An *In Vitro* Contraction Model in Mouse Primary Cultured Myotubes
Using Satellite Cells Originated from the EDL and Soleus Muscles

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**Purpose:** Skeletal muscle cell lines such as mouse C2C12 cells and rat L6 cells often show abnormal characteristics because of repeated-passage cultures and artificial culture conditions. Primary myotubes are considered to retain their *in vivo* properties. Here, satellite cells originating from the extensor digitorum longus (EDL) or soleus muscle were differentiated into primary myotubes and used for an *in vitro* contraction model.

**Materials and Methods:** Satellite cells from the mouse EDL or soleus were isolated by a single-fiber isolation method. We examined the formation of the sarcomere assemblies by α-actinin immunostaining in the differentiated myotubes. We also investigated the contractile characteristics of myotubes stimulated with an electric pulse and insulin induced-glucose uptake. C2C12 myotubes were used for comparison with the primary myotubes.

**Results and Discussion:** The sarcomere assemblies were observed in the primary myotubes but hardly observed in the C2C12 myotubes. The number of myotubes responding to stimulation by the electric pulse was increased in both the C2C12 and primary myotubes, although the movement in the primary myotubes was larger than that in C2C12 myotubes. The glucose uptake stimulated by insulin was significantly increased compared to the basal uptake in the primary myotubes and the C2C12 myotubes. These data suggest that the mouse primary myotubes, with their greater number of sarcomere assemblies and higher level of contractive activity, will be valuable as an *in vitro* contraction model that can be used in place of cell lines or human primary myotubes.

**Key words:** skeletal muscle, primary myotubes, contraction
Intramyocellular Lipid Accumulation After High-Fat Diet Is Associated with the Gene Expression Involved in Lipid Metabolism in Skeletal Muscle of Non-Obese Men

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Insulin resistance in skeletal muscle is one of the main features of metabolic syndrome, and it has been associated with lifestyle factors including diet 1) 2). Whereas the mechanisms underlying the development of insulin resistance have not been fully elucidated, the accumulation of intramyocellular lipid (IMCL) is recognized as an important determinant of insulin resistance, and is increased by a high-fat diet (HFD) 3) 4). The fat content of food is a determinant of the accumulation of IMCL. The effects of HFD on IMCL and insulin sensitivity are highly variable, although, it had shown that a short term (3-day) high-fat diet (HFD) in human increases the IMCL level and impairs insulin sensitivity in skeletal muscle3) 5).

The aim of this study was to identify the genes in muscle that are related to this inter-individual variation. Fifty non-obese healthy men were recruited for this study. Before and after HFD for 3 days, IMCL levels in the tibialis anterior were measured by 1H-magnetic resonance spectroscopy, and peripheral insulin sensitivity was evaluated by glucose infusion rate (GIR) during the euglycemic–hyperinsulinemic clamp. We observed a significant increase in TA-IMCL by HFD. GIR was significantly decreased by HFD. We also observed a negative correlation between changes in TA-IMCL and GIR by HFD (r = -0.37, p < 0.01). Subjects who showed a large increase in IMCL and a large decrease in GIR by HFD were classified as the high-responder (HR), and the subjects who showed a small increase in IMCL and a small decrease in GIR were classified as the low-responder (LR). In 5 subjects in each group, the gene expression profile of the vastus lateralis muscle was analyzed by DNA microarray analyses. Before HFD, gene expression profiles related to lipid metabolism were comparable between the 2 groups. Gene Set Enrichment Analysis demonstrated that 5 gene sets related to lipid metabolism were up-regulated by HFD in the HR group, but not in the LR group. Changes in gene expression patterns were confirmed by qRT-PCR using more samples (LR: n = 9; HR: n = 11). These results suggest that IMCL accumulation/impaired insulin sensitivity after HFD is closely associated with changes in the expression of genes related to lipid metabolism in muscle.

Key words: high fat diet, intramyocellular lipid, insulin sensitivity, skeletal muscle
References


Role of Exercise Intensity on Intramyocellular Lipid Level After Exercise in Subjects with Moderate Insulin Resistance

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It is known that the increased intramyocellular lipid (IMCL) levels observed in obese subjects are closely associated with insulin resistance (IR) in muscle1). Interestingly, some reports suggested low intensity exercise (LIE) decreased IMCL and improved IR2). On the other hand, a few reports showed vigorous intensity exercise (VIE) improved IR, but increased IMCL level3) 4). This phenomenon is a reminiscent of endurance-trained athletes, who possess a high oxidative capacity and enhanced insulin sensitivity, also have higher IMCL content known as athlete’s paradox (AP)5)6). From these findings, we hypothesized exercise intensity is one of the determinants of AP.

