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How is AMPK activity regulated in skeletal muscles during exercise?

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1. ABSTRACT

AMPK is a metabolic "master" controller activated in skeletal muscle by exercise in a time and intensity dependent manner, and has been implicated in regulating metabolic pathways in muscle during physical exercise. AMPK signaling in skeletal muscle is regulated by several systemic and intracellular factors and the regulation of skeletal muscle AMPK in response to exercise is the focus of this review. Specifically, the role of LKB1 and phosphatase PP2C in nucleotide-dependent activation of AMPK, and ionized calcium in CaMKK-dependent activation of AMPK in working muscle is discussed. We also discuss the influence of reactive oxygen species produced within the muscle as well as muscle glycogen and TAK1 in regulating AMPK during exercise. Currently, during intensive contraction, activation of alpha2-AMPK seems mainly to rely on AMP accumulating from ATP-hydrolysis whereas calcium signaling may have some importance during more gentle contraction conditions. Factors that regulate alpha1-AMPK during exercise are less clear but it appears, at least to some extent, to rely on an adenine nucleotide-dependent mechanism.

2. INTRODUCTION

The primary functions of skeletal muscle are to participate in regulation of whole-body glucose metabolism and to generate mechanical work where the latter requires a substantial amount of energy in the form of ATP. During low to moderate exercise intensities, ATP levels remain fairly stable despite the considerable contraction-induced increase in ATP turnover (109). To re-synthesize consumed ATP, skeletal muscle is dependent on availability of energy-containing substrates in addition to ATP-producing organelles and metabolic enzymes. The chief energy substrates for skeletal muscle are carbohydrates originating from plasma glucose and muscle glycogen as well as fatty acids originating from plasma and intramuscular lipid stores (47, 70, 97). During exercise, in addition to hormonal-induced changes in intracellular energy-substrates, the cytosolic content of several factors (e.g. Ca^{2+}, free AMP, P_i, creatine, H^+, NAD^+, reactive oxygen species, phosphatidic acid) can change. Many of these have been suggested to be initiators of signaling cascades that acutely increase substrate uptake and/or mobilize intracellular energy stores as well as in pathways inducing

chronic skeletal muscle adaptation. The biochemical regulation of proteins involved in these processes is likely to be under both allosteric and covalent control and this has been summarised in several reviews (70, 106, 125, 142). Since isoforms of mitogen activated protein kinases (MAPKs), Ca^{2+}/calmodulin-dependent kinases (CaMKs), conventional/novel PKCs and the 5´AMP-dependent protein kinase (AMPK) are activated or alter their subcellular location during muscle contraction, these molecules have all been proposed to be involved in regulating muscle metabolism during contraction and muscle adaptation to repeated exercise (4, 96, 103, 105, 145). Knowledge of how these signaling pathways are regulated is important as it may aid in the development of strategies to combat chronic metabolic disease and skeletal muscle dysfunction.

The role of AMPK in regulating muscle metabolism has received considerable attention over the past decade. AMPK is a ubiquitously expressed, multi-substrate heterotrimeric serine/threonine protein kinase and consists of one catalytic subunit (α) and two functionally and structurally different regulatory subunits (β, γ). Two isoforms of the α- and β-subunit (α1-2 and β1-2) and three isoforms of the γ-subunit (γ1-3) have been identified (69). AMPK, or perhaps more suitably the *AMPK system,* is hypothesised to function as a metabolic master regulator that aims to maintain cellular energy homeostasis by switching on catabolic pathways and turning off anabolic pathways (37, 67, 69). Studies of resting (non-contracting), fully differentiated rodent skeletal muscle and cultured muscle cells have shown that AMPK promotes ATP-synthesis by increasing GLUT4 translocation (13, 75, 136) and glucose uptake (45, 81, 85), as well as uptake and β-oxidation of fatty acids (11, 80, 81, 148). In addition, activation of AMPK in muscle aids in energy homeostasis by shutting down several energy consuming anabolic pathways such as glycogen synthesis (16, 62, 151) and ribosomal protein synthesis (10, 52, 58, 95). Furthermore, chronic activation of AMPK with chemical activators in resting muscle increases mitochondrial content (8, 64, 146, 163) and expression of proteins necessary for glucose uptake and processing (14, 48, 64) and regulation of these processes by AMPK may be viewed as a mechanism of the muscle cell to prepare for future metabolic challenges.

3. ACTIVATION OF SKELETAL MUSCLE AMPK BY EXERCISE

A number of studies have shown that AMPK is activated in rodent muscle by electrical stimulation *ex vivo*, and by motor nerve stimulation of both living animals and *in situ* perfused rat hindlimb (26, 45, 56, 139). Furthermore, *in vivo* exercise studies have shown that AMPK is activated in rat muscle during treadmill running and in human muscle during cycle exercise in a time and exercise-intensity-dependent manner (19, 32, 88, 126, 127, 135, 145, 153, 154). While studies of rodents using electrically stimulation to induce muscle contraction or *in vivo* exercise in general report activation of both skeletal muscle α1- and α2-AMPK, studies of human and rodent subjected to ergometer bicycle- and treadmill exercise find that α2-AMPK is activated by moderate exercise whereas α1-AMPK, if found to be activated, in general requires high exercise intensities (19, 32, 88, 89, 126, 154). Although this difference may relate to both intensity and fiber type recruitment, it has become apparent that the expression pattern of AMPK isoforms varies between rodent and human muscle and between muscle types as well (18, 28, 31, 150). A recent study has shown that of the three main AMPK complexes ($\alpha2\beta2\gamma1>>\alpha2\beta2\gamma3=\alpha1\beta2\gamma1$) expressed in mixed human vastus lateralis (150), it is essentially solely $\alpha2\beta2$ containing AMPK complexes which are activated during both sprint- and endurance bicycle exercise (9, 135). Finally, activation of muscle AMPK is higher in women compared with men during prolonged exercise (99) and is activated by resistance exercise in human muscle (27, 73).

