AMP-Activated Protein Kinase as a Drug Target

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Abstract

The AMP-activated protein kinase (AMPK) system is a regulator of energy balance at both the cellular and whole-body levels that, once activated by low energy status, effects a switch from ATP-consuming anabolic pathways to ATP-producing catabolic pathways. It now appears to be the major target for two existing classes of drug used to treat type 2 diabetes, i.e., the biguanides and thiazolidinediones. However, in both cases these activate AMPK indirectly, and an interesting question concerns whether a drug that directly activated AMPK would retain the therapeutic benefits of the existing drugs while eliminating unwanted side effects. AMPK activators also now have potential as anticancer drugs.

INTRODUCTION

Obesity and type 2 diabetes are becoming an epidemic in the developed world, and it is widely predicted that their long-term effects may cause life expectancy to fall for the first time in several centuries. In the United States, 30% of adults (60 million people) are now obese [body mass index (BMI) > 30 kg . m^{-2}; see **http://www.cdc.org**]. In the Nurse's Health Study (1), a BMI > 30 was found to increase the risk of developing type 2 diabetes in females by 20-fold, compared with lean controls (BMI < 23), with similar findings in smaller-scale studies of men (2). Although type 2 diabetes (characterized by a primary insulin resistance) is less acutely life-threatening than the type 1 form (characterized by a primary insulin deficiency), both forms carry the same risk of debilitating long-term complications, including retinal damage leading to blindness, kidney disease, nerve damage leading to foot amputations, and micro- and macrovascular disease. Recent trends in drug prescribing for type 2 diabetics have seen a move away from agents that stimulate insulin secretion, such as the sulfonylureas, and toward agents that increase insulin sensitivity, such as the biguanide, metformin, and the thiazolidinediones. An exciting recent development has been findings (3–6) that both of these latter classes of drug activate the AMP-activated protein kinase (AMPK).

Although it may appear to some people that AMPK has burst onto the scene relatively recently, studies on the system in fact go back a long way. With hindsight, its discovery can be traced to two independent findings reported in 1973 by the groups of Kim (7) and Gibson (8) that crude preparations of acetyl-CoA carboxylase and 3-hydroxy-3-methyl (HMG)-CoA reductase (the key regulatory enzymes of fatty acid and cholesterol biosynthesis, respectively) became inactivated when incubated with ATP. Both groups correctly surmised that the effects were due to phosphorylation of their respective enzyme by an endogenous protein kinase that contaminated their preparation, and Gibson's group went on to provide evidence that this protein kinase was itself activated by phosphorylation by an upstream kinase (9). In the 1970s, HMG-CoA reductase kinase (as it was then called) was only one of five or so protein kinases whose activity had been defined, and this was also only the second protein kinase cascade to have been discovered. However, it was not until 1987 that the author's laboratory reported that the inactivation of acetyl-CoA carboxylase and HMG-CoA reductase were both catalyzed by a single protein kinase (10). Because it soon became clear that this was a true multisubstrate kinase, we renamed it AMP-activated protein kinase after its allosteric activator, 5′-AMP (11). The realization that this kinase had a much more general role in regulating energy balance, both at the cellular and whole-body levels, and that it is a key target for drugs aimed at treatment of obesity and type 2 diabetes came much later and is the main focus of this review.

STRUCTURE AND REGULATION OF AMPK

AMPK is now known to exist as heterotrimeric complexes comprising a catalytic α subunit and regulatory β and γ subunits (**Figure 1**). Orthologues of all three subunits are found in all eukaryotic species where genome sequences are available (12, 13),

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suggesting that the system arose during an early stage of eukaryotic evolution. In mammals all three subunits are encoded by multiple genes (12) ($\alpha1$, $\alpha2$; $\beta1$, $\beta2$; $\gamma1$, $\gamma2$, $\gamma3$) and some of these are also subject to alternate splicing, so that a diverse collection of $\alpha\beta\gamma$ heterotrimers can exist. The catalytic subunit contains a conventional serine/threonine protein kinase domain at the N terminus (14) and a C-terminal region that is required for the formation of the complex with the other two subunits (15). The β subunit contains a C-terminal domain that is required for complex formation (16), and a central domain that is related to noncatalytic domains found in enzymes that metabolize the $\alpha1\rightarrow6$ branches in $\alpha1\rightarrow4$-linked glucans such as starch and glycogen. This domain causes AMPK to associate with glycogen particles in intact cells (16, 17) and it is the first region of AMPK for which a crystal structure is available (18). Its physiological function remains unclear, although an intriguing idea is that it allows the AMPK system to sense the availability of glycogen reserves in the cell. The three γ subunit isoforms contain variable N-terminal regions, followed by four tandem repeats of a sequence known as a CBS motif. First recognized by Bateman (19), the basic functional unit is now known to contain two repeats, which form a domain termed a Bateman domain (20) with a deep binding cleft between them (21). Bateman domains occur in a small number of proteins other than AMPK; our group showed that their common feature is that they bind ligands containing adenosine, i.e., AMP, ATP, or S-adenosyl methionine (21). The AMPK γ subunits are unusual in that they contain two Bateman domains in tandem, which bind two molecules of AMP or ATP in a strongly cooperative, but mutually exclusive, manner. Mutations in either the N- or the C-terminal domain reduce AMP binding and activation (suggesting that both sites must be occupied for AMP to activate the kinase), and also cause human heart disease, with some correlation between the severity of the disease and the severity of the defect in nucleotide binding (21, 22). The primary cause of these disorders appears to be the accumulation of abnormal deposits of glycogen, which lead to disruption of myofibril function (22) or, in milder forms, to the formation of abnormal conduction pathways between atria and ventricles that cause arrhythmias (23). Although the mutations abolish or reduce AMP activation, they also appear to increase the basal activity, probably because they also interfere with the binding of the inhibitory nucleotide, ATP (22). This gain-of-function effect explains why these mutations are dominant, with the full disease being present in heterozygotes.