To test this hypothesis, we recruited 20 men with moderate insulin resistance (HOMA-R > 1.6) and randomly assigned to LIE (40% VO2 peak) or VIE (70% VO2 peak) group. Each group performed with ergometer for 5 consecutive days. Before and 3-day after completion of protocol, IR was evaluated by glucose clamp. IMCL was measured by 1H-MRS. The IMCL was also evaluated immediately after the exercise at day 5. Our preliminary data showed that in VIE group IMCL level was not significantly changed after exercise at day 5. Although IMCL level was decreased at 3-day after last bout of exercise in LIE group, that in VIE group increased about 50% from baseline. Interestingly, insulin resistance was similarly improved in both groups. These data suggested exercise intensity is a determinant of change of IMCL.

Although, changes in IMCL level after exercise were opposite between LIE and VIE, the improvement of insulin resistance was similar. IMCL exists mostly as triacylglycerol (TAG), which may not impair insulin sensitivity in muscle. On the other hand, intramyocellular diacylglycerol (DAG) concentration is considered to induce insulin resistance, which is generally increased in parallel with the amount of intramyocellular TAG. It has been shown that one bout of aerobic exercise increased the expression level of diacylglycerol acyltransferase (DGAT)-1 in muscle and to prevent FFA-induced muscle DAG accumulation and insulin resistance in healthy humans. Thus, we speculated that DGAT1 expression and decreased DAG levels play roles in the mechanisms involved in the athlete’s paradox phenomenon seen in VIE group. Future analysis is clearly required to test this hypothesis.

References


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Exercise–Induced Transient Increase in IL-6 Stimulates GLUT4 Expression and Enhances Insulin Sensitivity in Mouse Skeletal Muscle

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A single bout of exercise induces transient increase in blood interleukin-6 (IL-6) level in human and rodents, however, the role of exercise–induced IL-6 is poorly understand. Prolonged, chronic increase in IL-6 reflects low–grade inflammation, which decrease insulin sensitivity in adipose tissue, liver and skeletal muscle. On the other hand, acute, short–period of IL-6 enhances insulin sensitivity. Because, the increase in IL-6 after exercise is transient, we hypothesized that transient increase in IL-6 after exercise enhances insulin sensitivity in skeletal muscle. C57BL6j mouse were i.v. injected normal IgG or IL-6 antibody before exercise. Twenty–four hours after a single bout of exercise (treadmill running: 20 m/min, 90 min with 10 degree incline), plantaris muscle was harvested and incubated in oxygenized KRB buffer to measure insulin–stimulated 2–deoxyglucose (2–DG) uptake. Compared with sedentary mouse, insulin–stimulated 2–DG uptake in plantaris muscle was increased 24 h after exercise in IgG–injected mouse, however, the increase induced by exercise was not observed in IL–6 antibody–injected mouse. Concomitant with this results, GLUT4 expression was increased 24 h after exercise in IgG–injected mouse, the increase was canceled in IL–6 antibody–injected mouse. Recombinant mouse IL–6 injection increased GLUT4 expression both fast–twitch plantaris muscle and slow–twitch soleus muscle in C57BL6j mouse. Furthermore, short period incubation of IL–6 (3–12 hours) increased GLUT4 expression in differentiated C2C12 myotubes, however long period (24 h) did not. These results suggests that exercise–induced transient increase in IL–6 affects skeletal muscle in autocrine/paracrine manner, which enhances GLUT4 expression leading to increase insulin sensitivity in skeletal muscle.
Potential Usefulness of Intrahepatic Lipid Accumulation and Liver Function Tests to Identify Insulin Resistance Phenotype in Non-Obese Type 2 Diabetes

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Despite low body mass index (BMI), Asian people often develop type 2 diabetes1)-3). In addition to reduced insulin secretion, etiological difference of insulin resistance (IR) between Caucasian and Asian might be involved in this phenomenon4)5). Previous data demonstrated that non-obese Asians easily develop non-alcoholic fatty liver disease (NAFLD)6)-8), which is considered as cause and result of IR9)-14). As well as fat accumulation in liver, liver enzymes, such as alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT), are easily elevated by small increase in BMI within normal limits in Asians15); however those are less observed in other ethnicities16). In addition, both ALT and GGT were correlated to insulin resistance independent of measures of adiposity17)18). These data suggested that intrahepatic lipid (IHL) accumulation and liver dysfunction could be markers of IR in non-obese type 2 diabetes.

To test this hypothesis, we recruited 16 non-obese (BMI < 25 kg/m²) type 2 diabetes (BMI 21.9 ± 2.0 kg/m², HbA1C 6.8 ± 0.5%, Diet and exercise or take α-glucosidase only). We measured IHL by 1H-magnetic resonance spectroscopy (MRS) at overnight fasting state. Total body fat content was measured by using the bioimpedance method. We also evaluated visceral fat and subcutaneous fat area by magnetic resonance imaging (MRI). Then, we performed euglycemic hyperinsulinenic clamp to measure insulin sensitivity (IS) in muscle and liver, respectively. We also measured serum liver function tests, such as AST, ALT and γ-GTP. Based on the upper limit of normal IHL level (4%) in general non-obese Japanese cohort, we divided the subjects into low IHL group (n = 11; 1.3 (0.46-2.39) %) and high IHL group (n = 5; 10.3 (6.26-12.7) %). Our preliminary data showed that compared with low IHL group, high IHL group showed lower muscle IS (6.79 (5.48-7.54) mg/kg/min vs 3.87 (3.84-5.66) mg/kg/min, p = 0.06). Correlation analysis in all subjects revealed that IHL was not significantly correlated to IS in muscle and liver, however, all liver function tests are significantly correlated to both hepatic and muscle IS, respectively.

