Significance of 5'AMP-activated protein kinase in metabolomic regulation by skeletal muscle contraction

Licht Miyamoto

*Laboratory of Pharmacology and Physiological Sciences, Frontier Laboratories for Pharmaceutical Sciences, Institute of Health Biosciences, The University of Tokushima Graduate School, 1-78-1 Shou-machi, Tokushima 770-8505, Japan
Department of Medical Pharmacology, Institute of Health Biosciences, The University of Tokushima Graduate School, 1-78-1 Shou-machi, Tokushima 770-8505, Japan*

Received: January 23, 2015 / Accepted: February 6, 2015

Abstract The number of patients exhibiting metabolic disorders has been pandemically increasing. Physical exercise is widely applied as an ideal therapeutic and preventive way against such metabolic diseases as diabetes due to its high potency of metabolic regulation. It evokes marked facilitation of glucose and lipid metabolism in contracting skeletal muscle. Recent investigations have suggested that 5'AMP-activated protein kinase (AMPK) should be a key molecule in contraction-caused metabolic changes. AMPK is activated in response to the rise in the AMP/ATP ratio following muscle contraction. The activated AMPK suppresses anabolic pathways, but increases catabolic ones to compensate for the ATP shortage. The various metabolic results of AMPK activation are similar to those in contracting skeletal muscle. However, some recent reports using genetically AMPK-disrupted animals reported that AMPK does not necessarily mediate metabolic control by muscle contraction, suggesting the significance of AMPK-independent metabolic pathways. Genetically manipulated animals without molecules of critical significance such as AMPK will tend to acquire compensatory mechanisms. Thus, another approach will be of great help to further understand the role of such key molecules. Thanks to the recent development of analytic systems, it is getting easier to obtain comprehensive information on metabolites. This metabolomics technology has been applied in studies on physical fitness and exercise little by little, providing us with novel findings; although it is not yet popular. Recent results of metabolomic studies in the field of physical exercise, including our investigation demonstrating metabolomic significance of AMPK in contracting skeletal muscle, will be introduced in this review.

Keywords : 5'AMP-activated protein kinase (AMPK), metabolomics, metabolome, muscle contraction, exercise, 5-aminoimidazole-4-carboxamide 1- β -D-ribofuranoside (AICAR)

Introduction

Metabolic disorders such as diabetes, obesity, hyperlipidemia and so on have become a global pandemic in recent years. A cluster of metabolic derangements often occur together, and are widely accepted as a pathological concept known as metabolic syndrome. Physical exercise is a potent regulator of glucose and lipid metabolism, and clinically applied in the treatment and prevention of such metabolic diseases due to its beneficial pleiotropic effects¹⁻⁶. As for glycemic control, for example, even a single bout of exercise can lower the blood glucose level by facilitating glucose uptake in contracting skeletal muscle. Muscle contraction as well as physical exercise has been known to evoke striking glucose uptake in skeletal muscle; but precise molecular mechanisms involved in

the muscle contraction-induced glucose uptake had been unclear for a long time until Drs. Hayashi, Goodyear, and their colleagues hypothesized, in 1998, that 5'AMP-activated protein kinase (AMPK) mediates glucose uptake in contracting skeletal muscle⁷. They found that muscle contraction activates AMPK, and that stimulation by an activator of AMPK, 5-aminoimidazole-4-carboxamide 1- β -D-ribofuranoside (AICAR), promotes glucose uptake in a similar manner as the contraction. They also reported that AMPK activities under various stresses, such as contraction, hypoxia and hyperosmolarity, correlate well with glucose uptake activity⁸. A number of investigations have provided evidential data demonstrating that AMPK plays broad pivotal roles in metabolic regulation in contracting skeletal muscle. However, there have been several reports showing that AMPK does not necessarily mediate all the metabolic modulation during muscle contraction⁹⁻¹². Even for glucose uptake, several studies dealing with

genetically manipulated animals denied the involvement of AMPK in muscle contraction-evoked glucose uptake facilitation. AMPK is a key enzyme regulating metabolic homeostasis, and genetically AMPK-impaired animals may acquire compensatory ways in order to live. Therefore, another approach should be taken to further understand the significance of AMPK in contracting skeletal muscle. Thus, we have performed comparative metabolomic analysis using an AMPK activator and electrical contraction *ex vivo* in isolated skeletal muscle as recent advances in analytical technologies enables comprehensive quantification of metabolites in the skeletal muscle. The current review will briefly introduce metabolic regulation in contracting skeletal muscle and current analytical technologies of metabolomics, and summarize the results of recent metabolomic reports on physical exercise including our study, discussing the significance of AMPK in metabolic regulation in contracting skeletal muscle in terms of metabolomics.

Acute effects of physical exercise on blood glucose regulation

Blood glucose levels are primarily controlled by pancreatic hormones such as insulin and glucagon. Insulin lowers blood glucose levels by facilitating glucose uptake in peripheral tissues, especially in skeletal muscles and adipose tissues, and suppressing hepatic glucose efflux; while glucagon increases blood glucose levels mainly by inducing the efflux. It is well known that even a single bout of physical exercise can decrease blood glucose levels mainly due to enhancement of glucose uptake in the contracting skeletal muscles independently of insulin. Furthermore, insulin sensitivity is improved by exercise. A large number of patients with metabolic disorders (not limited to diabetes) exhibit symptoms of insulin resistance. Thus, it should be an important strategy for the treatment of patients with metabolic disorders to ameliorate insulin sensitivity and augment insulin-independent glucose disposal. Hence, therapeutic exercise has been widely applied clinically to treat and prevent metabolic disorders, including diabetes, and authorized as a first-line therapy by official guidelines.