While experimental approaches such as *in situ* muscle perfusion and *ex vivo* incubation of isolated muscle indirectly show that activation of AMPK can be regulated by local factors within the muscle during contraction (26, 45, 65), this findings does not necessarily rule out some contribution of circulating factors during *in vivo* exercise. Several studies have shown that circulating factors such as adiponectin, leptin, interleukin-6 (IL6), ciliary neutrophic factor (CNTF) and catecholamines can activate AMPK in resting muscle (15, 82, 107, 141, 162). The role of catecholamines is particularly interesting because both adrenaline and noradrenaline are increased greatly in a time and intensity manner during *in vivo* exercise in human serum (22, 34). It may therefore be envisioned that activation of muscle AMPK during *in vivo* exercise, at least to some extent, is potentiated by circulating factors such as catecholamines. Even though studies of incubated rodent muscle and cultured muscle cells have shown that adreno-receptor agonists increase AMPK activity (57, 82), a recent human study by Kristensen and colleagues (74) questions an influence circulating factors, including catecholamines, on muscle AMPK activation during moderate *in vivo* exercise. In this study, muscle AMPK was activated by one-legged exercise and serum catecholamine levels were after 20 min of work further elevated by adding arm-cranking exercise to the one-legged exercise for an additional 20 min. The main findings was that AMPK activity in the non-contracting leg remained at basal during the entire exercise protocol in spite of a vast increase in serum catecholamines (up to 15-23 fold), and that a further increase in catecholamines induced by adding arm-cranking exercise did not increase AMPK activity further in the working leg. It was based on these findings suggested that physiological levels of adrenaline and noradrenaline do not activate AMPK in resting muscle and that activation of muscle AMPK during moderate *in vivo* exercise is not dependent on circulating factors but relies on local mechanisms within the working muscle (74). Whether these factors only constitute already known myofibrillar protein signaling cascades or if parameters such as myofibrillar pH, local blood flow or some degree of hypoxia play an additional role remains to be established.

As hypothesized by several research groups, it seems evident to attribute a role for AMPK in acute

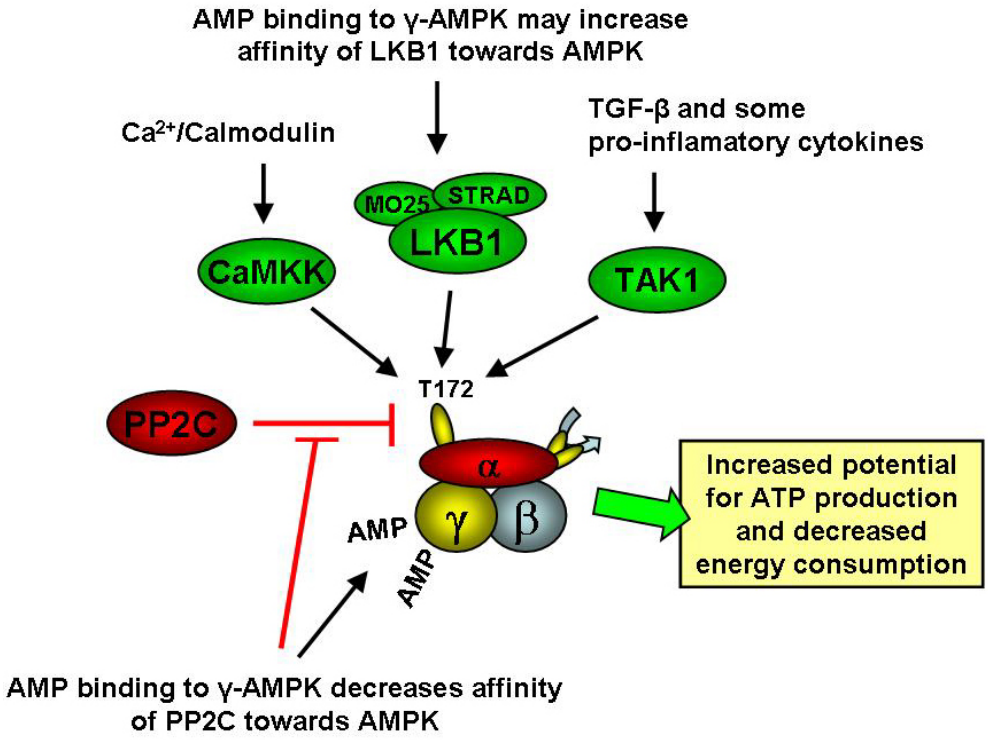

Figure 1. Schematic illustration of factors regulating AMPK activity. Binding of free AMP to the two Bateman domains on the γ-subunit induces a conformation change of the AMPK holoenzyme which directly increases AMPK activity allosterically as well as activating AMPK covalently by affecting the affinity of PP2C, and maybe also LKB1, towards AMPK α-T172. Binding of AMP to AMPK renders it a worse substrate for PP2C thus decreasing phosphatase activity towards α-T172 indirectly activating AMPK by conserving α-T172 phosphorylation. The activity of LKB1 is not believed to be regulated either covalently or allosterically in skeletal muscles but some observations suggest that the affinity of LKB1 towards α-T172 is increased with AMP. Several observations in particular *in vitro* and in cell culture have shown that the Ca^{2+}/calmodulin activated protein kinas kinase CaMKK is an additional AMPK kinase linking AMPK activation with cellular Ca^{2+} signaling. Finally, recent evidence has added the transforming growth factor-activated kinase (TAK1) to the list of AMPK kinases but knowledge of the role of TAK1 on muscle AMPK activation is still limited.

regulation of muscle metabolism in response to contraction. However, it has been surprisingly difficult to produce solid evidence demonstrating an executive role for AMPK in these processes. For instance, some observations (60, 85, 112) suggest that AMPK has a partial role in regulating glucose uptake in *ex vivo* contracting muscles whereas others (7, 33, 65) have found AMPK to be redundant. AMPK has also been speculated to play a role in adaptations of the metabolic "machinery" of skeletal muscle to exercise training. Studies using transgenic mice deficient in AMPK in general suggest that AMPK is not essential in initiating mitochondrial biogenesis and upregulating GLUT4 expression in response to exercise training (49, 64, 66, 98) whereas some (98), but not all (64), finding suggest some reliance on AMPK in training-induced expression of hexokinase II. These findings do in general not support a major role of AMPK in either acute or chronic metabolic regulations in response to exercise and exercise training. However, it is important to highlight that caution should be taken when evaluating these finding as present mouse models deficient in AMPK are still recognized by some level of residual AMPK activity. These areas will not be addressed further in this review and the reader is referred to other reviews (39, 63, 147).