The present study demonstrated that IHL accumulation and elevated liver enzymes were associated with impaired insulin sensitivity in non-obese Japanese type 2 diabetes. These data suggested the usefulness of those hepatic parameters as marker of impaired insulin sensitivity in non-obese Japanese type 2 diabetes. However, this study is preliminary analysis in small number of subjects; further analysis is clearly required to confirm these relationships.
References

Long–Lasting Effects of Early–Onset Exercise on the Prevention of Obesity and Its Related Lifestyle Diseases

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Decreased physical activity and increased obesity during childhood have recently emerged as significant social problems. Since long–term exposure to risk factors contributes to the development of lifestyle diseases, determining effective early–age prevention strategies is essential. Regular exercise and increased physical activity are well known to prevent obesity and insulin resistance in both animals and humans. However, although physical activity during childhood has a well-known direct effect on child health, the long–term consequences of childhood exercise on adult health and morbidity have not been well studied due to the difficulties of following subjects long–term. To address this, many researchers use rodents or other animal models. Previous studies have suggested that exercise has long–lasting effects on body weight after exercise cessation. In this review, studies examining the long–lasting effects of childhood exercise on obesity and metabolic diseases later in life using animal models are summarized, and the importance of exercise in childhood in preventing obesity and its related comorbidities is highlighted.

Key words: early–onset exercise, primordial prevention, long–lasting effect, obesity, metabolic disease

Introduction

The prevalence of obesity and other lifestyle diseases has dramatically increased in both adults and children in recent decades. According to a 2013 report by the Centers for Disease Control and Prevention in the U.S., 12.7 million children and adolescents aged 2–19 years old were considered obese (http://www.cdc.gov/obesity/data/childhood.html). In Japan, the prevalence of obesity increased threefold in Japanese children and adolescents aged 5–17 between 1978 and 20071). Since long–term exposure to risk factors contributes to the development of lifestyle diseases, determining effective early–age prevention strategies is essential. A number of previous studies have demonstrated that overweight children are more likely to be overweight as adults compared to normal weight children, with 40–60% of overweight children becoming overweight adults2 3). In addition, the Bogalusa heart study suggested that 77% of obese, and presumably sedentary, children remained obese during adulthood4). Therefore, maintaining a normal weight and body fat percentage during childhood may be important for long–term health.

Although the main risk factors of lifestyle diseases include genetic and environmental factors, diet and exercise are commonly recommended for the prevention and amelioration of obesity and lifestyle diseases5). However, the majority of dietary alterations fail in humans, and adults generally regain lost weight in the months and years following diet cessation6 7). Similarly, weight–reduced rats quickly regain lost weight when allowed free
access to food\textsuperscript{8–11}. Weight regain in humans and rodents may be partly attributed to the chronic reduction in resting metabolic rate associated with weight loss\textsuperscript{12}. On the other hand, exercise affects energy balance, and those who successfully maintain weight loss for several years have reported maintaining high levels of physical activity\textsuperscript{13, 14}. Although the control of energy balance by exercise and diet is still not well understood, access to a running wheel modulated both food intake and body weight in studies examining rodent obesity\textsuperscript{15}. In addition, in some rodent models, long-lasting effects of exercise on feeding and body weight have been identified\textsuperscript{16, 17}. Overall, these studies suggest that exercise is an effective way to maintain weight loss, and that physical activity levels have a strong influence on obesity and related lifestyle diseases.

Nonetheless, decreased physical activity in childhood has recently emerged as a significant social problem. However, little data exists regarding the effects of physical activity in childhood on adult health and mortality in humans. In addition, while multiple studies have examined the effects of exercise on energy homeostasis in adult rodents\textsuperscript{15, 18}, few have examined the effect of early-onset exercise in juvenile animals on the development of obesity\textsuperscript{16, 17, 19, 20}. The mechanisms by which exercise exerts long-term effects are also unknown at present. In this review, studies examining the long-lasting effects of childhood exercise on obesity and metabolic diseases using animal models are summarized, and the importance of exercise in childhood for the prevention of obesity and its related comorbidities is highlighted.

