AMPK: A Key Sensor of Fuel and Energy Status in Skeletal Muscle

Contraction induces marked metabolic changes in muscle, and the AMP-activated protein kinase (AMPK) is a good candidate to explain these effects. Recent work using a muscle-specific knockout of the upstream kinase, LKB1, has confirmed that the LKB1→AMPK cascade is the signaling pathway responsible for many of these effects.

It has been known for many years that exercise and contraction induces marked changes in the metabolism of skeletal muscle, including increases in glycogen breakdown, glycolysis, glucose uptake, and fatty acid oxidation, together with many changes in gene expression. The mechanism by which contraction triggers glycogen breakdown was established by Krebs and Fischer 50 years ago, when they reported that glycogen phosphorylase was activated by a Ca^{2+}-activated protein kinase [now termed phosphorylase kinase (70)]. However, the signaling pathways responsible for most of the other metabolic changes induced by exercise or contraction, including the increased glucose uptake and fatty acid oxidation, remained enigmatic. The first clue that the AMP-activated protein kinase (AMPK) might play an important role came from findings in Winder’s laboratory (116) that malonyl-CoA levels decreased during exercise in rat muscle. Since malonyl-CoA was known to be an inhibitor of fatty acid uptake into mitochondria [via inhibition of carnitine:palmitoyl-CoA acyl transferase (CPT1) on the outer surface of the inner mitochondrial membrane, a mechanism established by the late Dennis McGarry (78) and shown in FIGURE 1], this potentially explained the increase in fatty acid oxidation induced by exercise. However, what was the cause of the decrease in malonyl-CoA? This metabolite is produced by the enzyme acetyl-CoA carboxylase, which occurs as two isoforms, i.e., ACC1 and ACC2, thought to produce malonyl-CoA for fatty acid synthesis and for regulation of fatty acid oxidation, respectively, with ACC2 being the form expressed in muscle (2). ACC1 was already known to be phosphorylated and inactivated by AMPK in response to ATP depletion in liver cells (25, 27), suggesting that ACC2 might also be inactivated by AMPK during muscle contraction. Activation of AMPK in skeletal muscle, with simultaneous inactivation of ACC2 and decreased malonyl-CoA, was indeed demonstrated in response to both exercise in vivo (117) and contraction induced by electrical stimulation of the sciatic nerve in situ (55, 114).

A method to demonstrate a more direct, causal relationship came with the development of 5-aminoimidazole-4-carboxamide riboside (AICAR) as a pharmacological agent to activate AMPK in intact cells and tissues (23). Perfusion of rat hindlimb muscle with AICAR caused AMPK activation, and, as expected, this correlated with inactivation of acetyl-CoA carboxylase, decreased malonyl-CoA, and increased fatty acid oxidation (79) (FIGURE 1). In the same study, glucose uptake was also measured and found to increase (79), an effect that was subsequently shown to be due to increased transport across the plasma membrane (47) and to involve translocation of GLUT4 to the plasma membrane (72). These results demonstrated that activation of AMPK in muscle was sufficient for increased glucose uptake, but not that it was necessary for the increased glucose uptake observed during contraction. Before discussing the recent evidence that strongly supports that idea, we need to introduce the AMPK system itself in more detail.

AMPK: Early Studies

AMPK has been recently reviewed elsewhere (35, 38, 40, 65), and in this article we focus on studies particularly relevant to skeletal muscle. The kinase was first defined (20, 37) as a protein kinase that phosphorylated and inactivated both 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase and ACC1 (regulatory enzymes of cholesterol and fatty acid synthesis, respectively), although it was subsequently shown to inactivate ACC2 as well (119). As its name suggests, AMPK is allosterically activated by 5'-AMP (20), an effect antagonized by high concentrations of ATP (23). Because of the reaction catalyzed by adenylylate kinase (2ADP ↔ ATP + AMP), the AMP:ATP ratio varies approximately as the square of the ADP:AMP ratio (38), making the former ratio (essentially the parameter to which AMPK responds) a sensitive indicator of reduced cellular energy status. Consequently, any cellular or metabolic stress that either inhibits ATP synthesis [e.g., heat shock (22), hypoxia (77), ischemia (71), or glucose deprivation (102)] or that accelerates ATP consumption [e.g., contraction of skeletal muscle (55, 114, 117)] causes AMPK activation. It had been known for many years that muscle glycogen phosphorylase and phosphofructokinase (the key enzymes regulating glycogen breakdown and glycolysis, respectively) can also be activated allosterically by a rise in the AMP:ATP ratio.
(21, 91). The idea that this ratio was a key indicator of cellular energy status was therefore not new. However, the concept that a single protein kinase like AMPK could sense the same parameters and transmit the information to potentially hundreds of downstream targets was an important new development.