As first suggested by Gibson (9), AMPK is inactive unless phosphorylated by upstream kinases (the identity of which is discussed below), with the critical phosphorylation site being Thr-172 within the "activation loop" of the kinase domain on the α subunit (**Figure 1**) (24, 25). As its name suggests, 5′-AMP activates AMPK, but it does this via a complex mechanism involving three effects (26), all of which are thought to be due to binding to the Bateman domains on the γ subunit. This binding (*a*) promotes phosphorylation by the upstream kinase, (*b*) causes allosteric activation of the phosphorylated kinase, and (*c*) inhibits dephosphorylation of Thr-172 by protein phosphatases. All three effects are also antagonized by binding of ATP, which binds to the Bateman domains with a lower affinity than AMP and in a mutually exclusive manner (21). Because of the adenylate kinase reaction, which is maintained close to equilibrium in all eukaryotic cells, the cellular AMP:ATP ratio

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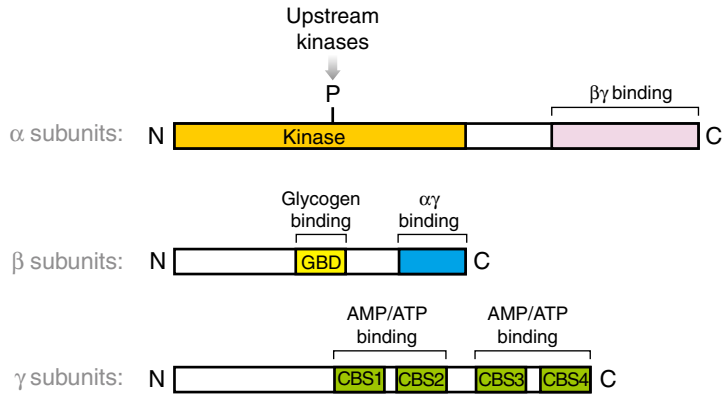

Figure 1

Domain structure of the three subunits of the AMPK heterotrimer. The colored boxes show regions of the sequences that are highly conserved in α, β, and γ subunit sequences from different species or between different isoforms in the same species. The functions of these domains are discussed in the text. Each of the two AMP/ATP-binding Bateman domains in the γ subunits are composed from two tandem repeats of a CBS motif.

varies approximately as the square of the ADP:ATP ratio (27), making the former ratio a very sensitive indicator of compromised cellular energy status.

For many years the upstream kinase(s) that phosphorylated Thr-172 on the α subunit of AMPK remained unidentified, but there has been recent rapid progress in this area. The major upstream kinase in most mammalian cells is a complex between the protein kinase LKB1 (28–30) and two accessory subunits, STRAD and MO25 (29). Intriguingly, LKB1 was first identified in humans as a gene carrying an autosomal dominant mutation in Peutz-Jeghers syndrome (PJS) (31, 32). Subjects with PJS develop numerous benign intestinal polyps, but also have a 15-fold increased risk of developing malignant tumors at other sites (33, 34). Somatic mutations in the LKB1 gene are also commonly found in lung adenocarcinomas (35), whereas some human tumor cell lines (e.g., HeLa cells) lack LKB1 expression, and its reintroduction into these cells inhibits their proliferation (36). Thus, LKB1 is a classical tumor suppressor and these findings suggested for the first time a link between AMPK and cancer. Although it was subsequently shown that LKB1 also acted upstream of at least 12 other AMPK-related kinases (37, 38), at present it seems likely that AMPK accounts for much of the tumor suppressor activity of LKB1, a view that is discussed further below.

The STRAD subunit is essential for the ability of the LKB1 complex to phosphorylate Thr-172 on AMPK (29). Interestingly, STRAD is one of the 50 pseudokinases among the 478 conventional protein kinases encoded in the human genome (39). It is classed as a pseudokinase because it has a domain related to a kinase domain, but with amino acids altered at some conserved positions such that it would be expected to be inactive. We have confirmed that STRAD is inactive as a protein kinase, although it binds ATP. However, mutations that greatly reduce ATP binding did not affect its

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ability to activate LKB1 (40). The MO25 subunit contains a helical repeat that is distantly related to the armadillo proteins and appears to stabilize the LKB1:STRAD complex (41).

Experiments in LKB1 (−/−) mouse embryo fibroblasts (29) and in skeletal muscle–specific mouse knockouts of LKB1 (42) show that LKB1 is essential for activation of AMPK by the biguanide phenformin (a close relative of metformin, formerly used to treat diabetes), by the AMPK-activating nucleoside 5-aminoimidazole-4-carboxamide riboside (AICAR), and by muscle contraction. As discussed in more detail below, we believe that all of these agents act by increasing the concentration of AMP (or an AMP analogue in the case of AICAR). However, some basal AMPK activity and phosphorylation of Thr-172 still occurs in LKB1 (−/−) mouse embryo fibroblasts, as well as in HeLa cells, which do not express LKB1, suggesting that there must be another upstream kinase phosphorylating Thr-172. In fact, several years earlier our laboratory had shown that calmodulin-dependent protein kinase kinases (CaMKKs), which are upstream kinases for calmodulin-dependent protein kinases I and IV, would also phosphorylate and activate AMPK (43), which at the time we did not believe to be physiologically relevant. However, when HeLa cells were incubated with the Ca^{2+} ionophore A23187, AMPK was dramatically activated, and this was blocked by the CaMKK inhibitor STO-609 and by siRNAs targeted at the β isoform of CaMKK (44). Other laboratories obtained similar findings (45, 46). We were also able to show that this pathway operated in a more physiological setting, i.e., in response to K^+-induced depolarization in rat brain slices (44).