The importance of physical activity in childhood

Physical inactivity (lack of physical activity) has been identified as leading cause of death, and leads to decreased physical fitness. Convincing evidence suggests that the risk for all-cause and cardiovascular-related disease mortality is increased in the absence of moderate or high levels of cardiorespiratory fitness in adults\textsuperscript{21}. In addition, decreased physical activity in children has emerged as a significant social problem. Maffeis \textit{et al.}\textsuperscript{22} reported that time spent on sedentary activities was positively correlated to body fat percentage in 9 year-old boys, and that 8–10 year-old obese children were more sedentary and spent less time performing non-sedentary activities compared to age-matched non-obese children\textsuperscript{23}. The World Health Organization (WHO) has recommended that children and adolescents aged 5–17 should accumulate at least 60 minutes of moderate- to vigorous-intensity physical activity daily. However, Troiano \textit{et al.}\textsuperscript{24} found that 58% of children aged 6–11 and 92% of adolescents fail to meet these recommendations.

Blair \textit{et al.}\textsuperscript{25} presented three possible models by which enhanced physical activity in childhood may improve health in adults: (1) childhood physical activity influences adult physical activity, which in turn can affect adult health, (2) childhood physical activity has a direct beneficial effect on childhood health, which predicts adult health, and (3) childhood physical activity has a direct beneficial effect on adult health. These models are supported by several other studies. A longitudinal study suggested that high levels of physical activity during childhood were positively correlated to physical activity patterns 21 years later\textsuperscript{26}. Moreover, a similar study revealed that individuals who had high levels of physical activity as adults were more likely to have lower waist circumferences, and were more likely to have been highly active during childhood\textsuperscript{27}. These results suggest that physical activity in childhood and adolescents has an indirect effect on abdominal obesity through the maintenance of physical activity during adulthood. Therefore, establishing high levels of physical activity during childhood, and continuing high levels of physical activity into adulthood may combat the development of lifestyle diseases. Although there have been many epidemiologic studies, including the ones described above, the effects of childhood physical activity on adult health are difficult to establish due to the challenges of following subjects through their entire lifespans.

Experimental models by using animal to study the effect of early-onset exercise

To examine whether exercise habits in childhood contribute to health or morbidity in later life, many researchers have used experimental animal models. In the case of rats, they are weaned from the mother at approximately 3 weeks of age, and are used for breeding from 10–12 weeks of age.
Although matching ages between species is difficult, Goto\textsuperscript{28} described that a 6-month-old rat is roughly equivalent to a 15-20-year-old human. Therefore, the first of rat’s life is roughly equivalent to human children/adolescence, so to examine the effects of early-onset exercise on health during adulthood, animals must engage in exercise during this period.

Experimental models of exercise cessation have often been used to explore how long-lasting the effects of early-onset exercise are and the mechanisms by which long-lasting effects occur. The rodent wheel lock (WL) model was developed by Rhodes \textit{et al.}\textsuperscript{29}, and involves housing young rats in cages equipped with voluntary running wheels. After a short period of voluntary running (3 to 6 weeks), the wheels are locked, removing the rats’ primary source of physical activity. Using this model, a number of researchers have examined how different organ systems in juvenile rats respond to cessation of daily physical activity. Roberts \textit{et al.}\textsuperscript{30} recently reviewed the evidence regarding the effects of exercise cessation on various physiological variables, including body weight, glucose and lipid metabolism, insulin resistance, and vascular function using the WL model\textsuperscript{30}. However, the longest studies using the WL model have only tracked rats for 173 hours after exercise cessation, and to determine the long-lasting effects of early-onset exercise, animals must be monitored over long periods of time. Several studies have examined the effects of exercise for more than 10 weeks using various models, and these results will be summarized in the next section.

The long-lasting effects of early-onset exercise on obesity and its related metabolic diseases

In a number of rodent obesity models, early-onset access to a running wheel and the subsequent increase in physical activity normalized body weight in diet-induced obesity (DIO) and type 2 diabetic, Otsuka Long-Evans Tokushima Fatty (OLETF), rats\textsuperscript{15,31,32}. OLETF rats represent a well-established animal model of obesity and type 2 diabetes, and are characterized by hyperphagia and obesity, which begin during early childhood\textsuperscript{33}. OLETF rats go on to develop hyperglycemia after 18 weeks of age. Since several reports have shown that running activity can prevent obesity in OLETF rats, but not Zucker fatty rats\textsuperscript{34}, OLETF rats have been used to examine the effects of exercise on the prevention of obesity and metabolic diseases.

