Doctoral Dissertation

STUDY ON THE PHYSIOLOGICAL ROLE OF ADMINISTERED ACETIC ACID IN RATS

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AYA ARAKI

The Graduate School of
Health and Welfare Science
Okayama Prefectural University

CONTENTS

Abbreviations

AMP	5’ adenosine monophosphate
AMPK	5’ AMP-activated protein kinase
OLETF	Otsuka Long-Evans Tokushima Fatty
HPLC	high performance liquid chromatography
BSA	bovine serum albumin
SE	standard error
ANOVA	analysis of variance
PGC1–α	peroxisome proliferator-activated receptor γ coactivator 1α
AceCS	acetyl-CoA synthetase
ACC	acetyl-CoA carboxylase
SIRT1	NAD^+-dependent type III deacetylase sirtuin-1
PCR	Real-time polymerase chain reaction

Chapter I Introduction

Acetic acid is an organic compound with the chemical formula CH_3COOH. Vinegar is roughly 4–8% acetic acid by volume, making acetic acid the main component of vinegar. Acetic acid has a distinctive sour taste and pungent smell. Ruminal micro-organisms convert cellulose and starch to fatty acids including acetic acid, propionic acid, and butyric acid (volatile fatty acids; VFA)[1-3]. Because VFA becomes a precursor of lipid and glucose, it is a valuable source of energy for ruminant animals[4]. Acetic acid accounts for 70-75% in VFA, and acetic acid is thought to play an important role as an energy source in ruminant animals[5]. While, acetic acid is formed as a final product of enhanced β-oxidation of fatty acids and utilized as a fuel in extra hepatic tissues under the starving condition of rats[6-13]. Under the fed condition, β-oxidation is suppressed and acetic acid production is decreased. When acetic acid was taken daily by obesity-linked type 2 diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats under the fed condition, it protected OLETF rats against obesity[14,15]. Furthermore, acetic acid contributed to protect from the accumulation of lipid in the liver as well as abdominal fat of OLETF rats[14]. Transcripts of lipogenic genes in the liver were decreased, while transcripts of myoglobin and Glut4 genes in abdominal muscles were increased in the acetic acid–administered OLETF rats[15]. Acetic acid administered under fed condition is converted to acetyl-CoA with concomitant formation of intracellular adenosine monophosphate (AMP) via the catalytic activity of acetyl-CoA synthetase 1 (AceCS1) in the cytosol[2, 16]. Increase in intracellular AMP concentration leads to the activation of AMP-activated protein kinase (AMPK), which acts as the master switch of energy metabolism[17-25]. It is indicated

that exogenously-administered acetic acid would have effects on lipid metabolisms both in liver and skeletal muscles via the activity of AMPK and have function that work against obesity and obesity-linked type 2 diabetes.

The function of acetic acid generated endogenously under starved conditions has already been clarified[26], however, the physiological role of administered acetic acid under different physiological conditions has not yet been investigated. In order to investigate the physiological role of administered acetic acid under different physiological conditions in skeletal muscles, changes in the AMP level and phosphorylated AMPK under fed or starved conditions were investigated (Chapter II).

Various cellular or metabolic stresses that either inhibit ATP synthesis (e.g., heat shock[27], hypoxia[28], or glucose starvation[29]) or that enhance ATP consumption such as physical exercise[30-36] are the other factors of increasing the concentration of AMP and phosphorylating AMPK. During exercise, AMPK is activated in skeletal muscle according to exercise intensity and it increases fatty acid oxidation. In Chapter III, effects of acetic acid and exercise training on lipid metabolism and exercise capacity was investigated with comparing to exercise training.

Chapter II Changes in the 5'-AMP concentration of skeletal muscles on acetic acid treatment under fed or starved conditions in rats.

Acetic acid is the main component of vinegar and is also found in ruminants as a product of bacterial fermentation[1-5]. It is usually found in considerably high concentrations in mammalian blood plasma after intake of alcohol[37-39].

Under starved conditions, fatty acids derived from adipose tissues are oxidized and converted to acetyl-CoA by β-oxidation, and acetic acid is produced from acetyl-CoA by the catalytic activity of acetyl-CoA hydrolase in liver mitochondria[6]. The acetic acid thus produced is utilized as a biological fuel via the action of AceCS in the mitochondria of extrahepatic tissues[7-13]. In contrast, under fed conditions, the acetic acid administered is converted to acetyl-CoA with concomitant formation of intracellular AMP via the catalytic activity of AceCS1 in the cytosol[2, 16].

Acetyl-CoA produced in the cytosol is utilized in diverse reactions such as acetylation of histone protein or transcription factors, and cholesterol synthesis[40-43] Increase in intracellular AMP concentration leads to the activation of AMPK, which acts as the master switch of energy metabolism[17-25]. Previously, we reported that orally administered acetic acid reduced pathological conditions in OLETF, which exhibit hyperglycemic obesity with hyperinsulinemia and insulin resistance, through the reduction of lipogenesis and protection against fat accumulation. Furthermore, in animals, the acetic acid administered accelerates the phosphorylation of AMPK, expression of myoglobin and GLUT4 in skeletal muscles, and oxygen consumption rate [14,15].

The function of acetic acid generated endogenously under starved conditions has already been clarified[26], however, the physiological role of administered acetic acid under different physiological conditions has not yet been investigated. The purpose of this study was to investigate the metabolic function of administered acetic acid in

skeletal muscles through monitoring changes in the AMP level under fed or starved conditions.

Materials and Methods

Experimental animals

Six-week-old male Sprague-Dawley (SD) rats (n=3-11) were fed a normal laboratory diet for 1 week to stabilize the metabolic condition. The rats were housed individually in an air-conditioned room at approximately 25°C with alternating 12-h periods of light and dark (light, 8:00-20:00). All the animals were allowed free access to water and an appropriate diet.

For examining tissue distribution of AceCS1 and AceCS2, rats were anesthetized by intraperitoneal injection of Nembutal (100 μl/ 100 g of body weight) under fed or starved (for 48 h) conditions. The skeletal muscles (e.g., soleus, gastrocnemius abdominal muscle), heart, brain cerebellum, liver, kidney, lung, and spleen were then immediately isolated, frozen in liquid nitrogen, and stored at -80°C for subsequent isolation of RNA.

For examining acetic acid metabolism, rats were administered 1% v/v acetic acid at 5 ml/kg body weight; 52.5 mg/kg body weight), and they were anesthetized by isoflurane in 2–60 min after injection of acetic acid, under fed or starved (48 h) conditions. Subsequently, the soleus and gastrocnemius muscles were obtained; they were freeze-clamped in liquid nitrogen and stored at -80°C for immunoblotting or lyophilized for high-performance liquid chromatography (HPLC) analysis.

The care and use of the animals in this study followed the guidelines of Okayama Prefectural University (No.27-3) and the laws and notifications of the Japanese

government.

Nucleotides analysis

Lyophilized samples were homogenized with ice-cold 0.5 N perchloric acid by using a Polytron homogenizer (YAMATO SCIENTIFIC, Tokyo, Japan). After centrifugation (at 700 G for 10 min at 4°C), the supernatant was neutralized with 5 N potassium hydroxide. The precipitate was removed by centrifugation. The concentrations of AMP, ADP, and ATP in the extracts of the skeletal muscle were determined by reverse-phase HPLC analysis with a liquid chromatography system equipped with a pump (LC-10AS), a UV/Vis detector (SPD-10A), and a column Shim-pack VP-ODS 4.6 × 250 mm (Shimadzu Corporation, Kyoto, Japan). The mobile phase consisted of 100 mM phosphate buffer (pH 6.3), containing 0.6% methanol. Column temperature was maintained at 25°C, the flow rate was 1.0 ml/min, and detection was performed at λ = 260 nm. Nucleotides were identified and quantified using the corresponding standard compounds (Oriental Yeast, Tokyo, Japan).