**AMPK: Subunit Structure and Regulation by Upstream Kinases**

AMPK is now known to exist as heterotrimeric complexes containing a catalytic α-subunit and regulatory β- and γ-subunits. Each subunit is encoded by multiple genes (α1, α2, β1, β2, γ1, γ2 and γ3), and some of the mRNAs are also subject to alternative splicing, giving rise to a large variety of possible heterotrimERIC combinations. The complex that predominates in most cell types appears to be the αβγ1γ3 combination, but skeletal muscle is unique in that it may be the only tissue that expresses all subunit isoforms, including γ3 (76). Different isoform combinations are stimulated to various extents by AMP and also appear to differ in their subcellular localization (101).

Although AMPK received its name because of the allosteric activation by AMP, this effect is rather small (5-fold or less) and insignificant compared with the allosteric activation by AMP, this effect is rather small. However, the biguanide drug metformin, and thiazolidinedione drugs like rosiglitazone (two of the currently most widely prescribed drugs for type 2 diabetes) have both been found to activate AMPK (33, 131). Whereas thiazolidinediones have at least one other target [the peroxisome proliferator-activated receptor-γ (PPAR-γ)], it seems likely that AMPK activation explains most, if not all, of the therapeutic actions of metformin. Metformin, the related biguanide phenformin, and the thiazolidinediones are all inhibitors of complex I of the mitochondrial respiratory chain (17, 28, 89). It seems likely that these drugs activate AMPK indirectly by increasing the cellular AMP:ATP ratio, which has indeed been demonstrated for rosiglitazone (33) and phenformin (45), although not for metformin (33, 44).

**Functions of the γ-Subunits and Mutations Causing Human Disorders**

AMPK is a highly conserved protein kinase, and genes encoding the α-, β-, and γ-subunits can be found in all eukaryotic genomes, including primitive protozoa, fungi, and plants, as well as mammals (40). The α-subunits are unstable on their own and the β- and γ-subunits are antagonized by high concentrations of ATP. These effects are entirely substrate mediated, i.e., they are due to binding of the nucleotides to the substrate (AMPK) and not to the upstream kinase or the protein phosphatase. Working together, the three effects of AMP mean that the system is activated by increases in the cellular AMP:ATP ratio in a highly sensitive manner (39).

Because a rise in AMP triggers phosphorylation and activation of AMPK, any metabolic poison that inhibits mitochondrial ATP synthesis (e.g., Refs. 22, 44, 120) will activate AMPK by increasing the cellular AMP:ATP ratio. Intriguingly, the biguanide drug metformin, and thiazolidinedione drugs like rosiglitazone (two of the currently most widely prescribed drugs for type 2 diabetes) have both been found to activate AMPK (33, 131). Whereas thiazolidinediones have at least one other target [the peroxisome proliferator-activated receptor-γ (PPAR-γ)], it seems likely that AMPK activation explains most, if not all, of the therapeutic actions of metformin. Metformin, the related biguanide phenformin, and the thiazolidinediones are all inhibitors of complex I of the mitochondrial respiratory chain (17, 28, 89). It seems likely that these drugs activate AMPK indirectly by increasing the cellular AMP:ATP ratio, which has indeed been demonstrated for rosiglitazone (33) and phenformin (45), although not for metformin (33, 44).

**FIGURE 1. Activation of muscle fatty acid oxidation by AMPK**

AMP-activated protein kinase (AMPK), whether activated by exercise or by S-aminomimidazole-4-carboxamide riboside (AICAR), phosphorylates and inactivates the ACC2 isoform of acetyl-CoA carboxylase (119) [which is associated with the mitochondrial membrane (1)], thus lowering malonyl-CoA. This relieves the inhibition of CPT1 (78), allowing fatty acids to enter mitochondria as carnitine esters (FA-carnitine). They are converted by carnitine-palmityl-CoA acyl transferase 2 (CPT2) back to fatty acyl-CoA (FA-CoA) esters in the mitochondrial matrix, where they are oxidized to generate ATP.
Our current hypothesis is that this high AMPK activity leads to a higher basal glucose uptake into the myocytes, with excessive glycogen storage being a consequence of this.

γ2 mutation that caused a much more severe form of heart disease (18). This mutation (R331Q) occurs at the same residue that is mutated in one of the inherited disorders (R331G). However, this mutation is not inherited because the subjects all died within days of birth and, in one case where both parental DNAs were available, they were normal. At autopsy the hearts of these infants were found to be grossly enlarged, with a very high glycogen content that was clearly disrupting the normal organization of the myofibrils, supporting the idea that the primary effect of γ2 mutations is to cause a glycogen-storage disorder. Interestingly, a mutation in the pig γ3 gene causes an amino acid replacement (R390Q) that is exactly equivalent to one of the mutations (R390Q) in human γ2 causing WPW syndrome (80). The γ3 gene appears to be expressed exclusively in skeletal muscle, and the R390Q mutation gives rise to elevated glycogen storage in that tissue. Unlike the harmful effects of elevated glycogen in cardiac muscle, in skeletal muscle it appears to be well tolerated and even increases resistance to fatigue (12).

