Topical Review

AMP-activated protein kinase – development of the energy sensor concept

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The LKB1→AMPK cascade is switched on by metabolic stresses that either inhibit ATP production (e.g. hypoxia, hypoglycaemia) or that accelerate ATP consumption (e.g. muscle contraction). Any decline in cellular energy status is accompanied by a rise in the cellular AMP : ATP ratio, and this activates AMPK by a complex and sensitive mechanism involving antagonistic binding of the nucleotides to two sites on the regulatory γ subunits of AMPK. Once activated by metabolic stress, AMPK activates catabolic pathways that generate ATP, while inhibiting cell growth and biosynthesis and other processes that consume ATP. While the AMPK system probably evolved in single-celled eukaryotes to maintain energy balance at the cellular level, in multicellular organisms its role has become adapted so that it is also involved in maintaining whole body energy balance. Thus, it is regulated by hormones and cytokines, especially the adipokines leptin and adiponectin, increasing whole body energy expenditure while regulating food intake. Some hormones may activate AMPK by an LKB1-independent mechanism involving Ca^{2+}/calmodulin dependent protein kinase kinases. Low levels of activation of AMPK are likely to play a role in the current global rise in obesity and Type 2 diabetes, and AMPK is the target for the widely used antidiabetic drug metformin.

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Introduction

A helpful analogy can be drawn between ATP and ADP and the chemicals in an electrical battery. Catabolism 'charges up the battery' by converting ADP to ATP, whereas most other tasks performed by the cell require energy and are driven, directly or indirectly, by hydrolysis of ATP to ADP or, less commonly, AMP. There is no *a priori* reason why these two opposing processes should always remain in balance, especially when the conditions experienced by the cell are fluctuating, but cells usually maintain their ATP : ADP ratio within rather narrow limits. How do they achieve this remarkable feat? While there are almost certainly multiple mechanisms, our central theme is that the AMP-activated protein kinase (AMPK) system is the key player.

With the benefit of hindsight, AMPK was discovered independently as soluble protein factors that, in the presence of ATP, caused time-dependent inactivation of the key regulatory enzymes of fatty acid and cholesterol synthesis, i.e. acetyl-CoA carboxylase (Carlson & Kim, 1973) and 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase (HMGR) (Beg *et al.* 1973). Although these factors were correctly surmised to be protein kinases, it was to be 14 years before it was realized that they were in fact the same entity. The HMG-CoA reductase kinase activity was initially reported to require ADP, as well as ATP, for activity (Brown *et al.* 1975), but this was probably an artefact caused by contamination by adenylate kinase, and it was later shown that 5′-AMP was the true activator (Ferrer *et al.* 1985). Another key finding was that HMG-CoA reductase kinase was inactivated by protein phosphatases and reactivated by an upstream kinase (Ingebritsen *et al.* 1978), making this only the second protein kinase cascade to be discovered.

In 1987 our laboratory reported that the acetyl-CoA carboxylase kinase and HMG-CoA reductase kinase activities were both functions of a single protein kinase (Carling *et al.* 1987). Since it soon became clear that it was a true multisubstrate protein kinase, we followed the precedent set by cyclic AMP-dependent protein kinase (which had originally been termed *phosphorylase kinase kinase*) and named it after its allosteric activator, AMP (Munday *et al.* 1988). Unfortunately, some workers refer to it erroneously as 'AMP-dependent protein kinase' rather

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than the correct 'AMP-activated protein kinase'. We were careful to avoid the former, partly because it does have a significant basal activity in the absence of AMP, and partly to avoid confusion with cyclic AMP-dependent protein kinase.

AMPK – structure and regulation

The modern era of research on AMPK arrived in 1994 with purification of the kinase to homogeneity, revealing that it contained three subunits (Davies *et al.* 1994; Mitchelhill *et al.* 1994), and the first cloning of DNA encoding a catalytic subunit (Woods *et al.* 1994). The kinase is now known to be a heterotrimeric complex comprising a catalytic α subunit and regulatory β and γ subunits. Each subunit is encoded by multiple genes ($\alpha 1$, $\alpha 2$; $\beta 1$, $\beta 2$; $\gamma 1$, $\gamma 2$, $\gamma 3$) yielding at least 12 heterotrimeric combinations, with splice variants further adding to the diversity. Obvious orthologues of the α, β and γ subunits occur in all eukaryotic species for which genome sequences have been completed, including plants and fungi as well as animals, and even very primitive protozoa like *Giardia lamblia* (Hardie *et al.* 2003). This suggests that the AMPK system arose very early during eukaryotic evolution. In budding yeast (*Saccharomyces cerevisiae*), all three subunits are required for the response to starvation for nutrients, especially glucose (Schmidt & McCartney, 2000), while in the primitive green plant *Physcomitrella patens* the catalytic subunits are required for survival during periods of darkness (Thelander *et al.* 2004), which is the equivalent of starvation for a photosynthetic organism.