Real-time polymerase chain reaction (PCR)

Total RNA was extracted from each tissue using Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan), and cDNA was synthesized from 0.5 μg RNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). Expression analysis of genes was performed by real-time PCR with the KAPA SYBR® FAST qPCR Kit (KAPA BIOSYSTEMS, Wilmington, MA) and iCyclerTM Real-Time PCR Detection System. β-actin (*Actb*) served as the endogenous control. The real-time PCR primers used were as follows:

AceCS2, forward primer 5′-ACTGTCTGGGACTGCGACTT-3′ and

reverse primer 5′-TCCTCACTTCTGTCCCAGGT-3′;

AceCS1, forward primer 5′-GGTGCTCAGAACTTGACGTG-3′ and

reverse primer 5′-GGCATGGGGTTTTCCAGTA-3′;

ß-actin, forward primer 5′-GGAGATTACTGCCCTGGCTCCTA-3′ and

reverse primer 5′-GACTCATCGTACTCCTGCTTGCTG-3′.

Western blotting

Rat tissues were cut into small pieces using scissors; suspended in solution containing 2.5 mM Tris, pH 8.0, 0.5 mM DTT, 1 mM EDTA, 10 mM $MgCl_2$, and 0.25 M sucrose; and homogenized with a Polytron homogenizer. The homogenate was centrifuged (3500 rpm, 10 min, 4°C) to remove the tissue debris. Protein concentration was determined with the Bradford assay, using bovine serum albumin (BSA) as a standard. The proteins run on the gel were transferred onto a polyvinylidene difluoride membrane (Merch, DA, Germany). After the membrane was treated for 1 h with 3% BSA in Tris-buffered saline containing 0.1% Tween 20 (TBST), it was incubated overnight at 4°C with the primary anti-AMPK antibody, pThr172 AMPK AceCS1 from Cell Signaling Technology (MA, USA), AceCS2 from Santa Cruz Biotechnology Inc. (CA, USA), or α-tubulin from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The primary antibody was diluted in Can Get Signal Immunoreaction Enhancer Solution 1 (TOYOBO). After three washes with TBST, the membrane was incubated with an HRP-conjugated secondary antibody for at least 1 h. After another set of three washes with TBST, the chemiluminescent reaction was performed for 5 min with ImmunoStar LD (Wako Pure Chemical Industries Ltd., Japan) according to the manufacturer's instructions, and

chemiluminescent signals were visualized and quantified with ImageQuant LAS-4000 and Multi Gauge V3.2 analyzing software (Fujifilm, Tokyo, Japan).

Statistical analysis

Data are expressed as mean ± standard error (SE). Statistical differences between the two groups were determined by the Student's t-test (Excel 2013), whereas multiple groups were compared by one-way analysis of variance (ANOVA) followed by Tukey-Kramer post hoc analysis (Mulcell 2005). Differences between groups were considered statistically significant at $p < 0.05$.

Results

Effect of acetic acid on the change in AMP level in skeletal muscles

Adenine nucleotide concentrations in perchloric acid extracts of the skeletal muscles were determined after intragastric injection of 52.5 mg/kg BW of acetic acid (Table 1, 2). Under fed conditions, the AMP content of the soleus muscle significantly increased ($p<0.05$) in 30 min and 60 min after injection of acetic acid (Fig.1Aa). In the gastrocnemius muscle it increased significantly in 10 min after injection of acetic acid (Fig.1Ba). In contrast, under starved conditions, the AMP content of the soleus muscle decreased in 2 min after the injection (Fig.1Ab), and in the gastrocnemius muscle and it was not significantly changed with the treatment of acetic acid under starved conditions. (Fig.1Bb).

Table.1 Adenosine nucleotides in soleus muscles after oral administration of acetic acid under fed or starved conditions

Adenosine nucleotides (μmol/g of protein) in soleus muscles of 7-week-old male SD rats orally administered 52.5 mg/kg of BW acetic acid for the indicated times under fed (a) or starved (b) conditions. Each data value is expressed as the mean ± SE (n=5~11). Significant differences among groups analyzed with one-way ANOVA followed by the Turkey- Kramer post hoc test. Groups without the same letter are significantly different ($p<0.05$).

a fed condition

Time (min)	ATP	ADP	AMP	Total
	μmol/g			
0	11.6±0.9	2.8±0.2	0.34±0.02[a]	14.7±1.0
2	11.8±0.8	2.6±0.4	0.36±0.03[a]	14.7±1.0
5	12.5±0.8	3.2±0.5	0.42±0.05[ab]	16.0±1.0
10	12.3±1.0	3.6±0.4	0.44±0.04[ab]	16.4±1.1
30	12.4±1.1	3.9±1.2	0.63±0.13[b]	16.9±0.5
60	11.4±0.9	2.7±0.5	0.59±0.08[b]	14.7±1.0

b starved condition

Time (min)	ATP	ADP	AMP	Total
	μmol/g			
0	11.5±0.9	3.3±0.2	0.62±0.08[a]	15.5±1.0
2	10.8±0.4	2.8±0.0	0.31±0.05[b]	14.0±0.3
5	11.9±0.5	2.7±0.1	0.22±0.00[b]	14.8±0.5
10	11.9±0.5	2.9±0.2	0.32±0.04[b]	15.1±0.7
30	11.2±0.4	3.0±0.2	0.33±0.05[b]	14.5±0.6
60	10.1±0.6	2.7±0.1	0.32±0.04[b]	13.1±0.6

Table.2 Adenosine nucleotides in gastrocnemius muscles after oral administration of acetic acid under fed and starved conditions

Adenosine nucleotides (μmol/g of protein) in gastrocnemius muscles of 7-week-old male SD rats orally administered 52.5 mg/kg of BW acetic acid for the indicated times under fed (a) or starved (b) conditions. Each data value is expressed as the mean ± SE (n=5~11). Significant differences among groups analyzed with one-way ANOVA followed by the Turkey- Kramer post hoc test. Groups without the same letter are significantly different ($p<0.05$).

a fed condition

Time (min)	ATP	ADP	AMP	Total
	μmol/g			
0	16.0±0.5	2.3±0.2	0.16±0.02[a]	16.2±2.4
2	15.5±1.0	2.7±0.2	0.15±0.02[a]	16.9±2.1
5	16.5±0.7	2.8±0.3	0.13±0.01[a]	19.4±0.9
10	16.3±0.9	3.1±0.3	0.26±0.03[b]	19.6±1.4
30	15.7±2.2	2.8±0.4	0.21±0.02[ab]	18.7±2.6
60	16.2±1.7	2.7±0.2	0.16±0.02[a]	19.1±1.8

b starved condition

Time (min)	ATP	ADP	AMP	Total
	μmol/g			
0	12.9±0.8	2.4±0.2	0.23±0.08	15.3±0.5
2	13.8±0.4	2.6±0.4	0.15±0.03	16.6±0.7
5	13.7±0.5	2.2±0.1	0.11±0.01	16.0±0.5
10	13.0±0.4	2.8±0.7	0.12±0.01	15.9±0.5
30	15.2±0.6	2.4±0.1	0.10±0.02	17.7±0.7
60	13.6±0.6	2.2±0.1	0.13±0.02	15.9±0.8