4. ALLOSTERIC AND COVALENT REGULATION OF AMPK ACTIVITY

Several biochemical and mutation studies have produced results describing mechanisms regulating AMPK activity. AMPK is allosterically activated by cooperative binding of at least two AMP molecules to the two "Bateman domains" on the γ-subunit motifs (20, 120). In addition, AMPK activity can be increased covalently by reversible phosphorylation on T172 on the activation loop of the catalytic α-subunit (41) (see Figure 1). Of these two means of activation, the covalent activation of AMPK increases kinase activity much more potently than the allosteric AMP activation (23, 84, 114, 143). Early *in vitro* studies by Hawley and colleagues showed that the Ca^{2+}/calmodulin sensitive CaMK kinase phosphorylates α-T172 leading to AMPK activation (43). More recent studies took advantage of the conservation of the Snf1/AMPK pathway in combination with the yeast genetic systems and identified two putative AMPK kinases by their function as Snf1-activating kinases. This genetic selection method yielded two authentic AMPK kinases, namely LKB1 and CaMKK (40, 42, 50, 51). Furthermore, recent studies

identified the transforming growth factor-activated kinase (TAK1), a member of the MAPK-kinase-kinase family, as an additional Snf1/AMPK kinase (83) (see Figure 1). AMPK activity can in addition be modulated by removal of activating phosphorylation, and isoforms of the protein phosphatase 2C (PP2C) dephosphorylate α-T172 thereby reducing AMPK activity (24). Modulation of phosphatase activity towards α-T172 is now thought to be of considerable importance as well (116, 128).

Binding of free AMP to the γ-subunit, in addition to its direct allosteric action, also affects covalent activation of AMPK. Previous reports suggested that AMP binding increases α-T172 phosphorylation by rendering AMPK a better substrate for LKB1, while simultaneously rendering it a worse substrate for deactivating phosphatases (23, 38). Recently, however, three independent studies have challenged this view by reporting that AMP binding activates AMPK only by rendering it a worse substrate for PP2C phosphatase activity (77, 116, 128) (see Figure 1). This notion favors a scenario where AMPK, at least in response to metabolic stress, is activated indirectly by conserving T172 phosphorylation of the catalytic α-AMPK subunit – a view which also seems to agree with the concept of LKB1 being a constitutively active kinase in skeletal muscle, as discussed below.

5. NUCLEOTIDE DEPENDENT ACTIVATION OF AMPK

It is well established that muscle contraction is associated with a vast increase in fuel turnover (>100 fold) and provides a major energetic challenge to the muscle fiber (110). During such conditions, AMP concentration increases are accompanied by only a small decrease in ATP concentration (109). Even though free cytosolic AMP content is heavily buffered by protein binding, estimates of the pool of free cytosolic AMP suggests that it increases in response to contraction and exercise, and that the increase is exercise-intensity dependent (79, 108, 137). The working muscle counteracts increases in the AMP ratio by converting AMP to inosine monophosphate (IMP) enzymatically by AMP deaminase (138). Accordingly, accumulation of muscle IMP indicates that ATP hydrolysis had exceeded ADP phosphorylation, and hence that the muscle was incapable of sustaining ATP resynthesis. Binding of AMP to the "Bateman domains" on the γ-subunit is antagonized by ATP, and the cellular $(AMP_{free})/(ATP)$ ratio is thus thought to be the best indicator of AMPK activation in response to a metabolic challenge (19, 23, 90). AMPK activation has also been linked to the phospho-creatine system. An earlier study suggests that the high-energy phosphate phospho-creatine inhibits AMPK activity allosterically, and the dramatic decrease in phospho-creatine during the onset of intensive exercise could thus be speculated to contribute to AMPK activation by a "relief of inhibition" mechanism (94). However, recent evidence has challenged this view by showing that phospho-creatine does not affect AMPK activity *in vitro* and activation of AMPK during intense exercise is not directly linked to changes in phospho-creatine levels (129). However, phospho-creatine is still an important element in buffering muscle ATP during intensive exercise, thereby diminishing AMP accumulation and potentially delaying AMPK activation.

So what AMPK kinase(s) are translating the metabolic challenge associated with muscle work into activation of AMPK? LKB1 is believed to be a crucial AMPK kinase in nucleotide-dependent activation of AMPK. LKB1 is a multi-substrate serine-threonine kinase which complexes with the two accessory subunits STRAD and MO25 and phosphorylates a related protein family of at least 13 kinases (5, 12, 78). LKB1 was originally identified as a tumor-suppressor protein that is mutated in patients with Peutz-Jeghers syndrome (2) and is expressed in skeletal muscle (111) with a higher content in slow-twitch rat muscle types compared with fast-twitch muscle types (130). Studies in rodent muscle have shown that LKB1 activity measured during well-defined *in vitro* conditions is not increased in response to *in situ* stimulated muscle contractions, or by *ex vivo* incubation with AICAR or phenformin (111). These data suggests that LKB1 activity is not covalently regulated in muscle in response to several stimuli, including muscle contraction. This notion is further supported by the finding that *in vitro* activity of AMPK-kinase(s) purified from rat liver is not reduced in response to phosphatase treatment (41) even though LKB1 is expressed in liver tissue and known to be phosphorylated at several specific sites (118, 119, 121). It could be argued that LKB1 is activated by allosteric factors following muscle contraction and that this regulation is lost when assessing LKB1 *in vitro*. This seems, however, to not be the case as activity of the LKB1 targets QSK, QIK, MARK 1/2 and MARK4 is not altered in response to muscle contraction, AICAR or phenformin, indicating that endogenous LKB1 kinase activity towards these targets is not altered (111). These findings indicate that LKB1 functions as a constitutively active component in phosphorylating AMPK at α-T172 in response to metabolic stress. Whether an increased rate of α-T172 phosphorylation is mainly because AMP binding to the γ-subunit increases the affinity of LKB1 towards AMPK, or in addition is dependent on AMP binding inhibits PP2C activity towards AMPK, is currently not clear (see Figure 1 & 2).