Thus, there appear to be at least two signaling pathways upstream of AMPK, one triggered by an increase in the AMP:ATP ratio and dependent on LKB1 and one triggered by an increase in Ca^{2+} and dependent on CaMKKβ (**Figure 2**). Because treatments that increase cellular AMP do not activate AMPK in HeLa cells despite the fact that they express CaMKKβ, binding of AMP to the γ subunits of AMPK (which triggers phosphorylation of Thr-172 by LKB1) does not appear to be sufficient to trigger phosphorylation by CaMKKβ. Although LKB1 is expressed ubiquitously, the CaMKKs are much more restricted, with neural tissue appearing to represent their main site of expression (47). However, a more comprehensive survey of the cell and tissue distribution of the CaMKK→AMPK pathway needs to be performed.

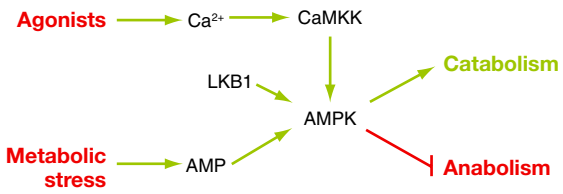

Figure 2

Two pathways upstream of the AMP-activated protein kinase (AMPK). AMPK is activated by phosphorylation at Thr-172 either by a metabolic stress that causes an increase in cellular AMP, resulting in phosphorylation by LKB1, or by agonists that cause an increase in cytosolic Ca^{2+}, resulting in phosphorylation by CaMKKs. Whatever the upstream pathway, AMPK switches off anabolic pathways that consume ATP while switching on catabolic pathways that generate ATP.

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Although AMPK is an excellent target for drug development (see below), we do not believe that the same is true of the upstream kinases. This is partly because both LKB1 and the CaMKKs also lie upstream of kinases other than AMPK, but also because, in the case of LKB1, it appears to be constitutively active in vivo (37, 48).

DOWNSTREAM TARGETS OF AMPK

Many downstream targets of AMPK were initially identified by activating the kinase in intact cells using the drug AICAR, an adenosine analogue that is taken up into cells via adenosine transporters (49) and converted to the monophosphorylated nucleotide, ZMP, by adenosine kinase (50). The use of AICAR to activate AMPK was developed by van den Berghe (51), but the author's laboratory showed that ZMP mimicked all three effects of AMP on the AMPK system, including increased phosphorylation of Thr-172 (52). AICAR is not completely specific for AMPK, because ZMP regulates other AMP-sensitive enzymes such as fructose-1,6-bisphosphatase (50) and muscle glycogen phosphorylase (53). Conclusions that derive from use of AICAR should therefore ideally be backed up by a molecular biological method such as the use of AMPK knockouts, siRNA, or dominant negative mutants. An AMPK inhibitor [compound C (3)] has recently become commercially available. It inhibits kinases other than AMPK (D.G. Hardie, unpublished), but if it reverses an effect of AICAR (which is not known to activate any other protein kinases), this provides good evidence for a role for AMPK.

AICAR was originally used to show that AMPK inhibited fatty acid synthesis in rat adipocytes (54) and both fatty acid and cholesterol synthesis in rat hepatocytes by inactivation of acetyl-CoA carboxylase and HMG-CoA reductase, respectively (51, 52). AMPK activation also inhibits other ATP-consuming, anabolic pathways, such as glycogen synthesis [via phosphorylation of muscle glycogen synthase (55, 56)], and protein synthesis. The latter occurs by multiple mechanisms, including inhibition of the target-of-rapamycin (TOR) pathway that stimulates translational initiation (57–60), and by activation of elongation factor-2 kinase, which inhibits the elongation step (61). In addition to these rapid, acute effects on anabolic pathways, AMPK activation also down-regulates anabolic pathways such as fatty acid synthesis (62, 63) and gluconeogenesis (64) at the level of gene expression. These effects may be due to its ability to down-regulate key transcription factors such as SREBP-1c (3), ChREBP (65), or HNF-4α (66), or by direct phosphorylation of transcriptional coactivators such as p300 (67) and TORC2. Phosphorylation of TORC2 anchors it in the cytoplasm as a complex with 14–3–3 proteins, and appears to be responsible for the inhibition of expression of gluconeogenic enzymes by AMPK (68, 69).

In addition to inhibiting anabolic pathways and thus conserving ATP, AMPK also stimulates catabolic pathways that generate ATP, including fatty acid oxidation (70, 71). Most fatty acids are oxidized in mitochondria, and their entry into the organelle is blocked by malonyl-CoA, which inhibits carnitine palmitoyl transferase-1 (CPT1), an enzyme required for the transport of fatty acids across the inner mitochondrial membrane (72). Malonyl-CoA is produced by one of the target enzymes for AMPK,

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acetyl-CoA carboxylase, which exists as two isoforms, ACC1 (α) and ACC2 (β). Both are phosphorylated and inactivated by AMPK (73, 74), but ACC1 is cytosolic and appears to be involved mainly with fatty acid synthesis, whereas ACC2 is associated with mitochondria and may have a special role in producing the malonyl-CoA that inhibits CPT1 (75, 76). As well as stimulating fatty acid oxidation by this mechanism, in cardiac muscle AMPK activation has been shown to stimulate fatty acid uptake by translocation of the CD38 (fatty acid translocase) protein to the plasma membrane (77).