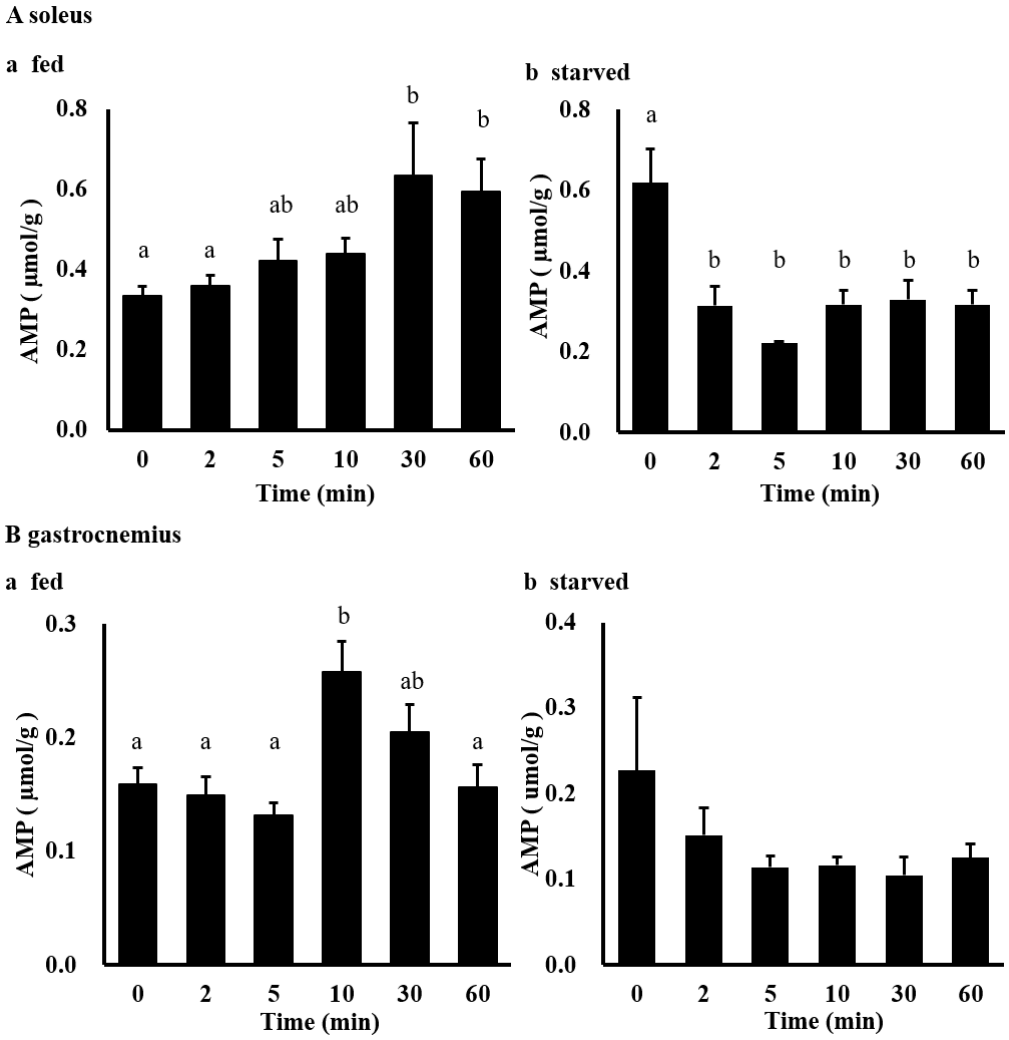


Fig.1 Change in AMP concentration in soleus (A) and gastrocnemius (B) muscles after oral administration of acetic acid under fed (a) or starved (b) conditions.

Acetic acid (52.5 mg/kg of BW) was orally injected into 7-week-old male SD rats under fed or starved condition. Each data value is expressed as the mean ± SE for five to eleven rats. Significant differences among groups analyzed by Turkey- Kramer. Groups without the same letter are significantly different ($p<0.05$). Reprinted by Annals of Obesity & Disorders Volume 1 Issue 3 October 14, 2016

Phosphorylation of AMPK on treatment with acetic acid

An increase in the AMP level in the cytosol induces phosphorylation and activation of AMPK[2, 16]. The phosphorylated AMPK in the soleus and gastrocnemius muscles of SD rats was analyzed by western blotting. Phosphorylation of AMPK significantly increased in 30-60 min after injection of acetic acid in soleus and gastrocnemius muscle under fed conditions (Fig.2Aa, Fig.2Ba). Under starved conditions, phosphorylation of AMPK remained unchanged even after administration of acetic acid (Fig.2Ab, Fig.2Bb).

Expression of AceCS in tissues

The *ACSS2* and *ACSS1* genes (AceCS1 and AceCS2) are expressed widely among tissues and associated with acetyl-CoA generation from acetic acid. Expression of the AceCS1 gene was found to be higher in the kidney among all tissues studied, while AceCS2 gene expression was higher in the heart and kidney (Fig.3A). Transcription of AceCS2 gene was induced under starved conditions in skeletal muscles (Fig.3B). The level of the protein encoded by AceCS2 gene increased in the soleus and gastrocnemius muscles under starved conditions (Fig.3C).

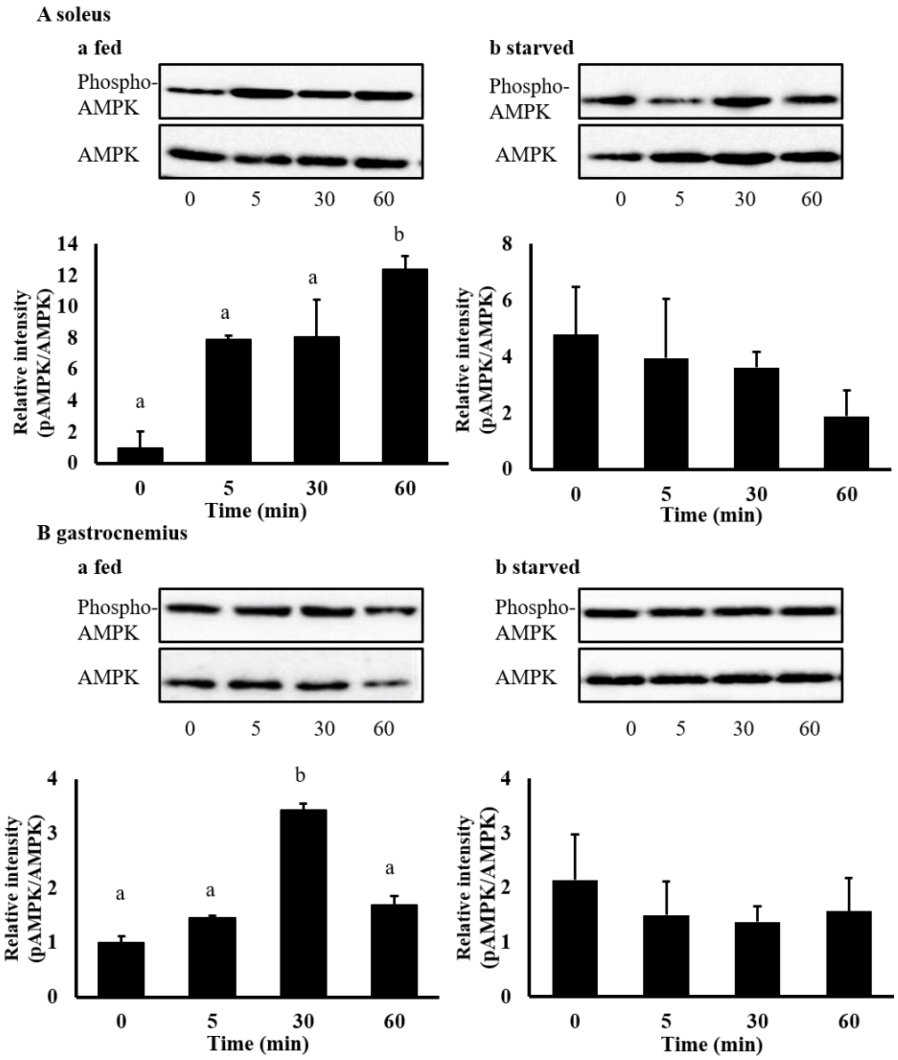


Fig.2 Effect of acetic acid administration on the phosphorylation of AMPK in s soleus (A) and gastrocnemius (B) muscles after oral administration of acetic acid under fed (a) or starved (b) conditions.