Physical exercise evokes various responses throughout the body including changes in blood flow, pressure, hormones, and so on. Nevertheless, muscle contraction itself appears as the most potent regulator of glucose and lipid metabolism. Muscle contraction caused by electrical stimulation of the sciatic nerve *in vivo*, as well as physical exercise, reduces blood glucose and lipid levels with facilitation of lipid oxidation and translocation of Glut4, an insulin-responsive glucose transporter causing glucose uptake in contracting skeletal muscles¹³⁻¹⁸). In contrast, skeletal muscle from another leg in the same animal does not induce Glut4 translocation, glucose uptake nor lipid oxidation. These metabolic responses are also found in

isolated skeletal muscle, which is led to contraction by electrical stimulation *ex vivo*.

AMPK activation in contracting skeletal muscle

AMPK is a heterotrimeric serine/threonine kinase, activated in response to a relative rise in AMP as it name designates. In other words, AMPK acts as an energy fuel sensor¹⁹). Muscle contraction, as well as physical exercise, strikingly activates AMPK in the contracting skeletal muscle, which is plausibly due to rapid elevation of AMP levels by consumption of ATP^{7,8,13}). AMPK is composed of catalytic alpha and two regulatory beta and gamma subunits. Two isoforms were identified for alpha and beta subunits each and three for gamma subunit in mammals²⁰⁻²³). Phosphorylation of threonine 172 residue of the alpha subunit results in AMPK activation. The rise in the AMP/ATP ratio brings about conformational changes subject to phosphorylation by an upstream kinase such as Liver kinase B1 (LKB1). LKB1-dependent phosphorylation of AMPK alpha is accepted as a dominant mechanism of AMPK activation by muscle contraction, as contraction-caused AMPK activation was not observed in LKB1-deficient skeletal muscle²⁴⁻²⁷). However, entry of a calcium ion coupled with contraction may also be involved in AMPK activation via another upstream kinase, calcium-calmodulin dependent protein kinase kinase beta (CaMKKb)²⁸⁻³⁰). Muscle contraction evoked by tetanic electrical stimulation causes both alpha1 and alpha2 isoforms-containing AMPK^{8,31,32}). Most aerobic exercises *in vivo*, which are relatively moderate, lead to the activation of the alpha2 isoform only³³⁻³⁶). In contrast, reactive oxygen and nitrogen species generated during contraction preferably activate the alpha1 isoform of AMPK independently of AMP levels^{37,38}). In addition, such metabolic hormones as leptin activate AMPK in the liver as well as in skeletal muscle^{39,40}).

AMPK and its metabolic regulation in skeletal muscle

Apart from its relevance in contracting skeletal muscle, AMPK activation has been demonstrated to modulate various metabolic responses closely related to the effects of physical exercise or muscle contraction. AMPK activation turns on catabolic processes such as lipid oxidation and the tricarboxylic acid (TCA) cycle (aka Krebs's cycle) and shut off anabolic reactions such as lipogenesis and gluconeogenesis, leading to an increase in ATP levels, in general. In this respect, AMPK should be a master switch of metabolic regulation as well as an energy fuel sensor.

AMPK activation facilitates glucose uptake in skeletal muscles as aforementioned. The precise mechanisms have not been revealed completely, but recent studies have suggested involvement of Akt substrates, Tbc1 domain family members 1 and 4 (Tbc1d1 and 4, also known as Akt substrate 160 or AS160), which possess GTPase activat-

ing proteins (GAPs) activity on Rab family small GTPase proteins controlling vesicle trafficking⁴¹⁻⁴⁸). AMPK phosphorylates Tbc1d1 and/or 4, which in turn brings about translocation of Glut4, possibly by suppression of Rab activities.

AMPK activation reduces lipid levels by increasing fatty acid oxidation while decreasing lipogenesis. Carnitine palmitoyltransferase 1 (CPT1) catalyzes a rate-limiting step of carnitine shuttle-mediating mitochondrial fatty acid transport. Acetyl-CoA carboxylase (ACC) is a typical substrate of AMPK and catalyzes malonyl-CoA formation, which is the first step of de novo lipogenesis. CPT1 activity is allosterically disturbed by malonyl-CoA. AMPK reduces the amount of malonyl-CoA mainly by curtailing ACC activity through direct phosphorylation, resulting in suppression of lipogenesis and augmentation of fatty acid oxidation⁴⁹⁻⁵¹). Furthermore, expression levels of CPT1 and ACC are also regulated by AMPK at the transcription level through their upstream factors such as peroxisome proliferator activated receptor (PPAR) alpha and sterol responsive element binding protein (SREBP) 1⁵²⁻⁵⁴).

Significance of the AMPK-dependent metabolic pathways including the typical examples introduced above has been well established, and many studies have supported the notion over the last two decades. Physical exercise and muscle contraction, indeed, are quite potent stimulators of AMPK activation in skeletal muscle. Nevertheless, several studies analyzing genetically manipulated animals have suggested that AMPK does not necessarily mediate a metabolic change in contracting skeletal muscle. Actually, we also found some discrepancies in glycogen metabolism³²). Glucose uptake activity in contracting skeletal muscle expressing dominant negative AMPK, or from AMPK knockout mice, is about the same or only partially lower than that of control animals⁹⁻¹¹). These results are noteworthy enough to raise doubt as to the importance of AMPK in contracting skeletal muscle. However, we cannot exclude the possibility that genetically manipulated animals without such viable key molecules acquire compensatory mechanisms to live on. Thus, another novel approach should be taken to further understand the relevance of important molecules. “Omics” technologies such as genomics and proteomics enable us to characterize and identify the aspects of the status of overall molecules within cells or tissues thanks to recent advances in analytical and computational techniques, while traditional approaches deal with activity and/or amount of one or only a few molecules; although omics technologies have not been so widely applied to investigations in skeletal muscle in terms of physical fitness and exercise. Metabolomics, examining small metabolites including intermediates, would especially be a powerful and novel technique in this field.