The use of transgenic mice not expressing LKB1 protein in heart and skeletal muscle has been a valuable tool to address the dependence of LKB1 in regulation of AMPK signaling. Three individual LKB1 knockout mice have been generated so far (72, 112, 131) and they in general report similar findings regarding regulation of α2-AMPK activity. Knockout of LKB1 is associated with an almost totally ablated α2-AMPK activity in resting muscle, and when AICAR is used as a surrogate for metabolic stress, lack of LKB1 completely prevents α2-AMPK activation (72, 112, 131). Interestingly, activation of α2-AMPK by electrically-induced contraction of incubated muscles and of hindlimb muscles is practically completely abolished in muscle lacking LKB1 (72, 112). Furthermore, AMPK signaling expressed as α-T172 and ACCβ S227 phosphorylation in general shows good agreement with α2-AMPK activity as virtually no signals are detected in rest

and in response to AICAR when LKB1 is deleted. During contraction, α-T172 phosphorylation is not increased, but some increase in ACCβ phosphorylation is still detected in both *ex vivo* and *in situ* models (72, 111, 131) which could reflect the isolated event of allosteric AMPK activation or other stress-sensitive ACC S227 kinases. Interestingly, α1-AMPK activity is normal or only modestly reduced in resting LKB1 knockout muscles and increases normally in response to AICAR exposure (72, 112). The role of LKB1 in regulating α1-AMPK activity during contraction is more difficult to address since different research groups provide conflicting evidence about whether α1-AMPK is activated by *ex vivo* contractions. While Sakamoto and colleagues did not observe activation of α1-AMPK with contraction (111), Koh and colleagues (72) reported that a 2 fold increase in α1-AMPK activity in response to *ex vivo* induced contraction in EDL muscle was reduced by 50% with deletion of LKB1. Interestingly, if subjecting LKB1 deficient heart muscle to ischemia and anoxia, activation of α2-AMPK is completely abolished and α1-AMPK is activated to near normal levels (113) which agree with the hypothesis that LKB1 is essential in α2-AMPK activation while only partially involved in α1-AMPK activation in response to metabolic stress.

Collectively, these findings depict LKB1 as the main kinase involved in α2-AMPK activation in resting muscle in response to AICAR, and during intensive electrically-induced contractions. α1-AMPK is also activated in muscle by conditions mimicking metabolic stress, and in several cases by contraction, and current observations suggest that LKB1 is not the only upstream α1-AMPK kinase. Furthermore, these findings suggest that changes in adenine nucleotide levels are important in activating AMPK signaling in contracting muscle. However, this idea is to a large degree based on studies using electrical stimulation protocol to induce contraction of rodent muscle and *in vitro* biochemical assays and one could question how these findings compare with observations in human muscle during *in vivo* exercise. If exercising non-trained humans at a moderate intensity (66% of VO_{2max}) for up to 120 min, both α1-AMPK and α2-AMPK are activated in a time-dependent manner and the degree of activations correlate with increases in the (AMP_{free})/(ATP) ratio (79). Also, the intensity dependent activation of especially α2-AMPK with increasing exercise intensities (40%, 59%, 79% of VO_{2max}) correlate with increases in the (AMP_{free})/(ATP) ratio (19). Finally, in humans subjected to a short-term exercise-training program which improves metabolic control without inducing fiber-type changes, both AMP accumulation and AMPK activation are diminished during a subsequent exercise bout compared to before training (79). Thus descriptive studies of humans using more physiological exercise strategies are in agreement with the proposal that AMP content has a significant role in muscle AMPK activation during prolonged exercise.

6. CALCIUM DEPENDENT ACTIVATION OF AMPK

In spite of a broad line of evidence that suggests an essential role for LKB1 and PP2C system in activating AMPK in response to metabolic stress, an increasing body of evidence implies that signaling molecules sensitive to ionized calcium (Ca^{2+}) also act as AMPK kinases, and that these perhaps also are involved in regulating AMPK activity in contracting skeletal muscle. The first evidence linking calcium signaling with AMPK was published by Hardie and colleagues (43) more than a decade ago and showed that purified CaMKK phosphorylates and activates AMPK *in vitro*. CaMKK is one of a family of Ca^{2+}/calmodulin-dependent protein kinases that also includes CaM kinases I, II and IV, elongation factor-2 kinase, myosin light chain kinases and phosphorylase kinase (for review, see (36)). There exist two isoforms of CaMKK (α and β) which are encoded by two separate genes with alternative splicing variants, and both isoforms are activated by Ca^{2+}-CaM (3, 29, 53). Knockdown of the β-CaMKK using siRNA in LKB1deficient HeLa cells reduces AMPK activation substantially, whereas less pronounced reductions are observed with an α-CaMKK knockdown (42, 55, 155). These findings are confirmed by the observation that *in vitro* incubation of AMPK with either CaMKK isoform activates AMPK (with β-CaMKK the most potent) and with the observation that over-expression of β-CaMKK, but not α- CaMKK, accentuates AMPK activation by the Ca^{2+} ionophore 23187 in CCL13 cells (42, 155). Furthermore, inhibition of CaMKK with the STO-609 compound in cells deficient in LKB1 significantly reduces AMPK activation by A23187 and ionomycin (42, 55, 155).

