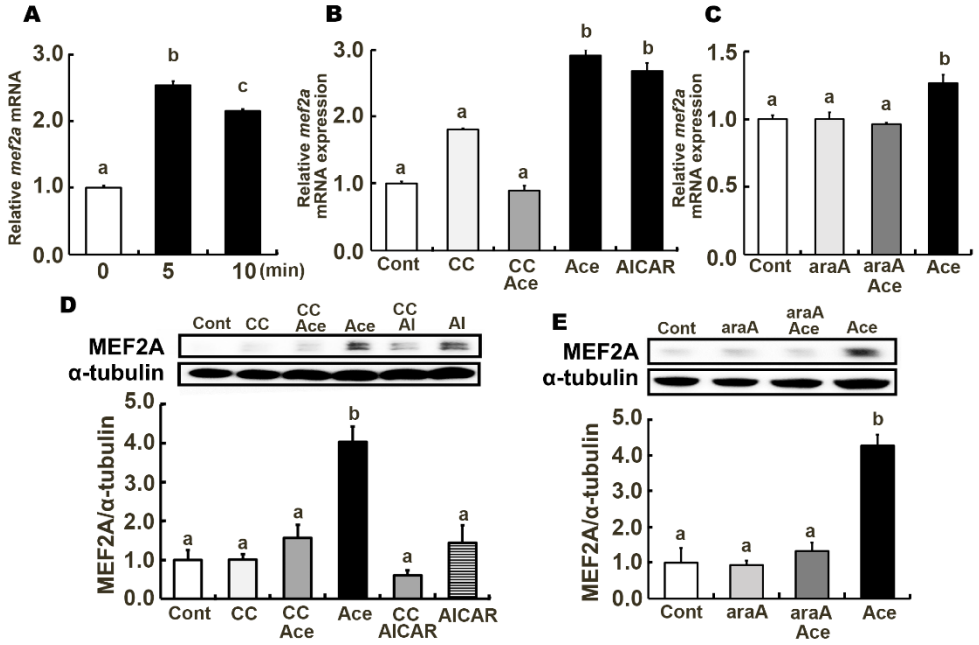

Fig 5. Effect of acetic acid on the expression of MEF2A in L6 myotube cells.

Total RNA was extracted from untreated L6 myotube cells or cells treated with 0.5 mM acetic acid for the indicated time period (A). L6 cells were treated with 0.5 mM acetic acid for 5 min, 0.5 mM AICAR for 12 hours, and pre-treated with 10 μM compound C for 30 min (B), and pre-treated with 2 mM araA for 20 min (C). Real-time PCR analysis was carried out for the determination of *mef2A* mRNA level in L6 myotube cells. MEF2A protein was analyzed by western blotting in 10 min treatment of 0.5 mM acetic acid, 0.5 mM AICAR for 12 hours, and 10 μM compound C for 30 min (D) or 2 mM araA for 20 min (E). Each bar represents the mean ± SE (n=3-6). Results were analyzed with one-way ANOVA followed by the Tukey-Kramer post hoc test for multiple comparisons. Groups without the same letter are significantly different ($p < 0.05$). Reprinted and revised by *PLOS ONE* 11(16): e0158055. 2016.

Acetic acid treatment increases mRNA and protein expression of PGC-1α in L6 myotube cells

PGC-1α is a transcriptional coactivator that mediates many biological processes related to energy metabolism. PGC-1α and MEF2 form a positive feedback loop [66]. Activation of AMPK has been implicated to be involved in the upregulation of *ppargc1a* expression [67]. Transcript levels of *ppargc1a* were significantly higher in cells treated with acetic acid than in non-treated control (Fig 6A). Pre-treatment with compound C or araA, and acetic acid completely suppressed the induction of *ppargc1a* mRNA (Figs 6B and C). Protein expression of PGC-1α was also increased upon treatment with acetic acid (Figs 6D and E).