In addition, some animal models suggest that early-onset exercise has long-lasting effects on body weight (Table 1). Interestingly, adult rats placed on similar exercise regimens did not sustain their weight loss after exercise cessation\textsuperscript{35}. Shima \textit{et al.}\textsuperscript{19} reported that exercise had long-lasting preventive effects on obesity and type 2 diabetes development in OLETF rats. Bi \textit{et al.}\textsuperscript{18} also demonstrated that the effects of exercise on body weight were long lasting, and that these effects might be mediated by central energy homeostasis regulating pathway, including neuropeptide Y (NPY) signaling in the dorsomedial hypothalamus (DMH). Patterson \textit{et al.}\textsuperscript{16} examined the duration of early running wheel activity necessary to produce sustained suppression of body weight and adiposity after exercise cessation, and determined that three weeks of exercise was sufficient to prevent obesity 10 weeks after wheel removal. In contrast, Chao \textit{et al.}\textsuperscript{17} examined the effects of a high-fat diet on body weight in OLETF rats that had prior access to running wheels for 4 weeks, and determined that the high-fat diet offset the long-lasting effects of exercise on body weight. In addition, Shindo \textit{et al.}\textsuperscript{20} observed the long-lasting effects of exercise on weight gain for the longest period, and determined that higher levels of citrate synthase (CS), succinate dehydrogenase (SDH), phosphofructokinase (PFK) activity and uncoupling protein (UCP-3) mRNA in skeletal muscle were found after long-term exercise cessation. Research from our own lab also suggests that the lower body weight and glucose levels are sustained after exercise cessation in OLETF rats compared with sedentary animals. In addition, we observed that exercise completely prevented increase in serum lipid parameters (e.g. triglyceride and total cholesterol), even after cessation (unpublished data).

Taken together, these studies suggest that early-onset exercise has long-lasting effects on the prevention of obesity and its related metabolic diseases through the regulation of central energy homeostasis pathway, and by increasing the activity of enzymes participating in energy metabolism. Further studies are needed to determine the underlying mechanisms by which early-onset exercise prevent obesity and...
Studies on the long-term effects of early-onset exercise and exercise cessation on obesity and lifestyle diseases development are difficult to perform in humans. Therefore, animal models provide a translational tool that can be used to identify preventative methods that can be utilized in children to prevent obesity and lifestyle diseases later in life. The information in this review establishes the importance and necessity of physical activity in childhood for the prevention of adult obesity and lifestyle diseases.

Summary

Studies on the long-term effects of early-onset exercise and exercise cessation on obesity and lifestyle disease development are difficult to perform in humans. Therefore, animal models provide a translational tool that can be used to identify preventative methods that can be utilized in children to prevent obesity and lifestyle diseases later in life. The information in this review establishes the importance and necessity of physical activity in childhood for the prevention of adult obesity and lifestyle diseases.

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Conflict of Interest

No conflicts of interest, financial or otherwise, are declared by the authors.

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3) Guo SS, Wu W, Chumlea WC, Roche AF: Predicting
Caffeine Increases Contraction-Stimulated 5'-'AMP-Activated Protein Kinase Activity and Insulin-Independent Glucose Transport in Rat Skeletal Muscle

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Objective: 5'-adenosine monophosphate-activated protein kinase (AMPK) has been identified as a key mediator of contraction-stimulated insulin-independent glucose transport in skeletal muscle. Caffeine acutely stimulates AMPK in resting skeletal muscle, but it is unknown whether caffeine affects AMPK in contracting muscle. In this study, we examined the effect of caffeine stimulation on contraction-stimulated AMPK activity and glucose transport.

Materials and Methods: (1) Isolated rat epitrochlearis muscle was preincubated and then incubated in the absence or presence of 3 mM caffeine for 30 min. Electrical stimulation (ES) was used to evoke tetanic contractions during the last 10 min of the incubation period. (2) Rats were given an intraperitoneal injection of caffeine (60 mg/kg body weight) or saline, and the extensor digitorum longus muscle was dissected 15 min later. ES of the sciatic nerve was performed to evoke tetanic contractions for 5 min before dissection.

Results: (1) The combination of caffeine plus contraction had additive effects on AMPKαThr 172 phosphorylation, α-isoform-specific AMPK activity, and 3-O-methylglucose (3MG) transport. Caffeine significantly delayed muscle fatigue during contraction, and the combination of caffeine and contraction additively decreased ATP and phosphocreatine contents. (2) Similar to the findings from isolated muscles incubated in vitro, the combination of caffeine plus contraction in vivo had additive effects on AMPK phosphorylation, AMPK activity, and 3MG transport.

Conclusions: These findings suggest that caffeine and contraction synergistically stimulate AMPK activity and insulin-independent glucose transport, at least in part by decreasing muscle fatigue and thereby promoting energy consumption during contraction.

Key words: 5'-AMP-activated protein kinase, muscle contraction, energy deprivation, muscle fatigue, glucose metabolism

Introduction

Skeletal muscle plays a major role in whole-body glucose metabolism in rodents and humans. Insulin and exercise (muscle contraction) are the physiologically important stimuli of glucose transport, the rate-limiting step in glucose utilization in skeletal muscle. Although both insulin and exercise elicit the translocation of glucose transporter 4 (GLUT4) from intracellular vesicle compartments to the sarcolemma and T-tubules, these stimuli activate specific signaling mechanisms. 5'-adenosine monophosphate-activated protein kinase (AMPK) has been identified as a signaling molecule involved in contraction-stimulated and insulin-independent glucose transport (reviewed in1,2). AMPK in skeletal muscle has also been implicated in a number of the metabolic effects of exercise such as

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increased insulin sensitivity\textsuperscript{7–9, 16} and GLUT4 expression\textsuperscript{17, 18}, inhibition of acetyl-CoA carboxylase and fatty acid oxidation\textsuperscript{7, 8}, modulation of glycogen synthesis\textsuperscript{9, 10}, mitochondrial biogenesis via peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α)\textsuperscript{11}, activation of sirtuin\textsuperscript{12, 13}, and the shift in the metabolic properties toward those of slow oxidative muscle fibers\textsuperscript{13}. These acute and chronic alterations in skeletal muscle suggest that AMPK is a metabolic enhancer that may prevent or delay the development of type 2 diabetes mellitus.