Analytical technology applied to metabolomics studies

The recent rapid development of analytical technologies provides us with novel powerful tools for comprehensive identification and quantification of metabolites within organisms. In other words, such metabolomics technologies enable us to capture a snapshot of whole metabolites, or “metabolome” as it is called.

There have been several ways developed for metabolomic analysis. Nuclear magnetic resonance (NMR) spectroscopy is appropriately applicable for aqueous samples, in general. Thus, it is suitable for detecting small metabolites in blood and urine. NMR spectroscopy raises multiple signals from each molecule, which makes it quite difficult to decode the obtained data. Though NMR spectroscopy has been a popular metabolomic technique, it may be a relatively less effective way to comprehend overall metabolites in skeletal muscle. However, it can be an interesting tool when used with isotopic labeling of molecules of interest.

Mass spectrometry (MS) is usually employed in combination with other analytical methods for metabolomics studies. Volatile samples are suitably separated by gas chromatography (GC), and GC-MS is widely used for metabolomic analysis on flavors, as well as secondary metabolites in plants. Liquid chromatography (LC)-MS also seems to be a popular method in metabolomics. One of the advantages of LC-MS is that it is easy to modify conditions of the LC phase, providing various alternative separation modes, although it is necessary to prepare another set of data library for annotation. Most lipidomics studies dealing with comprehensive lipid profiles adopt LC-MS. Capillary electrophoresis (CE) separates analytes based on their size and electrical charge. It is especially appropriate for separating small metabolites, most of which are ionic and/or polar compounds. Therefore, CE-MS appears to be the most suitable analytical method for metabolomic analysis of small metabolites within skeletal muscles at the moment; and we adopted capillary electrophoresis with the time of flight mass spectrophotometer (CE-TOFMS) for metabolomic analysis in our recent study³¹).

Application of metabolomics technology to research on physical exercise

There have not been so many studies applying the metabolomic approach for research on physical exercise. Moreover, most of the metabolomic investigations on physical exercise have not directly dealt with skeletal muscles, but with urine or blood. Proton NMR spectroscopy appears to be the most prevalent tool in this field. In one experiment, a bout of intensive short-term aerobic exercise acutely increased urinal excretion of lactate, pyruvate, alanine, acetate, succinate and hypoxanthine regardless of physical training background⁵⁵). Another group

also observed an increase in metabolites related to the TCA cycle and branched-chain amino acid metabolism including lactate, pyruvate, alanine and hypoxanthine in urine after acute repetition of sprint running⁵⁶. Mukherjee et al reported the elevation of urinal lactate excretion after acute submaximal endurance exercise in aged humans⁵⁷. These observations should indicate exercise-caused facilitation of glycolysis. A chronic multicomponent exercise program augmented urinal excretion of choline, guanidinoacetate and hypoxanthine, but decreased creatinine⁵⁸. Krug et al analyzed metabolome in the blood, urine and breath from healthy young men under various stress challenges, including 30-min bicycle ergometer exercise, at their anaerobic threshold levels using LC-tandem MS and proton NMR, and suggested significance of the beta oxidation-caused elevation of plasma acylcarnitine levels⁵⁹. Targeted GS-MS analysis revealed that high intensity interval training increased plasma intermediates related to the TCA cycle, such as succinic and citric acids, more than moderate intensity continuous exercise loading in well-trained athletes⁶⁰. The increase in the plasma concentration of the TCA cycle intermediates was also observed in full marathon runners⁶¹. It would be reasonable to assume that aerobic physical exercise facilitates the TCA cycle and beta oxidation, but the results were not consistent. Moderate exercise increased plasma succinic acid only, and citric and malonic acids were unchanged during the exercise; while acute ergometer loading at 80% $\dot{V}O_2$ max increased succinic, fumaric and malic acids, but decreased citrate in the blood^{60,62}. The metabolic intermediates should be subject to change according to time and challenge protocols.

Most of the experimental objects of metabolomics to date have been serum and urine even in animal studies. Duggan et al demonstrated a shift in overall metabolomic profiles obtained by proton NMR spectroscopy in the serum after semi-chronic wheel running exercise for 10 days in normal and high-fat diet-fed obese mice, and suggested less potency of the impact of exercise on the metabolome than that of the diet⁶³. They observed an exercise-induced decrease in serum acetate and glutamine, but no apparent change for the other molecules. The proton NMR spectroscopy-powered metabolomics demonstrated that duration of exercise harbors more potent metabolomic impact than velocity⁶⁴. This report also suggested the significance of allantoin, inosine and branched-chain amino acids as discriminant metabolites, and discussed that urine samples are more informative than blood in terms of metabolomics; but it should depend on what kinds of analytic methods we choose.