Acetic acid (52.5 mg/kg of BW) was orally injected into 7-week-old male SD rats (n=3) under fed or starved condition and phosphorylated AMPK was analyzed by western blotting. The relevant amount of the p-AMPK was normalized to the amount of AMPK. Each data value is expressed as the mean ± SE shown as relative intensity normalized to the value of 0 min group. Significant differences among groups analyzed by Turkey- Kramer. Groups without the same letter are significantly different ($p<0.05$). Reprinted by Annals of Obesity & Disorders Volume 1 Issue 3 October 14, 2016

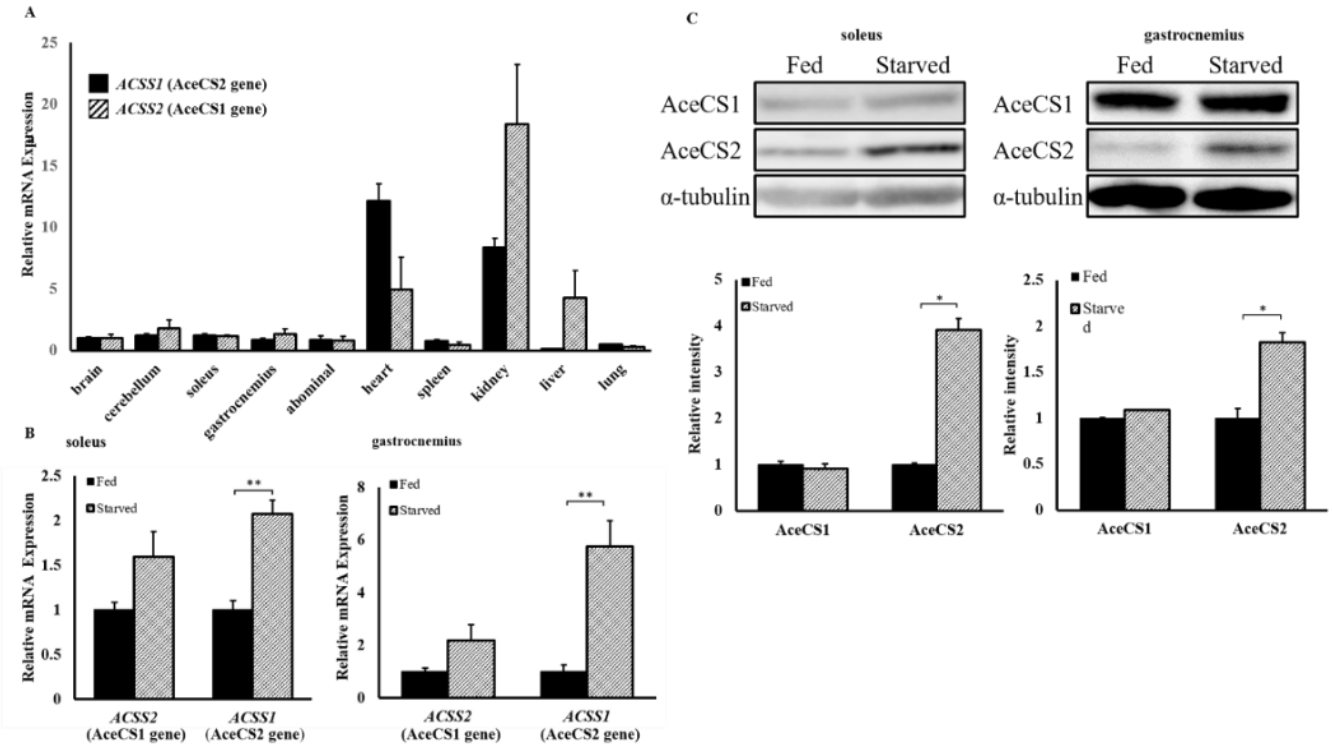


Fig.3 Expressions of Acetyl-CoA synthetase1 (AceCS1) or 2 (AceCS2) genes in tissues (A) and induction of AceCS2 gene (B) and protein (C) under fed or starved conditions.

Total RNA was isolated from soleus and gastrocnemius muscles in SD rats at 7–weeks age under fed or starved conditions, and analyzed mRNA expression levels of AceCS1 and AcsCS2 genes. A; AceCS1 and 2 genes were analyzed using tissues of rats under fed conditions. The relevant amount of AceCS1 or 2 transcripts was normalized to the amount of *actb*. Each data value is expressed as the mean ± SE shown as relative expression normalized to the value of brain mRNA expression. B; AceCS1 and 2 genes were analyzed using tissues of rats under fed or starved conditions. The relevant amount of AceCS1 or 2 transcripts was normalized to the amount of *actb*. C; Proteins were isolated from soleus and gastrocnemius muscles in SD rats at 7–weeks age under fed or starved conditions. Proteins were analyzed by western blotting. The relevant amount of the AceCS1 or 2 was normalized to the amount of α-tubulin. Each data value is expressed as the mean ± SE shown as relative expression normalized to the value of fed group. Significant differences among groups were analyzed by T-test (*: $p<0.05$ **: $p<0.01$). Reprinted by Annals of Obesity & Disorders Volume 1 Issue 3 October 14, 2016

Discussion

In our previous study, we reported that when acetic acid was orally administered to SD rats, it was readily taken up into the blood stream and was absorbed into tissues[34]. Acetic acid is converted to acetyl-CoA by the catalytic activity of AceCSs in tissues. There are two types of AceCSs: AceCS1, which localized in the cytosol, and AceCS2, which localized in the mitochondria[16, 42,43]. Acetic acid gets converted to acetyl-CoA in tissues where AceCS1 or AceCS2 proteins are expressed. The mRNA expression level of *ACSS1*, which converts acetic acid to acetyl-CoA in mitochondria, was significantly higher in the soleus and gastrocnemius muscles under starved conditions than under fed conditions (Fig.3).

Acetic acid converted to acetyl-CoA under starved conditions would be used mainly for biological fuel. In AceCS2-deficient mice, it was found that the ATP level was 50% lesser than that in wild-type mice and was associated with reduced body temperature and exercise capacity[44]. This observation indicates that AceCS2 plays an important role in ATP generation and thermogenesis under starved conditions.

AceCS1 is localized in the cytoplasm and is highly expressed in the liver and kidney [42, 43]. The results from our study indicate that AceCS1 is widely expressed in tissues in much smaller extent in addition to the liver and kidney (Fig.3), where acetyl-CoA generated may play a diverse role such as in cholesterol synthesis or histone acetylation. Under fed conditions, the acetic acid administered was converted to acetyl-CoA with concomitant formation of AMP. The AMP levels significantly increased in the soleus and gastrocnemius muscles after administration of acetic acid. However, under starved conditions, administration of acetic acid did not increase the AMP levels in those tissues

(Fig.1, 2). This finding indicates that AMP would accumulate in skeletal muscles on administration of acetic acid under fed conditions rather than starved conditions, and the generation might mainly be driven by the activity of cytosolic AceCS1 rather than that of mitochondrial AceCS2. Indeed, phosphorylated AMPK, which is localized in the cytosol, increased after administration of acetic acid in the gastrocnemius and soleus muscles under fed conditions (Fig.2). AMPK acts as the key metabolic master switch and regulates a number of enzymes involved in lipid homeostasis. Activation of AMPK occurs by an increase in AMP, leading to the inactivation of acetyl-CoA carboxylase by phosphorylation and blocking of fatty acid synthesis; furthermore, activation of fatty acid oxidation occurs via the decrease in intracellular malonyl-CoA to generate energy and enhance the energy expenditure system. In our previous study, we found that administeration of acetic acid under fed conditions resulted in suppression of lipid accumulation and lower weight gain for OLETF rats than water administered OLETF rats[14, 15].