AMPK is a heterotrimer comprising a catalytic α subunit, and regulatory β and γ subunits. There are two different α isoforms (α1 and α2); α1 is expressed ubiquitously, and α2 is expressed in skeletal muscle, heart, and liver. AMP binding results in the phosphorylation of α Thr\textsuperscript{172}, which is essential for kinase activation. Classically, AMPK acts as a signaling intermediary by monitoring cellular energy status\textsuperscript{14}. In isolated rat skeletal muscle incubated \textit{in vitro}, both α1-containing AMPK (AMPKα1) and α2-containing (AMPKα2) are stimulated by energy-decreasing (AMP-increasing) stressors including contraction, hypoxia, chemical inhibition of oxidative phosphorylation, and hyperosmolarity, all of which are potent stimulators of insulin-independent glucose transport\textsuperscript{15}.

We reported previously that incubation with caffeine (≥3 mM for ≥15 min) increased AMPKα Thr\textsuperscript{172} phosphorylation and AMPKα1 and α2 activities in isolated rat skeletal muscles and that these effects were accompanied by increased insulin-independent glucose transport\textsuperscript{16}. Caffeine-induced AMPK activation was also accompanied by decreased fuel status; for example, the phosphocreatine content was 23% lower in muscle stimulated with caffeine compared with the control\textsuperscript{16}. These results indicated that caffeine acts directly in skeletal muscle and has similar actions to those of contraction by acutely promoting AMPK activity with energy deprivation in skeletal muscle. It is notable that epidemiological studies have demonstrated that the intake of caffeinated beverages, including coffee and tea, is linked to a reduced risk of type 2 diabetes mellitus\textsuperscript{17–18}.

Caffeine increases force production during contraction by multiple mechanisms such as increased Ca\textsuperscript{2+} release and Ca\textsuperscript{2+} permeability in the sarcoplasmic reticulum, increased Ca\textsuperscript{2+} sensitivity, and slowing of the sarcoplasmic reticulum Ca\textsuperscript{2+} pump (reviewed in\textsuperscript{19–20}). Many researchers have reported that caffeine increases exercise performance and delays fatigue in rodents\textsuperscript{21–22} and humans\textsuperscript{23–26}. These ergogenic actions of caffeine led us to hypothesize that caffeine stimulates AMPK and glucose transport in contracting states by causing profound changes in the cellular energy status in skeletal muscle. To test our hypothesis, we examined the effect of caffeine stimulation on isolated rat skeletal muscle electrically stimulated \textit{in vitro}. We also explored the effect of systemic caffeine administration on contracting skeletal muscle in living rats.

\textbf{Materials and methods}

\textbf{1. Animals}

Male Sprague Dawley rats (150–160 g) were purchased from Shimizu Breeding Laboratories (Kyoto, Japan). Rats were fed a standard diet (Certified Diet MF; Oriental Koubo, Tokyo, Japan) and water \textit{ad libitum}, and fasted overnight before each experiment. Protocols for animal use and euthanasia were approved by the Kyoto University Graduate School of Human and Environmental Studies and Kyoto University Radioisotope Research Center.

\textbf{2. Muscle treatment \textit{in vitro}}

Muscles were treated as we described previously\textsuperscript{27} with some modifications. Isolated rat epitrochlearis muscle was preincubated in 7 ml of alpha minimum essential medium (αMEM) containing 1% penicillin/streptomycin for 40 min. The muscle was then incubated in 7 ml of fresh medium in the absence or presence of 3 mM caffeine for 30 min, 1 mM caffiene acid for 30 min, or 1 mM chlorogenic acid for 30 min. For tetanic contraction, the muscle was stimulated with an electric stimulator (SEN-3401; Nihon Kohden, Tokyo, Japan) during the last 10 min of the incubation period (train rate: 1/min, train duration: 10 s, pulse rate: 100 Hz, pulse duration: 0.1 ms, voltage: 10 V). Force was recorded with a force transducer (TRN001; Kent Scientific, Torrington, CT, USA) and a recorder (U-228-2P-500; Pantos, Kyoto, Japan). Control muscles were preincubated and incubated without contraction. Other muscle samples were used fresh in the 3-O-methyl-β-glucose (3MG) transport or
caffeine transport assay, or were immediately frozen in liquid nitrogen and subsequent analysis. All media were gassed with 95% O₂/5% CO₂ and maintained at 37°C.