Contrary to an earlier report (132), it has recently been shown that CaMKK is expressed in skeletal muscle (60, 104, 122, 144). Thus, it seems prudent to hypothesize some reliance on Ca^{2+}/calmodulin-CaMKK signaling in activating AMPK during exercise, given the well-described role of Ca^{2+} in excitation-contraction coupling. Indeed, over-expression of constitutively active α-CaMKK in fast-twitch mouse muscle increases basal AMPK phosphorylation in addition to basal α1- and α2-AMPK activities (149). Despite an earlier study did not reported activation of muscle AMPK using caffeine to raise cytosolic Ca^{2+} (158), a recent, more detailed study by Jensen & colleagues (59) demonstrated that while caffeine treatment does not alter total AMPK α-T172 phosphorylation or α2-AMPK phosphorylation, it does increase ACCβ- and α1-AMPK phosphorylation. It should be kept in mind when interpreting these findings that most strategies used to raise cytosolic Ca^{2+} aim to induce concentrations too low to cause muscle contraction, and that higher Ca^{2+} concentrations perhaps would result in more robust AMPK activation.

Recent work using chemical inhibitors of Ca^{2+}/CaM sensitive molecules have shed some light on whether CaMKK is involved in contraction-stimulated AMPK activation. Initial experiments aimed to determine if the Ca^{2+}/CaM-competitive inhibitor KN93, which in addition to inhibiting CaMK I, II and IV is assumed to inhibit CaMKK, was associated with impaired activation of AMPK in response to *ex vivo* electrically-induced contraction (60). Interestingly, in contrast to an earlier observation which showed no effect of CaMK inhibition on

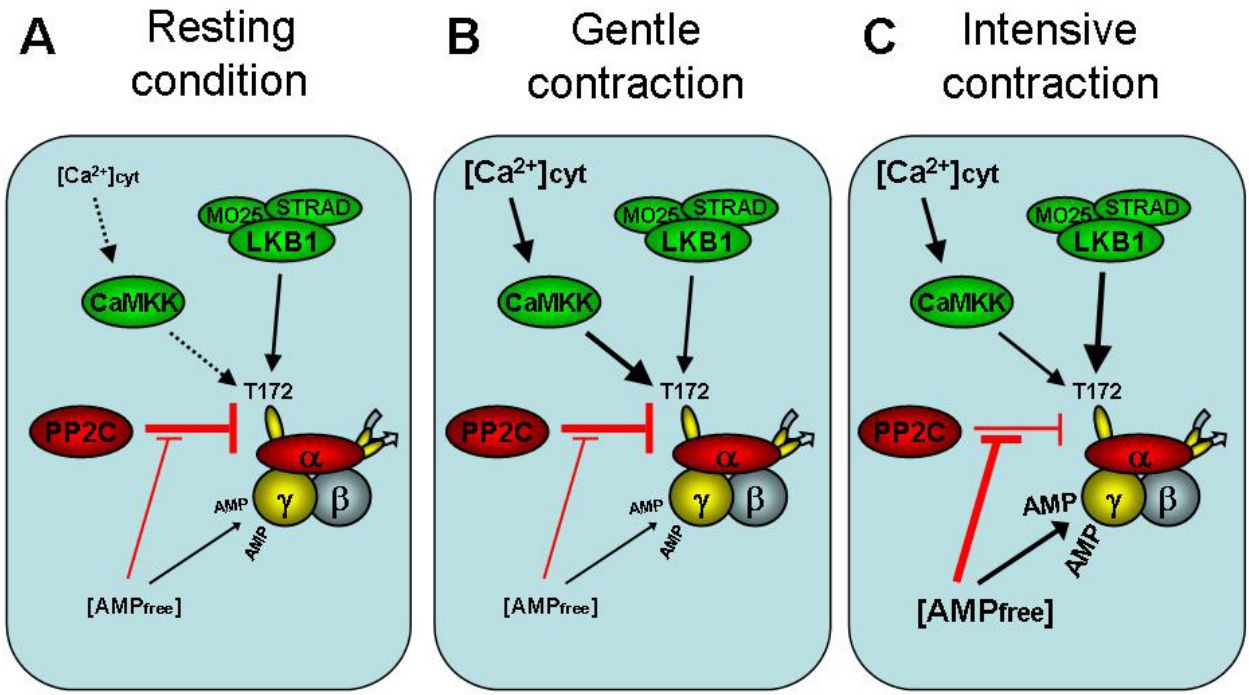

Figure 2. Hypothetical model of regulation of AMPK activity in muscle at rest and in response to gentle and intensive contractions based on observation in *ex vivo* electrically stimulated rodent skeletal muscle. A: Observations in LKB1 knockout mice may suggest that PP2C activity at resting conditions overrules LKB1 activity thereby preventing AMPK activation. As (Ca^{2+}) is low in non-contracting muscle CaMKK activity would be expected low as well B: During gentle contraction the muscle is fairly capable of maintaining the (AMP_{free})/(ATP) ratio. Recent evidence suggests that increased Ca^{2+} signaling during gentle contraction intensity activates CaMKK subsequently leading to increased AMPK activity. C: During intensive *ex vivo* electrically stimulated contraction muscle ATP is reduced paralleled by some increase in free AMP and close to an equimolar increase in IMP. The subsequent increase in the (AMP_{free})/(ATP) ratio leads to a potent activation of AMPK. The role of Ca^{2+} signaling during intensive contractions is less clear, but studies in LKB1 knockout mice and in mice over-expressing CaMKK indicate that AMPK activation during intensive contractions is not reliant on Ca^{2+}/CaM CaMKK signaling. Some evidence also suggests that the continual breakdown of muscle glycogen during prolonged exercise indirectly potentiates AMPK activation by a "relief of inhibition" resulting from lowered glycogen.

