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Review

AMP-activated protein kinase in skeletal muscle: From structure and localization to its role as a master regulator of cellular metabolism

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Abstract. The AMP-activated protein kinase (AMPK) is a metabolite sensing serine/threonine kinase that has been termed the master regulator of cellular energy metabolism due to its numerous roles in the regulation of glucose, lipid, and protein metabolism. In this review, we first summarize the current literature on a number of important aspects of AMPK in skeletal muscle. These include the following: (1) the structural components of the three AMPK subunits (i.e. AMPKα, β, and γ), and their differential localization in response to stimulation in muscle; (2) the biochemical regulation of AMPK by AMP, protein phosphatases, and its three known upstream kinases, LKB1, Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK), and transforming growth factor-β-activated kinase 1 (TAK1); (3) the pharmacological agents that are currently available for the activation and inhibition of AMPK; (4) the physiological stimuli that activate AMPK in muscle; and (5) the metabolic processes that AMPK regulates in skeletal muscle.

Keywords. Ca^{2+}/calmodulin-dependent protein kinase kinase, carbohydrate, glucose, lipid, LKB1, protein phosphatase, transforming growth factor-β-activated kinase 1.

Introduction

The AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase that has emerged as a master sensor of cellular energy balance in mammalian cells. The protein AMP-activated protein kinase was originally identified as an upstream regulator of two key mediators of lipid metabolism, acetyl-CoA carboxylase and 3-hydroxy-3-methylglutaryl CoA reductase [1, 2], although the name AMP-activated protein kinase (AMPK) was not adopted until 1988 [3]. Once the protein was identified it was found to be a homolog of the *Saccharomyces cerevisiae* metabolic stress sensing kinase, Snf1 kinase [4–6], a protein critical for yeast survival under conditions of glucose starvation. Studies over the last 20 years have now implicated AMPK in the regulation of numerous cellular functions in almost every tissue. This is particularly true for skeletal muscle where AMPK has been implicated in the regulation of glucose, lipid and protein metabolism, gene expression, growth, etc. In this review we provide an update on the structure, localization, biochemical regulation and physiological

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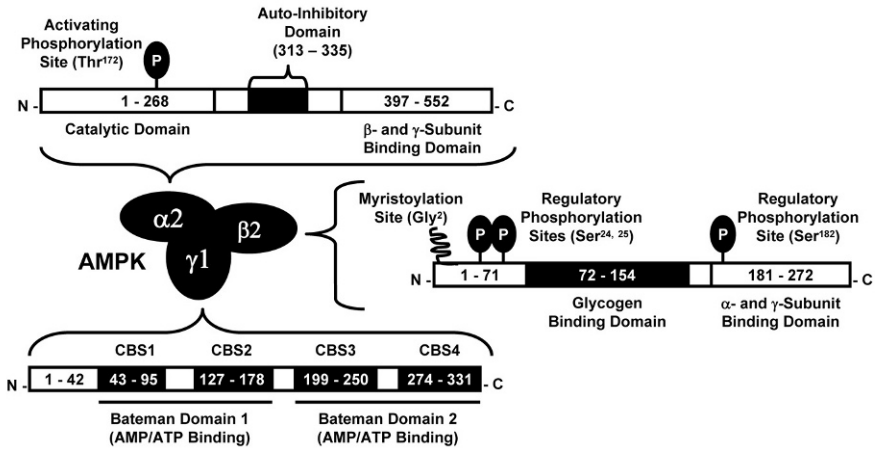

Figure 1. Structure of AMPK. The figure depicts the AMPK α2β2γ1 heterotrimer, one of the major AMPK heterotrimeric complexes expressed in skeletal muscle. The location of amino acids within the AMPKα2, β2, and γ1 subunits are from the human protein sequence.

functions that have been ascribed to the acute activation of AMPK in skeletal muscle.

Structure and subcellular localization of AMPK

The AMPK protein exists as a heterotrimer composed of a 63 kDa catalytic (α) subunit, and two non-catalytic regulatory subunits, β (30 kDa) and γ (38–63 kDa) [7, 8], in a ratio of 1α:1β:1γ (Fig. 1), and all three subunits are required for the formation of a stable and fully functional AMPK complex [9–11]. There are several isoforms for each of the three AMPK subunits, including α1, α2, β1, β2, γ1, γ2, and γ3 [11–15], and they are encoded by distinct genes. In human skeletal muscle, the α2β2γ3 complexes constitute the majority of AMPK heterotrimers [16], while in rat skeletal muscle the α2β2γ1 complexes are predominant [15]. The three subunits of AMPK each possess unique structural components that facilitate their differential roles in the regulation of AMPK activity and its physiological functions in mammalian cells. The AMPKα subunit possesses a highly conserved, N-terminal catalytic domain that contains the activating phosphorylation site (Thr^{172}), an autoinhibitory domain [17, 18], and a C-terminus that contains the domains required for binding with the β and γ subunits (Fig. 1) [18–21]. Interestingly, the two AMPKα variants, α1 and α2, have been shown to have a differential localization pattern in mammalian cells, with the AMPKα1 subunit being localized to the non-nuclear fraction and the AMPKα2 subunit localized to both the nucleus and non-nuclear fractions [22]. The AMPKβ subunit is a scaffold/docking subunit, and contains an N-terminal myristoylation site (i.e. Gly^2) that can target AMPK to membranes [23, 24], an internal glycogen-binding domain [25], and a C-terminal α- and γ-subunit binding domain that is sufficient to enable the formation of stable AMPK heterotrimers [9, 19, 21, 26] (Fig. 1). In addition, the β-subunit contains several regulatory phosphorylation sites that have been implicated in the nuclear localization of AMPK (i.e. Ser^{24}, Ser^{25}) [23] as well as the regulation of AMPK catalytic activity (i.e. Ser^{182}) [23]. Consistent with this finding, the AMPKβ subunits (β1 and β2) have been found in both nuclear and non-nuclear cell fractions [22]. The γ subunits possess a variable N-terminal region [21] followed by four highly conserved cystathionine-β-synthase sequence motifs [27, 28] (Fig. 1). In pairs, these cystathionine-β-synthase sequence motifs form two functional Bateman domains, Bateman domain 1 and Bateman domain 2 [21], structures that are capable of binding adenine nucleotides, such as AMP or ATP [27, 28] (Fig. 1). The γ1 subunit exhibits preferential nuclear localization over the other γ subunits [29].

The intracellular localization of the different AMPK subunits and their isoforms has suggested that some of these isoforms (i.e. α2, β2, and γ1) may be primarily involved in regulating gene expression while the remaining isoforms may be involved in regulating cytosolic and/or plasma membrane functions. Consistent with this theory, studies by McGee et al. demonstrated that in human skeletal muscle the AMPKα2 subunit translocated to the nucleus following 60 min of intense exercise [30], suggesting an increased role of AMPKα2 in regulating gene expression in skeletal muscle. In addition, recent studies by Kodiha et al. have shown that in HeLa cells, AMPKα1/2 and AMPKβ1/2 subunits translocated to the nucleus upon stimulation by agents that induce cellular stress (i.e. deoxyglucose and diethyl maleate) [31]. Thus, AMPK appears to be able to shift its intracellular localization to suit the needs of the cell.