These metabolomic investigations provide us with novel information indeed. Some reports demonstrated evidence that the metabolomic status in the plasma samples reflects metabolic conditions in skeletal muscle using cultured skeletal muscle cells^{61,65}. However, the consequent results would be relatively restricted as far as it does not

deal with contracting skeletal muscle, but with urine and blood, in terms of studies on physical exercise. Recently, lipidomic analysis using matrix-assisted laser desorption-ionization imaging mass spectrometry (MALDI-IMS) demonstrated an alteration in the composition of lipid profiles in murine transverse abdominal muscle that was electrically stimulated to contract acutely *in vivo*, or extensor digitorum longus muscle from rats after chronic treadmill training^{66,67}. MALDI-IMS is a relatively novel metabolomic approach, and will be a unique tool for labeling free visualization of the distribution of metabolites.

Metabolomic comparison between electrical contraction and AICAR stimulation in isolated skeletal muscle *ex vivo*

The comprehensive status of small metabolites in contracting skeletal muscle has yet to be revealed, although several previous studies dealing with physical exercise adopted metabolomic strategies as mentioned above. Furthermore, the entire role of AMPK in contracting skeletal muscle has not been clarified in spite of its potential significance. Thus, we conducted a metabolomic comparison between muscle contraction and pharmacological stimulation by AICAR to elucidate comprehensive metabolic alterations in contracting skeletal muscle and its relevance to AMPK³¹. We evaluated isolated rat epitrochlearis muscle stimulated *ex vivo*, where the activity of AMPK and glucose uptake are equivalently activated by AICAR stimulation and electrical contraction, to try and achieve a pure comparison avoiding secondary effects such as hormonal changes (Fig. 1, 2).

CE-TOFMS successfully annotated 132 small molecules from 184 detected peaks, and evidently demonstrated high resemblance of overall metabolome between AICAR stimulation and electrical contraction, suggesting the impact of AMPK activation on metabolism in contracting skeletal muscles (Fig. 3A). Subsequent sub analyses, classified by metabolic pathways, revealed close correlations in glucose and nucleic acid metabolism in a broad sense including glycolysis, the TCA cycle and metabolic pathways related to NAD, nucleic and ureic acid (Fig. 3B-E). In spite of the overall similarity, we noticed some discrepancies in amino acid metabolism. No correlations were found in the molecules related to branched chain amino acids, aspartate, alanine, lysine and aryl amino acids except for glycine, serine and cysteine (Fig. 3F-I). Aspartate, alanine and lysine, especially, out of 20 fundamental amino acids, were out of the correlation; and the resultant 16 amino acids exhibited high correlation ($r = 0.776$, $p < 0.001$, data not shown), suggesting the metabolic pathways associated with aspartate, alanine and/or lysine should be related to the AMPK-independent metabolomic shift in contracting skeletal muscle.

Principal component analysis (PCA), a popular nonbiased statistical method for analyzing many parameters,

Illustration of Skeletal Muscle Stimulation Ex Vivo

Fig. 1 Illustration of skeletal muscle stimulation ex vivo.

Isolated skeletal muscles were incubated in Krebs Ringer bicarbonate buffer with or without AICAR with continuous ventilation of 95% oxygen, 5% carbon dioxide. Ex vivo muscle contraction was induced by electrical stimulation in the buffer.

Fig. 2 AMPK and glucose uptake activities in AICAR-stimulated and contracting skeletal muscles.

Activities of AMPK alpha1 (A) and alpha2 (B), and 3-O-methylglucose uptake (C) in isolated epitrochlearis muscles in quiescence (open), stimulated by AICAR (closed) or electrical contraction (hatched).

extracted major (PC1) and minor (PC2) principal components, 63.8% and 36.2% respectively, and enabled us to project the characteristics of metabolome on PC1 and PC2 (Fig. 4). The major principal component, PC1 obviously distinguished both AICAR stimulation and electrical contraction from quiescent control, which corroborates the high similarity between AICAR stimulation and electrical contraction in terms of metabolome. In contrast,

the minor PC2 separated the characteristics between these two stimulations. Therefore, PC1 and PC2 should represent AMPK-dependent and -independent metabolomic changes in contracting skeletal muscles, respectively. Oxidative stress caused by physical exercise could activate AMPK, and AMPK activation has been believed to counteract oxidative stress^{37,68-70}). However, we also found that acute AMPK activation by AICAR stimulation decreases

Fig. 3 Correlation analyses on metabolomic changes between AICAR stimulation and electrical contraction.

Comparison between AICAR stimulation and muscle contraction on all molecules detected (A). Subanalyses on the molecules related to glycolysis and TCA cycle (B), NAD metabolism (C), nucleic acid metabolism (D), and ureic acid metabolism (E). Subanalyses on the molecules involved in amino acids metabolism related to branched chain amino acids (F), aspartate, alanine, and lysine (G), glycine and serine (H), and aryl amino acids (I). x-axis: fold changes by the AICAR stimulation, y-axis: fold changes by the electrical contraction.

Metabolomic Characteristics by Principal Component Analysis

Fig. 4 Metabolomic characteristics revealed by principal component analysis.

The primary (PC1) and secondary (PC2) principal component scores of quiescent control (open circle), AICAR stimulation (closed triangle) and electrical contraction (hatched square) are plotted. PC1 and PC2 represent 63.8% and 36.2%, respectively.

reduced glutathione levels, inducing the redox status to be oxidative³¹). The anti-oxidative responses following AMPK activation may be a kind of counteraction against this acute redox reaction. The change in redox status is supposed to underlie the common metabolomic effects as indicated by PCA and factor analysis³¹).