contraction-stimulated AMPK phosphorylation (157), it was shown that KN93 completely prevented activation of both α1-, and α2-AMPK in the mouse soleus muscle following 2 minutes of a relatively moderate contraction protocol (60). Of note, both AMPK isoforms increased normally with KN93 after 10 minutes of contraction (60). This study was then replicated using STO-609 at a concentration reported to specifically inhibit CaMKK activity (42). Using this compound, it was shown that contraction-induced α1-, and α2-AMPK activation was impaired after both 2 minutes and 10 minutes, in both soleus and EDL muscle (60). Results obtained using inhibitors are always associated with the risk of undesirable side effects on both related and unrelated molecules and it could be argued that STO-609 directly inhibited LKB1 or AMPK activity in spite of the low concentration (42). This possibility was tested using recombinant LKB1/MO25/STRAD and it was found that LKB1 activity was unaffected by STO-609, and that AICAR increased AMPK activity normally in the presence of 5μM STO-609. These findings suggest that the abolished AMPK activation during contraction with STO-609 was not due to undesirable inhibition of LKB1 but more likely due to inhibition of CaMKK *per se*. This in turn suggests that CaMKK signaling may play a role in regulating muscle AMPK activity during contractions, independent of LKB1. However, these findings are in contrast to earlier observations in LKB1 knockout mice suggesting that LKB1 is essential in activating α2-AMPK during contraction (72, 112). The discrepancies between observations in the LKB1 knockout mice and those with STO-609 treatment could be due to the more intensive protocols used to induce muscle contraction in the LKB1 knockout studies compared to the STO-609 inhibitor study. Thus, activation of AMPK during moderate intensity contraction may rely more on Ca^{2+}-signalling while activation of AMPK during intensive contraction may rely more heavily on increases in AMP (see Figure 2). In concert, in rat epitrochlearis muscle subjected to low-frequency *ex vivo* stimulation protocol not affecting the (AMP)/(ATP) ratio, α1-AMPK activity is still increased which supports the hypothesis that AMPK can be activated during muscle contraction by a mechanism not related to changes in adenine nucleotides (134). On the other hand, if mouse muscle is subjected to an intensive *ex vivo* stimulation protocol expected to increase the

(AMP_{free})/(ATP) ratio significantly (133, 134), incubation with STO-609 does not affect AMPK phosphorylation (149) which suggests that CaMKK does not contribute significantly to activation of AMPK during this condition.

Even though it is undoubtedly necessary to show that CaMKK activity is increased with muscle contraction similar to CaMKII (101, 103, 104) and to confirm observations based on the STO-609 inhibitor using e.g. transgenic animals, current findings may still suggest that CaMKK plays a role in activating muscle AMPK during contraction at less intensive contraction protocols of mouse muscle (see Figure 2). Noteworthy, observations in LKB1 knockout skeletal muscle could be interpreted to mean that CaMKK is mainly involved in regulating α1-AMPK but the finding that STO-609 inhibits activation of both catalytic AMPK subunits during contraction challenges this idea. If these ideas hold true for human muscle, α1-AMPK should be expected to be activated immediately with the onset of exercise as increased Ca^{2+}-signaling is required to facilitated actin-myosin cross-bridge formations of the muscle fiber. While the literature is not entirely clear regarding α1-AMPK activation during moderate to intensive exercise, it is unequivocal that neither α1-AMPK nor α2-AMPK are covalently activated during low intensity exercise (32, 79, 154) which do not supporting this notion. Furthermore, measures of AMPK activation after 30 and 120 sec of ergometer bicycle sprint exercise show that α2-AMPK is activated at both time points while α1-AMPK activity is actually decreased (9). Thus, while CaMKK may play a partial role in regulating AMPK activation in mouse muscle in response to electrical stimulation, current descriptive studies of exercising human may not favor such a relationship.

7. MUSCLE GLYCOGEN DEPENDENT ACTIVATION OF AMPK

Muscle glycogen appears to be an important controller of muscle AMPK activity but despite the strong negative relationship between muscle glycogen content and AMPK activity, little is known about the precise mechanistic link. Correlative studies of glycogen effects on metabolic processes in skeletal muscle from human and rodent have commonly used a combined exercise and diet protocol where muscle and liver glycogen is depleted by a single bout of prolonged exercise which is followed by a diet either high or low in carbohydrate. Feeding this diet subsequently gives experimental groups the next day with high and low muscle glycogen, respectively. Studies of fast-twitch and slow-twitch muscle types from rodents have shown that glycogen loaded muscles exhibit suppressed AMPK activation in response to electrically-induced contraction and AICAR (26, 151, 152). The negative correlation between glycogen content and AMPK activity is only significant for α2-AMPK activity, and not for α1-AMPK activity, suggesting that the effect of glycogen is mainly targeting α2-AMPK complexes (151). Human studies based on healthy untrained subjects generally support these findings as bicycle exercise for 30-60min at 65-70% of VO_{2max} is associated with greater activation of α2-AMPK in glycogen depleted than in glycogen loaded muscles (100, 152). As α2-AMPK in general is reported to be more sensitive to metabolic stress than α1-AMPK (114), an obvious explanation could be that glycogen depletion leads to a muscle more prone to develop metabolic stress during exercise because of reduced substrate availability for ATP re-synthesis. While measures such as ATP, AMP and phospho-creatine are not reported to be affected by glycogen manipulation in response to contraction and exercise (26, 79, 151, 152) the calculated (AMP_{free})/(ATP) ratio is increased to a greater extent during exercise in humans with low glycogen compared with a high glycogen situation (79). Thus, some lines of evidence suggest that the inverse relationship between glycogen content and AMPK activation during exercise can be explained by a greater increase in (AMP_{free})/(ATP) with low glycogen than high glycogen. However, as glycogen loading of rat muscle suppresses basal and AICAR-stimulated α2-AMPK activity in rat muscle not expected to be metabolically stressed (151), this relationship seems not to be solely explained by an adenine nucleotide dependent mechanism.