3. Muscle treatment in vivo

Caffeine dissolved in saline was injected intraperitoneally without anesthesia at 60 mg/kg body weight. The injection volume was 2 ml/kg body weight. Five minutes after caffeine or saline injection, rats were anaesthetized with intraperitoneal administration of pentobarbital sodium (75 mg/kg body weight), and electrodes (OM209-041; Unique Medical, Tokyo, Japan) were attached to the sciatic nerve on both sides. Fifteen minutes after caffeine or saline injection, the extensor digitorum longus (EDL) muscle was rapidly dissected. The muscle was used fresh to measure 3MG transport activity or other samples were immediately frozen in liquid nitrogen and subsequent analysis. For tetanic contraction, the sciatic nerves were stimulated during the last 5 minutes before dissection (train rate: 1/min, train duration: 10 s, pulse rate: 100 Hz, pulse duration: 0.1 ms, voltage: 2 V) using the SEN-3401 stimulator.

4. Analyses

Western blot analysis (27), isoform-specific AMPK activity assay (27), 3MG transport assay (27, 28), caffeine transport assay (29), and ATP and PCr assay (16) were performed as we described previously.

5. Statistical analysis

Results are presented as mean ± SE. Multiple means were compared using ANOVA followed by post hoc comparisons with Tukey’s test. Two means were compared using unpaired Student’s t test. Differences between groups were considered significant at p < 0.05.

Results and discussion

1. Caffeine and contraction additively stimulate AMPK and glucose transport in isolated skeletal muscle

Our previous study demonstrated that maximal activation of AMPK by caffeine is observed with a 30 min incubation at a concentration of 3 mM (16). We have also demonstrated previously that maximal activation of AMPK by contraction can be induced by 10 repeated 10 s tetanic contractions during 10 min and that there is no further increase in AMPK activity with 15 repeated 10 s tetanic contractions (20). However, tetanic contraction is not the strongest stimulus of AMPK activity in skeletal muscle. For instance, in incubated rat epitrochlearis muscle, dinitrophenol (0.5 mM for 20 min) increased AMPK activity by 6-fold compared with basal AMPK activity, and 10 tetanic contractions increased AMPKα2 activity only 4-fold (15). Therefore, even when AMPK activity is increased maximally by contraction in skeletal muscle, it may still be activated further by other stimuli. In the present study, the stimulatory effects of maximally effective caffeine and maximally effective contraction on AMPKα Thr(172) phosphorylation were partly but significantly additive (Figure-1A). The total AMPK content did not differ between the groups. The caffeine- and contraction-stimulated AMPKα1 and AMPKα2 activities were also significantly additive (Figure-1B). Treatment with 3 mM caffeine for 30 min and contraction increased the rate of 3MG transport by 2.4- and 4.4-fold compared with the basal level, respectively. The caffeine- and contraction-stimulated activity of 3MG transport was significantly additive (5.8-fold compared with the basal level) (Figure-1C). These results suggest that caffeine increases the maximal capacity of contraction-stimulated AMPK activation in skeletal muscle.

2. Caffeine and contraction additively decrease ATP and PCr contents in isolated skeletal muscle

To clarify whether the combined effect of caffeine and contraction on AMPK activity is associated with a change in energy status, we measured the muscle contents of ATP and PCr. Treatment with 3 mM caffeine for 30 min and contraction decreased the contents of ATP (Figure-2A) and PCr (Figure-2B). The effects of caffeine and contraction on ATP and PCr were partially additive (Figure-2A and B). Consistent with these findings, caffeine significantly mitigated muscle fatigue during contraction (Figure-2C), in association with an increase in the initial peak force (Figure-2D).

Caffeine can easily pass through the surface membrane of the muscle cell because of its hydro-
In this study, the intracellular concentration of caffeine reached a maximum by 30 min after the start of the exposure to caffeine and was not affected by contraction (Figure-1D). The ergogenic actions of caffeine may contribute to the decreased muscle fatigue and profound decrease in energy status in contracting skeletal muscle.

In the mechanism of energy reduction by caffeine, Miyazaki et al.32 demonstrated that 1-5 mmol/l of caffeine increased oxygen consumption acutely in frog skeletal muscles that were isolated and incubated in vitro. Those authors also found that the metabolic enhancement afforded by caffeine was associated with an increase in lactic acid content and decreases in ATP, ADP, and PCr contents in the muscle, without mechanical changes such as contracture formation. Thus, caffeine may act on the muscle energy status via acceleration of the energy supply, rather than via inhibition of mitochondrial function and suppression of ATP production.