Along with cellular fatty acid uptake, another key target for AMPK is glucose uptake. In muscle, AMPK activation stimulates glucose uptake (70) via translocation of GLUT4 to the plasma membrane (78). The detailed mechanism for this effect remains unclear, although it is distinct from that utilized by the insulin signaling pathway because it is not blocked by inhibitors of phosphatidylinositol 3-kinase, and the effects of insulin and AMPK activators are additive (79). Interestingly, AMPK activation in adipocytes causes a modest stimulation of glucose uptake, but (in contrast to muscle) it antagonizes the much larger stimulation of GLUT4-dependent glucose uptake observed in response to insulin (80). The mechanistic basis for these differences between muscle and adipocytes remains unknown, but the findings fit with the view that glucose uptake during exercise in muscle is mainly concerned with ATP generation and is therefore catabolic, whereas in adipocytes glucose uptake is mainly utilized for lipid synthesis and is therefore anabolic. In cells that do not express GLUT4, AMPK also upregulates glucose transport by GLUT1, by an unknown mechanism involving an activation of the transporter that is already located at the membrane, rather than translocation (81). In muscle, AMPK activation also increases GLUT4 expression by stimulating the binding of the transcription factor myocyte enhancer factor-2 (MEF-2) to the GLUT4 promoter (82). This would increase the capacity for glucose uptake in subsequent exercise sessions, and it represents one of the adaptations that occur during endurance training. Another response to regular endurance exercise is an upregulation of mitochondrial biogenesis (83), which is of medical relevance because subjects who are at risk of developing type 2 diabetes, such as older people and first-degree relatives of diabetics, appear to have a deficit in mitochondrial oxidative function (84). The master regulators of mitochondrial biogenesis are believed to be the peroxisome proliferator-activated receptor (PPAR)-γ coactivators-1α and -1β (PGC-1α/β), which are coactivators for several transcription factors, including NRF-1 and -2 and the nuclear hormone receptors PPAR-α, PPAR-δ, PPAR-γ, estrogen-related receptor-α, and thyroid hormone receptors (85). All of these upregulate expression of mitochondrial genes encoded in the nucleus, whereas NRF-1 and -2 also induce expression of TFAM, a protein required for the replication of mitochondrial DNA. Intriguingly, PGC-1α is upregulated in rat epitrochlearis muscle by prolonged low intensity exercise or by AICAR treatment (86), and results with transgenic mice expressing a dominant negative AMPK mutant in muscle suggest that AMPK is essential for upregulation of PGC-1α and mitochondrial biogenesis in response to ATP depletion triggered by feeding a creatine analogue (87). The molecular mechanism by which AMPK upregulates PGC-1α expression in muscle remains unclear.

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AMPK activation also stimulates glycolysis in some, but not all, tissues. Hue's group has shown that activation of AMPK can cause phosphorylation and activation of 6-phosphofructo-2-kinase (PFK2), the enzyme that synthesizes fructose-2, 6-bisphosphate, an allosteric activator of the key glycolytic enzyme 6-phosphofructo-1-kinase (PFK1). There are at least four isoforms of PFK2, but the AMPK phosphorylation site is present on only two of them, i.e., the cardiac (88) and inducible (iPFK2) forms (89), and not the liver or skeletal muscle forms. Activation of glycolysis in cardiac muscle represents a mechanism to generate ATP under hypoxic or ischemic conditions, and it is a novel explanation for the classical Pasteur effect, i.e., an increased rate of glycolysis in response to hypoxia (90). Upregulated expression of the iPFK2 isoform occurs in cells of the monocyte/macrophage lineage in response to proinflammatory stimuli, such as bacterial lipopolysaccharide. These cells, involved in the innate immune system, have to be able to operate at sites of infection or wounding where the conditions might be quite hypoxic, and this mechanism may allow them to generate ATP under these conditions by anaerobic glycolysis. Intriguingly, the iPFK2 isoform is also constitutively expressed in some tumor cells (91), and this could potentially explain their high rates of glycolysis (the so-called Warburg effect), which may help the tumors to survive in a relatively hypoxic state until a blood supply has been established.

Another metabolic target for AMPK is hormone-sensitive lipase (HSL). This enzyme had been thought to catalyze the initial, rate-limiting step in triglyceride breakdown, although studies with knockout mice show that triglyceride breakdown still occurs in its absence, with an accumulation of diglyceride (92). These results suggested that HSL catalyzes the removal of the second fatty acid, and led to the discovery of a novel adipocyte triglyceride lipase (ATGL), which appears to remove the first (93). Prior to this, with Steven Yeaman we had found that phosphorylation of HSL by AMPK antagonized its phosphorylation and activation by cyclic AMP-dependent protein kinase (94), and consistent with this, treatment of adipocytes with AICAR was found to antagonize activation of lipolysis by cyclic AMP-elevating agents (52, 54). These findings were challenged in a subsequent report that suggested that AMPK activation stimulated rather than inhibited lipolysis (95), but a more recent study has provided strong support for our original proposal (96). For example, in adipocytes from AMPK-α1-knockout mice both basal and isoproterenol-stimulated lipolysis were found to be elevated, and the cells were insensitive to the antilipolytic effects of AICAR. The adipocytes from these mice were also significantly smaller, consistent with increased lipolysis (96).