Several observations based on more biochemical and molecular strategies lean toward a scenario suggesting some reliance on a direct interaction between glycogen and AMPK. For instance, the AMPK β-subunit possesses a starch/glycogen-binding domain (GBD) that binds AMPK to glycogen in a cell free system which is evidenced by that mutations of key carbohydrate binding residues either partially or completely abolishes β-GBD binding to glycogen in cell free systems (92, 93). Furthermore, expression of this domain is essential in spatial targeting of AMPK to glycogen-containing granules in the cytoplasm of cultured human cell (54). On the other hand, a recent study used a refined glycogen purification protocol and reported that AMPK was not co-purified with glycogen, even though well-known glycogen associated proteins such as glycogen phosphorylase and glycogen debranching enzyme were co-purified with glycogen using this protocol (91). At first glance this finding does not support a direct interaction between AMPK and glycogen, but the finding could also simply reflect differing affinities for glycogen exhibited by AMPK and other glycogen associated proteins – that AMPK was lost during the purification. In line with the latter view, a more recent study reported that the dissociation constants of the AMPK β-GBD with oligosaccharide glycogen-mimicking structures do predict that AMPK does bind with glycogen, but also, that the of affinity AMPK towards glycogen is not very tight (71).

The majority of these data do suggest that AMPK binds directly with glycogen, but since binding of AMPK with glycogen particles purified from liver do not reduce AMPK activity *in vitro* the mechanism by which glycogen inhibits AMPK activation does not seem to involve a direct inhibition (54, 92). This idea is supported by observations in muscles from McArdle patients which are recognized by having chronically high muscle glycogen levels due to deficiency in glycogen phosphorylase. When these patients are exercised at a moderate intensity, α2-AMPK activity is not suppressed compared with control subjects in spite of high muscle glycogen levels. Thus, it may be envisioned that glycogen possibly functions as a

scaffolding factor bring AMPK together with factors regulating α-T172 phosphorylation rather than interfering directly with its kinase activity. Nevertheless, if the γ3-subunit is knocked out in mouse muscle the inverse relationship between glycogen content and AMPK activity is no longer seen (6) which could suggest that glycogen specifically targets γ3-containing AMPK complexes. The finding that primarily γ3-containing complexes are activated in response to bicycle exercise in human muscle (9) may increase the importance of glycogen breakdown in regulating AMPK activity in response to exercise. Interestingly, one may speculate that the reduction in muscle glycogen during exercise in part explains the steady increase in AMPK activity seen during prolonged bicycle exercise (102, 127, 153) by a gradual relief from a glycogen-related inhibition. Noteworthy, changes in AMPK activity in *in situ* contracting rat gastrocnemius muscle correlate more closely with changes in glycogen concentrations than changes of the $(AMP_{free})/(ATP)$ ratio. In particular, the increase in $(AMP_{free})/(ATP)$ ratio occurred before any changes in AMPK and ACC phosphorylation suggesting that glycogen may retard the ability of AMP to activate AMPK during contractions, at least in fast-twitch rat muscle. (Rose AJ, Alsted TJ & Richter EA, unpublished observation). Collectively, the mechanism by which glycogen regulates AMPK activity is still not clear but it does make sense in that a reduction in muscle glycogen leads to greater risk of developing metabolic stress during contraction/exercise which in turn leads to higher AMPK activation. On the other hand, since glycogen loading still suppresses AMPK activity in basal non-contracting muscle other mechanisms seem to be involved as well but it is at present not entirely clear if this is related to the β-subunit GBD dependent binding of AMPK to glycogen.

8. REACTIVE OXYGEN SPECIES DEPENDENT ACTIVATION OF AMPK

Low-grade chronic oxidative stress is normally associated with impaired insulin signaling in several tissues including skeletal muscle (46) but recent findings suggest that acute increases in skeletal muscle reactive oxygen species (ROS) levels activates AMPK (117, 133). Reactive oxygen species are mainly produced by the mitochondria, and xanthine oxidase may be a significant player in ROS formation in working skeletal muscle (35, 61, 86, 140). Observations based on incubated rodent muscle have shown that acute exposure to H_2O_2, a well-described ROS donor, increases glucose uptake in a time and concentration dependent manner, and that the increase can be prevented by the antioxidant *N*-acetyl-L-cysteine (NAC) (17, 117, 124, 133). Reactive oxygen species have also been linked to AMPK signaling as incubation of rat muscle with H_2O_2 or the super-oxide donor system hypoxanthine/xanthine oxidase specifically activates α1-AMPK but not α2-AMPK (133). An increase in AMP was not detected, but since ATP was decreased and IMP was increased, the mechanisms by which ROS activates α1-AMPK may rely, at least to some extent, on changes in muscle adenine nucleotide ratios in this set-up (133). In line with this study, perfusion of isolated rat heart with H_2O_2 sufficient to activate AMPK does not alter the (AMP)/(ATP) ratio but is associated with a significant increase in free creatine (76). Studies in other cell types support the hypothesis that AMPK is activated by oxidative stress as H_2O_2 exposure of both NIH-3T3 and smooth muscle cells is associated with increased α1-AMPK activity, and that this increase in the NIH-3T3 cells was tightly coupled with an increase in the (AMP)/(ATP) ratio (21). This notion is indirectly supported by the finding that transfection of CCL13 cells with LKB1 enhances H_2O_2-induced AMPK activation, and conversely that expression of dominant-negative LKB1 blunts the response, implying an essential role of LKB1 and thus probably reliance on changes in adenine nucleotides (156). On the other hand, studies of rat myotubes have shown that H_2O_2 in the lower μM range increase intracellular Ca^{2+} paralleled by increased CREB phosphorylation, suggesting that oxidative stress in muscle activates AMPK by a Ca^{2+} dependent mechanism (30). In addition, recent findings have shown that inhibition of CaMKK with STO-609 impairs ROS-induced AMPK activation in a LKB1 deficient cell line (115). Collectively, these observations show that treatment of rodent muscle with H_2O_2 leads to modest changes of the energy state and cell studies show that H_2O_2 exposure leads to increase Ca^{2+} signaling which apparently is translated to AMPK activation by a CaMKK dependent mechanism.