Our recent results demonstrated that AICAR stimulation should be feasible to closely mimic metabolomic changes in contracting skeletal muscles. AMPK activators are suggested to be exercise mimetic drugs in terms of metabolomics. In fact, AICAR stimulation was demonstrated to exhibit similar metabolic effects as physical exercise when used in combination with an agonist of peroxisome proliferator-activated receptor (PPAR) delta^{71,72}). However, AICAR stimulation alone could widely imitate various metabolic changes in contracting skeletal muscle metabolome.

Conclusion

Metabolomic techniques are quite informative and rapidly developing novel powerful tools in the field of metabolism. Our simple comparative study could clearly illustrate the comprehensive metabolic relevance of AMPK in contracting skeletal muscle. However, metabolomics has not yet been applied well for investigation of physical exercise and metabolites in skeletal muscle, especially in humans. Metabolomic variation among fiber types will also be of interest. Non-targeted metabolomic analysis might shed light on novel metabolic regulations or intracellular signal messengers. Metabolomics could be applied in combination with chemical modulators such as inhibitors and/or genetically manipulated animals in spite of the aforementioned demerits. Furthermore, metabolomic studies will provide us with a novel and more comprehensive understanding of metabolic regulation by physical exercise and muscle contraction, which would be of help in developing more efficient strategies on therapies and the prevention of prevailing metabolic diseases such as diabetes, obesity, and so on.

Conflict of Interests

The author declare that there is no conflict of interests regarding the publication of this article.

Acknowledgments

The author thanks Drs. Koichiro Tsuchiya, Tatsuya Hayashi, Toshiaki Tamaki, Tatsuro Egawa, Rieko Oshima and Taro Toyoda for helpful advice, and Dr. Omar M. Rodis for checking the manuscript.

References

- 1) Albright A, Franz M, Hornsby G, Kriska A, Marrero D, Ullrich I and Verity LS. 2000. American College of Sports Medicine position stand. Exercise and type 2 diabetes. *Med Sci Sports Exerc* 32: 1345-1360.
- 2) Berglund L, Brunzell JD, Goldberg AC, Goldberg IJ, Sacks F, Murad MH and Stalenhoef AF. 2012. Evaluation and treatment of hypertriglyceridemia: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab* 97: 2969-2989.
- 3) Jaques H. 2013. NICE guideline on lipid modification. *Eur Heart J* 34: 481-482.
- 4) Jaques H. 2013. NICE guideline on hypertension. *Eur Heart J* 34: 406-408.
- 5) Sim K (ed). 2012. Global Guideline for Type 2 Diabetes. International Diabetes Federation.
- 6) Young JC. 1995. Exercise prescription for individuals with metabolic disorders. Practical considerations. *Sports Med* 19: 43-54.
- 7) Hayashi T, Hirshman MF, Kurth EJ, Winder WW and Goodyear LJ. 1998. Evidence for 5' AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport. *Diabetes* 47: 1369-1373.
- 8) Hayashi T, Hirshman MF, Fujii N, Habinowski SA, Witters LA and Goodyear LJ. 2000. Metabolic stress and altered glucose transport: activation of AMP-activated protein kinase as a unifying coupling mechanism. *Diabetes* 49: 527-531.
- 9) Fujii N, Hirshman MF, Kane EM, Ho RC, Peter LE, Seifert MM and Goodyear LJ. 2005. AMP-activated protein kinase alpha2 activity is not essential for contraction- and hyperosmolarity-induced glucose transport in skeletal muscle. *J Biol Chem* 280: 39033-39041.
- 10) Jorgensen SB, Viollet B, Andreelli F, Frosig C, Birk JB, Schjerling P, Vaulont S, Richter EA and Wojtaszewski JF. 2004. Knockout of the alpha2 but not alpha1 5'-AMP-activated protein kinase isoform abolishes 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside but not contraction-induced glucose uptake in skeletal muscle. *J Biol Chem* 279: 1070-1079.
- 11) Mu J, Brozinick JT Jr, Valladares O, Bucan M and Birnbaum MJ. 2001. A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. *Mol Cell* 7: 1085-1094.
- 12) Miura S, Kai Y, Kamei Y, Bruce CR, Kubota N, Febbraio MA, Kadowaki T and Ezaki O. 2009. Alpha2-AMPK activity is not essential for an increase in fatty acid oxidation during low-intensity exercise. *Am J Physiol Endocrinol Metab* 296: E47-E55.
- 13) Hutber CA, Hardie DG and Winder WW. 1997. Electrical stimulation inactivates muscle acetyl-CoA carboxylase and increases AMP-activated protein kinase. *Am J Physiol* 272: E262-E266.
- 14) Berger M, Hagg S and Ruderman NB. 1975. Glucose metabolism in perfused skeletal muscle. Interaction of insulin and exercise on glucose uptake. *Biochem J* 146: 231-238.
- 15) Brozinick JT Jr, Etgen GJ Jr, Yaspelkis BB 3rd, Kang HY and Ivy JL. 1993. Effects of exercise training on muscle GLUT-4 protein content and translocation in obese Zucker rats. *Am J Physiol* 265: E419-E427.
- 16) Hespel P, Vergauwen L, Vandenbergh K and Richter EA. 1995. Important role of insulin and flow in stimulating glucose uptake in contracting skeletal muscle. *Diabetes* 44: 210-215.
- 17) Roy D, Johannsson E, Bonen A and Marette A. 1997. Electrical stimulation induces fiber type-specific translocation of