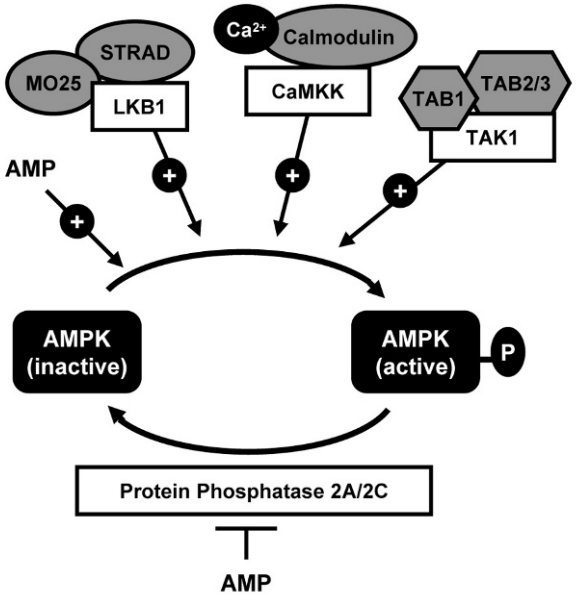

Figure 2. Biochemical Regulation of AMPK. The transition of the AMP-activated protein kinase (AMPK) from a non-phosphorylated, inactive state, to a phosphorylated active state can occur via several mechanisms. These include the following: 1) allosteric regulation by AMP; 2) phosphorylation by the serine/threonine protein kinase, LKB1, which exists in a heterotrimeric complex with its accessory proteins, mouse protein 25 (MO25) and Ste20-related adaptor protein (STRAD); 3) phosphorylation by the serine/threonine protein kinase, Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK); and 4) phosphorylation by the serine/threonine protein kinase, transforming growth factor-b-activated kinase 1 (TAK1), which exists in a complex with its accessory proteins, TAK-1 binding protein-1 (TAB1), and TAB2 or TAB3. The transition from an active state to a quiescent, inactive state is accomplished by dephosphorylation by protein phosphatase 2A or 2C.

Biochemical Regulation

The transition of AMPK from a quiescent state to a fully active state capable of phosphorylating substrates requires an increase in the phosphorylation of the AMPKα subunit on the activating threonine residue (i.e. Thr^{172}) found in the catalytic domain [32] (Fig. 2). Although this increase in AMPK phosphorylation was originally thought to occur solely via an AMP-dependent conformation change, studies have now shown that the regulation of AMPK is more complex and also involves phosphorylation by upstream kinases and decreased dephosphorylation by protein phosphatases. The following sections will describe the three main ways in which AMPK phosphorylation is regulated on a biochemical level.

Adenosine 5'-Monophosphate (5'-AMP). The AMPK protein was named "AMP-activated protein kinase" due to studies that demonstrated that its activity was sensitive to the presence of 5'-AMP [1, 2, 33]. As discussed in the previous section, the γ subunit of AMPK contains adenine nucleotide binding sites that facilitate the direct interaction of AMP with the AMPK heterotrimer (Fig. 2) [27, 28]. Thus, in mammalian cells an increase in AMP levels increases the binding of AMP to the γ subunit Bateman domains, induces a conformational change in the AMPK heterotrimer, and facilitates an increase in AMPK activity [28]. In addition, studies have shown that ATP antagonizes the binding of AMP to the γ subunit [28]. Thus, a decrease in the ratio of AMP:ATP would favor ATP binding to the Bateman domains and a decrease in AMPK activity [28, 34]. This allosteric regulation of AMPK by AMP and ATP nicely explains the exquisite sensitivity of AMPK to the small changes in AMP levels that are observed *in vivo*. Subsequent studies have also shown that AMP functions to regulate the activity of AMPK via an additional mechanism, the inhibition of AMPK dephosphorylation by protein phosphatases [35–37]. The regulation of AMPK phosphorylation by protein phosphatases will be the focus of one of the following sections.

AMPK kinases

In addition to allosteric regulation by AMP, AMPK is regulated by AMPK kinases, including the proteins LKB1, Ca^{2+}/calmodulin-dependent protein kinase kinase, and transforming growth factor-β-activated kinase 1. The following sections will provide a brief review of these proteins and their role in the regulation of AMPK.

LKB1. LKB1 is a serine/threonine protein kinase with considerable sequence and structural homology with the *Saccharomyces cerevisiae* Snf1 kinase kinases, Elm1, Pak1, and Tos3 [38, 39]. The human LKB1 gene encodes a single protein of 433 amino acids [40], comprised of an N-terminal nuclear localization domain (amino acids 38–43) [41, 42], a central catalytic domain (amino acids 49–309) [40], and a C-terminal prenylation site (Cys^{433}) that can target LKB1 to membranes [43]. In mammalian cells, LKB1 is associated with two proteins, Ste20-related adaptor protein (STRADα/β) [44], and mouse protein 25 (MO25α/β) [45], and together these proteins form a heterotrimeric complex [44, 45]. Skeletal muscle expresses LKB1, STRAD and MO25 [46], but currently there is no evidence regarding isoform specificity in this tissue.

Most studies suggest that LKB1 functions as a constitutively active kinase once bound to MO25 and STRAD. Work involving recombinant proteins for LKB1, MO25α/β and STRADα/β has shown that

LKB1 displays extremely low kinase activity in the absence of these accessory proteins [38], and that the association of LKB1 with STRAD increases LKB1 kinase activity [44, 45]. In agreement with these studies suggesting that LKB1 functions as a constitutively active kinase, to date no studies have demonstrated an increase in LKB1 activity from tissue or cell lysates in response to any stimulus [47], and the activity of none of the other LKB1 substrates present in muscle (i.e the AMPK-related kinases) increased in response to muscle contraction [47]. Although this lack of evidence does not completely rule out possible *in vivo* regulation of LKB1-MO25-STRAD association, it does suggest that, once associated, LKB1 is maximally activated.

Studies have shown that LKB1 functions as an AMPK kinase in cell-free systems [48] and in skeletal muscle [49] (Fig. 2). Using LKB1 isolated from rat liver extracts, studies have demonstrated that LKB1 phosphorylates and activates AMPK [48], and that this interaction occurs only in the presence of AMP [48]. Since LKB1 activity is not sensitive to allosteric regulation by AMP [36], this finding suggests that LKB1 can function as an AMPK kinase only when AMP enables the kinase access to the activating phosphorylation site of AMPK. However, recent studies using recombinant LKB1-MO25-STRAD complexes failed to demonstrate an AMP-sensitivity of LKB1 towards AMPK [50]. Thus, it appears that protein phosphatases were contaminating the LKB1 isolated from the rat liver extracts [50], and the AMP-sensitivity of LKB1-dependent phosphorylation was due to the ability of AMP, when bound to AMPK, to inhibit the dephosphorylation of AMPK by protein phosphatases [48]. In mouse skeletal muscle, loss of LKB1 expression resulted in a significant decease in the ability of the pharmacological AMP analogue, AICAR (5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside) to stimulate AMPK activity [49]. Thus, in skeletal muscle, LKB1 appears to be the primary AMPK kinase under conditions of high cellular energy stress.

Ca2+/calmodulin-dependent protein kinase kinase (CaMKK). The Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) is a Ca^{2+}/calmodulin-responsive kinase with considerable sequence and structural homology with the *Saccharomyces cerevisiae* Snf1 kinase kinases, Elm1, Pak1, and Tos3 [38, 51, 52], and the mammalian AMPK kinase, LKB1 [38, 39, 52]. There are two distinct genes for CaMKK, CaMKKα and CaMKKβ, and they share ~70% amino acid sequence homology with each other [53–58]. The rat CaMKKα and CaMKKβ genes encode a 505 and 587 amino acid amino protein, respectively [59] [60]. Both of these isoforms contain a central catalytic domain (amino acids 126–434 for CaMKKα; 162–470 for CaMKKβ) and a C-terminus that contains the overlapping Ca^{2+}/calmodulin binding and autoinhibitory domains (amino acids 435–505 for CaMKKα; 471–587 for CaMKKβ) [55, 58, 60]. The expression of CaMKKα protein in skeletal muscle is well accepted [61–63]. In contrast, the expression of CaMKKβ protein in skeletal muscle is controversial, with one group reporting detection of this isoform [62] and another reporting no detectable expression [55]. Interestingly, a recent report by McGee et al. demonstrated the emergence of detectable levels of CaMKKβ protein in skeletal muscle undergoing hypertrophic growth [63]. Thus, more experiments need to be done to determine the expression of CaMKKβ protein in stimulated and unstimulated skeletal muscle.