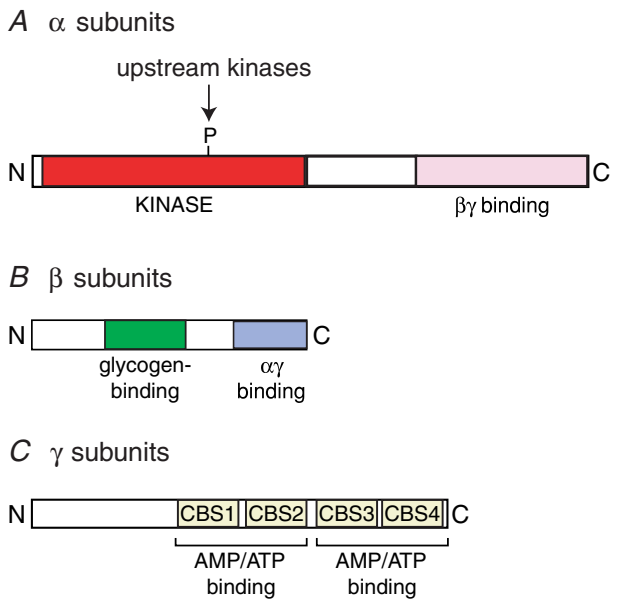

Figure 1. Typical domain structure of the α, β and γ subunits of AMPK and its homologues in lower eukaryotes

Thus, the response to starvation may have been an ancient and critical function of the system.

The three subunits of the kinase have a similar domain structure in all eukaryotes (Fig. 1). The α subunits contains a conventional serine/threonine kinase domain at the N-terminus, with the threonine residue whose phosphorylation is required for activity (Thr-172; Hawley *et al.* 1996) being located in the activation loop, the same region as in many other protein kinases that are activated by phosphorylation. The C-terminal region of the α subunit is required for formation of the complex with β and γ (Crute *et al.* 1998). The β subunits contain a C-terminal region required for complex formation, and a central '*N*-isoamylase domain' that causes AMPK complexes to bind to glycogen (Hudson *et al.* 2003; Polekhina *et al.* 2003). Although this domain is the first region of an AMPK complex for which a crystal structure has been determined (Polekhina *et al.* 2005), its physiological role remains unclear. The γ subunits contain variable N-terminal regions followed by four tandem repeats of a sequence known as a CBS motif (Bateman, 1997). These are now known to act in pairs to form two modules called Bateman domains (Kemp, 2004), the function of which is discussed further below.

Activation of the AMPK complex by AMP occurs by three independent mechanisms (Hardie *et al.* 1999): (i) allosteric activation of the phosphorylated enzyme; (ii) promotion of phosphorylation of Thr-172 by the upstream kinase; (iii) inhibition of dephosphorylation of Thr-172 by protein phosphatases. This triple mechanism means that the system is *ultrasensitive*, i.e. a small change in concentration of the input (AMP) can be converted into a much larger change in the final output (kinase activity) (Hardie *et al.* 1999). The Bateman domains on the γ subunits are now known to form the AMP binding sites: mutations in these domains in the $\gamma 2$ isoform cause hereditary heart disease, and prevent both AMP binding and activation by AMP (Scott *et al.* 2004). Since mutations in either the N-terminal or C-terminal Bateman domain can prevent activation, it seems likely that AMP has to be bound at both sites for activation to occur. Binding to the two sites is also highly co-operative, with a Hill coefficient close to 2. This implies that one site has no affinity for AMP until the nucleotide has bound at the other, and represents a further mechanism to increase the sensitivity of the system. The Bateman domains also bind ATP in a manner that is mutually exclusive with AMP, although their affinity for ATP is lower than that for AMP and much lower than that of the catalytic domain (Scott *et al.* 2004). Since ATP binding does not cause any of the three activating effects of AMP, high concentrations of ATP inhibit activation of AMPK by antagonizing binding of AMP.