3. Neither caffeic acid nor chlorogenic acid affects contraction-stimulated AMPKα Thr172 phosphorylation in isolated skeletal muscle

In addition to caffeine, caffeic acid and chlorogenic acid, which are the major constituents of coffee, also have antihyperglycemic properties.33-36 We previously reported that caffeic acid, but not chlorogenic acid, acutely promoted AMPK phosphorylation. In that study, the maximal activation of AMPK by caffeic acid was observed at 1 mM after a
30 min incubation in isolated rat epitrochlearis muscle. In the current study, we examined the effects of caffeic acid and chlorogenic acid on contraction-stimulated AMPK activity in skeletal muscle. AMPKα Thr\textsuperscript{172} phosphorylation was increased by caffeic acid (1 mM, 30 min) (Figure-3A), but not by chlorogenic acid (1 mM, 30 min) (Figure-3B). However, unlike caffeine (Figure-1A), incubation with caffeic acid or chlorogenic acid did not affect the contraction-stimulated AMPKα Thr\textsuperscript{172} phosphorylation (Figure-3A and B). The total AMPK content did not differ between the groups. These data show clearly that AMPK-activating agents do not necessarily have an additive effect on contraction-stimulated AMPK activity in skeletal muscle.

4. Intraperitoneal caffeine injection and contraction \textit{in situ} additively activate AMPK and glucose transport in skeletal muscle

To determine whether caffeine affects contraction-stimulated AMPK activity \textit{in vivo}, we measured the degree of phosphorylation of AMPKα Thr\textsuperscript{172} in EDL muscle dissected after intraperitoneal injection of caffeine or saline with or without contraction. AMPK phosphorylation was increased by caffeine and contraction, and the effects of caffeine and contraction were partially additive (Figure-4A). The total AMPK content did not differ between the groups. In the isoform-specific AMPK activity assay, caffeine and contraction increased both AMPKα1 and AMPKα2 activities, and the effects of caffeine and contraction were partially additive (Figure-4B). Caffeine injection
**Figure 3** Effect of caffeic acid and chlorogenic acid on AMPKα Thr172 phosphorylation in incubated rat skeletal muscle
Isolated epitrochlearis muscle was preincubated and incubated for 30 min in the absence (-) or presence (+) of 1 mM caffeic acid (A) or 1 mM chlorogenic acid (B). The muscle was tetanically contracted during the last 10 min of the incubation period and then subjected to Western blot analysis.
Values are mean ± SE; n=5-12 per group. *p<0.05, **p<0.01, ***p<0.001 vs. control; †††p<0.001 vs. contraction plus caffeic acid or chlorogenic acid; NS: not significant.

**Figure 4** Effect of intraperitoneal caffeine injection on contraction–stimulated AMPKα Thr172 phosphorylation, AMPK activity, and 3MG transport activity in rat skeletal muscle
Caffeine (60 mg/kg) or saline was injected intraperitoneally. Fifteen minutes after injection of caffeine or saline, the EDL was dissected and subjected to Western blot analysis (A), isoform–specific AMPK activity assay (B), or 3MG transport assay (C). Tetanic contraction was elicited by electrical stimulation of the sciatic nerve during the last 5 min before dissection. Representative immunoblots are shown.
Values are mean ± SE; n=4-11 per group. *p<0.05, **p<0.01, ***p<0.001 vs. control; ††p<0.01, †††p<0.001 vs. contraction plus caffeine.
and contraction also increased 3MG transport by 2.3- and 4.3-fold compared with the saline injection, respectively (Figure-4C). The effects of caffeine and contraction were partially additive (6.2-fold compared with the basal level) (Figure-4C).

5. Caffeine enhances contraction-stimulated Ca\(^{2+}\) /calmodulin-dependent protein kinase II (CaMKII) Thr\(^{286}\) phosphorylation in skeletal muscle

Ca\(^{2+}\) has been implicated in the activation of glucose transport through signaling pathways involving AMPK\(^{38}\). CaMKII has been used as an indicator of elevated cytosolic Ca\(^{2+}\) level in skeletal muscle\(^{39}\). Our previous study demonstrated that caffeine (3 mM, 15 min) significantly increased CaMKII Thr\(^{286}\) phosphorylation in isolated rat epitrochlearis muscle\(^{28}\), and tetanic contraction significantly increased CaMKII Thr\(^{286}\) phosphorylation in isolated rat epitrochlearis muscle\(^{40}\). To determine whether caffeine affects contraction-stimulated CaMKII Thr\(^{286}\) phosphorylation in skeletal muscle, we measured the degree of phosphorylation of CaMKII Thr\(^{286}\). In the present study, CaMKII Thr\(^{286}\) phosphorylation was increased by caffeine and contraction, and the effects of caffeine and contraction were partially additive (Figure-5A). Similarly, the combination of caffeine plus contraction in vivo had additive effects on CaMKII Thr\(^{286}\) phosphorylation (Figure-5B). The total CaMKII content did not differ between the groups. These results suggest that the elevating Ca\(^{2+}\) level is partially involved in the additive effects of the combination of caffeine plus contraction on AMPK activity.

In conclusion, our results suggest that caffeine and contraction synergistically stimulate AMPK activity and insulin-independent glucose transport, at least in part by elevating Ca\(^{2+}\) level and decreasing muscle fatigue, and thereby promoting energy consumption during contraction. We suggest that the ergogenicity of caffeine may contribute to the enhancement of the effect of exercise-induced promoting glucose metabolism by induction of profound activation of AMPK.

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References