The activity of CaMKKα/β is dependent upon increases in intracellular Ca^{2+} levels. When intracellular Ca^{2+} levels rise, Ca^{2+} associates with calmodulin, and the Ca^{2+}/calmodulin complex binds to CaMKK allowing for a release of the autoinhibitory domain and an increase in CaMKKα/β activity. Following this activation by Ca^{2+}/calmodulin, biochemical studies have shown that the CaMKKα and CaMKKβ isoforms are differentially regulated, with the α isoform exhibiting no Ca^{2+}/calmodulin-independent activity and the β isoform exhibiting substantial Ca^{2+}/calmodulin-independent activity [60]. Thus, when intracellular Ca^{2+} levels return to basal concentrations, CaMKKα activity also returns to basal levels, but CaMKKβ activity remains elevated.

In cell-free studies using CaMKK isolated from pig brain and AMPK isolated from rat liver, CaMKKα/β increased AMPK activity [48] (Fig. 2), and this increase was enhanced by the addition of AMP, or the combination of AMP, Ca^{2+}, and calmodulin [48], suggesting that the CaMKK-dependent activation of AMPK is sensitive to changes in AMP levels. However, since neither CaMKKα nor CaMKKβ activity is sensitive to changes in AMP levels [48], these findings were likely just due to protein phosphatase contamination of the CaMKK and AMPK preparations [36, 64]. This finding has now been confirmed by studies using recombinant proteins that demonstrated that the ability of CaMKKβ to phosphorylate AMPK is not sensitive to changes in AMP [36]. Thus, current results suggest that CaMKKs regulate AMPK in a Ca^{2+}/calmodulin-dependent, AMP-independent manner.

Transforming growth factor-β-activated kinase 1 (TAK1). Transforming growth factor-β-activated kinase 1 (TAK1) is a serine/threonine protein kinase that has been identified as an AMPK kinase in yeast based

on its ability to confer the Snf+ growth phenotype in yeast lacking expression of Elm1, Pak1, and Tos3 [65]. The TAK1 gene encodes a 578 amino acid protein [66] that contains an N-terminal catalytic domain (amino acids 1–300) that binds the TAK1-binding protein 1 (TAB1) [67], and a C-terminal association domain (amino acids 479–553) that binds the TAK-1 binding proteins, TAK1-binding protein 2 (TAB2) and TAK1-binding protein 3 (TAB3) [68, 69]. *In vivo*, TAK1 associates with the TAK-1 binding protein 1 (TAB1) and either TAB2 or TAB3 [69], to form the heterotrimeric complexes TAK1-TAB1-TAB2 or TAK1-TAB1-TAB3 [68] (Fig. 2). TAK1 and TAB1 protein have been detected in multiple tissues, including skeletal muscle [63, 67]. There are currently no reports on the expression of TAB2 and TAB3 in skeletal muscle.

The biochemical regulation of TAK1 activity is not completely understood, although studies have shown that activation of TAK1 requires the binding of TAB1 [70–72]. TAB1 binds directly to the catalytic domain of TAK1 [67] and promotes TAK1 autophosphorylation at Thr^{184}, Thr^{187} and Ser^{192}, residues in the kinase activation loop that have been associated with increases in TAK1 kinase activity [70–72]. In addition, protein phosphatase 2C dephosphorylates TAK1 and impairs the TAB1-mediated autophosphorylation of TAK1 [73]. Thus, intracellular mechanisms that impair protein phosphatase activity should increase TAK1 activity *in vivo*. The role of TAB2 and TAB3 in the regulation of TAK1 kinase activity is still not clear, as TAB2 is not required for TAK1 activity [74]. Thus, more research still needs to be done to completely understand the mechanisms underlying the regulation of TAK1 activity in vivo.

In mammalian cells, TAK1 is activated by a number of cytokines, such as transforming growth factor-β and tumor necrosis factor-α (TNF-α) [75], and is also activated by chemical agents that directly alter or mimic changes in AMP:ATP levels [i.e. AICAR and N,N-dimethyl-imido-dicarbon-imidic diamide (metformin) [76]]. Consistent with the activation of TAK1 by stimuli that alter AMP:ATP, TAK1 has recently been implicated as an AMPK kinase (Fig. 2). In cell-free studies, TAK1 directly phosphorylates AMPK on the same activation loop threonine residue (i.e. Thr^{172}) as LKB1 and CaMKK [65]. Although no studies have directly investigated the role of TAK1 in the regulation of AMPK in skeletal muscle, studies performed in cardiac muscle have demonstrated that loss of TAK1 prevents the phosphorylation of AMPK (Thr^{172}) induced by treatment with metformin [76]. The role of TAK1 in the physiological regulation of AMPK in skeletal muscle is a rich area for future investigation, especially in the regulation of AMPK induced by growth factors and cytokines.

Protein Phosphatase 2A and 2C (PP2A and PP2C). The role of protein phosphatases in the regulation of AMPK phosphorylation and activity is an important component of AMPK regulation. In cell-free studies, the protein phosphatases 2A and 2C dephosphorylated AMPK (Thr^{172}) [35, 36], and this effect was regulated by AMP and palmitate levels [64] (Fig. 2). Importantly, the ability of AMP to regulate the dephosphorylation of AMPK is not due to inhibition of protein phosphatase activity, but rather is due to the ability of AMP to allosterically inhibit the access of protein phosphatases to the activating threonine residue in AMPK [35]. Also, interestingly, work by Suter et al. has demonstrated that the ability of protein phosphatase to dephosphorylate the AMPKα1 isoform, but not the AMPKα2 isoform, was insensitive to changes in AMP concentrations [36]. Thus, collectively these results suggest that AMP does not directly regulate protein phosphatase activity, but instead regulates the access of protein phosphatases to the activating threonine residue in the AMPKα subunit when it is bound to the Bateman domains in the γ-subunit.

Chemical activators and inhibitors of AMPK. Understanding of the physiological role of AMPK in skeletal muscle has been greatly enhanced by the use of several pharmacological activators and inhibitors of AMPK. These include the AMP mimetic, AICAR; the mitochondrial toxins, dinitrophenol and rotenone; the small molecule activator, A-769662; and the small molecular inhibitor, Compound C. In addition, two classes of drugs that are widely prescribed for the treatment of type 2 diabetes, the biguanides (e.g. metformin) and thiazolidinediones (e.g. rosiglitazone and pioglitazone), also function as AMPK activators in skeletal muscle. The following sections will provide a brief review of the mechanism of action of these compounds in the activation or inhibition of AMPK.

AMPK Activators

AICAR. To date, the greatest information about the role of AMPK in skeletal muscle has been gained by the use of the pharmacological compound, AICAR (5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside). AICAR is transported into cells by the adenosine transporter, and then metabolized by the enzyme adenosine kinase into 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranosyl monophosphate (ZMP), an AMP analogue [34, 77]. ZMP then functions like

endogenous AMP by binding to the Bateman domains of AMPK and promoting allosteric activation of the kinase [78]. Importantly, AICAR does not alter endogenous levels of AMP or ATP [79]. In addition, similar to endogenous AMP, ZMP would be likely to prevent the dephosphorylation of AMPK by inhibition of AMP-sensitive phosphatases, although there is currently no data in support of this postulation. Thus, the AICAR metabolite ZMP is an AMPK activator via its ability to serve as an AMP mimetic. In isolated skeletal muscle preparations, AICAR increases AMPK phosphorylation [80, 81], making it a useful chemical probe for understanding the role of acute AMPK activation in the regulation of glucose, protein and lipid metabolism. However, it is important to note that AICAR is not a selective activator of AMPK and can also activate other AMP-sensitive enzymes, including glycogen phosphorylase [82] and fructose-1,6-bisphosphatase [83]. Thus, studies using AICAR to assess the role of AMPK in physiological functions should be cautious when interpreting data due to the possible activation of other proteins.

Mitochondrial Toxins. Dinitrophenol and rotenone are cellular metabolic poisons that activate AMPK in numerous cell types, including skeletal muscle [81, 84]. Dinitrophenol is a membrane-permeable, benzene-based compound that acts as a proton ionophore in the mitochondrial membrane, and uncouples the proton gradient across the inner mitochondrial membrane. This destroys the proton motive force that cells use to generate ATP, thus resulting in a loss of cellular energy to heat production, and an overall decrease in intracellular ATP levels. Rotenone is also a membrane-permeable compound but, unlike dinitrophneol, it acts as a mitochondrial toxin by inhibiting the transfer of electrons from mitochondrial complex I to ubiquinone. This prevents nicotinamide adenine dinucleotide from being converted into ATP, thus causing a decrease in intracellular ATP levels. In isolated rodent skeletal muscles, Hayashi et al. and Fujii et al. observed a significant increase in AMPKα1 and AMPKα2 activities in response to treatment with dinitrophenol or rotenone [81, 84]. However, interestingly, only dinitrophenol significantly decreased intracellular ATP levels [84]. Thus, despite the suggested mechanism for rotenone activation of AMPK, there is still some controversy.