What is the effect of these mutations on the binding of AMP to AMPK and its regulation by the nucleotide? Consistent with the idea that the Bateman domains are the regulatory AMP-binding sites, all of the mutations affect both binding of AMP to the isolated Bateman domains and activation of the αβγ-complexes by AMP (24, 104). Moreover, the order of severity of the mutations is the same for both effects (18, 104). This provides strong evidence that the Bateman domains are responsible for the activating effects of AMP. It is also noticeable that the mutation that has the greatest effect on AMP and ATP binding (R331Q) also causes the most severe clinical defect (18). Four of the mutations (R331Q, H383R, R331G/R331Q) affect basic residues, and modeling of the Bateman domains suggests that their positively charged side chains bind the negatively charged phosphate group of AMP (104). The fact that mutations in both Bateman domains can affect activation by AMP suggests that both sites need to be occupied before AMPK is activated, and since binding to the two sites is highly cooperative, this would be another mechanism to increase the sensitivity of the AMPK system. The Bateman domains also bind ATP (albeit at lower affinity than AMP), with binding of AMP and ATP being mutually exclusive. This is consistent with the previous findings that high concentrations of ATP inhibit all three activating effects of AMP on the AMPK system (23, 26, 43).

One puzzling feature of the γ-subunit mutations is that, although lack of activation by AMP represents a loss of function, the mutations are dominant in their effect. However, the mutations also reduce binding of the inhibitor, ATP, as well as the activator, AMP, so the overall effect will depend on the relative concentrations of the two nucleotides in vivo (104). In fact, coexpression of the R331G and R331Q mutants with α1 and γ1 in human embryonic kidney 293 cells shows that, although the mutant complexes are no longer activated by cellular stresses, their basal level of phosphorylation and activity is higher than that of the wild type, possibly because inhibition of phosphorylation by basal levels of ATP is relieved (18). Our current hypothesis is that this high AMPK activity leads to a higher basal glucose uptake into the myocytes, with excessive glycogen storage being a consequence of this.

The γ-subunits of AMPK are not the only muscle proteins containing Bateman domains. The nine members of the human CIC chloride channel family
all contain a single Bateman domain at the COOH terminus, which is cytoplasmic. We have recently shown that the Bateman domain of CIC2 (an isoform expressed in neurons and other cells) binds ATP and that a mutation in CIC2 associated with idiopathic generalized epilepsy, as well as a different mutation equivalent to one found in the muscle isoform, CIC1, drastically reduce this binding (104). The mutation in CIC1 causes congenital myotonia, a form of muscle stiffness characterized by a delayed relaxation after contraction. Very recently, Bennetts et al. (13) have shown that ATP binding reduces the open probability of CIC1 at resting membrane potentials. Since CIC1 is found at the plasmalemma and in T tubules, opening of its anion channel would stabilize the resting membrane potential and repolarize the plasmalemma following excitation by acetylcholine. Bennetts et al. (13) propose that the opening of the CIC1 channel would represent a mechanism to limit muscle contraction as ATP levels fall and may therefore be a factor in muscle fatigue. This feedback mechanism would be defective in the subjects with congenital myotonia. Although this effect is not mediated by AMPK, it involves an “energy-sensing” domain very similar to those found on the γ-subunits of AMPK.

**Functions of the β-Subunits and Regulation of AMPK by Glycogen**

As well as a COOH-terminal domain that is required for binding to the α- and γ-subunits (53, 60), all β-subunits contain a conserved central domain that has been shown to bind glycogen (53, 93). Although this glycogen-binding domain (GBD) is conserved across all eukaryotic species and certainly causes interactions between AMPK and glycogen particles in intact cells, its physiological function remains unclear. Glycogen synthase appears to be a physiological target for AMPK in skeletal muscle (see below), and one possibility is that the GBD colocalizes AMPK with this key substrate. A second intriguing possibility, not necessarily mutually exclusive with the first, is that the AMPK system can act as a sensor not just of the short-term energy status of the cell in the form of ATP but also of the medium-term reserves of energy in the form of glycogen. There is some evidence in favor of this idea, in that a high level of muscle glycogen represses the activation of AMPK by AICAR in perfused rat muscle (122) and by exercise in human muscle (121).

