Physical exercise is a powerful physiological stimulus that activates glucose, lipid, and energy metabolism in an acute and chronic manner. The beneficial effect of physical exercise is broadly applied as exercise therapy in a clinical setting for the treatment of “life-style-related diseases” such as type 2 diabetes and metabolic syndrome. Recent reports suggest that 5′-AMP-activated protein kinase (AMPK) in skeletal muscle contributes to the multiple benefits of exercise: enhancement of insulin sensitivity, increase in insulin-independent glucose uptake, increase in fatty-acid oxidation, modification of mitochondrial function by interaction with the peroxisome proliferator-activated receptor-γ (PPAR-γ) transcriptional coactivator 1-alpha (PGC-1α) and with the anti-aging molecule sirtuin, and in shifting muscle metabolic properties toward slow muscle. In this manuscript, we will review our recent publications and related articles and discuss AMPK activation in skeletal muscle during exercise.

AMPK structure and activation mechanisms

AMPK is a heterotrimeric kinase comprising a catalytic α subunit and two regulatory subunits, β and γ. There are two distinct α isoforms (α1 and α2): α1 is expressed ubiquitously, whereas α2 is dominant in skeletal muscle, heart, and liver. Henceforth, we will refer to α1-containing AMPK as AMPKα1 and to α2-containing AMPK as AMPKα2, as the different α isoforms give distinct activating patterns. The binding of AMP to the BATMAN domains of the γ subunit of AMPK causes allosteric activation of AMPK and induces phosphorylation of the Thr172 residue of the α subunit, which is essential for maximal kinase activity. The level of phosphorylation depends on the balance of the activities of upstream kinases, including LKB1 and the calcium/calmodulin-dependent protein kinase kinase (CaMKK) and protein phosphatases such as protein phosphatase 2C (PP2C). Because of its AMP dependency, AMPK is classically considered a signaling molecule in muscle cells by monitoring cellular energy levels, such as the AMP to ATP ratio.

Studies showing predominant AMPKα2 activation in skeletal muscle

In 1996, Winder et al. demonstrated for the first time that AMPK is activated during exercise in skeletal muscle. However, the authors measured AMPK activity...
in rat hind-limb muscle homogenates fractionated using ammonia sulfate precipitation and did not determine the \( \alpha \)-isoform specificity. The following year, Vavvas et al.\(^4\) first discriminated AMPK\( \alpha1 \) from AMPK\( \alpha2 \) activity in skeletal muscle during muscle contraction. Rat hind limbs were electrically stimulated to contract via the sciatic nerve at a frequency of 5 times/s (5 Hz) under anesthesia. Muscles were contracted for up to 5 min, followed by rapid isolation of the muscles for processing. Specific antibodies to the \( \alpha1 \) or \( \alpha2 \) subunit were added to muscle homogenates and AMPK activity was measured in each immunoprecipitate. The results of this experiment showed that the increased AMPK activity in contracted muscle was due to AMPK\( \alpha2 \), whereas AMPK\( \alpha1 \) activity was unchanged at every time point during the 5-min muscle contraction.

Selective activation of AMPK\( \alpha2 \) by exercise was also shown by Fujii et al. in 2000\(^{13}\), which was the first report of AMPK activity in human skeletal muscle. Healthy subjects performed a cycling exercise and their vastus lateralis muscles were biopsied. In muscle homogenates, exercise at 50\% \( \dot{V}O_{\text{max}} \) for 20 min did not increase the activity of AMPK\( \alpha1 \) or AMPK\( \alpha2 \); whereas exercise at 70\% \( \dot{V}O_{\text{max}} \) for 20 min increased the activity of AMPK\( \alpha2 \), but not that of AMPK\( \alpha1 \). In the same year, Wojtaszewski et al.\(^{14}\) reported supportive data in healthy subjects: a cycle exercise at 50\% \( \dot{V}O_{\text{max}} \) for 90 min did not increase the activity of AMPK\( \alpha1 \) or AMPK\( \alpha2 \); whereas an exercise at 75\% \( \dot{V}O_{\text{max}} \) for 60 min increased the activity of AMPK\( \alpha2 \) but not AMPK\( \alpha1 \). Moreover, in the same year, Chen et al.\(^{15}\) reported that a very vigorous sprint cycling exercise that elicited exhaustion in 30 s, increased the activity of both AMPK\( \alpha1 \) and AMPK\( \alpha2 \) by approximately two- to three-fold in the vastus lateralis muscle in healthy humans.