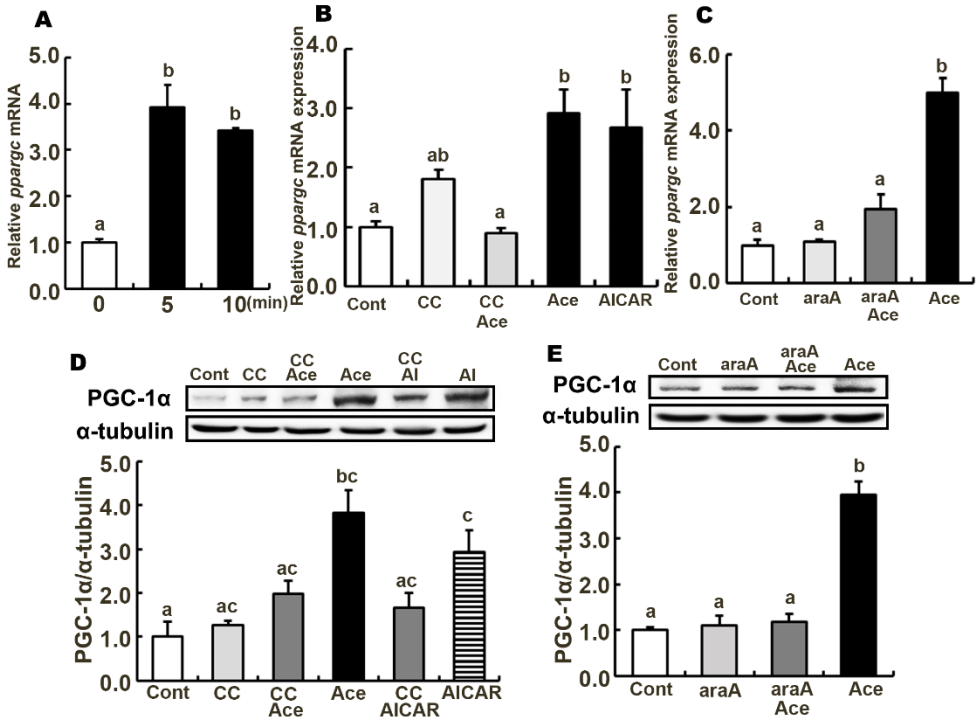

Fig 6. Effect of acetic acid on the expression of PGC-1α in L6 myotube cells.
Total RNA was extracted from untreated L6 myotube cells or cells treated with 0.5 mM acetic acid for the indicated time period (A). L6 cells were treated with 0.5 mM acetic acid for 5 min, 0.5 mM AICAR for 12 hours, and pre-treated with 10 μM compound C for 30 min (B) or 2 mM araA for 20 min (C), and total RNA were isolated. Real-time PCR analysis was carried out for the determination of *ppargc1a* mRNA level in L6 myotube cells. PGC-1α protein level was analyzed by western blotting in 5 min treatment with 0.5 mM acetic acid, 0.5 mM AICAR for 12 hours, and 10 μM compound C for 30 min (D) or 2 mM araA for 20 min (E). Each bar represents the mean ± SE (n=3-6). Reprinted and revised by *PLOS ONE* 11(16): e0158055, 2016.

Acetic acid induces nuclear localization of MEF2A

The nuclear MEF2A protein level was measured by western blotting to see the nuclear localization of MEF2A. Nuclear MEF2A was significantly increased at 5 min (2.1-fold), 10 min (3.3-fold) and 30 min (2.3-fold) after treatment with acetic acid compared to non-treated control (Fig 7A). In contrast, there was no change in the cytosolic MEF2A levels (Fig 7B). However, nuclear localization of MEF2A was significantly reduced in the presence of compound C (Fig 7A). Furthermore, we analyzed L6 myotube cells by confocal immunofluorescence. Fig 7C is the typical image showing that MEF2A was localized in nucleus in L6 cells treated with acetic acid, while it was localized in the cytosol in the presence of compound C and in non-treated cells (Fig 7C). And the nuclear localization rate of MEF2A was significantly increased by the treatment of acetic acid (Fig 7D).