**Identification of Upstream Kinases**

A major breakthrough in the study of the regulation of AMPK in the past couple of years has been the first definitive identification of upstream kinases that phosphorylate Thr\(^{172}\) on the α-subunit and thus activate the kinase. Initial advances came when various genome-wide screening approaches identified three kinases, i.e., Elm1, Pak1 and Tros3, that act upstream of the yeast ortholog of AMPK, the SNF1 complex (52, 85, 109). The nearest relatives of these yeast kinases in mammals were LKB1 and the β-isofrom of calmodulin-dependent protein kinase (CaMKK-β). Subsequent work has shown that LKB1 and CaMKK-β (and perhaps also CaMKK-α) can indeed be physiological activators of AMPK under different circumstances in vivo.

Intriguingly, LKB1 was originally identified as the gene mutated in the rare autosomal dominant human genetic disorder, Peutz-Jeghers syndrome (PJS) (48, 59). PJS subjects develop numerous benign tumors (classed as hamartomas) in the gastrointestinal tract and have a 20-fold-increased risk of developing malignant tumors at other sites (34), whereas mutations in the LKB1 gene are also seen in some sporadic cancers, especially adenocarcinoma of the lung (103). LKB1 is thus a classical tumor suppressor. The functional form of LKB1 is a complex with two accessory subunits, Ste20-related adapter protein (STRAD) (11) and Mouse protein-25 (MO25) (15), both of which exist as two isoforms (α and β) encoded by distinct genes. One of us (D. G. Hardie) had partially purified an upstream kinase for AMPK from rat liver (43), and following the new findings in the yeast system our laboratories were able to show that this was a complex between LKB1, STRAD-α, and MO25-α (42). LKB1 also exists as a complex with STRAD-β and MO25-α in skeletal muscle (K. Sakamoto, J. Boudeau, and D. Alessi, unpublished observation). Experiments with cultured cells in which the expression of LKB1 was manipulated in various ways proved that it was both necessary and sufficient for the activation of AMPK by AICAR and phenformin (42, 107, 124). Experiments revealing the important role of LKB1 in muscle will be discussed in a later section.

Does activation of AMPK explain the tumor-suppressor effects of LKB1? This seems possible, because activation of AMPK has been reported to inhibit the target of rapamycin (TOR) pathway, thus potentially inhibiting cell growth and hypertrophy (see below) while it also blocks the G1→S phase transition in the cell cycle (61). However, LKB1 acts upstream of a family of at least 12 AMPK-related kinases (58, 73), and some of these could also be involved in the tumor-suppressor effects of LKB1. Another interesting question is whether PJS patients display any metabolic disorder. To date, about 150 mutations in LKB1 have been identified in PJS patients and sporadic cancers (4, 16). The majority of these result in substantial truncations of the catalytic domain and would be expected to impair LKB1 catalytic activity. It would be interesting to find out if the activity of AMPK in tissues, including muscle and liver, is reduced in PJS patients, and if so whether this would affect their metabolic responses during exercise.
Recently, three groups simultaneously reported evidence that the CaMKKs can act upstream of AMPK, at least in some cell types (45, 54, 123). In cells lacking expression of LKB1, phosphorylation of AMPK at Thr172 and consequent activation did not occur in response to AICAR or phenformin but still occurred in response to Ca2+ ionophores. Experiments using pharmacological inhibitors and short interfering RNAs directed against the CaMKKs suggest that the latter, and especially CaMKK-β (45, 123), are responsible for activation of AMPK via phosphorylation of Thr172 under these circumstances. The failure of AICAR or phenformin to activate AMPK in cells lacking LKB1, but expressing CaMKKs, shows that LKB1 is essential for activation of AMPK mediated by a rise in the AMP:ATP ratio. It also suggests that a rise in AMP is not sufficient to stimulate phosphorylation of AMPK by the CaMKKs, which are activated instead by elevated Ca2+ concentrations.

What is the physiological relevance of these observations? The CaMKKs have a rather restricted tissue distribution and are mainly expressed in cells of neural origin (5). In rat brain slices, depolarization of neurons induced by increased medium K+ concentration (known to cause an influx of Ca2+ by voltage-gated channels) caused activation of AMPK that was blocked by a CaMKK inhibitor and was not associated with changes in the cellular AMP:ATP ratio. By contrast, activation of AMPK by phenformin in the same system was accompanied by increases in AMP:ATP and was unaffected by the CaMKK inhibitor (45). The CaMKKs are not expressed significantly in skeletal muscle, and experiments with muscle-specific knockouts of LKB1 (see below) suggest that the CaMKK–AMPK pathway cannot be a major player in mediating the effects of muscle contraction, despite the fact that increases in cytosolic Ca2+ obviously occur. In cells where the CaMKKs are significantly expressed, such as neurons, activation of AMPK may be a mechanism for anticipating the increased demand for ATP that always accompanies rises in cytosolic Ca2+, due to the fact that the Ca2+ must be pumped out of the cytoplasm to restore basal levels.