In 2001, Musi et al.\(^{16}\) measured AMPK activity in skeletal muscle during exercise in patients with type 2 diabetes and age-matched healthy controls. A cycling exercise of 45 min at 70\% of maximum workload increased AMPK\( \alpha2 \) activity by 2.7-fold in both groups; however, there was no effect of exercise on the activity of AMPK\( \alpha1 \). In the same year, this group showed that a low-intensity treadmill running exercise did not increase AMPK\( \alpha1 \) or AMPK\( \alpha2 \) activity, whereas a high-intensity exercise increased the activity of AMPK\( \alpha2 \), but had no effect on that of AMPK\( \alpha1 \), in rat skeletal muscle\(^{17}\), which supported the previous findings of Fujii et al.\(^{13}\) and Wojtaszewski et al.\(^{14}\) in humans.

These observations fit the hypothesis that AMPK\( \alpha2 \) is activated by low- to middle-intensity exercise and that AMPK\( \alpha1 \) is only activated by high-intensity exercise, i.e., that an increase in exercise intensity leads to the activation of AMPK in skeletal muscle in the order of AMPK\( \alpha2 \) at lower and AMPK\( \alpha1 \) at higher levels (Fig. 1A). This idea was considered reasonable and was widely accepted because AMPK\( \alpha2 \) activity increased in response to AMP more dramatically than AMPK\( \alpha1 \), suggesting that AMPK\( \alpha2 \) is more AMP dependent than AMPK\( \alpha1 \). However, there are important cases that do not fit this theory which we will address.

**Studies showing simultaneous activation of AMPK\( \alpha1 \) and AMPK\( \alpha2 \) in skeletal muscle**

Exercise includes dynamic changes in multiple intracellular and extracellular events in skeletal muscle. In 2000, Hayashi et al.\(^{19}\) used electrical stimulation to contract isolated skeletal muscle as a simple *in vitro* model of exercise to investigate AMPK activation during muscle contraction. Epitrochlearis muscles isolated from rats were incubated in Krebs buffer, which was electrified to induce tetanic contraction. The authors found that tetanic contraction increased the activity of AMPK\( \alpha2 \) and AMPK\( \alpha1 \) simultaneously. In addition, in 2001 Musi et al.\(^{17}\) reported that tetanic contraction increased in parallel the activity of AMPK\( \alpha2 \) and AMPK\( \alpha1 \).
both AMPKα1 and AMPKα2 in a dose-dependent manner and that, in the period after contraction, the activity of the two AMPKs decreased in a time-dependent manner. Thus, preferential AMPKα2 activation was not observed when an isolated muscle was incubated and stimulated to tetanically contract *ex vivo*.

In 2006, Toyoda et al. reported that AMPKα1, but not AMPKα2, was activated immediately as a postmortem artifact during the dissection procedure. AMPK activity was compared in the rat epitrochlearis muscle immediately after isolation and after incubation in Krebs buffer; it was discovered that AMPKα1 was highly activated immediately after isolation, but that its activity decreased and stabilized after incubation for 60 min (Fig. 2). The activity of AMPKα1 in muscle immediately after isolation was comparable to its activity in muscle maximally stimulated by tetanic contraction. In contrast, the activity of AMPKα2 did not differ between conditions.