A-769662. To better understand how AMPK activation occurs and how it regulates skeletal muscle metabolism, researchers have pursued the identification and development of more specific chemical AMPK activators. Recently over 700 000 compounds were screened to select for molecules that can function as activators of AMPK in cell-free assays and in primary rat hepatocytes [85]. This screen yielded a non-nucleoside thienopyrididone, named A-769662, as a potent and reversible, small molecule activator of AMPK [85]. In cell free assays, A-769662 activated AMPK purified from rat liver with an EC_{50} of 0.7 μM [85], a concentration well below that required by AMP ($EC_{50} = 112$ μM) [85]. The mechanism by which A-769662 activates AMPK is presently unclear. Although studies have shown that A-769662 allosterically regulates AMPK activity [85, 86], and inhibits the activity of protein phosphatases [86], it does not elicit these effects by binding to the γ subunit Bateman domains [85, 86], altering intracellular AMP, ADP or ATP levels [85], or by binding to any known ligand binding site on AMPK [86]. Thus, A-769662 activates AMPK by binding to a currently uncharacterized site. Importantly, although the mechanism by which A-769662 activates AMPK is currently unknown, the fact that it directly binds to and regulates AMPK activity is very encouraging, and promises to be a useful tool for researchers attempting to fully elucidate the role of AMPK in the regulation of skeletal muscle metabolism.

Metformin. Metformin (N,N-dimethylimidodicarbonimidic diamide) is one of the most commonly prescribed drugs for the treatment of type 2 diabetes, and increases the activity of AMPK in skeletal muscle [87]. The biochemical mechanism by which metformin activates AMPK is currently not clear. Studies have shown that metformin is an inhibitor of mitochondrial complex I [88, 89], a key complex involved in the regulation of cellular respiration and the generation of ATP. Thus, metformin could activate AMPK via an increase in AMP:ATP levels. Interestingly, while studies have demonstrated that phenformin, another biguanide compound, increases intracellular AMP:ATP and ADP:ATP levels in mouse skeletal muscle [49], metformin does not [90, 91], suggesting that the metformin-induced inhibition of mitochondrial complex I may not be the mechanism underlying its activation of AMPK in skeletal muscle. It is possible that metformin activates AMPK via one of several other mechanisms, including direct binding and allosteric regulation, activation of AMPK kinases, increasing intracellular Ca^{2+} levels, and/or inhibition of protein phosphatases. However, studies have shown that metformin, or its derivative phenformin, does not alter LKB1 activity [47], does not increase intracellular Ca^{2+} levels [92], and does not inhibit protein phosphatase activity [90].

Interestingly, in cardiac muscle Xie et al. demonstrated that loss of TAK1 protein prevented the metformin-induced activation of AMPK, suggest-

ing that TAK1 is necessary for the actions of metformin on AMPK [76]. The role of TAK1 in regulating metformin-induced increases in AMPK in skeletal muscle has not yet been explored. In contrast, studies in the skeletal muscle from LKB1 knockout mice demonstrated an almost complete inhibition of the phenformin-induced activation of AMPKα2 [49] suggesting that LKB1 is necessary for the actions of phenformin in this tissue. Clearly, more work needs to be done to sort out the mechanism(s) by which metformin and phenformin activate AMPK in skeletal muscle.

Thiazolidinediones. Rosiglitazone (5-((4-(2-(methyl-2-pyridinylamino)ethoxy)phenyl)methyl)-2,4-thiazol-idinedione) and pioglitazone (5-((4-(2-(5-ethyl-2-pyridinyl)ethoxy)-phenyl)methyl)-(+)-2,4-thiazolidinedione) are two additional drugs that are used to treat individuals with type 2 diabetes. These compounds belong to a class of drugs known as thiazolidinediones, and have been shown to reduce blood glucose levels in rodents [93], and in humans [94]. Part of the mechanism by which thiazolidinediones ameliorate hyperglycemia is via the activation of AMPK in skeletal muscle. In isolated rodent skeletal muscle, thiazolidinediones dose-dependently and rapidly (~5 min) increased AMPK activity [91, 95]. In addition, chronic treatment of obese Zucker rats with rosiglitazone for six weeks normalized the decrease in basal AMPK activation that is commonly observed in the skeletal muscle from obese rats [96]. Thus, thiazolidinediones are acute activators of AMPK in skeletal muscle and can prevent obesity-induced impairments in AMPK activity. The mechanism by which thiazolidinediones increase AMPK in skeletal muscle is controversial. Two studies have reported an increase in the intracellular ratio of AMP:ATP following thiazolidinedione treatment [91, 95], while another study reported no change in the AMP:ATP ratio [96]. Thus, more work will need to be done to precisely determine the manner in which thiazolidinediones activate AMPK in skeletal muscle.

AMPK Inhibitor

Compound C. Compound C (6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl)]-3-pyridin-4-yl-pyrrazolo[1,5-a]-pyrimidine), is a cell-permeable pyrrazolopyrimidine compound that can act as a reversible and ATP-competitive inhibitor of AMPK [87, 97]. However, it does not inhibit AMPK activation in response to all stimuli. In H-2K^b skeletal muscle cells, Compound C inhibited the AICAR-induced activation of AMPK [97], but did not inhibit dinitrophenol-induced increases in AMPK activity [97], suggesting that Compound C blocked the conversion of AICAR into its AMP-like metabolite, ZMP. Further investigation showed that Compound C inhibits the adenosine transporter [97], the primary transporter for the uptake of AICAR into cells. Thus, Compound C can inhibit AICAR-induced activation of AMPK, but will likely not be effective in inhibiting AMPK activation by compounds that do not rely on the adenosine transport system.

In addition, Compound C is not a specific inhibitor of AMPK and has also been shown to significantly inhibit a number of other protein kinases, including extracellular-signal-regulated kinase 8 (ERK8), protein kinase C-related kinase 2 (PRK2), mitogen-activated protein kinase-integrating protein kinase 1 (MNK1), CaMKKα, phosphorylase kinase (PHK), Aurora B, brain-specific kinase 2 (BRSK2), casein kinase 1δ (CK1δ), dual-specificity tyrosine-phosphorylated and -regulated kinase 1A (DYRK1A), DYRK2, DYRK3, homeodomain-interacting protein kinase 2 (HIPK2), sarcoma kinase (Src), lymphocyte cell-specific protein-tyrosine kinase (LCK), and tumor-necrosis-factor-receptor-associated factor family member associated nuclear factor κB activators-binding kinase 1 (TBK1) [98]. Thus, care should be taken in attributing experimental data obtained with Compound C solely to AMPK.

Physiological stimulators

In addition to activation by pharmacological agents, AMPK activity is increased in mammalian cells/tissues by several endogenous stimuli including exercise/muscle contractile activity, cytokines/growth factors, nutrient deprivation, ischemia/hypoxia, and heat shock. While all of these factors can activate AMPK in mammalian cells, the role of some of these stimuli in the physiological regulation of AMPK in skeletal muscle is questionable, and will not be discussed in this review. Thus, in the following sections we will provide a summary of the current literature on the regulation of AMPK in response to exercise/muscle contraction in skeletal muscle.

Exercise/muscle contraction

Exercise/skeletal muscle contraction is a multifactorial process involving changes in cellular energy status (i.e. increased AMP:ATP levels), increases in intracellular Ca^{2+} levels, generation of reactive oxygen species, release of hormone/cytokines/growth factors, etc., and it is presently not clear which and/or how

many of these factors contribute to the activation of AMPK in contracting skeletal muscle. Thus, in the following paragraphs we will attempt to dissect out the different stimuli and provide evidence for their individual involvement in the regulation of skeletal muscle AMPK activity in response to contractile activity.