We started this review by arguing that the AMPK system was a sensor of cellular energy status, but why does it

respond to AMP and ATP rather than ADP and ATP? Eukaryotic cells universally express the enzyme adenylate kinase, which catalyses the reaction 2ADP $\rightleftharpoons$ ATP + AMP and maintains it close to equilibrium at all times. It is easy to show (Hardie *et al.* 2003) that this causes the cellular AMP : ATP ratio to vary approximately as the square of the ADP : ATP ratio. The former ratio is thus a much more sensitive indicator of compromised energy status than the latter. It is interesting that many of the features of regulation of the AMPK system seem to be geared to maximizing its sensitivity.

Identification of the upstream kinases

Although evidence was reported in 1978 that AMPK (then called HMG-CoA reductase kinase) was activated by an upstream kinase (Ingebritsen *et al.* 1978), it was to be 25 years before the latter was identified. An 'AMP-activated protein kinase kinase' (AMPKK) was partially purified in 1996 (Hawley *et al.* 1996), but attempts to purify it to homogeneity proved frustrating. However, after the completion of the budding yeast genome project, three protein kinases acting upstream of the yeast homologue of AMPK (the SNF1 complex) were identified by various genome-wide screening approaches (Hong *et al.* 2003; Sutherland *et al.* 2003). While none had a clear and obvious mammalian homologue, their kinase domains were most closely related to those of the protein kinase LKB1, and the calmodulin-dependent protein kinase kinases, CaMKKα and CaMKKβ. In a rapid flurry of activity, evidence was obtained that all three, but especially LKB1 and CaMKKβ, can phosphorylate Thr-172 and thus activate AMPK in intact cells and *in vivo* (Hawley *et al.* 2003, 2005; Woods *et al.* 2003, 2005; Shaw *et al.* 2004; Hurley *et al.* 2005; Sakamoto *et al.* 2005, 2006).

LKB1 exists as a complex with two accessory subunits, STRAD and MO25, and the AMPKK partially purified earlier (Hawley *et al.* 1996) was shown to be a complex between LKB1 and the α isoforms of STRAD and MO25 (Hawley *et al.* 2003). Intriguingly, LKB1 had been originally identified in humans as a tumour suppressor, indicating for the first time a link between AMPK and cancer. LKB1 is the gene mutated in the rare autosomal dominant human genetic disorder, Peutz–Jeghers syndrome (PJS) (Hemminki *et al.* 1998; Jenne *et al.* 1998). PJS subjects develop numerous benign tumours in the gastrointestinal tract, but also have a 20-fold increased risk of developing malignant tumours at other sites (Giardiello *et al.* 2000). Moreover, mutations in the LKB1 gene are also seen in some sporadic cancers, especially adenocarcinoma of the lung (Sanchez-Cespedes *et al.* 2002). LKB1 is required for activation of AMPK in response to treatments that elevate AMP or AMP mimetic agents, both in cultured cells (Hawley *et al.* 2003) and in skeletal muscle *in vivo* (Sakamoto *et al.* 2006). However, the LKB1 complex itself is not regulated by AMP and appears to be constitutively active (Lizcano *et al.* 2004; Sakamoto *et al.* 2004), with activation of the cascade being produced by the binding of AMP to AMPK causing it to become a better substrate for LKB1 (Hawley *et al.* 2003) (Fig. 2).

Surprisingly, increases in AMP do not stimulate phosphorylation of Thr-172 by the CaMKKs, which is triggered instead by a rise in Ca^{2+} (Fig. 2) (Hawley *et al.* 2005; Hurley *et al.* 2005; Woods *et al.* 2005). While LKB1 is ubiquitously expressed, a limited survey of different tissues suggest that the CaMKKs are mainly expressed in neural tissues (Anderson *et al.* 1998). Experiments with muscle-specific knockouts of LKB1 in mice suggest that the Ca^{2+}-mediated pathway cannot be a significant player in skeletal muscle (Sakamoto *et al.* 2005), but a more thorough investigation of the distribution of the Ca^{2+}-mediated pathway needs to be performed.