Lipolysis might be regarded as a catabolic pathway, so at first sight these findings would seem to contradict the paradigm that AMPK inhibits anabolism and stimulates catabolism. However, if fatty acids released by lipolysis are not removed from the cell, they recycle back into triglyceride with the consumption of ATP and generation of AMP, thus activating AMPK. We therefore proposed (97) that inhibition of HSL by AMPK was a mechanism to limit this recycling and ensure that the rate at which fatty acids were released by lipolysis did not exceed the rate at which they could be disposed of by export or by internal oxidation. Interestingly, AMPK is activated by cyclic AMP-elevating agents such as isoproterenol in adipocytes (95, 96, 98), but

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not in other cell types. We suspect that activation by cyclic AMP-elevating agents in adipocytes is secondary to their ability to stimulate lipolysis, and consequently to cause recycling of fatty acids back into triglyceride. It will be interesting to see whether AMPK inhibits lipolysis catalyzed by ATGL.

As well as the numerous metabolic targets of AMPK for which it is best known, it is becoming increasingly clear that it has many other downstream effects. Of particular relevance to this review are recent findings suggesting that AMPK inhibits cell growth and proliferation, and also regulates apoptosis. These findings are not surprising given that cell growth, DNA replication, and mitosis are all major consumers of ATP, and also that the upstream regulator of AMPK, LKB1, is known to be a tumor suppressor. Several groups have reported that AMPK activation caused a G1/S phase cell cycle arrest in different cell lines, and that this is associated with accumulation of the tumor suppressor p53 and of the cyclin-dependent kinase inhibitors p21 and p27, which act downstream of p53 (99–102). This appears to be associated with phosphorylation of p53 on Ser-15 (99, 101), although it has not been shown that this is a direct phosphorylation. Another potential mechanism to explain these effects is that AMPK has been found to reduce the cytoplasmic:nuclear ratio of the RNA-binding protein HuR, reducing its ability to stabilize mRNAs encoding critical cell cycle regulators such as cyclins A and B1 (103).

AMPK activation may also inhibit cell proliferation owing to its general effects on biosynthesis, including its ability to inhibit fatty acid synthesis [which is elevated in many cancer cells (102)] and the TOR pathway (57, 60). The TOR pathway is a critical activator of cell growth and proliferation that is activated by growth factors such as insulin and IGF1 via the phosphatidylinositol (PI) 3-kinase and PKB/Akt signaling pathway (**Figure 3**). The TSC1:TSC2 complex is a GTPase activator protein (GAP), which inactivates the small G protein Rheb, an upstream activator of TOR (104). Akt/PKB phosphorylates TSC2 at sites that are believed to inhibit its Rheb-GAP activity (105), whereas AMPK is thought to have the opposite effect (58). Intriguingly, heterozygous loss-of-function mutations in TSC1 or TSC2, in PTEN (a lipid phosphatase that reverses the effects of PI 3-kinase), or in LKB1 all cause hereditary cancer syndromes in humans (tuberous sclerosis complex, Cowden's syndrome, and Peutz-Jeghers syndrome, respectively). These diseases are all characterized by benign tumors that are classified as hamartomas, in which cells grow abnormally but retain their normal differentiated state. All of these proteins normally oppose activation of the TOR pathway (**Figure 3**), and in the intestinal lesions in PJS the TOR pathway appears to be hyperactivated (106). The natural product rapamycin (sirolimus), a pharmacological inhibitor of TOR through which the pathway was originally discovered and named, is undergoing trials for treatment of human cancers (107).

The effects of AMPK on apoptosis are complex. In some situations AMPK appears to prevent the process (30, 108–110), whereas in others AMPK appears to induce it (111–116). Successful apoptosis requires ATP, and one explanation for these conflicting findings may be that AMPK activation sometimes allows cells to recover from an energy crisis that would otherwise have triggered cell death. However, if the damaged or metabolically depleted cells go past the point of no return, AMPK may become

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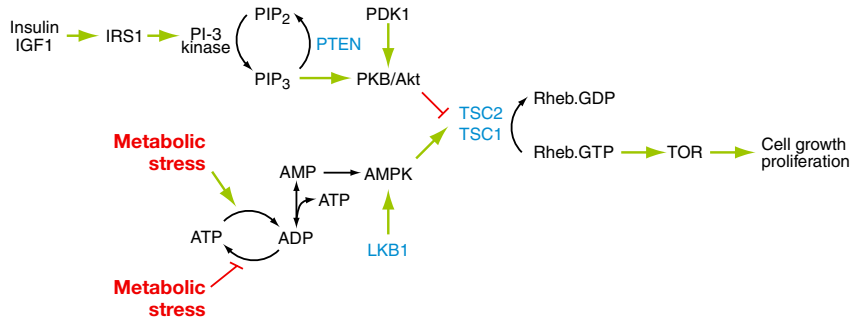