- GLUT-4 to T tubules in skeletal muscle. *Am J Physiol* 273: E688-E694.
- 18) Wojtaszewski JF, Laustsen JL, Derave W and Richter EA. 1998. Hypoxia and contractions do not utilize the same signaling mechanism in stimulating skeletal muscle glucose transport. *Biochim Biophys Acta* 1380: 396-404.
 - 19) Hardie DG and Carling D. 1997. The AMP-activated protein kinase--fuel gauge of the mammalian cell? *Eur J Biochem* 246: 259-273.
 - 20) Gao G, Fernandez CS, Stapleton D, Auster AS, Widmer J, Dyck JR, Kemp BE and Witters LA. 1996. Non-catalytic beta- and gamma-subunit isoforms of the 5'-AMP-activated protein kinase. *J Biol Chem* 271: 8675-8681.
 - 21) Gao G, Widmer J, Stapleton D, Teh T, Cox T, Kemp BE and Witters LA. 1995. Catalytic subunits of the porcine and rat 5'-AMP-activated protein kinase are members of the SNF1 protein kinase family. *Biochim Biophys Acta* 1266: 73-82.
 - 22) Stapleton D, Mitchelhill KI, Gao G, Widmer J, Michell BJ, Teh T, House CM, Fernandez CS, Cox T, Witters LA and Kemp BE. 1996. Mammalian AMP-activated protein kinase subfamily. *J Biol Chem* 271: 611-614.
 - 23) Stapleton D, Woollatt E, Mitchelhill KI, Nicholl JK, Fernandez CS, Michell BJ, Witters LA, Power DA, Sutherland GR and Kemp BE. 1997. AMP-activated protein kinase isoenzyme family: subunit structure and chromosomal location. *FEBS Lett* 409: 452-456.
 - 24) Hong SP, Leiper FC, Woods A, Carling D and Carlson M. 2003. Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases. *Proc Natl Acad Sci USA* 100: 8839-8843.
 - 25) Woods A, Johnstone SR, Dickerson K, Leiper FC, Fryer LG, Neumann D, Schlattner U, Wallimann T, Carlson M and Carling D. 2003. LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Curr Biol* 13: 2004-2008.
 - 26) Sakamoto K, McCarthy A, Smith D, Green KA, Grahame Hardie D, Ashworth A and Alessi DR. 2005. Deficiency of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake during contraction. *EMBO J* 24: 1810-1820.
 - 27) Shaw RJ, Lamia KA, Vasquez D, Koo SH, Bardeesy N, Depinho RA, Montminy M and Cantley LC. 2005. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. *Science* 310: 1642-1646.
 - 28) Hawley SA, Pan DA, Mustard KJ, Ross L, Bain J, Edelman AM, Frenguelli BG and Hardie DG. 2005. Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. *Cell Metab* 2: 9-19.
 - 29) Hurley RL, Anderson KA, Franzoni JM, Kemp BE, Means AR and Witters LA. 2005. The Ca²⁺/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases. *J Biol Chem* 280: 29060-29066.
 - 30) Woods A, Dickerson K, Heath R, Hong SP, Momcilovic M, Johnstone SR, Carlson M and Carling D. 2005. Ca²⁺/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells. *Cell Metab* 2: 21-33.
 - 31) Miyamoto L, Egawa T, Oshima R, Kurogi E, Tomida Y, Tsuchiya K and Hayashi T. 2013. AICAR stimulation metabolome widely mimics electrical contraction in isolated rat epitrochlearis muscle. *Am J Physiol Cell Physiol* 305: C1214-C1222.
 - 32) Miyamoto L, Toyoda T, Hayashi T, Yonemitsu S, Nakano M, Tanaka S, Ebihara K, Masuzaki H, Hosoda K, Ogawa Y, Inoue G, Fushiki T and Nakao K. 2007. Effect of acute activation of 5'-AMP-activated protein kinase on glycogen regulation in isolated rat skeletal muscle. *J Appl Physiol* (1985) 102: 1007-1013.
 - 33) Fujii N, Hayashi T, Hirshman MF, Smith JT, Habinowski SA, Kaijser L, Mu J, Ljungqvist O, Bimbaum MJ, Witters LA, Thorell A and Goodyear LJ. 2000. Exercise induces isoform-specific increase in 5'-AMP-activated protein kinase activity in human skeletal muscle. *Biochem Biophys Res Commun* 273: 1150-1155.
 - 34) Nakano M, Hamada T, Hayashi T, Yonemitsu S, Miyamoto L, Toyoda T, Tanaka S, Masuzaki H, Ebihara K, Ogawa Y, Hosoda K, Inoue G, Yoshimasa Y, Otaka A, Fushiki T and Nakao K. 2006. alpha2 isoform-specific activation of 5'-adenosine monophosphate-activated protein kinase by 5-aminoimidazole-4-carboxamide-1-beta-D-ribose nucleoside at a physiological level activates glucose transport and increases glucose transporter 4 in mouse skeletal muscle. *Metabolism* 55: 300-308.
 - 35) Wojtaszewski JF, Nielsen P, Hansen BF, Richter EA and Kiens B. 2000. Isoform-specific and exercise intensity-dependent activation of 5'-AMP-activated protein kinase in human skeletal muscle. *J Physiol* 528 (Pt 1): 221-226.
 - 36) Stephens TJ, Chen ZP, Canny BJ, Michell BJ, Kemp BE and McConell GK. 2002. Progressive increase in human skeletal muscle AMPKalpha2 activity and ACC phosphorylation during exercise. *Am J Physiol Endocrinol Metab* 282: E688-E694.
 - 37) Toyoda T, Hayashi T, Miyamoto L, Yonemitsu S, Nakano M, Tanaka S, Ebihara K, Masuzaki H, Hosoda K, Inoue G, Otaka A, Sato K, Fushiki T and Nakao K. 2004. Possible involvement of the alpha1 isoform of 5'-AMP-activated protein kinase in oxidative stress-stimulated glucose transport in skeletal muscle. *Am J Physiol Endocrinol Metab* 287: E166-E173.
 - 38) Toyoda T, Tanaka S, Ebihara K, Masuzaki H, Hosoda K, Sato K, Fushiki T, Nakao K and Hayashi T. 2006. Low-intensity contraction activates the alpha1-isoform of 5'-AMP-activated protein kinase in rat skeletal muscle. *Am J Physiol Endocrinol Metab* 290: E583-E590.
 - 39) Miyamoto L, Ebihara K, Kusakabe T, Aotani D, Yamamoto-Kataoka S, Sakai T, Aizawa-Abe M, Yamamoto Y, Fujikura J, Hayashi T, Hosoda K and Nakao K. 2012. Leptin activates hepatic 5'-AMP-activated protein kinase through sympathetic nervous system and alpha1-adrenergic receptor: a potential mechanism for improvement of fatty liver in lipodystrophy by leptin. *J Biol Chem* 287: 40441-40447.
 - 40) Minokoshi Y, Kim YB, Peroni OD, Fryer LG, Muller C, Carling D and Kahn BB. 2002. Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. *Nature* 415: 339-343.
 - 41) Chavez JA, Roach WG, Keller SR, Lane WS and Lienhard GE. 2008. Inhibition of GLUT4 translocation by Tbc1d1, a Rab GTPase-activating protein abundant in skeletal muscle, is partially relieved by AMP-activated protein kinase activation. *J Biol Chem* 283: 9187-9195.
 - 42) Chen S, Murphy J, Toth R, Campbell DG, Morrice NA and Mackintosh C. 2008. Complementary regulation of TBC1D1 and AS160 by growth factors, insulin and AMPK activators. *Biochem J* 409: 449-459.
 - 43) Funai K and Cartee GD. 2008. Contraction-stimulated glucose transport in rat skeletal muscle is sustained despite