Metabolic stresses that activate AMPK in intact cells and *in vivo*

Arguments discussed above suggest that the LKB1→AMPK cascade will be activated in a highly sensitive manner by even a very small decrease in cellular energy status. What conditions cause this to happen? Similar to its homologues in single celled eukaryotes like *S. cerevisiae* (Wilson *et al.* 1996), AMPK can be activated in cultured mammalian cells by glucose deprivation (Salt *et al.* 1998). However, regulation of AMPK by glucose *in vivo* may be normally restricted to specialized cells, including pancreatic β cells that secrete insulin in response to the level of glucose in the portal circulation, and cells in the hypothalamus that initiate feeding behaviour in response to hypoglycaemia. Like β cells, cells

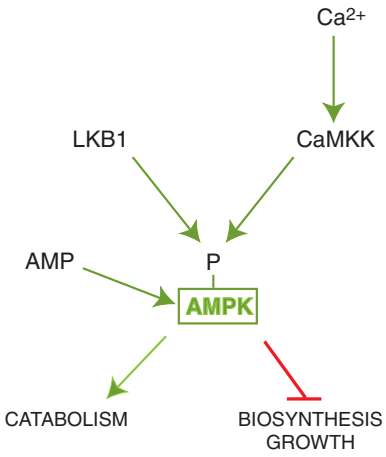

Figure 2. Upstream regulators of AMPK
LKB1 and CaMKKs phosphorylate the same residue (Thr-172) on the α subunit of AMPK. Phosphorylation by LKB1, but not by CaMKKs, is stimulated by binding of AMP to AMPK.

in the hypothalamus are unusual in that they express the high capacity glucose transporter, GLUT2, and the high Km isoform of hexokinase known as hexokinase IV or glucokinase (Kang *et al.* 2006). Thus, in these cell types the rate of glucose metabolism responds to physiological fluctuations in blood glucose, whereas in most other cells production of ATP from glucose would only decrease when blood glucose dropped to pathologically low levels. Both in β cells and in the hypothalamus there is evidence that AMPK activity correlates inversely with glucose over a physiologically relevant concentration range (Salt *et al.* 1998; da Silva Xavier *et al.* 2003; Minokoshi *et al.* 2004). Moreover, activation of AMPK by pharmacological means, or by over-expression of activated AMPK mutants, inhibits insulin release by pancreatic β cells, while in the hypothalamus it stimulates feeding behaviour (Salt *et al.* 1998; da Silva Xavier *et al.* 2003; Andersson *et al.* 2004; Minokoshi *et al.* 2004). These results suggest that the AMPK system is part of the mechanism by which these specialized cells sense fluctuations in glucose, although other mechanisms like the binding of ATP to K_{ATP} channels (Antcliff *et al.* 2005) are certainly also involved.

Other metabolic stresses that activate AMPK include ischaemia (Kudo *et al.* 1995) or hypoxia (Marsin *et al.* 2000), which have been shown to activate the kinase in heart muscle. Like regulation by glucose, activation of AMPK by hypoxia probably only occurs in many tissues under pathological conditions. However, just as there are specialized glucose-sensing cells in the pancreas and hypothalamus, there are specialized oxygen-sensing cells where regulation of AMPK by hypoxia may be a more normal physiological event. These include pulmonary artery smooth muscle cells, which contract in response to hypoxia and thus divert blood flow to oxygen-rich areas of the lung, and Type 1 cells in the carotid body, which stimulate afferent fibres leading to the brain in response to hypoxia, causing a compensatory increase in the rate of breathing (Gonzalez *et al.* 1994; Lopez-Barneo *et al.* 2001). In both cases, there is evidence that AMPK is involved in the oxygen sensing mechanism (Evans *et al.* 2005), a topic that is discussed in more detail elsewhere in this issue.

Another key metabolic stress that activates AMPK is exercise or contraction in skeletal muscle (Hardie & Sakamoto, 2006). The degree of activation of AMPK seems to be dependent on the level of metabolic stress caused by the contraction. For example, prior endurance training reduces the effect of the same intensity of exercise both in rodents (Durante *et al.* 2002) and humans (Nielsen *et al.* 2003). Interestingly, AMPK has also been found to be activated by strength exercise in endurance-trained humans, and by endurance exercise in strength-trained humans, but not vice versa (Coffey *et al.* 2006). This implies that the AMPK system may be particularly important during exercise of an intensity that is greater than that to which the individual is normally adapted. It seems likely, although not conclusively proven, that AMPK is activated by the increases in cellular AMP : ATP ratio that accompany such exercise. In mice, contraction of leg muscles produced by electrical stimulation of the sciatic nerve *in situ* causes increases in the ADP : ATP ratio and (as expected) even larger changes in the AMP : ATP ratio (Sakamoto *et al.* 2005). Intriguingly, the changes in nucleotide ratios are even greater in mice in which expression of the upstream kinase LKB1 in muscle is knocked out. In these mice the basal activity of the $\alpha 2$ isoform of AMPK is completely abolished, and the activities of the $\alpha 1$ and $\alpha 2$ isoforms do not increase in response to contraction (Sakamoto *et al.* 2005). These results confirm that the AMPK system helps to protect muscle against the metabolic stresses caused by contraction, in which the demand for ATP can be increased by more than 100-fold within seconds.