Figure 3

Two pathways regulate the target-of-rapamycin (TOR) pathway, a key regulator of cell growth and proliferation. Growth factors that stimulate PI 3-kinase, such as insulin or IGF-1 acting via insulin receptor substrate-1 (IRS1), stimulate the phosphorylation and activation of the PKB/Akt kinase by its upstream kinase, PDK1. PKB/Akt inhibits the TSC1:TSC2 complex, switching off its ability to convert the G protein Rheb to its inactive GDP-bound state. The Rheb-GTP complex then stimulates TOR, and hence cell growth and proliferation. Conversely, the AMPK pathway is activated by metabolic stresses via phosphorylation and activation by LKB1. This stimulates the GTPase activator protein (GAP) activity of TSC1:TSC2, causing conversion of Rheb to its inactive GDP form and switching off the TOR pathway. Loss-of-function mutations in LKB1, PTEN, TSC1, or TSC2 (shown in *blue*, all of which oppose TOR activation) all cause human cancer syndromes associated with the formation of benign tumors known as hamartomas.

proapoptotic. Interestingly, it has been proposed that increased cellular AMP:ATP and AMPK activity contributes to the senescence observed in human fibroblasts after a high number of passages in culture (117).

PHYSIOLOGICAL ACTIVATION OF AMPK

The first treatments that were shown to activate AMPK in intact cells were metabolic stresses that caused ATP depletion, such as incubation of rat hepatocytes with high fructose (118); heat shock (119); or metabolic inhibitors such as arsenite, dinitrophenol, antimycin A, and azide (119, 120). It was also shown to be activated in cardiac muscle and myocytes by hypoxia or ischemia (88, 121). Hypoxia in cardiac muscle may only occur under pathological circumstances, but there is now evidence that physiologically relevant levels of hypoxia can activate AMPK in specialized oxygen-sensing cells such as pulmonary artery smooth muscle, where it appears to trigger contraction and thus divert blood flow to oxygen-rich areas of the lung, and carotid body glomus cells, where it is involved in regulating breathing and hence in the response to whole-body hypoxia (122). In cultured cells, AMPK is also activated by glucose deprivation (123), which was an interesting observation because the SNF1 complex, the budding yeast orthologue of AMPK, is required for the response of that organism to glucose starvation (124). Most cells express low Km forms of hexokinase, and ATP levels will only start to fall (and AMPK become activated) when extracellular glucose drops to unphysiologically low levels. However, pancreatic β cells (125) and

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glucose-sensing cells in the hypothalamus (126) express the high Km glucokinase isoform. In these cells, AMPK appears to be modulated by variations in glucose within the physiological range and to be involved in regulation of insulin secretion and appetite, respectively (123, 127).

The single finding that probably sparked the most interest in the AMPK system was the observation that it was activated by exercise in rat skeletal muscle (128), which was subsequently reproduced in humans (129, 130). Studies in mice with a specific muscle knockout of LKB1 show that AMPK activation in response to contraction is completely dependent on LKB1 and is associated with an increase in the cellular AMP:ATP ratio that is presumed to be the trigger for the activation (42). Indeed, in the LKB1-deficient mice the increases in AMP:ATP ratio in response to contraction were much larger (42), supporting previous proposals (119) that the AMPK system protects cells against stresses that cause ATP depletion.

AMPK clearly evolved in single-celled eukaryotes, and the findings discussed in this section so far are all compatible with the idea that the AMPK system acts mainly in a cell-autonomous fashion, protecting the cell that expresses the kinase against stresses that caused depletion of ATP within that cell, even in multicellular eukaryotes. However, in the past few years it has become apparent that the AMPK system is also regulated by hormones and cytokines involved in the regulation of whole-body energy balance. The first suggestions for this came with findings that AMPK was activated by receptors that are coupled via the G protein, Gq, to PI-specific phospholipase C, such as the receptors for platelet-activating factor, norepinephrine (α_1-adrenergic), and bradykinin (131). Because activation of these receptors would trigger release of inositol-1,4,5-trisphosphate and intracellular Ca^{2+}, the CaMKK→AMPK pathway most likely mediates this process, although this has not yet been proven conclusively. Subsequently, it was shown that the cytokine leptin, which is released by adipocytes and inhibits food intake via effects on the hypothalamus of the brain, caused a biphasic effect to activate AMPK in skeletal muscle (132). There was a rapid but transient effect that appeared to be a direct effect on the muscle and could be reproduced in a muscle cell line, and a slower but more prolonged effect that required administration of leptin into the brain and appeared to be mediated by sympathetic nerves via α_1-adrenergic activation. Whatever the mechanism, AMPK activation could explain the ability of leptin to stimulate energy expenditure by activating fatty acid oxidation in muscle. Remarkably, it was also reported that inhibition of AMPK in the hypothalamus could also explain the ability of leptin to inhibit food intake (127). Conversely, activation of AMPK in the hypothalamus, achieved by expressing activated mutants of the γ1 subunit (127), or by intraventricular injection of AICAR (133) stimulated food intake in rats. Consistent with these findings, several other anorexic treatments, such as insulin, melanocortin receptor agonists, high glucose, or refeeding, were found to inhibit hypothalamic AMPK, whereas orexigenic agents, such as ghrelin, agouti-related protein, and cannabinoids, were found to stimulate it (127, 133, 134). How leptin modulates AMPK activity, and how it has opposite effects in different tissues (127, 132) [a phenomenon also observed with cannabinoids (134)] remains unclear at present.