- reversal of increased PAS-phosphorylation of AS160 and TBC1D1. *J Appl Physiol* (1985) 105: 1788-1795.
- 44) Kane S, Sano H, Liu SC, Asara JM, Lane WS, Garner CC and Lienhard GE. 2002. A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain. *J Biol Chem* 277: 22115-22118.
 - 45) Kramer HF, Witezak CA, Taylor EB, Fujii N, Hirshman MF and Goodyear LJ. 2006. AS160 regulates insulin- and contraction-stimulated glucose uptake in mouse skeletal muscle. *J Biol Chem* 281: 31478-31485.
 - 46) Ma X, Iwanaka N, Masuda S, Karaike K, Egawa T, Hamada T, Toyoda T, Miyamoto L, Nakao K and Hayashi T. 2009. Morus alba leaf extract stimulates 5'-AMP-activated protein kinase in isolated rat skeletal muscle. *J Ethnopharmacol* 122: 54-59.
 - 47) Sano H, Kane S, Sano E, Miinea CP, Asara JM, Lane WS, Garner CW and Lienhard GE. 2003. Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. *J Biol Chem* 278: 14599-14602.
 - 48) Taylor EB, An D, Kramer HF, Yu H, Fujii NL, Roeckl KS, Bowles N, Hirshman MF, Xie J, Feener EP and Goodyear LJ. 2008. Discovery of TBC1D1 as an insulin-, AICAR-, and contraction-stimulated signaling nexus in mouse skeletal muscle. *J Biol Chem* 283: 9787-9796.
 - 49) Hardie DG, Corton J, Ching YP, Davies SP and Hawley S. 1997. Regulation of lipid metabolism by the AMP-activated protein kinase. *Biochem Soc Trans* 25: 1229-1231.
 - 50) Winder WW and Hardie DG. 1996. Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise. *Am J Physiol* 270: E299-E304.
 - 51) Winder WW, Wilson HA, Hardie DG, Rasmussen BB, Hutber CA, Call GB, Clayton RD, Conley LM, Yoon S and Zhou B. 1997. Phosphorylation of rat muscle acetyl-CoA carboxylase by AMP-activated protein kinase and protein kinase A. *J Appl Physiol* 82: 219-225.
 - 52) Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ and Moller DE. 2001. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* 108: 1167-1174.
 - 53) You M, Matsumoto M, Pacold CM, Cho WK and Crabb DW. 2004. The role of AMP-activated protein kinase in the action of ethanol in the liver. *Gastroenterology* 127: 1798-1808.
 - 54) Lee WJ, Kim M, Park HS, Kim HS, Jeon MJ, Oh KS, Koh EH, Won JC, Kim MS, Oh GT, Yoon M, Lee KU and Park JY. 2006. AMPK activation increases fatty acid oxidation in skeletal muscle by activating PPARalpha and PGC-1. *Biochem Biophys Res Commun* 340: 291-295.
 - 55) Enea C, Seguin F, Petitpas-Mulliez J, Boildieu N, Boisseau N, Delpech N, Diaz V, Eugene M and Dugue B. 2010. (1) H NMR-based metabolomics approach for exploring urinary metabolome modifications after acute and chronic physical exercise. *Anal Bioanal Chem* 396: 1167-1176.
 - 56) Pechlivanis A, Kostidis S, Sarasilanidis P, Petridou A, Tsalis G, Mougios V, Gika HG, Mikros E and Theodoridis GA. 2010. (1)H NMR-based metabolomic investigation of the effect of two different exercise sessions on the metabolic fingerprint of human urine. *J Proteome Res* 9: 6405-6416.
 - 57) Mukherjee K, Edgett BA, Burrows HW, Castro C, Griffin JL, Schwertani AG, Gurd BJ and Funk CD. 2014. Whole blood transcriptomics and urinary metabolomics to define adaptive biochemical pathways of high-intensity exercise in 50-60 year old masters athletes. *PLoS One* 9: e92031.
 - 58) Sheedy JR, Gooley PR, Nahid A, Tull DL, McConville MJ, Kukuljan S, Nowson CA, Daly RM and Ebeling PR. 2014. (1)H-NMR analysis of the human urinary metabolome in response to an 18-month multi-component exercise program and calcium-vitamin-D3 supplementation in older men. *Appl Physiol Nutr Metab* 39: 1294-1304.