Cellular Energy Status – AMP. In skeletal muscle, contractile activity increases energy demand by more than 100-fold over resting muscle [99]. When the consumption of ATP exceeds the rate of ATP generation, part of the adenine nucleotide pool is deaminated to inosine monophosphate, which can then either be reaminated back to AMP or further degraded into the metabolites, hypoxanthine, xanthine and urate [99]. This increase in ATP breakdown is known to induce measurable increases in AMP, but no detectable changes in ATP levels, thus resulting in an overall increase in the intracellular ratio of AMP:ATP.

As discussed earlier, an increase in AMP levels can regulate AMPK via allosteric activation [27, 28], and inhibition of the ability of protein phosphatases to dephosphorylate AMPK [35, 36, 64], and the biochemical processes underlying these mechanisms are discussed in greater detail in an earlier section. Studies have suggested that LKB1 is the main AMPK kinase in skeletal muscle in response to changes in cellular energy status. In mouse skeletal muscle, ablation of LKB1 results in a significant decrease in AMPKα2 activity [49, 100], but a significant increase in AMP:ATP and ADP:ATP levels [49], in response to high intensity, tetanic contractions, suggesting that LKB1 is the predominant AMPKα2 kinase in muscle in response to cellular energy stress. Interestingly, some studies have reported no significant change in AMPKα1 activity in response to exercise/muscle contraction [49, 81], and have suggested that the AMPKα1 activity assayed from whole muscle homogenates is not derived from the myocytes but, instead, is coming from non-myocyte cell contamination. Consistent with this data, muscle-specific LKB1 knockout mice did not exhibit significant decreases in AMPKα1 activity in the basal state [49, 100]. However, other studies have reported increases in AMPKα1 activity in response to contractile activity [100, 101], and studies by Koh et al. did report decreases in the contraction-induced activation of AMPKα1 in muscle-specific LKB1 knockout mice [100]. Thus, the role of LKB1 as the primary upstream regulator of AMPKα1 in skeletal muscle is still controversial.

Intracellular Ca^{2+} Levels. Increases in intracellular Ca^{2+} levels are a fundamental part of the molecular signals underlying muscle contraction as direct Ca^{2+} binding to contractile proteins (i.e. troponin C) initiates the molecular processes by which muscle fibers generate force [reviewed in [102]]. Thus, increases in intracellular Ca^{2+} levels are a necessary component of muscle contraction and no studies investigating the regulation of AMPK by exercise/muscle contraction can completely discount putative inputs by Ca^{2+}.

The main mechanism by which increases in intracellular Ca^{2+} levels are currently thought to regulate AMPK is via the activation of CaMKKs. As discussed earlier, increases in intracellular Ca^{2+} and the interaction of Ca^{2+} with calmodulin stimulate CaMKKs, proteins that can phosphorylate and activate AMPK *in vitro* [48] and *in vivo* [61] (Fig. 2). However, despite this simple scheme for Ca^{2+} and CaMKK-dependent regulation of AMPK by contractile activity, the role of CaMKKs as physiological regulators of AMPK in skeletal muscle is controversial. In mouse skeletal muscle, expression of a constitutively active form of CaMKKα significantly increased AMPKα1 and AMPKα2 activities [61], suggesting that CaMKKs are regulators of both AMPK catalytic subunits in skeletal muscle. However, subsequent studies using the chemical Ca^{2+}/calmodulin competitive inhibitor, KN-93 (N-[2-[[[3-(4'-chlorophenyl)-2-propenyl]-methylamino]-methyl]phenyl]-N-(2-hydroxyethyl)-4'-methoxy-benzene-sulfon-amide phosphate salt), or the CaMKK inhibitor, STO-609 (7-oxo-7H-benzimidazo[2,1-a]-benz[de]-isoquinoline-3-carboxylic acid, acetate), have produced conflicting results regarding the physiological regulation of AMPK by CaMKKs. In one study, treatment of mouse skeletal muscles with STO-609 did not inhibit contraction-stimulated AMPK (Thr^{172}) phosphorylation [61], while in another study treatment of mouse muscles with KN-93 or STO-609 resulted in a significant impairment in contraction-induced AMPKα1 and AMPKα2 activity [62]. In addition, studies that treated mouse skeletal muscle with the sarcoplasmic reticulum Ca^{2+} store-releasing agent, caffeine, demonstrated a significant inhibition of caffeine-induced AMPKα1, but not AMPKα2 activity when treated with STO-609 [103], suggesting that CaMKK regulates AMPKα1 but not AMPKα2 activity in skeletal muscle. Thus, future studies involving the use of transgenic or knockout models will need to be performed in order to fully understand the role of CaMKK as physiological regulators of AMPK in response to contractile activity in skeletal muscle.

Reactive oxygen species. Reactive oxygen species are small, unstable forms of oxygen that are highly reactive with other molecules due to the presence of unpaired valence shell electrons. They are continually

produced in the mitochondria as a metabolic by-product of aerobic respiration, and their rate of production increases in skeletal muscle during exercise/muscle contraction [reviewed in [104]]. Recent evidence has suggested that reactive oxygen species may play a key role in regulating AMPK activity in skeletal muscle in response to contractile activity [105, 106]. In rat epitrochlearis muscle, incubation with the oxidant, hydrogen peroxide, dose-dependently increased AMPKα1 activity [106], and this effect was prevented by treatment with the antioxidant, N-acetyl-L-cysteine [106]. In mouse extensor digitorum longus muscle, contraction-induced increases in AMPK activity were inhibited (~50 %) by N-acetyl-L-cysteine treatment [105], providing more evidence linking reactive oxygen species to contraction-induced increases in AMPK activity.

The mechanism underlying the increase in AMPKα1 activity in skeletal muscle in response to oxidant stress is currently unknown, although studies have shown that treatment of rat epitrochlearis muscles with hydrogen peroxide does not increase AMP:ATP levels [106]. Thus, more research needs to be done in order to sort out the individual contribution of reactive oxygen species to AMPK activation in contracting skeletal muscle.

Hormones/Cytokines/Growth Factors. In addition to the effects of changes in intracellular levels of AMP:ATP, Ca^{2+} or reactive oxygen species on AMPK activation in skeletal muscle, exercise/muscle contractions have also been shown to induce increases in the levels of circulating hormones, cytokines and growth factors that may also contribute to the regulation AMPK in response to contractile activity. The most widely studied of these factors towards the regulation of AMPK in skeletal muscle is the cytokine, interleukin-6.

Interleukin-6 is a pleiotropic cytokine that has been associated with both pro-inflammatory and anti-inflammatory effects [107]. Interleukin-6 levels increase in the blood in response to muscle contractile activity [108, 109], and this increase in circulating interleukin-6 has been linked with the activation of AMPK in skeletal muscle [110]. In isolated rat skeletal muscle, interleukin-6 increased AMPK (Thr^{172}) phosphorylation [110]. In addition, basal and muscle contraction-induced increases in AMPK (Thr^{172}) phosphorylation were blunted in gastrocnemius muscles from interleukin-6 knockout mice [110]. Collectively, these data strongly suggest that interleukin-6 plays a role in regulating AMPK activation in response to contractile activity.

The mechanism by which interleukin-6 increases AMPK phosphorylation in skeletal muscle is presently unknown, although recent work suggests that it may act via changes in intracellular Ca^{2+} levels. In C2C12 mouse skeletal muscle cells, treatment with interleukin-6 increased intracellular Ca^{2+} levels [111], suggesting that interleukin-6 may activate AMPK via a Ca^{2+}/calmodulin and CaMKK-dependent mechanism. However, to date no studies have investigated this hypothesis. Thus, further studies will need to be done in order to more clearly define the relationship between elevations in circulating interleukin-6, muscle contractile activity, and the activation of AMPK in skeletal muscle.