Another key cytokine in regulating whole-body energy balance is adiponectin (135). Paradoxically, although released by adipocytes and therefore referred to as an

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adipokine, plasma adiponectin concentrations are negatively correlated with obesity and type 2 diabetes (136, 137). In 2002, two groups showed that adiponectin stimulates glucose uptake and fat oxidation in skeletal muscle via activation of AMPK, whereas in the liver it also inhibits gluconeogenesis, in both cases via AMPK activation (138, 139). Expression of a dominant negative mutant of AMPK in the liver using an adenoviral vector greatly reduced the hypoglycemic effects of adiponectin in vivo, suggesting that the major effect of adiponectin on plasma glucose is to inhibit glucose production by the liver via AMPK activation (139). Although AMPK is clearly a critical regulator of metabolism in the liver, adiponectin is the only known physiological regulator of the kinase in this organ at present.

Another adipokine released by adipocytes in rodents (although not in humans) is resistin, which generally appears to have effects opposite to those of adiponectin. Interestingly, in the livers of resistin (−/−) mice AMPK phosphorylation at Thr-172 is elevated, suggesting that resistin may inhibit AMPK in the liver (140).

Finally, recent work by the laboratory of Cantrell in collaboration with the author has shown that AMPK is transiently activated in T lymphocytes by stimulation of the T cell antigen receptor. Interestingly, this appears to be mediated by the CaMKK pathway following elevation of intracellular Ca^{2+} (140a). Stimulation of the antigen receptor activates a dramatic program of growth, proliferation, and differentiation in these cells; all of these will create a large demand for energy. We speculate that activation of AMPK may help to metabolically prepare the cells for this increased demand (see **Table 1** for a summary of this section).

PHARMACOLOGICAL ACTIVATION OF AMPK

The growing realization that AMPK could switch metabolism from an anabolic state, favoring the synthesis and storage of glucose and fatty acids, to a catabolic state, favoring the oxidation of these fuel molecules, led Winder and the author to propose in 1999 that AMPK activators might be effective treatments for type 2 diabetes (141). This was soon tested by in vivo treatment with AICAR of various animal models of insulin resistance, such as genetically obese mice (ob/ob) and rats (fa/fa), or rats fed a high-fat diet. Encouragingly, the drug was found to reverse most, if not all, of the metabolic abnormalities of these animals. Thus, it caused improvements in glucose tolerance, decreases in plasma fatty acids and triglycerides, decreases in hepatic glucose output and blood pressure, increases in glucose disposal and HDL cholesterol, and even a tendency toward reduction of abdominal fat (142–145). Further support for the idea that AMPK was a potential target for antidiabetic drugs came with reports that two major classes of existing antidiabetic drugs, i.e., the biguanides (metformin and phenformin) and the thiazolidinediones (e.g., rosiglitazone, troglitazone, and pioglitazone) activated AMPK in intact cells and/or in vivo (3–6, 29). However, these reports left open the question as to whether AMPK activation was responsible for the therapeutic effects of these drugs.

In the case of metformin, one issue was its potency. The concentrations of metformin (between 100 μM and 2 mM) that were required to stimulate glucose transport in isolated human muscle (146) and to activate AMPK in cultured cells (3–5), were

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Table 1 Treatments that activate AMPK in intact cells*

Treatment	Cell type/organ system	Effect	Reference
Treatments that interfere with ATP synthesis			
Arsenite	Rat hepatocytes	↑	(119)
Dinitrophenol	Fao hepatoma cells	↑	(120)
Antimycin A	Fao hepatoma cells	↑	(120)
Azide	Fao hepatoma cells	↑	(120)
Oligomycin	H4IIE hepatoma cells	↑	(5)
Oligomycin	Perfused rat heart	↑	(88)
Hypoxia	Perfused rat heart	↑	(88)
Hypoxia	Pulmonary artery smooth muscle	↑	(122)
Ischemia	Perfused rat heart	↑	(121)
Glucose starvation	Budding yeast (SNF1 complex)	↑	(159)
Glucose starvation	Pancreatic β cells (INS1 cells)	↑	(123)
Glucose starvation/hypoglycemia	Neuroblastoma cells/hypothalamus	↑	(127, 160)
Treatments that accelerate ATP breakdown			
High [fructose]	Rat hepatocytes	↑	(118)
High [deoxyglucose]	CHO cells	↑	(161)
Heat shock**	Rat hepatocytes	↑	(119)
Exercise	Rat muscle	↑	(128)
Electrical stimulation	Rat muscle	↑	(162, 163)
Exercise	Human muscle	↑	(129, 130)
Hormones/cytokines			
α1-adrenergic agonists	CHO expressing α1 receptor	↑	(131)
Platelet-activating factor (PAF)	CHO expressing PAF receptor	↑	(131)
Bradykinin	CHO expressing bradykinin receptor	↑	(131)
Leptin	Mouse skeletal muscle/H-2K^b cells	↑	(132)
Leptin	Mouse/rat hypothalamus	↓	(127, 133)
Agouti-related protein	Mouse hypothalamus	↑	(127)
Ghrelin	Rat hypothalamus, heart	↑	(133, 134)
Ghrelin	Rat liver, adipose tissue	↓	(134)
Cannabinoids	Rat hypothalamus, heart	↑	(134)
Cannabinoids	Rat liver, adipose tissue	↓	(134)
Other treatments			
Antigen	T lymphocytes	↑	(140a)
Pharmacological agents			
AICAR	Rat hepatocytes, adipocytes	↑	(51, 52)
Metformin	Rat liver, muscle	↑	(3)
Thiazolidinediones (rosiglitazone)	Mouse muscle (H-2K^b) cells	↑	(4)
Thiazolidinediones (pioglitazone)	Rat liver, adipocytes	↑	(6)

*This is not a comprehensive list, but an attempt has been made to cite the first publication that demonstrated the effect.