**Activation of AMPK in Muscle**

Since the original observations that AMPK was activated in muscle during exercise (117) and electrically stimulated contraction (55, 114), it has generally been assumed that the activation is caused by an increase in the cellular AMP:ATP ratio caused by increased ATP consumption. Increases in this ratio have indeed been observed during in situ electrical stimulation of mouse muscle (99), although not after exercise in rat muscle in vivo (117). However, any changes in the latter case may have been lost during the time it took to remove the muscle for analysis. Unfortunately, levels of muscle AMP are too low to be measured noninvasively by 31P nuclear magnetic resonance.

Even if an increase in AMP:ATP ratio is the primary signal for AMPK activation during exercise, this does not rule out the possibility of additional mechanisms. The cytokine interleukin-6 (IL-6) is released from muscle during exercise (29), and it has recently been reported that it activates AMPK in isolated rat muscles (66). Moreover, both basal and exercise-stimulated AMPK activity were reduced in the gastrocnemius muscle of IL-6 knockout mice, although the degree of stimulation of AMPK by exercise was similar (66). These results suggest that IL-6 may amplify the effects of exercise on AMPK via an autocrine mechanism.

Other important recent results have shown that AMPK is activated in muscle by the adipokines leptin (82) and adiponectin (112, 128) and that this may be responsible for the increase in fatty acid oxidation, and hence (at least in part) the increase in energy expenditure, induced by these cytokines. Coupled with findings that leptin regulates AMPK in the hypothalamus and that hypothalamic AMPK regulates food intake (6, 81), these results show that AMPK is a key player in regulation of energy balance not only at the cellular level, but also at the whole body level.

**Downstream Targets of AMPK**

This topic has been discussed in several recent reviews (35, 40, 65), and in this article we will focus on a small number of recently identified targets that are particularly relevant to skeletal muscle. Metabolic changes induced by AMPK in muscle (summarized in **Figure 2**) are both acute changes due to direct phosphorylation of metabolic enzymes and chronic changes due to effects on gene expression. In general, the metabolic changes induced by AMPK are similar to those produced by endurance exercise (e.g., distance running), such as increased uptake and oxidation of plasma glucose and fatty acids and increased expression of the glucose transporter GLUT4 and hexokinase (HKII) (50). By contrast, the changes that occur in response to resistance exercise (e.g., weightlifting), such as increased glycogen breakdown and glycolysis, are caused by regulation of phosphorylase and phosphofructokinase by established mechanisms that do not require AMPK.

An important question, which has not been completely answered, is whether AMPK is preferentially activated by endurance rather than resistance exercise. Most of the protocols that have been used to study regulation of AMPK in humans have involved endurance exercise, and, perhaps surprisingly, no direct comparisons of endurance vs. resistance exercise have been performed. However, studies of rat muscle using electrical stimulation favor the idea that activation of AMPK occurs primarily in response to endurance exercise. Firstly, AMPK activation by continuous low-frequency stimulation of rat gastroc-
mius/plantar is rather slow, occurring in minutes rather than seconds (55). Secondly, a recent study of ex vivo stimulation of rat extensor digitorum longus and soleus muscles using protocols designed to simulate resistance or endurance exercise showed that AMPK was not activated by the former (9). The “resistance exercise” protocol involved short bursts of high-frequency stimulation followed by rest periods, and it may be that any tendency for AMPK to become activated during the bursts of stimulation was reversed during the rest periods as ATP levels recovered. It would be of interest to confirm these findings in human muscle using different exercise protocols.

Taking everything together, AMPK appears not to be required for anaerobic metabolism of endogenous glycogen but is required instead for the switch to aerobic oxidation of blood-borne fuels. There are obvious analogies between this and the role of the yeast ortholog of AMPK, the SNF1 complex. Genetic analysis shows that the SNF1 complex is required for the switch from anaerobic (fermentative) metabolism of glucose to the oxidative metabolism of glucose and other fuels (36).