Summary

While cell-free and isolated skeletal muscle incubation experiments have highlighted the individual contribution of AMP, changes in intracellular Ca^{2+} levels, reactive oxygen species and interleukin-6 levels toward the activation of AMPK, it is presently not possible to tease apart the relative contribution of all of these independent factors towards the activation of AMPK in response to exercise/muscle contraction *in vivo*. For example, changes in intracellular Ca^{2+} levels are a fundamental component of muscle contraction due to the requirement of Ca^{2+} for muscle cross-bridge formation. In skeletal muscle, increases in intracellular Ca^{2+} levels initiate the stimulation of several intracellular Ca^{2+} pumps (i.e. the plasma membrane and sarco-endoplasmic reticulum Ca^{2+}-ATPases) that utilize ATP to maintain Ca^{2+} homeostasis. Thus, any stimulus that increases intracellular Ca^{2+} levels has the potential to also decrease ATP levels and subsequently cause an increase in the intracellular ratio of AMP:ATP. Experimentally, this has been observed in isolated mouse skeletal muscle where treatment with caffeine increased intracellular Ca^{2+} levels [112], increased AMPKα1 activity and also increased AMP:ATP levels [103]. Thus, during exercise/muscle contraction it is likely that a complex and highly coordinated orchestration of events, including increases in intracellular Ca^{2+} levels, increases in the ratio of AMP:ATP, the generation of reactive oxygen species, and elevations in circulating factors, may all contribute to the regulation of AMPK activity in skeletal muscle.

Physiological regulation of cellular metabolism in skeletal muscle. AMPK has been termed the master regulator of cellular metabolism due to its varied roles in glucose, protein and lipid metabolism. In the following sections, we will summarize the current literature on the role of AMPK in the acute regulation of these metabolic processes in skeletal muscle,

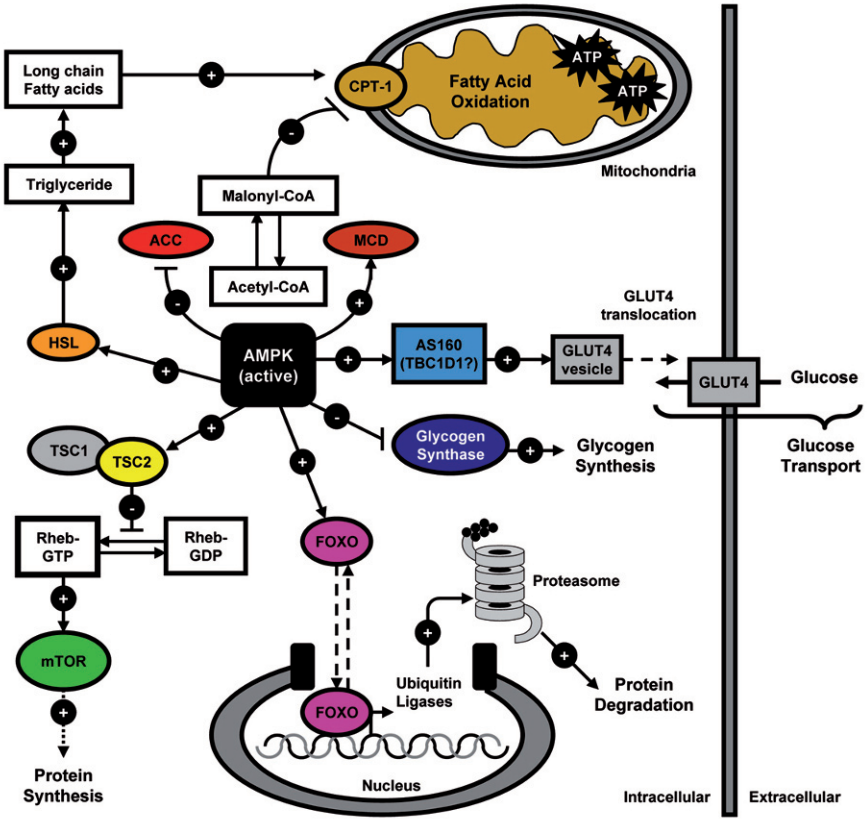

Figure 3. AMPK and the Regulation of Skeletal Muscle Metabolism. Proposed model of the interaction of AMPK in the regulation of glucose, lipid and protein metabolism in skeletal muscle. [Legend: ACC = acetyl-CoA carboxylase; AMPK = AMP-activated protein kinase; AS160 = Akt substrate of 160 kDa; CPT-1 = carnitine palmitoyl transferase-1; FOXO = forkhead transcription factor; GLUT4 = glucose transporter 4; HSL = hormone sensitive lipase; MCD = malonyl-CoA decarboxylase; mTOR = mammalian target of rapamycin; TSC1, TSC2 = tuberous sclerosis complex 1 or 2.]

focusing on the role of AMPK in glucose transport, glycogen synthesis, protein synthesis and degradation, fatty acid oxidation and lipolysis.

Glucose metabolism. Glucose metabolism comprises the processes involved in the transport of glucose from the blood into cells, and the breakdown and resynthesis of simple sugars, oligosaccharides and polysaccharides. The activation of AMPK in skeletal muscle has been linked with both an increase in glucose transport and an increase in the synthesis of glycogen. Thus, the following sections will focus on the experimental evidence that is currently available implicating AMPK in these processes (Fig. 3).

Glucose transport. Glucose transport is the process by which glucose is taken up into skeletal muscle from the blood. The glucose transporter isoform 4 (GLUT4) is the main glucose transporter isoform expressed in skeletal muscle, and the translocation of GLUT4 from intracellular locations to the plasma membrane and transverse-tubules is the primary mechanism through which stimuli increase glucose transport into skeletal muscle [113, 114]. Insulin and exercise/muscle contraction are the two most physiologically relevant stimulators of glucose transport into muscle, but importantly the proximal signaling proteins that regulate GLUT4 translocation in response to insulin and exercise are distinct. Insulin mediates muscle glucose transport via a signaling pathway that is fairly well-characterized, and involves the binding of insulin to the insulin receptor, tyrosine phosphorylation of insulin receptor substrate-1/2 (IRS-1/2), activation of phosphatidylinositol 3-kinase (PI3-kinase), phosphorylation of Akt, and phosphorylation of the Akt substrate of 160 kDa (AS160) [reviewed in [115]]. In contrast, the signaling pathway(s) by which muscle contraction stimulates GLUT4 translocation and glucose transport are still relatively unknown, although studies have shown that neither ablation of the insulin receptor in skeletal muscle [116], nor inhibition of PI3-kinase, had any effect on contraction-induced glucose transport [117, 118]. These results clearly demonstrate that the early signals mediating contraction-induced glucose transport are separate from those mediated by insulin.

The most widely studied protein in the regulation of contraction-stimulated skeletal muscle glucose transport is arguably AMPK. Incubation of rodent skeletal muscle with AICAR or dinitrophenol resulted in a significant increase in AMPKα1/α2 activity and muscle glucose transport [81, 119, 120], demonstrating

that activation of AMPK is positively correlated with increases in muscle glucose transport. In addition, inhibition of AMPKα2, the predominant AMPK catalytic isoform in skeletal muscle, resulted in a complete inhibition of AICAR-induced increases in glucose transport [81, 119]. Collectively these results suggest that in skeletal muscle, activation of AMPK with agents that elicit, or mimic, changes in AMP:ATP levels are responsible for increases in muscle glucose transport.

A direct role of AMPK in the regulation of exercise/contraction-induced muscle glucose transport has produced conflicting data regarding the role of AMPK in the regulation of muscle glucose transport. Using several different transgenic mouse models of skeletal muscle AMPK inhibition (e.g. AMPKα2 knockout, AMPKα2 inactive, and AMPKγ3 knockout mice), numerous studies have shown that inhibition of AMPK activity does not impair contraction-induced muscle glucose transport [81, 119, 121]. Although these studies demonstrated that AMPK is not essential for contraction-stimulated glucose uptake, AMPK may still function in this process. This idea stems from the central hypothesis that exercise/muscle contraction activates multiple, redundant signaling pathways, all of which are individually capable of stimulating GLUT4 translocation and glucose uptake. Thus, while selective activation of any one of these signaling pathways is capable of increasing muscle glucose transport, inhibition of only one signaling pathway is not sufficient to significantly impair contraction-induced glucose transport. To observe a significant impairment, two or more contraction-stimulated signaling pathways would need to be simultaneously inhibited. Thus, in the transgenic mouse models of AMPK inhibition, it is possible that other isoforms of AMPK (e.g. AMPKα1), or other signaling proteins [e.g. Ca^{2+}/calmodulin-dependent protein kinases and/or protein kinase C isoforms] have altered their expression or activity levels to compensate for the loss of AMPKα2 activity. Elucidation of these signaling proteins is a high priority for researchers interested in discerning the molecular mechanisms for contraction-induced glucose transport in skeletal muscle.