 - 59) Krug S, Kastenmuller G, Stuckler F, Rist MJ, Skurk T, Sailer M, Raffler J, Romisch-Margl W, Adamski J, Prehn C, Frank T, Engel KH, Hofmann T, Luy B, Zimmermann R, Moritz F, Schmitt-Kopplin P, Krumsiek J, Kremer W, Huber F, Oeh U, Theis FJ, Szymczak W, Hauner H, Suhre K and Daniel H. 2012. The dynamic range of the human metabolome revealed by challenges. *FASEB J* 26: 2607-2619.
 - 60) Peake JM, Tan SJ, Markworth JF, Broadbent JA, Skinner TL and Cameron-Smith D. 2014. Metabolic and hormonal responses to isoenergetic high-intensity interval exercise and continuous moderate-intensity exercise. *Am J Physiol Endocrinol Metab* 307: E539-E552.
 - 61) Lewis GD, Farrell L, Wood MJ, Martinovic M, Arany Z, Rowe GC, Souza A, Cheng S, McCabe EL, Yang E, Shi X, Deo R, Roth FP, Asnani A, Rhee EP, Systrom DM, Semigran MJ, Vasan RS, Carr SA, Wang TJ, Sabatine MS, Clish CB and Gerszten RE. 2010. Metabolic signatures of exercise in human plasma. *Sci Transl Med* 2: 33ra37.
 - 62) Brugnara L, Vinaixa M, Murillo S, Samino S, Rodriguez MA, Beltran A, Lerin C, Davison G, Correig X and Novials A. 2012. Metabolomics approach for analyzing the effects of exercise in subjects with type 1 diabetes mellitus. *PLoS One* 7: e40600.
 - 63) Duggan GE, Hittel DS, Sensen CW, Weljie AM, Vogel HJ and Shearer J. 2011. Metabolomic response to exercise training in lean and diet-induced obese mice. *J Appl Physiol* (1985) 110: 1311-1318.
 - 64) Le Moyec L, Mille-Hamard L, Triba MN, Breuneval C, Petot H and Billat VL. 2012. NMR metabolomics for assessment of exercise effects with mouse biofluids. *Anal Bioanal Chem* 404: 593-602.
 - 65) Lehmann R, Zhao X, Weigert C, Simon P, Fehrenbach E, Fritsche J, Machann J, Schick F, Wang J, Hoene M, Schleicher ED, Haring HU, Xu G and Niess AM. 2010. Medium chain acylcarnitines dominate the metabolite pattern in humans under moderate intensity exercise and support lipid oxidation. *PLoS One* 5: e11519.
 - 66) Goto-Inoue N, Manabe Y, Miyatake S, Ogino S, Morishita A, Hayasaka T, Masaki N, Setou M and Fujii NL. 2012. Visualization of dynamic change in contraction-induced lipid composition in mouse skeletal muscle by matrix-assisted laser desorption/ionization imaging mass spectrometry. *Anal Bioanal Chem* 403: 1863-1871.
 - 67) Goto-Inoue N, Yamada K, Inagaki A, Furuichi Y, Ogino S, Manabe Y, Setou M and Fujii NL. 2013. Lipidomics analysis revealed the phospholipid compositional changes in muscle by chronic exercise and high-fat diet. *Sci Rep* 3: 3267.
 - 68) Sandstrom ME, Zhang SJ, Bruton J, Silva JP, Reid MB, Westerblad H and Katz A. 2006. Role of reactive oxygen species in contraction-mediated glucose transport in mouse skeletal muscle. *J Physiol* 575: 251-262.

- 69) Sandstrom ME, Zhang SJ, Westerblad H and Katz A. 2007. Mechanical load plays little role in contraction-mediated glucose transport in mouse skeletal muscle. *J Physiol* 579: 527-534.
- 70) Zmijewski JW, Banerjee S, Bae H, Friggeri A, Lazarowski ER and Abraham E. 2010. Exposure to hydrogen peroxide induces oxidation and activation of AMP-activated protein kinase. *J Biol Chem* 285: 33154-33164.
- 71) Bueno Junior CR, Pantaleao LC, Voltarelli VA, Bozi LH, Brum PC and Zatz M. 2012. Combined effect of AMPK/PPAR agonists and exercise training in mdx mice functional performance. *PLoS One* 7: e45699.
- 72) Narkar VA, Downes M, Yu RT, Embler E, Wang YX, Banayo E, Mihaylova MM, Nelson MC, Zou Y, Juguilon H, Kang H, Shaw RJ and Evans RM. 2008. AMPK and PPARdelta agonists are exercise mimetics. *Cell* 134: 405-415.