One consequence of resistance exercise training that is not seen in response to endurance exercise is hypertrophy, i.e., increased muscle bulk due to increases in protein synthesis and muscle fiber volume. Relevant to this, a recently identified target for AMPK is the TOR protein kinase pathway, which is activated by insulin, growth factors, and amino acids and stimulates protein synthesis, and hence cell growth and hypertrophy. Using the phosphorylation of ribosomal protein S6 kinase as a marker for TOR activation, AMPK has been found to inhibit its activation in several cell types including skeletal muscle (14, 68, 69), probably via phosphorylation of TSC2 (tuberin) (57). The latter forms a complex with TSC1 (hamartin) and contains a GTPase activator protein (GAP) domain that promotes conversion of the small G protein Rheb to its GDP-bound form, which no longer activates TOR (110) (FIGURE 3). Ribosomal protein S6 kinase is activated by an in vivo electrical stimulation protocol designed to simulate resistance exercise and produce hypertrophy in rat muscle (10), while studies using the TOR inhibitor rapamycin (95) and mice in which S6 kinase is knocked out (87) suggest that activation of TOR and S6 kinase are both required for muscle hypertrophy. The ability of AMPK to inhibit TOR thus provides a potential explanation for the lack of muscle hypertrophy induced by endurance exercise training. Muscles of mice with
knockouts of the α1 (63, 64) or α2 (115) subunits of AMPK or of LKB1 (99) do not display obvious hypertrophy, although their responses to resistance exercise or growth factor treatment have not been studied. It is also interesting that loss-of-function mutations in LKB1 (which acts upstream of AMPK) and of TSC1 or TSC2 (which act downstream of AMPK) both lead to a high incidence of benign tumors classed as hamartomas in humans; in the case of TSC1 and TSC2 the condition is known as tuberous sclerosis complex (90). Hamartomas are benign tumors that grow abnormally but retain their differentiated status, indicating that their defect is in regulation of cell growth.

A single bout of endurance exercise is capable of increasing the insulin sensitivity of muscle, as first demonstrated in 1982 (94). This effect of exercise can be mimicked by activation of AMPK using AICA riboside both in vivo (56) and in vitro (30). Although the mechanism for this effect is not known, one intriguing possibility is that it involves the ability of AMPK to inhibit the TOR pathway. Recent studies in which the activity of the TOR→ ribosomal protein S6 kinase I pathway was ablated by various approaches suggest that that pathway downregulates the insulin signaling pathway via phosphorylation of the insulin receptor substrate-1 (IRS1). This phosphorylation downregulates IRS1 function by reducing its synthesis, by increasing its degradation, and/or by reducing its ability to activate phosphatidylinositol-3-kinase (41, 106, 113). AMPK activation has the potential to reverse this feedback regulation on insulin signaling via its ability to inhibit the TOR pathway.

One other consequence of endurance exercise training is an increase in oxidative capacity via increased mitochondrial biogenesis, a phenomenon first observed by Holloszy in 1967 (49). It is important to know how this occurs, because subjects at risk of developing type 2 diabetes appear to have a relative deficit in oxidative capacity (74, 83, 92). The first evidence that the mechanism might involve AMPK came from studies in which the AMPK-activating drug AICAR was found to cause upregulation of mitochondrial enzymes in rat muscle (118). A transcriptional coactivator that is a key regulator of mitochondrial biogenesis is PPAR-γ coactivator-1α (PGC-1α) (126). Intriguingly, mRNA encoding PGC-1α is upregulated in rat epitrochlearis muscle by low-intensity swimming exercise in vivo or by AICAR treatment in vitro (111). These results suggested that AMPK may be the sensor involved in the mechanism that detects a deficit in oxidative capacity and switches on mitochondrial biogenesis, a hypothesis that is discussed further below. The mechanism by which AMPK upregulates PGC-1α expression remains unknown.

The mechanism by which AMPK activation upregulates GLUT4 also remains unclear, although it has recently been reported that for myocyte enhancer factor-2 (MEF-2) and GLUT4 enhancer factor (GEF) [two transcription factors required for GLUT4 expression (88)], both the nuclear content and the binding to DNA were increased in response to AICAR treatment of rats in vivo (51). In the same study it was reported that AMPK phosphorylates GEF in cell-free assays, although the site was not identified and there is no evidence that this occurs in vivo.

As well the ability of AMPK to stimulate muscle glucose uptake as discussed earlier, it also appears to stimulate fatty acid uptake. Thus incubation of rat cardiomyocytes with AICAR, or the ATP synthase inhibitor oligomycin, activated AMPK and increased fatty acid uptake due to translocation of the FAT/CD36 transporter to the membrane (75). AMPK therefore appears to stimulate both uptake and oxidation of fatty acids, at least in cardiac muscle.