Since skeletal muscle continuously produces ROS, and since the production is increased by muscular work both *ex vivo* and *in vivo* (35, 86, 140) and during electrical stimulation of cultured rodent cells (30, 123), it seems evident to hypothesize some reliance on ROS formation in activating AMPK during exercise (117). A recent study by Sandström and colleagues addressed this topic and showed that the presence of the antioxidants NAC and ebselen almost completely prevented increases in ROS, and reduced AMPK phosphorylation by ~50% in response to electrically-stimulated contraction (117). These results suggest that activation of AMPK during *ex vivo* muscle contraction is partially dependent on ROS production. The mechanism by which ROS formation attenuates AMPK activation during muscle contractions is not clear. If the mechanism entirely relies on altered adenine nucleotide signaling, then treatment of contracting muscle with antioxidants should be expected to reduce ATP and phospho-creatine depletion. Nevertheless, treatment of isolated rat diaphragm muscle with antioxidants during repeated contraction had no effect on the depletion of these two measures (159). This may suggest that the partial role of ROS in activating AMPK during *ex vivo* muscle contracting, as reported by Sandström and colleagues (117), is not regulated by an adenine nucleotide independent mechanism as suggest by the authors. Using an *ex vivo* system where muscle oxygenation depends on simple diffusion rather than capillary delivery, may cause a relatively high build-up of ROS compared with *in vivo* conditions. An obvious question is therefore if ROS plays a significant role in activating AMPK during more physiological exercise regimes. It is well known that ROS are produced in skeletal muscle during *in vivo* exercise but due to methodological issues it is difficult to obtain quantitative measures of ROS production or the individual ROS species produced, and hence difficult to compare *ex vivo* and *in vivo* conditions (68). Although speculative, as

increased respiration is believed to correlate with increased ROS production, the contribution of ROS in AMPK activation during contraction would be expected to be greater during intensive *ex vivo* electrically stimulated contractions than during *in vivo* exercise.

9. TAK1 - A CYTOKINE DEPENDENT AMPK REGULATOR?

The TAK1 protein kinase has recently been reported to be an upstream AMPK kinase. TAK1 was identified as a mediator of TGF-β signaling and is also shown to convey signaling of several pro-inflamatory cytokines such as TNF-α, IL-1 and bacterial LPS (25, 161). A recent study showed that TAK1 activates AMPK in response to metabolic stress in cultured heart cells and embryonic mouse fibroblasts via an LKB1 dependent mechanism (160). Even though current findings in general suggest that LKB1 activity is neither regulated covalently nor allosterically, this study addresses the possibility that TAK1, in addition to LKB1, is directly involved in activation of AMPK in response to metabolic stress. However, it remains to be established whether TAK1 has a role in activating AMPK in skeletal muscle and if skeletal muscle TAK1 signaling is sensitive to exercise and contraction.

10. CONCLUDING REMARKS

Our understanding of the mechanisms regulating AMPK activity acutely in working muscle has increased over the years, but recent studies have also (re)introduced new potential players such as CaMKK, PP2C and perhaps also TAK1 into the field. Both human and rodent studies agree on that α2-AMPK is activated in a time and intensity dependent manner during both physiological *in vivo* exercise and in response to electrical stimulation. Human studies in addition suggest that it is mainly α2β2-containing AMPK complexes which are activated by exercise. Regulation of α1-AMPK is still intricate to address as α1-AMPK in some studies is reported to be activated by electrical stimulation of rodent muscle and physiological *in vivo* exercise of human and rodent (1, 19, 33, 44, 64-66, 72, 79, 154) and other studies do not report activation regardless of type of stimuli (9, 32, 87, 88, 100, 111, 112, 126, 152). Interestingly, although several hormones can activate AMPK in muscle recent findings suggests that activation of AMPK during exercise mainly relies on local factors within the muscle.

While initial studies depicted regulation of muscle AMPK as mainly being dictated by the $(AMP_{free})/(ATP)$ ratio via LKB1 and PP2C, more recent observations have linked Ca^{2+} signaling via CaMKK to AMPK activation. Studies of rodent muscles support that LKB1 is a crucial factor in activating AMPK in response to both metabolic stress and intensive *ex vivo* electrically-induced contraction via an adenine nucleotide dependent mechanism and may also suggest that CaMKK signaling have a role in regulating AMPK during more moderate *ex vivo* contraction intensities. Even though it is tempting to attribute a significant role of PP2C in adenine nucleotide dependent AMPK activation during exercise/contraction there exists at present no direct data proving such a connection but this is clearly an area for further research. Descriptive studies of humans using more physiological *in vivo* exercise models supports that changes in adenine nucleotide are important in activating muscle AMPK but does so far not supported that CaMKK signaling has an important function.

It has been understood for several years that muscle glycogen content correlates negatively with α2-AMPK activation (but not α1) during exercise/contraction and that this relationship is apparent in both human and rat muscle. The mechanism is still far from clear but may in part be related to AMPK binding to glycogen targeted by the β-subunit GBD, and in part related to a greater level of energy stress in glycogen-depleted muscle during contraction. Finally, it is important to take into consideration that even though many animal models are superior in revealing potential molecular interactions compared with more descriptive human studies, they are still recognized by some limitations and direct extrapolation of results based on e.g. *ex vivo* incubated muscles and transgenic manipulations to human should be done with caution.

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