Recent work has led to the identification of two possible downstream effectors of AMPK in the regulation of skeletal muscle glucose transport, the AS160 and its homolog TBC1D1. AS160 is a Rab-GTPase activating protein that regulates the translocation of GLUT4 from intracellular vesicles to the plasma membrane by maintaining small G-proteins, known as Rabs, in a GDP-bound state [122]. The phosphorylation of AS160 at specific target sites is thought to disrupt GTP-ase activity, thus allowing for the release of AS160/TBC1D1 inhibition on GLUT4 vesicle intracellular localization, and the initiation of vesicle exocytosis [122]. In rodent skeletal muscle, stimulation of AMPK with AICAR or exercise/muscle contraction, increased AS160 phosphorylation [123, 124], suggesting that AMPK may regulate GLUT4 exocytosis via the phosphorylation and regulation of AS160. Further investigation has shown that mutation of four critical phosphorylation sites on AS160 can impair contraction-induced glucose transport in mouse skeletal muscle [125]. Thus, AS160 has emerged as a key component of the signaling cascade by which AMPK regulates glucose transport in skeletal muscle. TBC1D1 is also a Rab-GTPase activating protein and recent work has shown that it is also regulated by AMPK in skeletal muscle. In L6 rat skeletal muscle myotubes, and rodent skeletal muscle, TBC1D1 phosphorylation is enhanced following treatment with AICAR [126, 127], suggesting that TBC1D1 may also mediate AMPK-induced increases in skeletal muscle glucose transport. Investigating the role of TBC1D1 in the regulation of GLUT4 translocation and glucose uptake in skeletal muscle in response to AMPK activation is currently an important research area for diabetes investigators.

Glycogen synthesis. Once glucose has been transported into the skeletal muscle cell, it can either be metabolized or stored as glycogen, the major carbohydrate storage form in mammalian cells. Glycogen is a highly branched polysaccharide of glucose, and is synthesized from UDP-glucose by the enzyme, glycogen synthase. Since the conversion of UDP-glucose to glycogen is currently thought to be the rate-limiting step in glycogen synthesis [128], the regulation of glycogen synthase is a key component of glycogen metabolism.

The regulation of glycogen synthase activity is complex and involves allosteric regulation by glucose-6-phosphate, and phosphorylation and dephosphorylation by upstream kinases and phosphatases [129]. Over the last 20 years, researchers have provided evidence demonstrating that AMPK is one of those upstream kinases. In cell-free studies, AMPK has been shown to directly phosphorylate glycogen synthase on Ser^7 [130], one of several phosphorylation sites known to inhibit glycogen synthase activity [129]. Consistent with this finding suggesting a role of AMPK in the deactivation of glycogen synthesis, treatment of isolated mouse skeletal muscles with AICAR resulted in a significant decrease in glycogen synthase activity [131], and this effect was blocked in the skeletal muscle of AMPKα2 knockout mice [131]. Thus, acute activation of AMPK is associated with decreases in

glycogen synthesis via decreased activation of glycogen synthase.

Studies of mutations in the AMPKγ3 subunit have suggested that chronic alterations in AMPK activity also have significant effects on skeletal muscle glycogen metabolism [121, 132, 133]. In transgenic mice that overexpress the AMPKγ3 subunit, or express a mutated form of the AMPKγ3 subunit (i.e. R225Q AMPKγ3), there was an ~2-fold increase in skeletal muscle glycogen levels [121, 133], and this was associated with significant increases in glycogen synthase activity [133]. Interestingly, there was a dissociation between glycogen synthase activity and AMPK activity in these two transgenic mouse models as the AMPKγ3 R225Q mutant mice exhibited decreased AMPKα2 activity and the AMPKγ3 overexpressing mice exhibited no change in AMPK phosphorylation [121], or either AMPKα1 or AMPKα2 activity [133]. Collectively, these data demonstrate that the increases in skeletal muscle glycogen content in the AMPKγ3 transgenic mice may not solely be due to decreases in AMPK activity. Consistent with these findings demonstrating a dissociation between changes in AMPK activity and skeletal muscle glycogen levels, transgenic mice that express a dominant negative form of the AMPKα2 subunit have ~50% less skeletal muscle glycogen content [134]. Interestingly, these mice do not exhibit changes in glycogen synthase or glycogen phosphorylase activity [134], again demonstrating that the chronic effects of AMPK on muscle glycogen metabolism are not solely due to phosphorylation by AMPK.

Protein metabolism

Protein metabolism comprises the biochemical processes responsible for the synthesis of amino acids and proteins, and the degradation of proteins. In skeletal muscle, the activation of AMPK has been implicated in both the inhibition of protein synthesis, and the stimulation of protein degradation. The following sections will summarize the current literature that implicates AMPK in the regulation of these processes.

Protein synthesis. Protein synthesis is the process in which messenger RNA is translated into protein via the direction of ribosomes and ribosomal enzymes. In skeletal muscle, the primary stimuli responsible for the regulation of protein synthesis are growth factors and hormones (e.g. insulin; insulin-like growth factor), and these factors activate protein synthesis via a fairly well-characterized cascade mediated via the PI3-kinase/Akt signaling pathway. Insulin and insulin-like growth factor bind sarcolemmal receptors, which stimulate tyrosine phosphorylation of the receptor and IRS-1/2, activation of PI3-kinase, and activation of Akt [reviewed in [115]]. Stimulation of Akt then promotes protein synthesis via phosphorylation of the tuberous sclerosis complex 2 (TSC2), a protein that together with its partner tuberous sclerosis complex 1 (TSC1) act as GTP-ase activating proteins for the small G-protein, Rheb [135]. Phosphorylation of TSC2 likely inhibits Rheb GTP-ase activity, allowing Rheb-GTP to accumulate in the cytosol, although direct experimental evidence for this is lacking. Increases in Rheb-GTP stimulate mTOR kinase activity [136], thus resulting in an increase in protein synthesis.

In addition to the upstream control by growth factors and hormones, mTOR-dependent signaling is also regulated by changes in cellular energy status (Fig. 3). A recent study demonstrated that treatment of C2C12 myotubes with AICAR decreased total cellular protein content and increased the degradation of myofibrillar proteins [137], suggesting that AMPK activation inhibits protein synthesis in skeletal muscle. Consistent with these findings, treatment of isolated rat skeletal muscle with AICAR activates AMPK and causes an inactivation of mTOR and mTOR-dependent signaling proteins [138, 139]. This impairment in mTOR signaling by AMPK is likely due to direct phosphorylation of TSC2 Thr^{1227} and Ser^{1345} residues by AMPK [140]. Thus, activation of AMPK signaling with AICAR is associated with the negative regulation of protein synthesis in skeletal muscle [138]. Surprisingly, recent work has shown that skeletal muscle hypertrophy, induced by synergist ablation, increases the phosphorylation of mTOR [141], but also activates AMPK [142]. Since acute AMPK activation promotes overall muscle protein breakdown, this interesting finding suggests a complex and intriguing role for AMPK in the regulation of protein synthesis in skeletal muscle.