Although the muscle isoform of glycogen synthase (GS) was shown to be phosphorylated by AMPK in cell-free assays over 15 years ago (19), evidence that it is a physiological target has only emerged recently. AMPK phosphorylates GS at site 2 [Ser1 (19)], which causes inactivation of the enzyme but also primes phosphorylation at the neighboring site 2a (Ser10) by casein kinase-1, which causes further inactivation (31, 130). Effects of phosphorylation at these sites on activity are overridden by high concentrations of glucose-6-phosphate. One of us (D. G. Hardie) recently made phosphospecific antibodies that recognize GS phosphorylated at site 2, or at sites 2 + 2a, and these have now been used to show that AICAR treatment of mouse muscle leads to phosphorylation of site 2 and, to a lesser extent, sites 2 + 2a, with a concomitant inactivation of the enzyme. Moreover, these effects are lost in muscles from mice (described further in the next section) in which the α2 isoform of AMPK has been knocked out (62). These results strongly suggest that
GS is a physiological target for AMPK in skeletal muscle. At first sight they appear to run counter to other findings that muscle GS is activated rather than inactivated during exercise, an effect that requires the function of the glycogen-bound form of protein phosphatase-1 (8). However, the level of glycogen appears to have a dominant effect on the phosphorylation state of GS, and activation of GS during exercise could be due to glycogen depletion and might mask any underlying effect of AMPK. This interpretation is supported by studies of patients with McArdle’s syndrome, a hereditary defect in glycogen phosphorylase. Since these subjects cannot break down muscle glycogen, the effect of glycogen depletion on GS activity is lost, and in these subjects exercise causes a marked activation of AMPK and inactivation of GS, as opposed to the activation of GS seen in control subjects (86).

**Physiological Role of AMPK in Muscle: Studies with Transgenic Mice**

The most convincing evidence that AMPK is required for the metabolic changes that occur in response to endurance exercise comes from gene-targeting studies in mice. Global knockouts of both catalytic subunit genes of AMPK (α1 and α2) have been constructed (63, 115). In the α2 knockout mice, but not the α1 knockout mice, the effects of AICAR on glucose uptake, and on expression of mRNAs encoding PGC-1α and HKII in skeletal muscle, were completely abolished, although surprisingly the effects of contraction or exercise on these processes were not (63, 64). At first sight this suggests that these effects of contraction are not mediated by AMPK, but a caveat with this approach is that there is clearly some redundancy between the α1 and α2 isoforms of AMPK, and double knockouts have not yet been studied. An additional complication is that in muscle from α2 knockout mice the expression of α1 is upregulated twofold in skeletal muscle (63, 115), which may provide some compensation for the lack of α2.

A possible route around this problem of redundancy came with the development of mice in which muscle AMPK was downregulated by expression of an inactive, dominant negative mutant of the α2-subunit from a strong promoter [muscle creatine kinase (MCK)] (84). Although mRNA encoding the α2 mutant was overexpressed 50-fold in skeletal muscle, the expression of total α-subunit protein was unchanged and the endogenous, active α-subunits appeared to have been entirely replaced by the inactive mutant. The explanation for this behavior may be that the α-subunits are unstable in the absence of β and γ, so that the level of expression of α-subunits is limited by the availability of β- and γ-subunits. Interestingly, the effects of AICAR and hypoxia on glucose uptake were completely eliminated in these mice, but the effects of contraction were only partially abolished, suggesting that AMPK contributes to the latter but is not the whole story (84). However, an important caveat with this dominant negative approach concerns whether the expression of the endogenous protein is completely eliminated. The Western blots in the original paper indicate that the loss of the endogenous α-subunits was substantial, but α1 and α2 were not assayed independently and this did not rule out the possibility that a small residual activity remained. Indeed, in another paper using the same mice to study cardiac muscle (where the MCK promoter is also expressed), α1 activity remained unchanged (97). In a second mouse line in which the inactive α2 subunit was expressed from a cardiac muscle-specific (myosin heavy chain) promoter, a careful study of AMPK activity showed that α2 activity was reduced but not completely abolished, whereas α1 activity was unaffected (127).

Presumably because of their metabolic defects, the mice expressing the dominant negative AMPK in skeletal and cardiac muscle took much less voluntary exercise (84). For this reason, it was not possible to perform studies of the long-term effects of exercise training in these mice. As a surrogate for this, the mice were fed β-guanidinopropionic acid (GPA), a creatine analog that depletes muscle ATP by interfering with its replenishment from phosphocreatine. Feeding GPA mimics many of the effects of endurance exercise in skeletal muscle, including induction of PGC-1α and mitochondrial biogenesis, but these effects were abolished in the dominant negative mice (132). Thus, at least when using GPA as a surrogate for endurance exercise, AMPK appears to be essential for the upregulation of mitochondrial biogenesis.