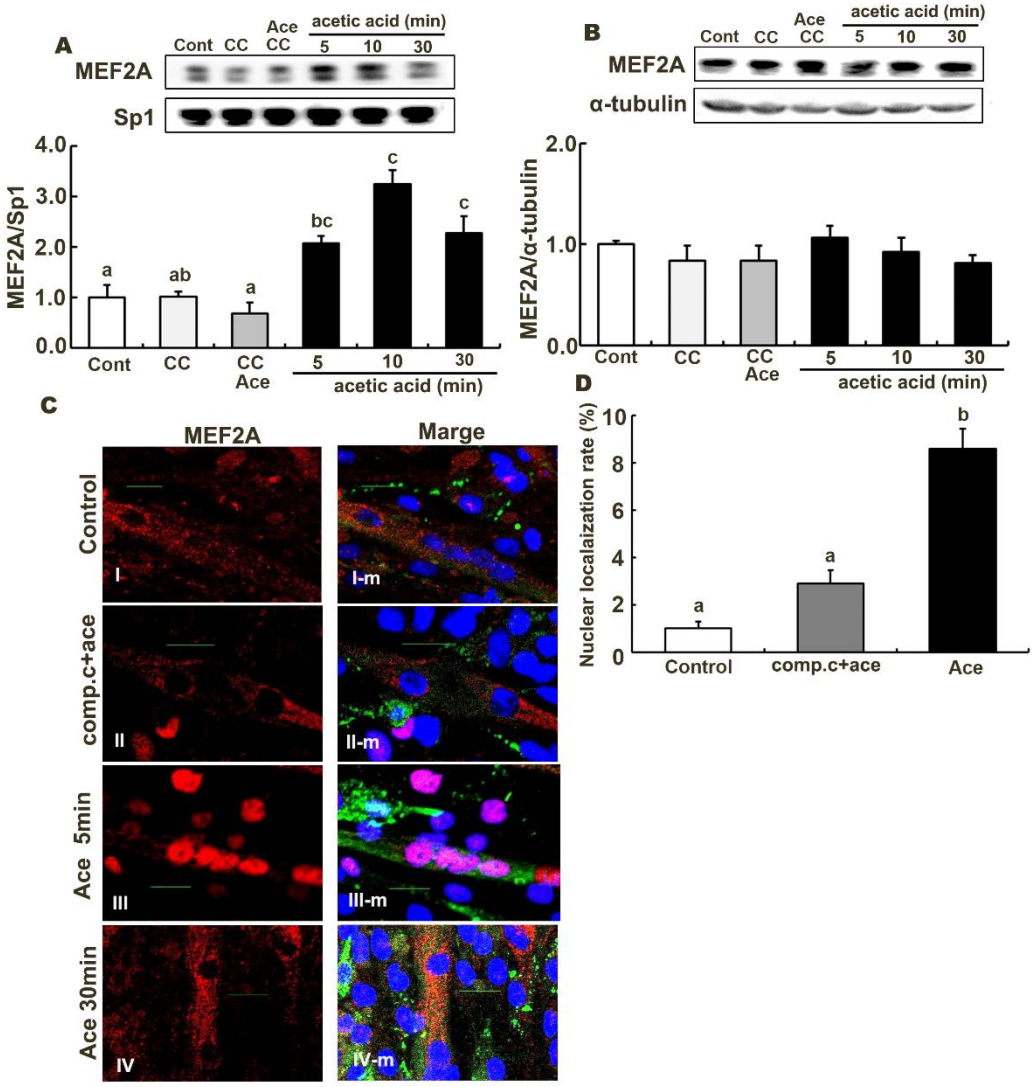

Fig 7. Effect of acetic acid treatment on nuclear MEF2A expression in L6 myotube cells.

L6 myotube cells were treated with 0.5 mM acetic acid, and nuclear fraction (A) and cytosolic fraction (B) were separated. MEF2A level was examined by western blotting as described in materials and methods. L6 myotube cells were cultured on glass cover slips coated with poly-L-lysine and treated with 0.5 mM acetic acid in the presence or absence of 10 μM compound C (C). Then cells were fixed and nuclear DNA was stained by Hoechst 33258 (blue). Cells were immunostained for MEF2A (red) and myosin (green). Scale bar = 20 μm. The nucleus immunostained with anti-MEF2A antibody were counted (8 mm^2 area, n=3) and the rate of nuclear localization of MEF2A was calculated (D). Each bar represents the mean ± SE (n=3-4).