**Heat shock accelerates ATP turnover because protein refolding via heat shock proteins requires ATP hydrolysis; however, it may also inhibit ATP synthesis.

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1 to 2 orders of magnitude higher than those (10–40 μM) estimated to occur in peripheral plasma after therapeutic doses in humans. However, the major hypoglycemic effects of the drug are believed to occur via inhibition of hepatic gluconeogenesis (147, 148) and being supplied directly from the gut by the portal vein, the liver would experience a higher concentration of orally administered metformin than peripheral tissues. An important recent paper studied mice where AMPK activation by metformin in liver was prevented by a knockout of LKB1, in which the hypoglycemic effect of metformin was completely abolished (68). This not only confirmed that the major effect of metformin (at least in mice) is to reduce hepatic gluconeogenesis, but also suggested that the effect is mediated by AMPK activation. Strictly speaking, these results do not rule out the possibility that a kinase downstream of LKB1 other than AMPK [including SIK1, which is known to regulate expression of gluconeogenic genes in the liver (69)] could be involved in the mechanism of metformin action. However, this seems unlikely because none of the AMPK-related kinases, including SIK1 (SIK), SIK2 (QIK), or SIK3 (QSK) are activated by phenformin in mouse embryo fibroblasts or rat skeletal muscle (37, 48).

By what mechanism do biguanides activate AMPK? Although metformin is excreted unchanged in the urine and there is no evidence that it is metabolized, it nevertheless appears to act indirectly because it does not activate AMPK, or affect its phosphorylation by LKB1 or its dephosphorylation by protein phosphatases, in cell-free assays (5). Both metformin and phenformin have been reported to be inhibitors of complex I of the respiratory chain (149, 150), suggesting that they may activate AMPK by increasing cellular AMP:ATP ratios. This has been difficult to demonstrate in the case of metformin (4, 5), but increases in cellular AMP:ATP can be readily demonstrated in response to phenformin (44), which is a more rapid and potent activator of AMPK in intact cells. Inhibition of the respiratory chain may explain the life-threatening cases of lactic acidosis that caused phenformin to be withdrawn from clinical use (150a). Because cells lining the gut would also experience the highest concentrations of the drug, this may also explain the gastrointestinal side effects of metformin (diarrhea, nausea, abdominal discomfort, anorexia), which limit its use in many patients. There is evidence that the small increases in lactate often observed in response to metformin are mainly derived from the gut (151).

In 1995, the thiazolidinediones were reported to be high-affinity ligands for the nuclear hormone receptor family member PPAR-γ (152), and it has generally been assumed that this is their key target. PPAR-γ is primarily expressed in adipocytes and is involved in adipocyte differentiation (153). An intriguing question has been how a drug target whose action is apparently confined to adipocytes can improve insulin sensitivity in other organs, especially skeletal muscle. A potential answer came with findings that thiazolidinediones stimulate the expression and release of adiponectin from adipocytes (154). Indeed, recent studies in adiponectin knockout mice suggest that at least some of the effects of thiazolidinediones are mediated by adiponectin (154a). As discussed above, adiponectin appears to exert its effects in both skeletal muscle (where it stimulates glucose uptake and fatty acid oxidation) and liver (where it stimulates fatty acid oxidation and inhibits expression of gluconeogenic enzymes) via activation of AMPK (138, 139). Indeed, inhibition of rat liver AMPK in vivo via

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adenoviral expression of a dominant negative mutant blunts the hypoglycemic effect of adiponectin (139).

Although activation of AMPK following adiponectin release may account for many of the long-term effects of thiazolidinediones, there is also evidence that they can activate AMPK more directly. Thus, they activate AMPK in the mouse muscle H-2K^b cell line; in isolated rat muscle; and in rat muscle, liver, and adipose tissue in vivo within 15–30 minutes and at doses as low as 5 μM/10 mg kg^{-1} (4, 155). Moreover, thiazolidinediones rapidly activate AMPK in Swiss-3T3 fibroblasts that do not express levels of PPAR-γ sufficient to induce thiazolidinedione-dependent gene expression (155). These adiponectin-independent effects of thiazolidinediones on AMPK are probably explained, similar to those of biguanides, by their ability to inhibit Complex 1 of the respiratory chain (156) and thus increase cellular AMP:ATP ratios (4, 155).

CONCLUSIONS AND PERSPECTIVES

The suggestion that activators of AMPK may be useful for treatment of type 2 diabetes (141) has been supported by subsequent findings, discussed above. Indeed, two existing major classes of insulin-sensitizing drug, i.e., the biguanides and the thiazolidinediones, may exert many if not all of their therapeutic effects by activating the AMPK system. However, both of these drug classes appear to activate AMPK indirectly, either by inhibiting the respiratory chain or (in the case of thiazolidinediones) by stimulating release of adiponectin. This leaves open the question as to whether a compound that directly activates AMPK might yield the same beneficial therapeutic effects of existing drugs while avoiding unwanted side effects (such as the gastrointestinal effects observed with metformin and the weight gain observed with thiazolidinediones). To answer this question, the AMP-binding domains on the γ subunit, and possibly the glycogen-binding domain on the β subunit, are perhaps the most promising targets for the development of new drugs. Another interesting area for future development of drugs targeted at AMPK is in the arena of cancer. Intriguingly, it has recently been reported in two studies that type 2 diabetics treated with metformin have a lower incidence of cancer than those treated with other agents (157, 158).

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