Under starved conditions, fatty acids derived from adipose tissue are oxidized by β-oxidation to form ketone bodies and acetic acid in the liver[26]. Acetic acid is excreted into the blood stream and utilized as a physiological fuel in extrahepatic tissues in a similar manner as ketone bodies. In this study, acetic acid administered to rats under starved conditions was found to lead the much lesser accumulation of AMP in skeletal muscles than that in fed condition. Moreover, the levels of phosphorylated AMPK remained unchanged in these tissues under starved conditions. It is suggested that (a) acetic acid gets converted to acetyl-CoA with formation of AMP in mitochondria because of the catalytic activity of AceCS2 under starved conditions, (b) the AMP formed in mitochondria might be converted to ADP rapidly by the action of adenylate

kinase with ATP in mitochondria, which would be reacted with much easier than that in cytosolic reaction, and (c) the ADP would be utilized for generation of ATP[45,46]. Under fed conditions, administration of acetic acid led to the generation of AMP, which might be produced mainly in cytosol, and led to the activation of AMPK. On the basis of these observations, we suggest that acetic acid has a possible role in lipid metabolism in muscles and may also play a role in fighting obesity and obesity-linked type 2 diabetes through the activation of AMPK.

Chapter III Effect of exercise training with intake of acetic acid on lipid metabolism and endurance performance.

Previously, we reported that oral administered acetic acid contributed to suppression of lipogenesis in the liver and to the reduction of lipid accumulation in adipose tissue of Otsuka Long-Evans Tokushima Fatty (OLETF) rats, which exhibit hyper glycemic obesity with hyperinsulinemia and insulin resistance[14]. In addition, OLETF rats administered with acetic acid showed a higher rate of oxygen consumption and a smaller size of lipid droplets in white adipose tissues[15]. Under fed condition, orally administered acetic acid is immediately taken up from the digestive organs and excreted into the blood stream in rats[14, 47, 48]. Then, acetic acid is absorbed by tissues such as skeletal muscle or liver, and converted to acetyl-CoA with concomitant formation of AMP by the catalytic activity of acetyl-CoA synthetase (AceCS) in the cytosol[2,16]. We observed that the AMP/ATP ratio increased in the liver and muscle of acetic acid administered OLETF rats, which enhanced phosphorylation and activation of AMPK in both tissues[14, 15].

AMPK is a heterotrimeric protein kinase which has been found to play a key role in regulation of whole-body energy by phosphorylating key metabolic enzymes in both biosynthetic and oxidative pathways[49]. Various cellular or metabolic stresses that either inhibit ATP synthesis (e.g., heat shock[27], hypoxia[28], or glucose starvation[29]) or that enhance ATP consumption such as physical exercise[30-36] increase the intracellular AMP/ATP ratio, leading to AMPK activation. Well known function of AMPK is an inactivation of acetyl-CoA carboxylase (ACC) which is the rate-limiting enzyme of fatty acid synthesis. Activated AMPK phosphorylates ACC, which leads to decrease of intracellular malonyl-CoA, block of fatty acid synthesis, activation of carnitine palmitoyl-CoA transferase I, increase of fatty acid oxidation to generate energy, and enhancement of energy expenditure system[49]. In addition, activated AMPK

phosphorylates peroxisome-proliferator-activated receptor γ coactivator 1α (PGC-1α) and activates its downstream gene targets such as PGC-1α itself, GLUT4, cytochrome c and UCP-3[50]. Activated AMPK also activates the NAD+-dependent type III deacetylase sirtuin-1 (SIRT1) by increasing intracellular NAD+/NADH ratio via accelerating NAD+ synthesis[51], leading to deacetylation and activation of PGC-1α[52]. In skeletal muscle, activation of AMPK-SIRT1-PGC-1α axis resulting from either muscle contraction or chemical stimulator[52] leads to increase of slow twitch muscle fiber via enhancement of mitochondrial biogenesis, oxidative metabolism, and type I myofibers.

During exercise, AMPK is activated in skeletal muscle according to exercise intensity and it increases fatty acid oxidation. In this study, in order to investigate the interactive effects of acetic acid and exercise training on lipid metabolism and exercise capacity, we examined endurance performance and lipid metabolism during exercise in rats administered acetic acid with exercise training in the preliminary study.

Materials and Methods

As the experimental animals, 6 weeks old SD rats were used. Rats were fed normal laboratory diet for 1 week to stabilize the metabolic condition. Rats were randomly assigned to four groups: water-injected (rest-water group), acetic acid-injected (rest-ace group), exercise-trained after injection of water (water-ex group), exercise-trained after injection of acetic acid (ace-ex group). Rest-water group was given distilled water at 5 ml/kg of body weight, and acetic acid group was given 1% (v/v) acetic acid of 5 ml/kg body weight daily 5 days a week for 4 weeks.

During resting or exercise training, oxygen consumption of rats were measured by an O_2/CO_2 metabolism measuring system (Muromachi Kikai). This system monitors VO_2 and VCO_2 at 3-min intervals and calculates the respiratory quotient (RQ) ratio (VCO_2/VO_2). Each rat was in a sealed chamber with a constant air flow (of 1.5 l/min) for 24 h at 25˚C with free access to water and diet. Measurement was performed during the dark or light period. The consumed oxygen concentration (VO_2), RQ ratio, and energy consumption were calculated. To examine spontaneous physical activity, each rat was housed in sealed chamber equipped with an infrared sensor and the activity was measured using a Supermex system (Muromachi Kikai) concomitantly with measurement of VO_2 and VCO_2. The water-ex and acetic acid-ex groups were exercise-trained by air-tight treadmill (Muromachi Kikai) for 30 min at 18 m/min after injection of water or acetic acid daily 5 days a week for 4 weeks.

The substrate utilization rate and energy production rate were calculated using the formula (15), that is, the rate of glucose oxidation (mg/min) = $4.55VCO_2$ (ml/min) - $3.21VO_2$ (ml/min) - 2.87N(mg/min), the rate of lipid oxidation (mg/min)=

1.67(VO_2-VCO_2) - 1.92N, and the rate of energy production (kcal/min)=(1.07 ×RQ+ 3.98) × VO_2, in this formula N is the rate of urinary nitrogen excretion used to estimate protein oxidation. However, the contributions of protein oxidation were ignored as it was considering that only a small portion of resting and exercise energy expenditure arisen from protein oxidation.

To examine exercise capacity, we carried out exercise tolerance test. Rats were housed in an air-tight treadmill (Muromachi Kikai) and the rats were challenged at 18 m/min. The speed increased 1m/min every min until exhaustion, which is defined as touching on the shocker in the rear part of treadmill more than 10 times for 1 min.

Food consumption and body weight were recorded every day. After one month of experiment, the rats were anesthetized by intraperitoneal injection of Nembutal, and abdominal, gastrocnemius, and soleus muscles were immediately isolated, weighed, frozen in liquid nitrogen, and stored at –80˚C for subsequent analysis of mRNA.

Real-time polymerase chain reaction (PCR)

Total RNA was prepared from isolated skeletal muscles by using Sepasol-RNA super I (Nacalai Tesque) and reverse-transcribed by using PrimeScript® RT reagent Kit with gDNA Eraser (Takara Bio) according to the manufacturer's instructions. To determine mRNA expression levels, quantitative real-time PCR analyses were performed by using the iQ5 (Bio-Rad) with KAPA SYBR Fast qPCR Kit (Nippon Genetics). The primer sequences used for the amplification were as follows:

β-actin (*actb*), forward : 5'-GGAGATTACTGCCCTGGCTCCTA-3',

reverse : 5'-GACTCATCGTACTCCTGCTTGCTG-3',

cytochrome c (*cycs*), forward : 5'-AGCGGGACGTCTCCCTAAGA-3',

reverse : 5’-CTTCCGCCCAAACAGACCA-3’,

MHC1 (*myh7*), forward : 5’-AGAGGAAGACAGGAAGAACCTAC-3’,

reverse : 5’-GGCTTCACAGGCATCCTTAG-3’, MHC2b (*myh4*),

forward : 5’-GAGGACCGCAAGAACGTG-3’,

reverse : 5’-TGTGTGATTTCTTCTGTCACC-3’,

PGC-1α (*ppargc1a*), forward : 5’-GACCCCAGAGTCACCAAATGA-3’,

reverse : 5’-GGCCTGCAGTTCCAGAGAGT-3’. Data were normalized for actb mRNA and expressed relative to that in muscles of rest-water group.