The response to metabolic stresses, such as starvation, is probably the key role for AMPK in single celled eukaryotes, and may have been its original *raison d'être*. However, it is now becoming clear that, during the course of evolution of multicellular organisms, hormones and cytokines have acquired the ability to regulate the AMPK system. Particularly interesting were findings that AMPK is activated by the adipokines, leptin in skeletal muscle (Minokoshi *et al.* 2002), and adiponectin in skeletal muscle and liver (Tomas *et al.* 2002; Yamauchi *et al.* 2002). This increases energy expenditure in muscle by increasing fatty acid oxidation and up-regulating mitochondrial biogenesis (see below). Conversely, leptin *inhibits* AMPK in the hypothalamus, consistent with the ability of the adipokine to inhibit food intake (Minokoshi *et al.* 2004). These results suggest that AMPK also has a key role in the regulation of body weight and whole body energy balance.

Downstream targets of AMPK

Some of the key effects of AMPK on energy metabolism are illustrated in Fig. 3. Many of the downstream effects were originally demonstrated using the compound 5-aminoimidazole-4-carboxamide (AICA) riboside, a nucleoside that is taken up into cells and converted to an AMP analogue, AICA riboside monophosphate (ZMP), which mimics all three effects of AMP on the AMPK system (Corton *et al.* 1995). As discussed above, the first targets of AMPK to be identified were acetyl-CoA carboxylase and HMG-CoA reductase, and using AICA riboside it was demonstrated that activation of AMPK caused consequent inhibition of fatty acid and cholesterol synthesis in heptocytes (Corton *et al.* 1995; Henin *et al.* 1995). AMPK activation also inhibits muscle glycogen synthesis via phosphorylation of glycogen synthase (Carling & Hardie, 1989; Jorgensen *et al.* 2004), protein synthesis in many cells via inhibition of the target-of-rapamycin (TOR) pathway

(Inoki *et al.* 2003) and activation of elongation factor-2 kinase (Horman *et al.* 2002), and *de novo* glucose synthesis (gluconeogenesis) in the liver via phosphorylation of the transcriptional coactivator TORC2, which inhibits expression of genes involved in this pathway (Lochhead *et al.* 2000; Koo *et al.* 2005). This generalized inhibition of biosynthesis by AMPK (particularly its ability to inhibit the growth-promoting TOR pathway) may partly explain the tumour suppressor effects of the upstream kinase LKB1. In addition, AMPK activation appears to cause a G1 phase cell cycle arrest that is dependent on another tumour suppressor, p53 (Imamura *et al.* 2001; Jones *et al.* 2005). This arrest would prevent entry into S phase of the cell cycle when DNA replication takes place, the latter being another costly process in terms of ATP turnover.

As well as conserving ATP by inhibiting biosynthetic pathways, AMPK activation stimulates catabolic pathways that generate ATP. In skeletal muscle it stimulates glucose uptake (Merrill *et al.* 1997), both via translocation of the glucose transporter GLUT4 to the plasma membrane (Kurth-Kraczek *et al.* 1999) and, in the longer term, by increasing its expression (Holmes *et al.* 1999). Studies of mice in which AMPK activation has been knocked out by various means show that AMPK is at least partly responsible for the increased glucose uptake during muscle contraction (Mu *et al.* 2001; Sakamoto *et al.* 2005). In other cells, AMPK activation increases the intrinsic activity of the glucose transporter GLUT1 by an unknown mechanism (Barnes *et al.* 2002). In some cell types such as cardiac myocytes (Marsin *et al.* 2000) and monocytes and macrophages (Marsin *et al.* 2002), AMPK phosphorylates and activates 6-phosphofructo-2-kinase, thus increasing the glycolytic activator fructose-2,6-bisphosphate, stimulating ATP production by glycolysis in response to hypoxia. By phosphorylation of the ACC-2 (-β) isoform of acetyl-CoA carboxylase AMPK lowers malonyl-CoA, relieving inhibition of uptake of fatty acids into mitochondria via the carnitine carrier system, and thus stimulating fatty acid oxidation (Merrill *et al.* 1997). As well as these acute effects on glucose and fatty acid oxidation, AMPK also up-regulates mitochondrial biogenesis (Winder *et al.* 2000), thus increasing the capacity of tissues for aerobic production of ATP. This appears to involve increased expression of the transcriptional coactivator PGC-1α (Zong *et al.* 2002).