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DISCUSSION

Skeletal muscle is one of the most important insulin-responsive organs in the body [29,30], and an accumulation of locally derived fat metabolites in skeletal muscle is an important factor contributing to insulin resistance [29]. It has been demonstrated that AMPK plays a key role in regulating fat oxidation and glucose metabolism via upregulation of mitochondrial proteins, GLUT4, and several metabolic enzymes in mice, rats, or human skeletal muscles [23–25,28,60–62]. Present data indicate that acetic acid functions as an activator of AMPK and might be able to suppress lipogenesis via enhancing lipid metabolism. When acetic acid is taken up into tissues, it is converted to acetyl-CoA with concomitant formation of AMP by the catalytic activity of acetyl-CoA synthetase [68–73]. Acetic acid is easily absorbed by L6 myotube cells, and increases the AMP/ATP ratio in the cells. An increase in AMP/ATP ratio stimulates the phosphorylation of AMPK. Phosphorylated AMPK increased in a dose-dependent manner with the concentration of acetic acid (Fig 2A), which was in the range of physiological blood concentration of acetic acid. Pre-treatment of cells with compound C inhibited phosphorylation of AMPK by acetic acid (Fig 2C). Moreover, other acids such as lactic acid or citric acid were unable to increase the phosphorylation of AMPK (Fig 2E). Activated AMPK leads to an inactivation of ACC via phosphorylation, a decrease in malonyl-CoA content, a subsequent increase in fatty acid oxidation, and a block in fatty acid synthesis. Phosphorylation of ACC significantly increased at 10 min of acetic acid treatment (Fig 2F) and it was inhibited by pre-treatment with compound C and araA (Figs 2F and 2G). In our previous study, chronic intake of acetic acid induced gene expression of myoglobin and GLUT4 in skeletal muscle of rats, and the rats showed a higher rate of oxygen consumption and a smaller size of lipid droplets in white adipose tissue [63]. In this study, transcripts and protein levels of myoglobin and GLUT4 were increased in

acetic acid-treated L6 myotube cells. Pre-treatment of acetic acid treated cells with compound C and araA completely suppressed the increase of myoglobin and GLUT4 gene and protein expression (Figs 3C, E, D-J), suggesting that acetic acid induces myoglobin and GLUT4 transcription through the AMPK-mediated signaling pathway in L6 myotube cells. Myoglobin is expressed in cardiac and skeletal myocytes, and it plays a role in the diffusion of oxygen and to maintain mitochondrial respiration [37]. The myoglobin gene is expressed depending on myofiber subtypes, and the expression is regulated by environmental stimuli such as chronic hypoxia and endurance exercise training [38–41]. GLUT4 is one of the glucose transport proteins and is responsible for insulin-mediated glucose uptake in muscle and adipose tissue. It was shown that glucose homeostasis depends on the level of GLUT4 expression [74–76].

Transcription of myoglobin and GLUT4 genes is regulated by MEF2, which is a transcription factor involved in skeletal muscle differentiation [42–44,47,49,50,77]. The MEF2 family is comprised of four members, MEF2A, B, C and D [46,78], and belongs to the MCM1-agamous-deficiens-serum response factor (MADS) supergene family of DNA binding proteins [78–80]. MEF2B is a unique member of the MEF2 gene family [81] and its expression is restricted to myogenic lineages during early embryo development [82]. MEF2D is expressed in proliferating myoblasts prior to the onset of differentiation [46,83]. MEF2C is expressed late in the differentiation program, and MEF2A protein appears in cells entering the differentiation pathway and the expression is regulated at multiple levels during development and differentiation. The predominant MEF2 DNA-binding complex in muscle cells is composed of MEF2A homodimers [46]. There is a MEF2 binding site in GLUT4 promoters that contains the binding site of a MEF2A-MEF2D heterodimer. Reduced MEF2A expression accounts for the reduction in DNA binding activity and directly correlates with the decrease in GLUT4 gene expression [84]. MEF2A is a substrate for p38 mitogen-activated protein (MAP) kinase (MAPK),

and 312 and 319 within the transcription activation domain of MEF2A are phosphorylated by p38 [85]. Phosphorylated MEF2A enhances MEF2-dependent gene expression. MAPK-independent pathways such as the AMPK-associated pathway have also been implicated in the regulation of MEF2 [48]. Treatment of human skeletal muscle cells with AICAR, a pharmacological activator of AMPK, stimulated MEF2 DNA binding activity [48]. AICAR-mediated MEF2 DNA binding was independent of p38 MAPK activation, and it was completely inhibited by an AMPK inhibitor, compound C [48]. AICAR-injected rats showed increased nuclear MEF2 DNA binding activity, and chronic AICAR treatment dramatically increased the expression of glucose transport protein GLUT4 in muscle [86]. In this study, we observed that MEF2A nuclear localization was increased upon treatment with acetic acid and it was inhibited by pre-treatment with compound C (Fig 7). Furthermore, transcripts of MEF2A and PGC-1α genes were significantly increased in cells treated with acetic acid (Figs 5A-C, 6A-C). PGC-1α plays a key role in the regulation of mitochondrial biogenesis and oxidative metabolism and its activity has been reported to be regulated by AMPK [87]. MEF2A and PGC-1α contain a MEF2A binding site in their promoter sequences [66,88] and their gene expression was coordinated with one another [66]. Those findings implicate that acetic acid induced expression of both MEF2A and PGC-1α as well as of myoglobin and GLUT4 genes might be caused by an increase in nuclear localization of MEF2A via the activation of AMPK.