Statistical analysis

Data are expressed as mean ± standard error (SE). Statistical differences were compared by one-way analysis of variance (ANOVA) followed by Tukey-Kramer post hoc analysis (Mulcell 2005). Differences between groups were considered statistically significant at $P < 0.05$.

RESULTS

Body weight gain tended to be lower in rest-ace, water-ex, and ace-ex groups than that of rest-water group during experimental period (Fig. 1). Effects of treatments with acetic acid and exercise training on body weight gain, food intake, food efficiency, and abdominal fat content were shown in Fig. 2. The total body weight gain was significantly lower in rest-ace and ace-ex groups than that of rest-water group. While, total amount of food intake was not significantly changed among 4 groups. Food efficiencies of rest-ace, water-ex, and ace-ex groups were significantly lower as compared with rest-water group. Abdominal fat contents of rest-ace, water-ex, and ace-ex groups were significantly lower by about 60%, 50% and 50%, respectively than that of the rest-water group.

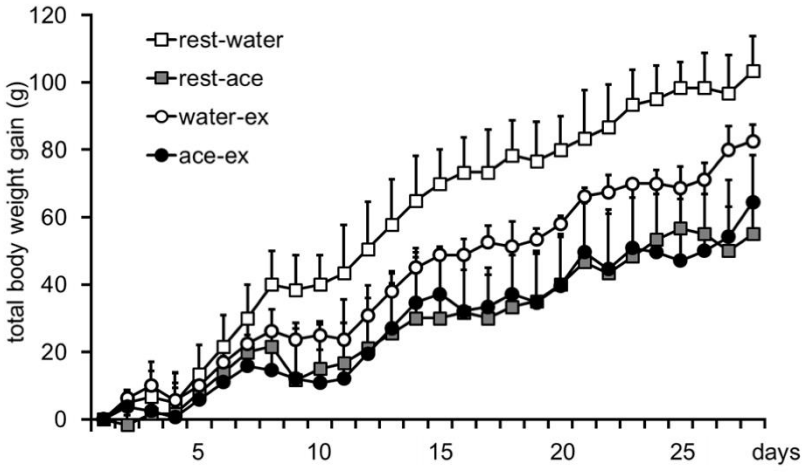


Figure 1, Total body weight gain

Time course of body weight change of rats at 6 weeks of age administrated distilled water (rest-water) or acetic acid (rest-ace) during resting period or administrated distilled water (water-ex) or acetic acid (ace-ex) before exercise. Values are shown as arithmetic mean ±SE of 3 rats of each group. n=3

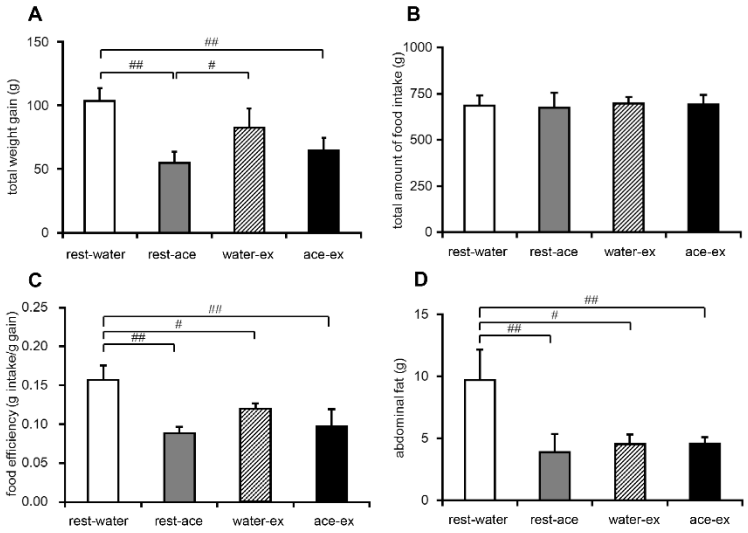


Figure 2, Effect of acetic acid ingestion on total weight gain, total amount of food intake, food efficiency, and abdominal fat content.

Total body weight gain (A), total amount of food intake (B), food efficiency (C), and abdominal fat content (D) of rats administered water or acetic acid during rest period and before exercise. Values are shown as arithmetic mean ±SE of 3 rats of each group. Statistical differences are shown as # $P<0.05$, ## $P<0.01$ by Tukey-Kramer's post hoc test. n=3

Activation of AMPK by treatments of acetic acid and exercise training may increase lipid oxidation [15, 31] In order to determine whether acetic acid and exercise training change in the energy metabolic rate in the sedentary state, oxygen consumption was measured and RQ ratio was calculated (Fig. 3). Energy consumption rates in water-ex and ace-ex groups revealed significantly higher than that in rest-water group. Motor activity levels were significantly lower in rest-ace, water-ex, and ace-ex groups than that in rest-water group.

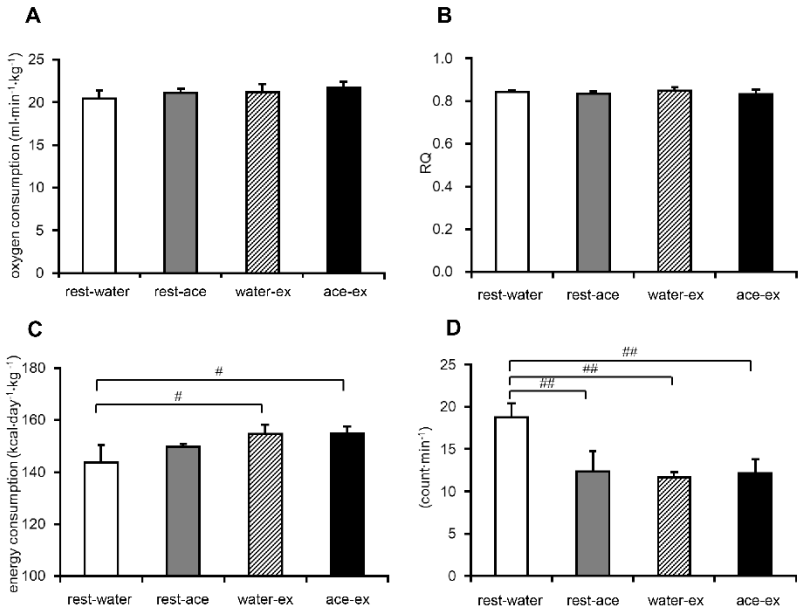


Figure 3, Effect of acetic acid ingestion on oxygen consumption, RQ, energy consumption, and spontaneous motor activity in the sedentary state.

Oxygen consumption and carbon dioxide production were monitored using on O_2/CO_2 metabolism measuring system for small animals in air tight chamber. The rats of energy consumption were calculated using energy consumption (kcal/day/kg)= light cycle (kcal/h/kg)*12 + dark cycle (kcal/h/kg)*12 (A), oxygen consumption, (B), RQ, (C), energy consumption, and (D), spontaneous motor activity of rats.

Values are shown as arithmetic mean ±SE of 3 rats of each group. Statistical differences are shown as # $P<0.05$, ## $P<0.01$ by Tukey-Kramer's post hoc test. n=3

In order to investigate the effect of acetic acid and exercise training on exercise capacity and fuel utilization during exercise, rats were started off running on a treadmill at 18 m/min, and then the speed was increased by the rate of 1 m/min until exhaustion. Exercise capacity was shown to be increased in water-ex and ace-ex groups as compared with rest-water group (Fig. 4A). Running duration was significantly longer in water-ex and ace-ex groups than that in rest-water group (Fig. 4B).