Although many of the targets initially identified for AMPK are involved in energy metabolism, examples of targets involved in other processes are increasingly being found. For example, ion channels have the potential to initiate very significant ATP turnover, due to their ability to rapidly dissipate concentration gradients across membranes when in the open state. Transepithelial NaCl transport, an energetically costly process for epithelial cells, is inhibited by effects of AMPK both on the cystic fibrosis transmembrane regulator Cl^- channel (CFTR) (Hallows *et al.* 2000, 2003*a*,*b*) and the amiloride-sensitive Na^+ channel (ENaC) (Carattino *et al.* 2005; Woollhead *et al.* 2005). In our view there are likely to be many other undiscovered targets for AMPK within ion channels and pumps, and this is an area that is ripe for further exploration.

Conclusions and medical perspectives

To summarize, AMPK is activated by metabolic stresses that either inhibit ATP production (e.g. hypoxia, hypoglycaemia) or accelerate ATP consumption (e.g. muscle contraction). Once activated by such metabolic stresses, it switches on catabolic pathways that generate ATP, while switching off biosynthetic pathways and other processes that consume ATP. Its key roles in maintaining energy balance, both at the single cell and the whole body levels, suggest that it will also be an important player in the derangements of energy metabolism that occur in conditions like obesity, Type 2 diabetes and the metabolic syndrome. There is no current evidence that mutations or altered expression of AMPK is a common cause of Type 2 diabetes in humans, but the latter is strongly correlated with obesity and a sedentary lifestyle, and a low activation state of AMPK in the periphery, due to over-nutrition and lack of exercise, may be a contributory factor in its onset. Consistent with this, two of the major classes of drug currently widely used to treat Type 2 diabetes, i.e. biguanides like metformin (Zhou *et al.* 2001) and the thiazolidinediones (Fryer *et al.* 2002), have been reported to activate AMPK. Both appear to do this indirectly by inhibiting complex I of the respiratory chain (El-Mir *et al.* 2000; Owen *et al.* 2000; Brunmair *et al.*

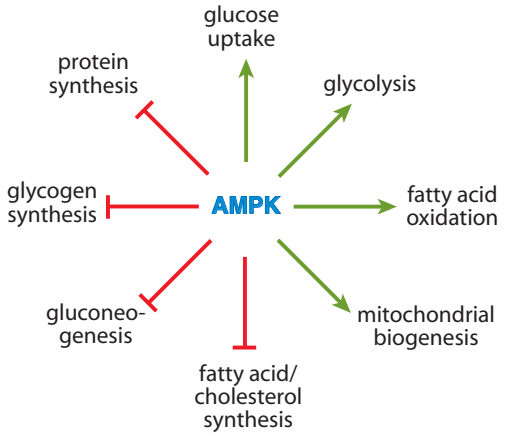

Figure 3. Key processes of energy metabolism that are regulated by AMPK
A green arrow indicates activation, whereas a red line with a bar at the end indicates inhibition. Some of these processes are regulated by multiple effects of AMPK. For example, glucose uptake and fatty acid synthesis are regulated both acutely (with no change in gene expression) and chronically (via effects on gene expression).

2004). The thiazolidinediones also have other effects, but there is now good evidence, from studies of mice that are deficient in the upstream kinase LKB1 in the liver, that the blood glucose lowering effects of metformin are mediated entirely by AMPK (Shaw *et al.* 2005). Given the increasing prevalence of Type 2 diabetes, with estimates of almost 200 million people (5% of the adult population) suffering from the condition worldwide in 2003 (www.idf.org), and given that metformin is used for treatment of over 120 million, the growing attention on the AMPK system is likely to continue for the foreseeable future.

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