De Angelis et al. showed that transforming growth factor β (TGF-β) inhibited myogenesis and prevented the activation of the transcriptional complex related to MEF2A through localization of MEF2A in the cytoplasm [89]. Furthermore, activated AMPK inhibited TGF-β, Smad3 gene expression, and TGF-β-induced myofibroblast differentiation [90–92]. In this study, MEF2A, which contains a nuclear localization sequence at its C-terminus [46,93], was localized in the nucleus shortly after treatment with acetic acid and exported to the cytoplasm 30 min after treatment. Investigation of

the transport mechanism of MEF2A upon treatment with acetic acid is currently underway.

mRNA and protein expression levels of MEF2A were significantly increased upon treatment with acetic acid in L6 myotube cells (Fig 5). In addition, pre-treatment of acetic acid-treated cells with compound C completely suppressed the increase in MEF2A mRNA and protein expression levels (Figs 5B and D). A ChIP assay revealed that the MEF2A promoter contains a MEF2A binding site and that MEF2A would associate with the site to control the MEF2A transcription [88]. These results implicate that increased expression of MEF2A by acetic acid might be caused by increased nuclear localized MEF2A via the activation of AMPK.

Activation of AMPK is linked to lipid catabolism and improvement of insulin sensitivity [62]. Numerous AMPK activators have been described including adiponectin or berberine. Adiponectin, which is an adipocytokine preventing metabolic syndrome or atherosclerosis, activates AMPK in skeletal muscle and improves insulin sensitivity [67,94] Berberine, a food component, has an effect on AMPK activation, adipose tissues, and macrophages [95,96]. In this study, acetic acid activated AMPK, induced gene and protein expression of myoglobin and GLUT4, stimulated glucose incorporation, and suppressed lipid accumulation in L6 cells. Thus, acetic acid has the potential to prevent metabolic disorders through the activation of AMPK.

Chapter IV Conclusion Remarks

Diabetes is one of the most emergent health problem of the world in the 21st century. Diabetes continues to increase in most parts of the world. Furthermore, Asians, including Japanese, had the highest postprandial glycemia and lowest insulin sensitivity [97]. Thus, it is one of the most important issue to solve. Several studies reported that acetic acid has been such effects as a suppression of increased postprandial glycemia [98], protection against high-fat diet induced obesity and insulin resistance [99], appetite suppression [100] and activated AMPK in the liver and adipose tissue [101] etc. AMPK is a key regulator of nutrient metabolism and energy homeostasis [10,102]. AMPK functions as an energy sensor at the cellular, and when it is activated, it leads to an increase of fatty acid oxidation, glucose uptake independently with insulin, glycolysis, mitochondrial biogenesis, and decrease lipogenesis [62,102]. Therefore, increased AMPK activation is important to prevent and cure of obesity and type 2 diabetes. Actually, it is known that the mechanism of action of metformin, used drug for treatment of type 2 diabetes, is due to the activation of AMPK.

In this study, chronic intake of acetic acid induced gene expression of myoglobin and GLUT4 in skeletal muscle of rats, and the rats showed a higher rate of oxygen consumption and a smaller size of lipid droplets in white adipose tissue. Transcripts and protein levels of myoglobin and GLUT4 were increased in acetic acid-treated rats and L6 myotube cells. Pre-treatment of acetic acid treated cells with compound C and araA completely suppressed the increase of myoglobin and GLUT4 gene and protein expression, suggesting that acetic acid induces myoglobin and GLUT4 transcription through the AMPK-mediated signaling pathway. Treatment of acetic acid increased MEF2A and PGC-1α genes and proteins and it was appeared that those expressions were led by activation of AMPK. Those findings implicate that acetic acid induces expression of both MEF2A and PGC-1α as well as of myoglobin and GLUT4 genes and it could result, stimulations of lipid oxidation and glucose uptake.

Activation of AMPK is involved in improving lipid metabolism and insulin sensitivity. Thus, acetic acid may contribute to the prevention of metabolic disorders such as obesity and diabetes. It is useful to develop the easy way to take vinegar in the future study that will help for health promotion.

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