A third approach that our laboratories recently introduced (99) is a muscle-specific knockout of the upstream kinase LKB1 using Cre-loxP technology. An advantage of this approach is that there is only one LKB1 gene, eliminating problems of redundancy when knocking out individual AMPK α-subunits. A global LKB1 knockout is an embryonic lethal mutation (129), but Ashworth’s laboratory created a line of mice with loxP sites in the LKB1 gene. In these “floxed” mice the expression and activity of LKB1 was already reduced in skeletal muscle by ~90%, whereas the activities of the α1- and α2 isoforms of AMPK were reduced by ~60% (99). This fortuitous effect of the loxP sites on LKB1 expression turned out to be advantageous, because the deletion of genes in skeletal muscle using the Cre-loxP system is not always complete. Myocytes are formed by fusion of many precursor cells, and if the recombination induced by Cre recombinase has failed in some of the precursors, residual expression of the target gene may remain. By starting with a “floxed” strain where the expression of LKB1 was already decreased by 10-fold, and crossing with a deleter strain that expressed Cre recombinase from a MCK promoter, this resulted in a total loss of LKB1 activity...
and expression in skeletal muscle (99). The activity of the α2 isoform of AMPK and its stimulation by contraction, AICAR or phenformin was also abolished. Surprisingly, the low level of α1 activity in the “floxed” mice was not reduced further on crossing with the deleter strain, indicating either that this residual α1 was derived from nonmuscle cells (e.g., fibroblasts, endothelial cells) or that there is an alternate upstream kinase for α1. At least in cardiac muscle, the latter explanation seems to be correct, because there is a significant activity of the α1 isoform even in isolated cardiac myocytes prepared from the “floxed” mice expressing Cre recombinase, which are completely deficient in LKB1 (100). These results show that LKB1 is absolutely required for the activity of the α2 isoform in skeletal muscle and for full activation of α1, but the possibility of an alternate upstream kinase for the α1 isoform in skeletal muscle requires further investigation.

Significantly, the effects of AICAR and contraction on glucose uptake were normal in muscle from the “floxed” mice (in which AMPK activity was reduced by 60%) but were greatly reduced in muscles from “floxed” mice crossed with the deleter strain (in which α2 activity was completely abolished). This shows that the LKB1 → AMPK cascade is the primary signaling pathway causing the increased glucose uptake in response to contraction. However, in neither the mice expressing the dominant negative AMPK mutant (84) nor the LKB1 muscle knockouts (99) were the effects of contraction on glucose uptake completely eliminated, which suggests that another signaling pathway also functions in this process. Hollosy’s laboratory have provided evidence that in rat muscle a Ca²⁺-mediated pathway, possibly involving calmodulin-dependent protein kinase II (CaMKII), is also involved (125). CaMKII has also been found to be activated by cycle ergometer exercise in human muscle (96).

In muscle from the mice in which LKB1 expression was completely eliminated, phosphorylation of acetyl-CoA carboxylase in response to AICAR or contraction was also almost abolished, confirming that AMPK is the kinase mainly responsible for phosphorylation of this target, and the consequent stimulation of fatty acid oxidation during exercise. Another interesting finding with muscle from these mice was that the increases in the ADP:ATP and AMP:ATP ratios induced by contraction were much larger than those seen with muscle from the wild-type controls. This provides direct genetic evidence for the idea, first suggested many years ago (22), that AMPK protects cells against stresses that deplete ATP.

One caveat with the studies of the LKB1 knockout mice is that LKB1 also acts upstream of several other AMPK-related protein kinases (73), one or more of which could also be required for the effects of contraction on glucose uptake. However, this seems rather unlikely, because although several of the AMPK-related kinases are expressed in skeletal muscle, none of them, other than AMPK, are activated by contraction (98). Nevertheless, we recently found that the activity of several of them, including QSK and MARK4, were profoundly reduced in mice with a muscle-specific knockout of LKB1, both in skeletal (3) and heart (K. Sakamoto, J. Boudeau, and D. Alessi, unpublished observation) muscle. Thus we cannot completely rule out the possibility that these kinases are involved in some of the metabolic effects of the LKB1 signaling pathway.

Conclusions and Perspectives

The studies with knockout mice described in the previous section, particularly those using muscle-specific LKB1 knockouts (99), suggest that AMPK is the “most valuable,” although not the only, player determining the increased glucose uptake, fatty acid oxidation, and inhibition of glycolysis synthesis in skeletal muscle during exercise, hence maintaining cellular energy homeostasis. Thus many of the acute adaptations of muscle to exercise appear to be mediated by this system. Given that mice with defects in AMPK seem to be intolerant of exercise (84), it may be more difficult to study longer-term, chronic effects, such as the increased mitochondrial biogenesis. However, the studies using feeding of β-GP to mice expressing the dominant negative mutant of AMPK (132) already suggest that the pathway may be crucial in those effects as well. This reinforces the idea that AMPK signaling pathway may be a good target for development of drugs aimed at treatment of obesity and type 2 diabetes. Given that existing agents like metformin appear to work indirectly by inhibiting mitochondrial respiration, the AMP-binding domains on the γ-subunits represent attractive new targets for drug development.

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