The findings of Toyoda et al. suggest that results need to be interpreted carefully when measuring AMPKα1 activity in muscle samples collected without stabilization of AMPKα1 activity. If AMPKα1 activity is increased by the isolation process and reaches a level that is higher than the “true” AMPKα1 activity *in vivo*, an increase in activity will not be detectable, even if exercise increases AMPKα1 activity. These authors speculate that the predominant activation of AMPKα2 during exercise shown in humans and animals (mentioned above) is due to the fact that “true” AMPKα1 activation was masked by the isolation process. In addition, this concept could explain the observation by Chen et al. of increased AMPKα1 activity after a 30 s bicycle sprint exercise: the exercise was extremely intense and the increase in AMPKα1 activity associated with exercise overcame the increase associated with the isolation process.

**Studies showing preferential AMPKα1 activation in skeletal muscle**

Studies using isolated skeletal muscle reported by Hayashi et al. and Musi et al. showed that both AMPKα1 and AMPKα2 were activated in response to tetanic contraction. However, tetanic contraction is very strong and the muscle contraction that humans and animals naturally experience is much weaker than tetanic contraction. Therefore, Toyoda et al. investigated AMPK activity in isolated and incubated rat epitrochlearis muscles stimulated by twitch contraction at a frequency of 1 or 2 Hz. The result was different from the above-mentioned activation pattern (parallel activation of AMPKα1 and AMPKα2) as AMPKα2 was not activated and only AMPKα1 was activated (Fig. 3A). It is important to note that the 1 or 2 Hz contraction was not accompanied by a decrease in energy status, such as a decrease in AMP concentration or an increase in the AMP/ATP ratio (Fig. 3B). In addition, at a frequency over 5 Hz, twitch contraction increased AMPKα2 activity, which was accompanied by a decrease in energy status.

Preferential or exclusive activation of AMPKα1 was observed for AMPK-activating stimuli other than muscle contraction. In 2004, Toyoda et al. incubated isolated rat epitrochlearis muscles in Krebs buffer containing 1-3 mM H$_2$O$_2$ for 20 min to investigate whether oxidative stress induced AMPK activation. H$_2$O$_2$ activated AMPKα1 exclusively and this activation was inhibited by addition of the antioxidant *N*-acetyl-l-cysteine (NAC) (Fig. 4). In addition, AMPKα1 was activated exclusively when muscle was stimulated by the reactive-oxygen-generating system hypoxanthine and xanthine oxidase (HX–XO). Recently, Egawa et al. investigated the effect of caffeine on isolated epitrochlearis muscles and found that a low concentration of caffeine (1 mM, 15 min incubation) activated AMPKα1, but not AMPKα2; whereas a high concentration of caffeine (3 mM, 15 min incubation) activated both AMPKα1 and AMPKα2 (Fig. 5). Moreover, injection of a physiological dose of caffeine (5 mg/kg body weight) via the tail vein activated AMPKα1, but not AMPKα2, in the rat epitrochlearis muscle *in vivo*.

Increased enzymatic activity without a concomitant significant change in energy status markers was a common feature of AMPKα1 activation by low-intensity muscle contraction, H$_2$O$_2$, and caffeine. If the observation of AMPK activity in muscles incubated in Krebs buffer...
reflects the activity of AMPK in vivo, then AMPKα1 is the molecule that is activated by low-intensity exercise, such as activities of daily life, in which the energy status is balanced for muscle contractility (Fig. 6). In contrast, AMPKα1 and AMPKα2 are both activated by middle- to high-intensity exercise, accompanied by an obvious decrease in energy status (Fig. 1B).

The preferential activation of AMPKα1 accompanied by Thr172 phosphorylation suggests the involvement of covalent modification via upstream kinases. LKB1 is believed to be a crucial AMPK kinase; however, it is constitutively active and is not activated directly by AMP, and the dephosphorylation process may be inhibited by AMP. The preferential activation of AMPKα1 was not accompanied by energy depletion, suggesting that LKB1 is not the main AMPK kinase. In contrast, Jensen et al. found that caffeine-induced AMPKα1 activation in mouse skeletal muscle was blocked by the CaMKK inhibitor STO-609. This result indicates that CaMKK is an upstream kinase that is responsible for caffeine-induced AMPK activation. However, a low dose of caffeine (1 mM) activated AMPKα1 without a concomitant increase in the phosphorylation of CaMKI, which is a downstream target of CaMKK, suggesting that CaMKK is not likely to be involved in preferential AMPKα1 activation. Thus, it is possible that other enzymes such as PP2C are involved in the regulation of AMPKα1 in skeletal muscle.