Protein degradation. Proteolysis, or protein degradation, is the process responsible for the directed breakdown of proteins within a cell. Protein degradation is regulated by three major proteolytic systems: the Ca^{2+}-dependent cysteine protease, calpain system [reviewed in [143]]; the lysosomal system [reviewed in [144]]; and the ATP-dependent ubiquitin-proteasome system [reviewed in [145]]. In skeletal muscle, the ubiquitin-proteasome system is the major proteolytic pathway responsible for the regulation of protein degradation under a variety of catabolic conditions, including fasting, diabetes, denervation (i.e. loss of nerve activity), etc. [137, 146]. In the ubiquitin proteasome system, proteins are first marked for

degradation by the covalent attachment of chains of ubiquitin, a 76 amino acid polypeptide that is recognized by the proteasome [147]. The ubiquitination of proteins is regulated by at least three major enzymes: the E1 ubiquitin-activating proteins, the E2 ubiquitin-carrier proteins, and the E3 ubiquitin protein ligases [146]; and all of these enzymes play distinct and critical roles in the ubiquitin labeling of proteins. Following ubiquitination, proteins are targeted to the proteasome complex where they are digested into small peptides by the proteasome [148, 149], and then released back into the cytosol for subsequent hydrolysis into amino acids.

The ubiquitin proteasome system for protein degradation is an ATP consuming process [149], with the E1 ubiquitin activating proteins and the proteasome itself requiring ATP for their enzymatic functions [150, 151]. Thus, the entire process of protein degradation can create a shift in cellular energy status leading to an increase in AMP:ATP levels. Not surprisingly, this has led researchers to speculate that AMPK may play a role in the regulation of protein degradation. In C2C12 myotubes, AICAR significantly increased the rate of protein degradation and increased the expression of FOXO1 and FOXO3a [137], transcription factors that regulate the expression of the E3 ubiquitin ligases, atrogin-1 and muscle-specific RING-finger protein-1 (MuRF1), in skeletal muscle [152, 153]. In agreement with these findings, other work has shown that the AICAR and metformin-induced increase in the expression of the ubiquitin ligases, atrogin-1 and MuFR1 expression was blocked by treatment with the AMPK inhibitor, Compound C [154]. Thus, these data provide more evidence in support of a direct role for AMPK in the regulation of protein degradation via the ubiquitin-proteasome system.

Lipid metabolism

The complete oxidation of long chain fatty acids requires the breakdown of intramyocellular triacylglycerols, the transport of long chain fatty acids into the mitochondria matrix for β-oxidation, and the entry of acetyl-CoA groups into the citric acid cycle for oxidation. The data describing the role of AMPK in the regulation of fatty acid oxidation and skeletal muscle lipolysis will be described below.

Fatty acid oxidation. In skeletal muscle, long chain fatty acids are metabolized in the mitochondria, by a process known as β-oxidation, to produce the ATP needed to sustain cellular life. Three key enzymes that regulate the uptake of fatty acids into the mitochondria are acetyl-CoA carboxylase (ACC), malonyl-CoA decarboxylase, and carnitine palmitoyl transferase-1 (CPT-1). Acetyl-CoA carboxylase catalyzes the carboxylation of acetyl-CoA to produce the metabolite, malonyl-CoA. In skeletal muscle, the production of malonyl-CoA inhibits CPT-1, the enzyme responsible for the transfer of fatty acyl groups from acyl-CoA to cartinine. Thus, the overall effects of activating ACC are an increase in the production of malonyl-CoA, a decrease in CPT-1 activity, and a reduction in the uptake and oxidation of fatty acids in the mitochondria (Fig. 3).

Research over the last 20 years has demonstrated that AMPK regulates fatty acid oxidation in skeletal muscle via its phosphorylation and inactivation of ACC. In cell-free studies, AMPK directly phosphorylates and inhibits ACC [155]. In rodent skeletal muscle, stimulation of AMPK with AICAR, increased AMPK activity and increased the oxidation of palmitate [120, 156, 157], providing strong correlative evidence linking AMPK activation with increases in fatty acid oxidation in skeletal muscle. Interestingly, in humans, a direct link between increased AMPK activity, malonyl-CoA levels and skeletal muscle fatty acid oxidation during exercise is not as conclusive. Wojtaszewski et al. reported a disassociation between AMPK activity and ACC (Ser^{221}) phosphorylation in humans during a low intensity (45 % $VO_{2\ max}$), but exhaustive, cycling exercise bout [158]. In addition, Roepstorff et al. observed similar changes in ACC and malonyl-CoA levels in the skeletal muscle of humans, despite vastly different AMPK activities [159]. Thus, in human skeletal muscle, the connection between AMPK activation, malonyl-CoA levels, and skeletal muscle fatty acid oxidation is more complex.

Recent studies using transgenic mice null for the AMPK kinase, LKB1, now clearly indicate that AMPK cannot be the sole regulator of ACC in skeletal muscle [49, 100, 160]. In skeletal muscles from LKB1 knockout mice, muscle contraction did not significantly increase AMPK (Thr^{172}) phosphorylation, but did still increase ACC (Ser^{221}) phosphorylation [49, 100, 160], although this effect was severely blunted compared to wild-type mice [49, 100, 160]. In addition, Smith et al. have shown that stimulation of isolated rat soleus muscle strips with AICAR plus high intensity, tetanic contractions resulted in greater increases in fatty acid oxidation compared to high intensity, tetanic contraction alone [161]. Taken together, these data suggest that AMPK is not the sole regulator of skeletal muscle fatty acid oxidation during exercise/muscle contraction in rodents or in humans. Elucidation of the other signaling proteins that may be responsible for regulating fatty acid oxidation in skeletal muscle is currently a high priority area of investigation for many researchers.

Skeletal muscle lipolysis. Triacylglycerols in skeletal muscle represent a key intracellular energy source as the breakdown of intramuscular triglycerides into free fatty acids can be used to fuel β-oxidation and the generation of ATP. Intramuscular triglyceride breakdown is regulated by the activity of the enzyme, hormone sensitive lipase (HSL), which hydrolyzes the conversion of triglycerides into free fatty acids and glycerols. Since the conversion of triglyceride to free fatty acids is currently thought to represent the rate-limiting step in the regulation of skeletal muscle lipolysis [162], the factors that regulate HSL activity are critical to the regulation of lipid breakdown in skeletal muscle.

Studies have suggested that AMPK may be a direct upstream regulator of HSL activity in skeletal muscle. In cell-free studies, Garton et al. demonstrated that AMPK directly phosphorylates HSL on Ser^{565} (Site 2) [163, 164], one of five known phosphorylation sites that can either directly regulate the activity, or alter the sensitivity of HSL to activation by other protein kinases. However, directly connecting AMPK activation with intramuscular triglyceride breakdown in skeletal muscle has proven difficult and, to date, no study has directly linked AMPK activation with HSL activity and intramuscular triglyceride breakdown. In isolated rat soleus muscle, tetanic contractions increased AMPK activity, and increased the Ser^{565} phosphorylation and activity of HSL [165], suggesting that AMPK may regulate HSL activity in response to muscle contraction. However, further investigation showed that treatment of muscles with the protein kinase C inhibitor, calphostin C, completely inhibited the contraction-induced increases in HSL activity without affecting HSL (Ser^{565}) phosphorylation [165]. In addition, Roepstorff et al. demonstrated that in human skeletal muscle, bicycling exercise (65 % VO_2 max) increased AMPKα2 activity and HSL (Ser^{565}) phosphorylation, with no concomitant increase in HSL activity or intramuscular triglyceride hydrolysis [164]. Thus, collectively these data suggest a potential role for AMPK in mediating intramuscular triclyceride breakdown in skeletal muscle via phosphorylation of HSL; however, more mechanistic studies using AMPK transgenic mice need to be done in order to more directly assess the physiological connection between AMPK, HSL activity, and skeletal muscle lipolysis.

Concluding remarks

Over the last 20 years, AMPK has emerged as a key mediator of glucose, protein and lipid metabolism in skeletal muscle. As a critical sensor of intracellular energy status, the activation of AMPK has been linked with physiological processes responsible for the activation of ATP generating pathways (e.g. glucose transport) and the deactivation of ATP consuming pathways (e.g. glycogen synthesis), an overall role that is key for maintaining cellular life. Although this research has resulted in a large increase in our overall understanding of numerous aspects of AMPK, including its structure, localization, biochemical and physiological regulation, and numerous physiological effects, there is still much more to be learned.

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