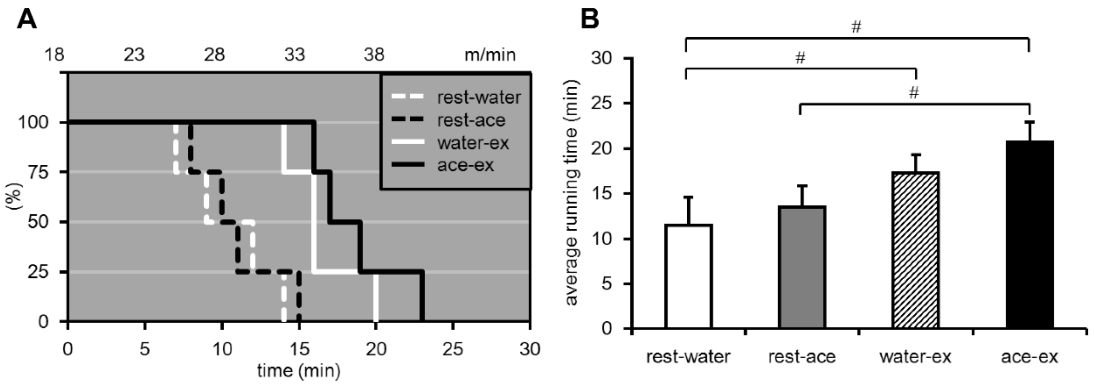


Figure 4, Effect of acetic acid ingestion on exercise tolerance.

Rats were administered water or acetic acid daily (5 days a week) for 4 weeks and then they were exercised by forced running on a treadmill at 18 m/min. The speed increased by 1 m/min every 1 min until exhaustion. Exhaustion is defined as touching on the shocker more than 10 times for 1 min. Exercise tolerance is shown as a Kaplan-Meier survival curve (A). Average running duration is shown in panel B. Values are shown as arithmetic mean ±SE of 4 rats of each group. Statistical differences are shown as # $P<0.05$ by Tukey-Kramer's post hoc test.

To investigate the fuel utilization during exercise, VO_2 and VCO_2 were monitored simultaneously until exhaustion (Fig. 5). Oxygen consumptions in all four groups were increased as speed increased until exhaustion. RQ ratios of all groups during exercise were increased as the speed of treadmill increased, however the ratio in rest-ace and water-ex groups tended to be lower than that of rest-water group, and the RQ ratio in ace-ex group was significantly lower in 3, 9, 12 min after beginning of the run than that of rest-water group (Fig. 5).

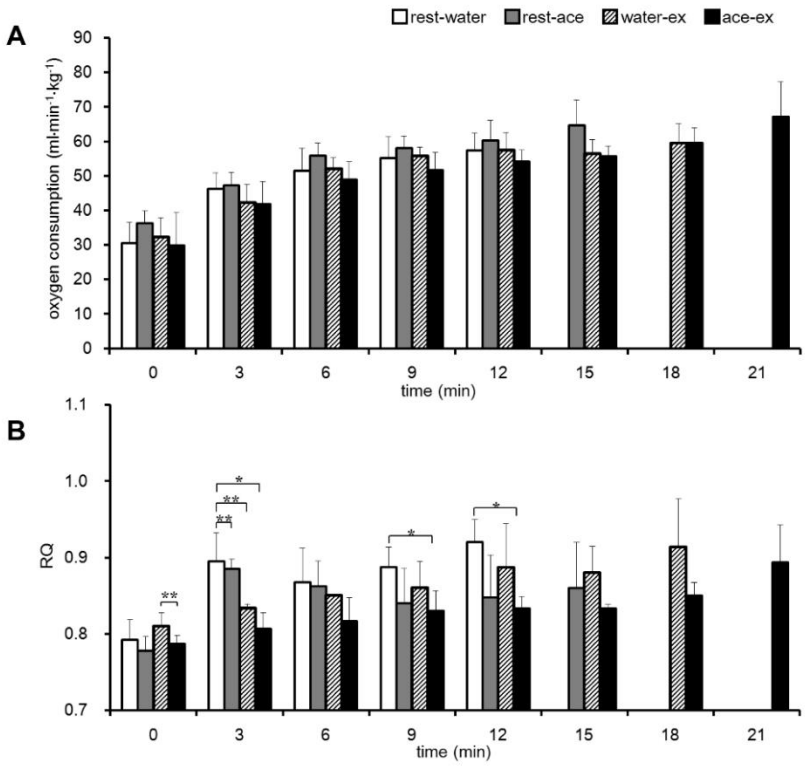


Figure 5, Effect of acetic acid ingestion on the oxygen consumption and RQ during exercise tolerance test.

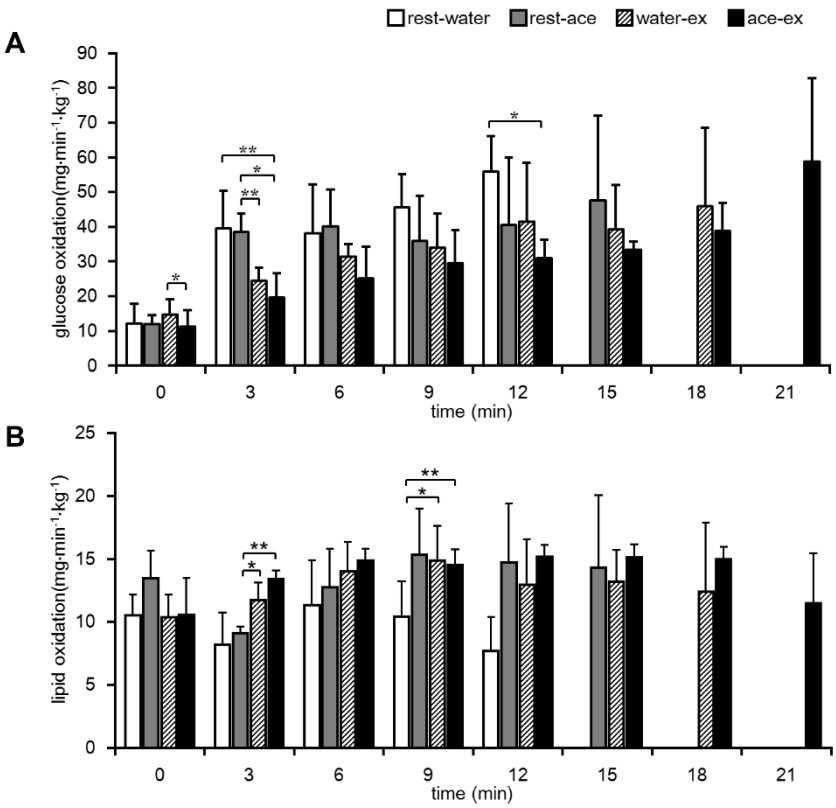
During exercise tolerance test, oxygen consumption and carbon dioxide production were monitored using on O_2/CO_2 metabolism measuring system for small animals in air tight chamber. (A), Oxygen consumption. (B), RQ. Values are shown as arithmetic mean ±SE of 3-4 rats of each group.

Statistical differences are shown as *P<0.05 **P<0.01 vs rest-water by student t-test. rest-water n=4, rest-ace n=4, water-ex n=3, ace-ex n=3

The calculated level of glucose oxidation during exercise tended to be higher in rest-water group, while glucose oxidation in ace-ex group was relatively lower, and it was significantly lower in 3 min and 12 min after start running than that of rest-water group (Fig. 6A). While, the calculated level of lipid oxidation during exercise tended to be higher in rest-ace, water-ex and ace-ex groups as compared with rest-water group,

and it was significantly higher in water-ex and ace-ex groups in 9 min after start running than that in rest-water group (Fig. 6B).

Figure 6, Effect of acetic acid ingestion on glucose and lipid oxidation during exercise tolerance test.