**Fig. 3** Low-frequency electrical stimulation increases preferentially AMPKα1 activity in a contraction frequency-dependent manner in rat epitrochlearis muscles. Isolated muscles were electrically stimulated (50 V) to contract at the frequencies indicated for 2 min. To tetanically contract the muscles, muscles were stimulated for 10 sec each min, repeated 10 times. (A) Isoform-specific AMPK activity was determined in anti-α1 or anti-α2 subunit of AMPK immunoprecipitates. (B) Intracellular AMP concentration and ATP concentration were determined by HPLC, and the AMP:ATP ratio were calculated. (A) Values are means ± SE (A: n = 9–29 per group, B: n = 8–9 per group). **P < 0.01 vs Basal values. Reproduced from Ref. 19 with permission.

**Fig. 4** Antioxidant NAC inhibits H2O2-stimulated AMPKα1 activity. Isolated rat epitrochlearis muscles were preincubated in the presence or absence of 20 mM NAC for 60 min and then treated with H2O2 (1 and 3 mM) for 20 min. Isoform-specific AMPK activity was determined in anti-AMPKα1 and α2 immunoprecipitates. Values are means ± SE; n = 5–19 per group. **P < 0.01. Reproduced from Ref. 20 with permission.

**Fig. 5** Caffeine activates preferentially AMPKα1 activity in incubated rat epitrochlearis muscles. Isolated muscles were incubated in the absence (Control) or presence of caffeine for 15 min. Isoform-specific AMPK activity was determined in anti-AMPKα1 and -AMPKα2 immunoprecipitates. Fold increases are expressed relative to the activity of muscles in the control group. Values are mean ± SE; n = 6–14 per group. *P < 0.05, **P < 0.01 vs. control. Reproduced from Ref. 21 with permission.
AMPK and oxidative stress during and after exercise

The finding that AMPKα1 is activated by H\textsubscript{2}O\textsubscript{2} gives rise to the possibility that oxidative stress that occurs during exercise is a triggering signal to activate AMPKα1 in skeletal muscle. Skeletal muscle is an organ that consumes vast amounts of oxygen and is exposed to oxidative stress during exercise. The mitochondrial electron transport system is considered the major intracellular source of reactive oxygen species (ROS). Together with ROS production, skeletal muscle cells continuously consume oxygen and produce ATP in the mitochondria. Exercise and muscle contraction require an increase in ATP production, resulting in an increase in the production of oxidants and leading to a shift in the prooxidant/antioxidant balance toward oxidants, i.e., oxidative stress. Presumably, oxidative stress occurs ahead of an energy shortage, which may predict an energy shortage in the near future as ROS production can increase even when the level of ATP is maintained (Fig. 6). Therefore, it is reasonable to think that low-intensity twitch contraction activates AMPKα1 for the regulation of the metabolism via oxidative stress. Notably, health-promoting properties, including the increase of insulin sensitivity and the upregulation of PGC1α, are reduced in individuals exposed to physical activity and co-treated with antioxidant supplements\textsuperscript{26}.

Lack of exercise and AMPK

We think that “lack of exercise” is partly defined by “lack of AMPK activation in skeletal muscle”. Therefore, what type of lifestyle can prevent or avoid “lack of AMPK activation in skeletal muscle”? Evidently, it is better to schedule time for physical exercise. However, is it necessary to allocate reserve time just for physical exercise? Is it insufficient to simply engage in regular physical activity derived from daily life? In other words, is it a problem to be completely sedentary after 1 h of adequate exercise? To answer these questions, we need to elucidate the significance and molecular mechanisms of “continuous low-intensity exercise” in daily activities. In this case, it is probably meaningful to focus on molecules that are able to respond to low-intensity exercise. We think AMPKα1 is one of the potent candidate molecules that satisfy these criteria.

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References