Glucose (A) and lipid oxidation (B) during exercise tolerance test were calculated as described in "Materials and Methods". Values are shown as arithmetic mean ±SE of 3-4 rats of each group. Statistical differences are shown as *P<0.05 **P<0.01 vs rest-water by student t-test. rest-water n=4, rest-ace n=4, water-ex n=3, ace-ex n=3

To determine the effects of treatment of acetic acid and exercise training on mRNA levels related to lipid metabolism in skeletal muscles, mRNA expressions of ppargc1a, *cycs, myh4*, and *myh7* which were related to metabolism and muscle fiber types were analyzed by quantitative real-time RT-PCR. Transcript of PGC-1α was tended to be

increased in gastrocnemius muscle of ace-ex groups, whereas it was not changed by the treatment of acetic acid or excise in soleus muscle (Fig. 7A). Expressions of mRNA, *cycs, myh4,* and *myh7* genes in gastrocnemius muscle were analyzed. Expression of cycs was tended to be increased in rest-ace and water-ex groups, and it was significantly induced higher in ace-ex group as compared with that in rest-water group (Fig. 7B). MHC1 expression, which is expressed much in slow twitch oxidative fiber, was significantly increased in rest-ace and ace-ex groups (Fig. 7C). On the other hand, the expression of MHC2b, which is expressed in fast twitch muscle fiber, was not changed among 4 groups (Fig. 7D).

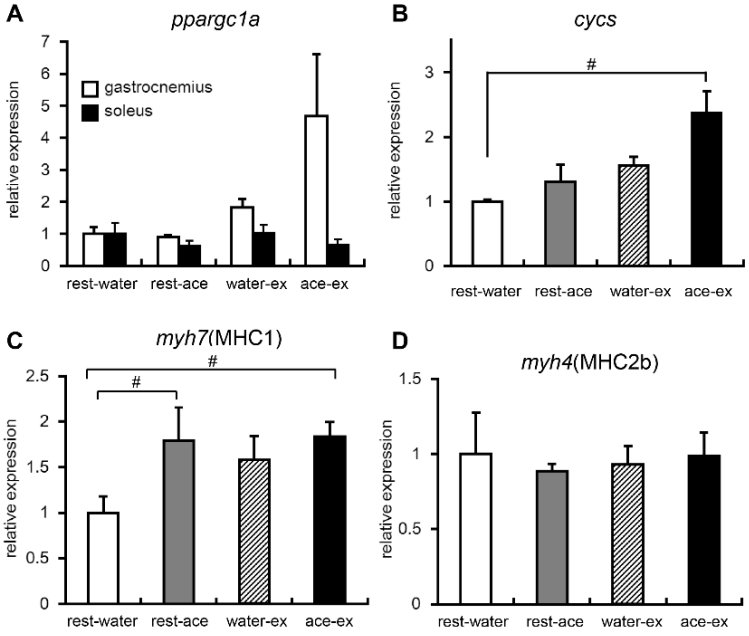


Figure 7, Effect of chronic intake of acetic acid and exercise training on gene expressions in skeletal muscles.Quantitative RT-PCR analysis was performed using total RNA isolated from gastrocnemius and soleus muscle and of rats administered distilled water or acetic aicd with sedentary or exercise training. (A), ppargc1a expression in gastrocnemius muscle and soleus muscle. (B-D), cycs, myh7, myh4 expressions in gastrocnemius muscle. Values are shown as arithmetic mean ±SE of 3-5 rats of each group. Statistical differences are shown as # $P<0.05$ by Tukey-Kramer's post hoc test.

Discussion

In our previous study, we showed that chronic intake of acetic acid induced gene expressions of myoglobin and GLUT4 and also increased energy consumption in rats[15]. Orally administered acetic acid was taken up from the intestine, absorbed by liver and skeletal muscles, and it increased AMP/ATP ratio in those cells[14, 15]. An increase in the AMP/ATP ratio leads to the phosphorylation of AMPK. Treatment of acetic acid increased AMP/ATP ratio and promoted phosphorylation of AMPK in skeletal muscle of rats. Physical exercise is well known factor to increase AMP/ATP ratio and to activate AMPK[30, 31]. In this study, we examined the effects of acetic acid and exercise training on the endurance performance and lipid metabolism of rats during exercise. Treatments of chronic intake of acetic acid and exercise training led to the reduction of abdominal fat content and result in lower weight gain. Energy consumption in rats treated with exercise training (water-ex and ace-ex groups) were higher, while, those motor activities were lower than control group, suggesting that energy consumption that may be derived from increased basal energy metabolism was stimulated by exercise training. Endurance performance was promoted in exercise training groups. During endurance exercise, glucose utilization was lower and lipid utilization was higher in rats treated with exercise training groups than that of ret-water group. These data indicate that the treatment both of chronic intake of acetic acid and exercise training increases fat oxidation and decrease glucose oxidation during endurance exercise and it is promoted in fiber type switching to oxidative fiber in gastrocnemius muscle.

Exercise training induce muscle remodeling and mitochondrial proliferation in skeletal muscle, resulting fiber-type switch from glycolytic to oxidative fibers and enhancing

lipid oxidative capacity[55-58]. *Ppargc1a* expression is increased in muscle by an acute exercise or long-term exercise training[59-64]. Increased *ppargc1a* expression in muscle showed an enhanced ability to exercise and improvement of peak oxygen uptake[58]. Furthermore, AMPK is also activated by exercise, and activated AMPK phosphorylates PGC-1α, then its downstream genes targets are upregulated[50]. In this study we observed that skeletal muscle of rats treated with acetic acid and exercise training induced mRNA expression of cytochrome c gene and tended to increase PGC1α gene as compared with those in water group. Exercise-trained rats showed an increase of exercise capacity, additionally with acetic acid treatment, glucose oxidation was reduced, and lipid oxidation was promoted during exercise tolerance test. Those results indicate that treatments both of acetic acid and exercise training would contribute to enhancement of lipid metabolism and improvement of exercise capacity, which may be through the activation of AMPK and PGC1α. These treatments have a potential to prevent life-style related diseases and increase life span.

Chapter IV Conclusion

Physiological role of administered acetic acid under different physiological conditions such as fed and starved has not yet been investigated. In this study, we investigated the metabolic function of administered acetic acid in skeletal muscles under fed or starved conditions.

Under fed conditions, the acetic acid administered is converted to acetyl-CoA with concomitant formation of AMP. Indeed, the AMP content of the skeletal muscles increased after injection of acetic acid under fed condition. In contrast, under starved conditions, the AMP content decreased after the injection. This finding indicates that AMP would accumulate in skeletal muscles on administration of acetic acid under fed conditions rather than starved conditions. An increase in intracellular AMP concentration leads to the activation of AMPK, which acts as stimulates fatty acid oxidation. Phosphorylation of AMPK significantly increased after injection of acetic acid under fed conditions. While under starved conditions, it was found to lead much lesser extent of phosphorylated AMPK.

Physical exercise is well known factor to increase AMP to activate AMPK. We examined whether acetic acid and exercise training have a synergy effect on endurance performance and lipid metabolism.

Treatments of chronic intake of acetic acid and exercise training led to the reduction of abdominal fat content and result in lower weight gain. That may be derived from increased basal fatty metabolism stimulated by acetic acid. During endurance exercise, lipid utilization was higher in rats treated with acetic acid than that of water group. These data indicate that fiber type switching to oxidative fiber in skeletal muscle by these treatments. Furthermore, we observed that skeletal muscle of rats treated with acetic acid induced mRNA expression of cytochrome c gene and tended to increase

PGC-1α gene as compared with those in water group. Those results indicate that treatments both of acetic acid and exercise training would contribute to enhancement of lipid metabolism and improvement of exercise capacity, which may be through the activation of AMPK. On the basis of these observations, we suggest that acetic acid has a possible role in lipid oxidation in muscles and may also play roles in fighting obesity and obesity-linked type 2 diabetes.

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